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ABSTRACTS

ACUTE LEUKEMIAS XV

Biology and Treatment Strategies

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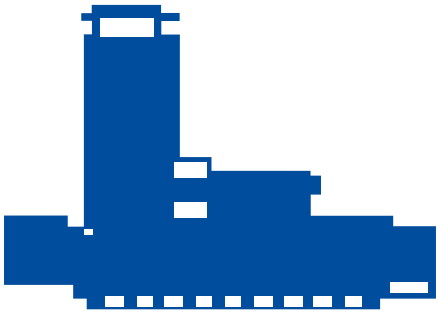

International Symposium

ACUTE LEUKEMIAS XV
Biology and Treatment Strategies

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Biology and Treatment Strategies

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February 22 – 25, 2015, Munich, Germany

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Conventional and Non-Culture Based Microbiological Diagnostics
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SATELLITE SYMPOSIA

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How Can We Improve Induction Strategies in AML?
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SATELLITE SYMPOSIUM II
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(20) ASXL1 Mutations Associated with Cytogenetic Findings in *DE NOVO* and Secondary Acute Myeloid Leukemia

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(21) The Role of STAT5 in FLT3-Mediated Leukemogenesis

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(22) Prevalence of PML/RARA, AML1/ETO, CBFB/MYH11 Translocations and Expression Level of Myeloid CD Markers amongst AML PATIENTS of Assam, India

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(23) The Mutatome of CBFB/MYH11 Rearranged Acute Myeloid Leukemia (AML)

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(24) A Leukemia Associated CD34/CD123/ CD25/CD99-Positive Immunophenotype Identifies FLT3-Mutated Clones in Acute Myeloid Leukemia

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(25) Leukemic Bone Marrow Cells Derived Microvesicles Increase MicroRNA21 Expression in Umbilical Cord Blood Hematopoietic Stem Cells *(IN VITRO)*

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(26) Visualization of AML-Specific FLT3 Mutants and Differential Downstream Signaling

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(27) Hypoxia Regulates FLT3 Expression and Function in Patient Samples and in the BA/F3 Model

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(28) Systems Biology of Mixed Lineage Leukemia

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(29) *IN VIVO* Imaging Facilitates Reliable and Sensitive Monitoring of Preclinical IN VIVO Treatment Trials in the Individualized Xenograft Mouse Model of AML

B. Vick[1-3], M. Rothenberg[4], N. Sandhöfer[1-4], M. Carlet[1], C. Finkenzeller[1], C. Krupka[1,4], M. Grunert[1], A. Trumpp[2,3,5], S. Corbacioglu[6], M. Ebinger[2,3,7], M.C. André[7,8], W. Hiddemann[2-4], M. Subklewe[1-4], S. Schneider[4], K.H. Metzeler[1-4], K. Spiekermann[1-4], I. Jeremias[1-3,9]
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(30) Detection of Chromosome 9q Deletion in Acute Myeloid Leukemia (AML) Patients Using Targeted Sequencing Data

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(31) Evaluation of DNA Repair Gene Polymorphisms in Acute Myeloid Leukemia

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(32) Acute Myeloid Leukemia Preceded by t-MDS after Succesful Treatment of Chronic Lymphocytic Leukemia with FC-R Regimen: Secondary Leukemia? Another Myeloid Neoplasm? - A Case Report

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(33) Mimicking the Bone Marrow Niche IN VITRO: Establishment of a 3D Perfusion Culture System for Mesenchymal Stromal Cells

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(34) The DNA-Repair Machinery as a Novel Target in a Humanized T(8;21) + Pre-Leukemia Model with Activated C-KIT

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III. AML – THERAPY

(35) Evaluation of New DNA Methyltransferases Inhibitors in AML

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(36) New Therapeutic Targets for MLL+ AML Identified via MLL-AF9 Depletion

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(37) Localized Biliar Myeloid Sarcoma Followed by Acute Myeloid Leukemia Treated with Chemotherapy and Consequent Allogeneic Stem Cell Transplantation: A Case Report

B. Nicolino[1], S. Aydin[1], C. Frairia[1], B. Allione[1], E. Audisio[1], A. Busca[2], S. D'Ardia[1], C. Dellacasa[2], F. Manfredotti[3], F. Marmont[2], F. Moro[3], U. Vitolo[1]
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(38) A Fluorescence In-Situ Hybridization-Based Screen Allows Rapid Detection of Adverse Cytogenetics in Patients (PTS) with Acute Myeloid Leukemia (AML)

N. Sandhöfer*[1], K. Metzeler*[1], P. Kakadia[2], N. Huk[1], N. Konstandin[1], A. Dufour[1], M. Subklewe[1], W. Hiddemann[1], K. Spiekermann[1], S. Bohlander[2], S. Schneider*[1], nd J. Braess*[3]
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(39) The Method of Risk-Adapted Therapy Adult Acute Myeloid Leukemia Based on the Monitoring Minimal Residual Disease by Multicolor Flow Cytometry

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(40) The Hedgehog Pathway Mediator GLI Represents a Negative Prognostic Marker in Acute Myeloid Leukemia and its Inhibition Mediates Anti-Leukemic Effects *IN VITRO*

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IV. ALL – BIOLOGY

(41) Influence of Submicroscopic Genomic Rearrangements on Gene Expression in T-CELL Large Granular Lymphocyte Leukemia

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(42) Knockdown of XIAP Sensitizes B-ALL Cells towards Chemotherapy in an Innovative, Dual Luciferase IN VIVO Competition Assay

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(43) Clonality-Analysis in BCRABL-Induced Leukemia by Genetic Barcodes

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(44) Drug Sensitivity of Patient-Derived ALL Tumor Cells is Impaired by Co-Culture with Feeder Cells Mimicking the *IN VIVO* Micro-Environment

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(45) Single Cell Clones from a Patient with ALL Show Different Drug Sensitivity and Growth Behavior *IN VIVO*

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(46) PH-like ALL Is Associated with Homogenous Molecular Alterations and Inferior Outcome in Adults

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(47) Myelodysplasia in Adults with Near-Tetraploidy and Lymphoid (ALL) or Ambiguous Lineage (ALAL) Acute Leukemias

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(48) Overcoming Apoptosis Resistance in Acute Lymphoblastic Leukemia in a Preclinical ALL Xenograft Model

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(49) Prognostic Importance of MN1-Transcript Levels in Patients with Acute Lymphoblastic Leukemia (ALL) Treated According to the Protocol ALL-MB-2008

N. Migal, I. Pakhomova, I. Emelianova, A. Kustanovich, and O. Aleinikova.
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(50) Incidence of IKZF1 Deletions in Russian Cohort of Pediatric B-Precursor ALL. Results of the Russian Moscow-Berlin Study Group

A. Panfyorova, Yu. Olshanskaya, A. Kazakova, E.Matveeva, Eu. Aprelova, Yu.Chekmeneva, S. Lagoiko, J. Rumiantseva, D. Litvinov, O. Bydanov, G. Novichkova, A. Karachunskii
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(51) AXITINIB Targets Gatekeeper-Mutant BCR-ABL1(T315I) -Driven Leukemia in a Distinct and Selective Fashion

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(52) The Metabolic Stress Sensor MLXIP Mediates Malignancy of cALL *IN VIVO*

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V. ALL – THERAPY

(53) International Validation of a Harmonized NGS PGM Assay at Clinical Laboratories in the US and the EU

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(54) A Phase III Multicenter, Randomized, Study Evaluating Efficacy and Safety of Erythrocyte Encapsulated L-ASPARAGINASE (ERYASP) versus Native L-ASPARAGINASE (L-ASP) in Combination with COOPRALL Regimen in Patients with First Relapse of Acute Lymphoblastic Leukemia (ALL)

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(55) *IN VIVO* Response to Remission Induction Poly-Chemotherapy Modeled in NOD/SCID Mice Reflects Patient Risk and Outcome

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(56) Effective *IN VIVO* Targeting of BCP-ALL in a NOD/SCID/huALL Mouse Model by CD70 Directed Immunotherapy

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VI. APL – THERAPY

(57) Proteasome Activity Is Dispensible for the Degradation of PML-RARA: Efficacy of Bortezomib along with Arsenic Trioxide in the Treatment of ATO Sensitive and Resistant Acute Promyelocytic Leukemia

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VII. PEDIATRIC ACUTE LEUKEMIAS

(58) Single Cell Developmental Classification of B Cell Precursor Acute Lymphoblastic Leukemia (BCP ALL) Reveals Link between Phenotype, Signaling and Drug Response

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(59) Primary Induction Failure and Early Relapses in Children with AML

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(60) GATA1-Centered Genetic Network on Chromosome 21 Drives Down Syndrome Acute Megakaryoblastic Leukemia

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(61) Early T Precursor (ETP) Phenotype Impact on Outcome of Pediatric Acute Lymphoblastic Leukemia (ALL)

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(62) Monitoring of Asparagine in CSF after Administration of Pegylated E.COLI Asparaginase within the AIEOP-BFM ALL 2009 Trial

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(63) Outcome of Pediatric Acute Myeloid Leukemia Treated with AML-BFM 98 Protocol in a Developing Country: Lessons and Challenges

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(64) Loss of Fusion Gene in Leukemia with MLL-Rearrangement Provides Insight to Development of 3-Way-Translocations

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(65) Therapy Adaption in the Genetically High Risk Group in Childhood Acute Myeloid Leukemia: A Report of AML-BFM-Study 2004

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(66) Expression of Mecom Transcripts in Childhood Acute Leukemia

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(67) Tumor Cells' Immunophenotype Predicts the Presence of MLL Gene Rearrangements in Infant Acute Leukemia

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(68) New Aspects of Genetic High Risk Stratification in Pediatric AML: A Report of AML-BFM-Study 2004

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(69) Development of Patient-Specific Assay for MRD Detection in Pediatric Patients with CEBPA-Positive NK AML

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(70) Minimal Residual Disease in Peripheral Blood and Bone Marrow of Infants with MLL-Rearranged Acute Lymphoblastic Leukemia. Concordance and Prognostic Significance

G. Tsaur, A. Popov, T. Riger, A. Solodovnikov, T. Nasedkina, A.Kustanovich, O. Aleinikova, E. Shorikov, O. Streneva, L. Saveliev, L. Fechina
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VIII. IMMUNOTHERAPY OF ACUTE LEUKEMIAS

(71) Targeting Acute Myeloid Leukemias with TCR-Transgenic T Cells

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(72) PD-1/PD-L1 Blocking Enhances CD33/CD3-BISPECIFIC BiTE® Antibody Construct (AMG 330) Mediated Lysis of Primary AML Cells

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IX. STEM CELL TRANSPLANTATION

(73) Autologous Hematopoietic Stem Cell Transplantation for Adults with Acute Myeloid Leukemia: A Single-Centre Experience

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(74) Haploidentical 2nd Allo-HSCT for Treatment of Acute Leukemia (AL) Relapse after 1st Allo-HSCT: Retrospective Registry Analysis of 63 PTS

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(75) Allogeneic Transplantation in Elderly Patients with AML and MDS Comparing Two Reduced-Intensity Regimes

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(76) New Strategy in Stem Cell Transplantation for AML: CD96 Antibody TH-111 Removes Leukemic Stem Cells from Autografts

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(77) Monitoring of Bone Marrow Chimerism after Allogeneic HSCT – Early Relapse Detection by a Quantitative PCR Approach

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LATE SUBMISSIONS

AGIHO - EDUCATIONAL SYMPOSIUM

Epidemiology of Lung Infiltrates in Leukemia and Stem Cell Transplant Patients

J. Carratalà

Department of Infectious Diseases, Hospital Universitari de Bellvitge-IDIBELL, University of Barcelona, Barcelona, Spain

MAIN SESSION IV

FLT3 Inhibitors in AML

Richard M. Stone
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ACUTE LEUKEMIAS XV

Biology and Treatment Strategies
Munich, Germany, February 22– 25, 2015

Special Lecture

Updates on CARs for Leukemia and Beyond

C.H. June
Abraham Cancer Center, Perelman School of Medicine, Philadelphia, PA, USA

It is now realized that T cell immune surveillance of cancer occurs in many cases. One approach that has now been developed to deal with tolerance to tumors has been checkpoint blockade, which can enable the natural immune response to respond to antigens encountered in the tumor microenvironment. In this seminar I will outline recent results in the area of synthetic biology, which uses engineered T cells to overcome immune tolerance. There are three types of T cell therapy that are being actively tested in the clinic (1). In one case tumor infiltrating lymphocytes are obtained from surgical biopsies. This therapy is most advanced in melanoma, and trials for patients with metastatic melanoma are currently underway with phase 3 trials in Europe. Gene transfer technologies can also be used to produce engineered T cell therapies. One approach is a chimeric antigen receptor or CAR T cell approach. Another is the use of engineered T cells that express T cell receptors of known specificity and affinity.

There are number of considerations for successful T cell therapy with adoptively transferred T cells. It is necessary to have an adequate number of T cells in order to eradicate large numbers of tumor cells. If a kilogram of tumor represents approximately $1x10^{12}$ T cell tumor cells, then it is likely that a similar number of T cells will be required for an equivalent effector to target ratio in vivo. The failure to achieve a critical mass of T cells numerically may explain many previous trials with disappointing results following therapeutic vaccines. There are two potential solutions to overcome inadequate effector T cell function in cancer patients with adopted cell transfer therapy. On the one hand it is possible to infuse very large numbers of T cells, as has been done in tumor infiltrating lymphocyte therapy [2]. The other approach is to infuse smaller numbers of cells that are programmed to expand extensively in the patient[3,4].

There are three essential factors to consider when developing engineered T cell therapies (**Fig1**).

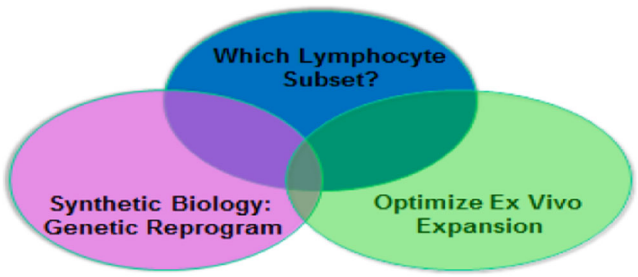

Fig1: Essential factors for augmenting adoptive cellular immunotherapy

One issue under consideration is the nature of the lymphocyte subset to be infused. At present it is not known whether the optimal cell type is a memory cell or a naïve cell. Related to this is the question of the proportion of CD4 helper cells and CTLs. Secondly it is important to consider the aspects of genetic engineering that are used to reprogram the cells [5]. Finally it is necessary to have an optimized approach for the manufacturing of the T cells.

The design of CAR T cells has become increasingly sophisticated (**Fig2**).

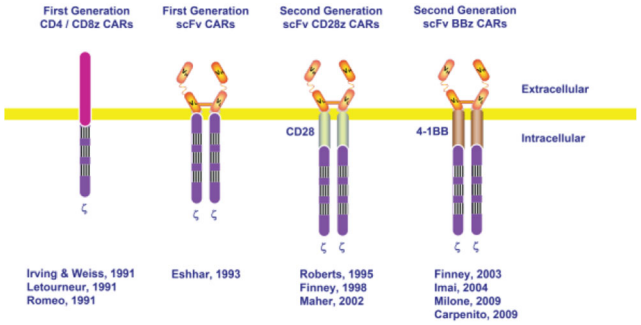

Fig2: Design of CAR T Cells

The initial CAR T cells were so-called first-generation CARs and only had signaling from the T cell receptor zeta chain or related molecules. More recently second and third generation CAR T cells have been designed, incorporating progressively more complicated signaling domains. A number of strategies have been used to make CARs more potent or to enhance their proliferative capacity or their cytokine secretion. For example it has been shown that so-called Signal 1 from the Lafferty model [6] can be enhanced by encoding a ubiquitin-resistant linker of activated T cells (LAT) [7]. A number of costimulatory domains have been used to augment Signal 2. These include CD28, and the tumor necrosis family members 4-1BB, Ox40, and CD27, for example. Recent studies have shown that the ICOS signaling domain, a molecule related to CD28, provides CAR T cells with enhanced persistence in preclinical animal models. These studies were initiated by Crystal Paulos and showed that when T cells were activated through their natural ICOS receptor molecule with agonistic antibodies, that this promoted the proliferation of TH 17 cells and T follicular helper cells that had highly potent effector functions (**Fig 3**).

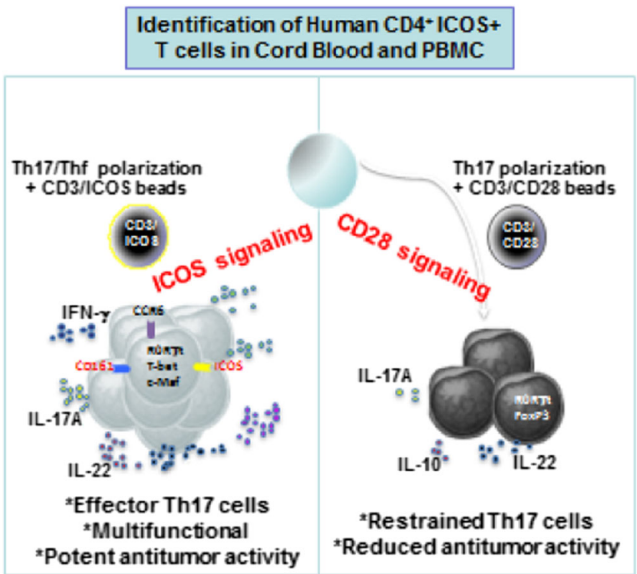

Fig.3: Distinct Cosignaling by CD28 and ICOS: Implications for ACT therapy

In contrast, if the T cells were costimulated with a CD28 dominant costimulatory signal, then the TH17 cells were restrained in their antitumor activity and inflammatory properties [8]. More recently, Sonia Guedan has demonstrated that when ICOS is expressed in CD4 cells by encoding the cytosolic domain in the CAR endodomain that this leads to enhanced

persistence of the CD4 cells and that those cells promote the persistence of CD8 CAR T cells [9]. This latter effect is independent of the costimulatory domain it is expressed in the CD8 CAR T cell. Other studies in our laboratory have recently identified examples of CAR designs that have either a continuous and ligand-independent growth phenotype, or are quiescent in the absence of stimulation by surrogate antigen. Finally, studies by Yangbing Zhou have recently shown that it is possible to increase the therapeutic index of CARs by affinity-tuning of the single chain variable fragments.

Ongoing research is defining the characteristics of CAR design that lead to a continuously active CAR. These T cells are capable of proliferating for months without stimulation or addition of exogenous cytokines. Factors that dictate ligand independent growth of the CAR T cells depend on the particular scFv used, and so far they have only been observed with CD28 endodomains. The growth is independent of exogenous ligands and there is evidence for constitutive signaling through CD28 involving AKT, NF-kappaB and the MAP kinase pathway. The continuous CAR T cells have constitutive secretion of Th1 and Th2 cytokines, and they tend to highly express T-bet, Eomes, GATA3, and Bcl-xL. Transformation not observed, and surprisingly, CAR T cells with the continuous growth phenotype are less effective in pre-clinical tumor models. One implication of this research is that it is important to screen newly designed CAR T cells for constitutive activity.

The initial CAR T cell trials were conducted in patients with HIV, using a first-generation CAR (10). At the University of Pennsylvania CAR T cell trials in cancer patients targeting CD19 were initiated in 2010 [11]. There were three unique aspects of this the CAR, termed CART19, that were used in this trial that have not been tested previously. First a lentiviral vector was used to express the CAR. Secondly, a short duration T cell culture process using antibody-coated beads was used for T cell manufacturing. Thirdly, the CAR employed a TNF family member for co-stimulation, CD1 37, also referred to as 4-1BB. The initial patients that were treated had refractory chronic lymphocytic leukemia [12]. All three of the initial patients responded to the CART19 therapy. The patients had large bulky tumors and it was possible to conclude that each CART19 cell or its progeny through cell division killed more than 1000 tumor cells. As of December 2014 the patients continue to express the CARs and the remissions have been durable in two of the three patients having no evidence of leukemia for more than 4 years. The level of CAR T cell proliferation in vivo is a predictive biomarker of clinically beneficial antitumor responses. In more recent studies the CART19 T cell approach has been used to treat patients with acute lymphoblastic leukemia 9ALL). We recently reported the first 30 patients on this phase 1 trial and a striking 90% rate of complete remission was observed in both pediatric and adult cases [13]. Updated results as of December 1, 2014 are shown in **Fig4**. Durable B cell aplasia is associated with a low risk of relapse of ALL following CART19 T cell therapy.

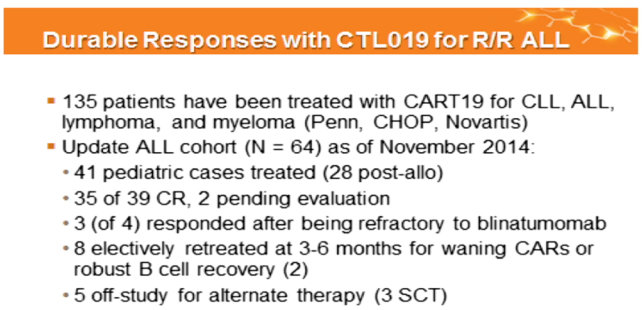

Fig4: Updated results of pendiatric r/r ALL trial with CART19. Results updated from Maude et al, NEJM 2014

There have been a number of toxicities associated with CAR T cell therapy. B cell aplasia is observed in responding patients following therapy with CD19 or CD20 specific CARs. Tumor lysis syndrome has been observed in many patients, and this is managed as per standard oncology practices and the unique aspect of this is that it may be delayed for 20 to 50 days after infusion of the CAR T cells. Cytokine release syndrome characterized initially with fever occurs in many patients, and this is related to the tumor burden in the patient. Finally, a subset of patients develops macrophage activation syndrome which is characterized in the serum by very high levels of ferritin, C-reactive protein and evidence of coagulopathy. The hallmark of this syndrome is very high levels of IL-6 and interferon gamma in the serum. This syndrome is rapidly reversed with the IL-6 receptor antibody tocilizumab, indicating that this inflammatory disorder is dependent on IL-6 (**Fig5**).

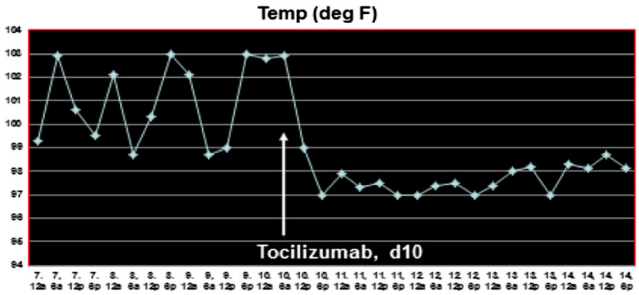

Fig5: Tocilizumab Anti-Cytokine Therapy for Cytokine Release Syndrome. A patient with leukemia was given tocilizumab on day 10 after CART19 therapy, with immediate resolution of fever

In summary, engineered T cells are finally on the path towards widespread use. A number of trials are now underway that should lead to commercial approval in the United States and in Japan.

Conflict of Interest: Intellectual property rights licensed by University of Pennsylvania to Novartis. Author also declares that he has sponsored research grant from Novartis.

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Main Sessions I-XI

(authors in alphabetical order)

Targeting complementary bone marrow niches in AML

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Abstract

The bone marrow niche is a preeminent factor in the maintenance and propagation of acute myeloid leukemia (AML). The interactions between the microenvironment and AML stem cells are believed to offer protection against therapy and drive relapse in refractory disease. Forthcoming therapies that target the linkage between leukemia cells and surrounding shelter offer promise for improved outcomes in AML. We report here a limited review of emerging therapies that target two separate elements of the bone marrow niche: the CXCR4/CXCL12 axis and E-selectin. In addition, we discuss a novel approach to studying the bone marrow microenvironment in vivo, using a humanized xenotransplantation bone marrow model in mice. We report results from the added use of cancellous bone chips as scaffolding for subcutaneous bone formation, and concomitant addition of AML cells with implantation.

Introduction

The interactions between the bone marrow niche and AML stem cells may offer niche-specific protection against therapy and lead to relapse (1, 2). Forthcoming therapies that target the molecular connections between leukemia cells and surrounding shelter offer promise for eradication of residual AML cells and cures (3).

The marrow is composed of at least two separate environmental compartments relevant to hematopoietic cells: the endosteal (osteoblastic) niche and the endothelial (vascular) niche. Research has supported the notion that both are involved in normal hematopoietic stem cell function. Both niches also appear to be instrumental in abnormal leukemia stem cell (LSC) maintenance, in a way that likely mimics their role in normal blood cell function (2, 4-7). Given that leukemia initiating cells are present in more than one hematopoietic progenitor phenotypic compartment, and that various leukemia stem cell phenotypes have been identified in AML, it is conceivable that each niche supports different leukemia stem/progenitor cell states (8, 9). Interactions that occur between various niche cells and LSC encompass a complex interplay that relies on a multitude of cells, signals, conditions, and contacts (**Fig1**).

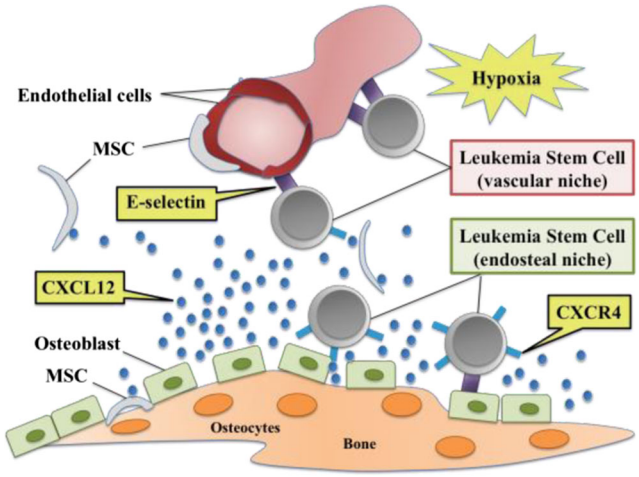

Fig1: Complexity of complementary niches. Leukemia stem cells may reside in the endosteal niche and/or the endothelial niche. Both rely on microenvironment signals (such as CXCL12) and cell surface receptors (such as CXCR4 and E-selectin) to guide and maintain stem cell states. The proliferation of leukemia cells in and around the niche promotes a hypoxic environment that may metabolically favor LSC survival. Other cellular components of the marrow niche, such as mesenchymal stem cells (MSC) and osteoblasts, also communicate with and contact niche leukemic cells. A particular target for mobilization is not restricted to a singular niche.

Emerging therapies target complementary elements of the bone marrow niche, and thus may also target different stem cell fractions in AML. Targeting the CXCR4/CXCL12 axis or E-selectin are both viable strategies for helping mobilize and eliminate leukemia cells. To further evaluate the microenvironment in vivo, we have incorporated a humanized bone marrow model in mice. The purpose of the current study was to determine whether human cancellous bone chips could serve as scaffolding in a model using subcutaneous implantation of mesenchymal stem cells (MSC), endothelial colony forming cells (ECFC), and matrigel, along with concomittant addition of bulk or FACS-sorted subpopulations of leukemia cells.

Novel approaches to anti-CXCR4 therapy

Identifying the elements linking leukemia cells and the microenvironment, and understanding those interactions mechanistically, is critical for disruption of leukemia resistance. One interaction that occurs between LSC and the bone marrow microenvironment is through C-X-C chemokine receptor type 4 (CXCR4, or CD184) on the leukemic cell, and its attractant ligand C-X-C chemokine receptor 12 (CXCL12, or stromal cell-derived factor-1α, SDF-1) in the marrow niche (10-12). Interestingly, the level of CXCR4 expression on AML cells correlates with worse outcomes (13, 14).

CXCR4/CXCL12 inhibition is a known mobilizer of marrow stem cells, and the CXCR4 inhibitor plerixafor is approved by the US Food and Drug Administration for mobilization of CD34+ cells prior to autologous stem cell transplantation for non-Hodgkin's lymphoma and multiple myeloma (15). Inducing mobilization of LSC is a method that is also being used to treat leukemia. In pre-clinical in vivo leukemia models, inhibition of CXCR4 resulted in mobilization of leukemia into peripheral blood and subsequent sensitization to cytotoxic chemotherapy (16). A recent phase 1 study demonstrated this same approach was useful in humans, where AML patients treated with plerixafor prior to chemotherapy demonstrated modest (two-fold) leukemia mobilization and increased sensitivity to a standard ara-

C plus anthracycline regimen (17). A more recent study used plerixafor plus G-CSF in the pre-allogeneic stem cell transplant setting for patients with myeloid malignancies (18). Although outcomes were not superior compared to historical patients, efficient mobilization of leukemic clones occurred (18). Other CXCR4 inhibitors are under development including a monoclonal antibody (PF-06747143) (19), peptide inhibitors (BL-8040) (20), and other small molecule inhibitors (such as LY2510924) (21). These agents by themselves exerted anti-leukemic effects, with complete remissions and reduction of bone marrow blasts in pre-clinical and clinical studies.

The concept of CXCR4 inhibition was initially developed with the aim of releasing leukemic cells from the niche, and thus removing microenvironmental protection from LSC. Combinations of CXCR4-inhibitors with other agents are being investigated, but optimized synergistic lethality with other AML therapies has yet to be determined. This concept was complemented by the observation that CXCR4 inhibition can lower the apoptotic threshold of AML cells, independent from mobilization, through an axis consisting of the SDF-1-receptor CXCR4, the transcription factor Yin Yang 1, the microRNA let7a, and its targets BCL-XL and c-MYC (22).

Combinations with other mobilizing agents such as granulocyte-colony stimulating factor (G-CSF), are designed to potentiate the effects of CXCR4 inhibition. G-CSF itself works through several mechanisms to promote mobilization (23). These agents may be combined also with other targeted therapies, such as FLT3 inhibitors. A trial that investigated the combination of plerixafor, G-CSF, and sorafenib in relapsed/ refractory FLT3-mutated AML resulted in 36% CR/p rates, an overall response rate of 62% and some durable responses (24).

E-selectin inhibitors for the vascular niche

Recent work has revealed the interactions between HSCs and the vascular niche by way of E-selectin binding and signaling, and in particular the effects of E-selectin on stem cell function (25). Accumulating evidence suggests that inhibition of this pathway also induces AML stem cell mobilization and blocks protection from the vascular niche (26, 27). The E-selectin inhibitor GMI-1271 is progressing into clinical trials for patients with AML.

Targeting the vascular niche to eradicate LSC may become a complementary strategy to the targeting of other niche components. The interactions between AML stem cells and the endothelium is not fully understood (28). Interestingly, hematopoietic and endothelial cells arise from the same primitive embryonic precursors (the hemogenic endothelium), and many genes important in the hematopoietic and endothelial differentiation process are known to be involved in AML (29). A special relationship may be forged between leukemia stem cells and the endothelium that has yet to be understood and exploited for treatment and prevention of relapse.

In order to better understand how the leukemia niche functions, it may be studied using a variety of experimental tools. For example, the leukemia niche has been shown to be hypoxic compared to normal bone marrow. Treatments that capitalize on the hypoxic microenvironment, such as hypoxia-activated pro-drugs, may be particularly useful as another niche-directed therapy (30). New findings suggest that leukemic cells and LSC are generating energy not through the Warburg effect, but through oxidative phosphorylation (Oxphos). Thus LSC scavenge oxygen and adapt to thrive under hypoxic conditions (31). Hypoxic culture conditions are beneficial to the maintenance of a stem cell state (32), and for the growth and expansion of MSC.

Methods

Recently, advances have been made by creating models to study the leukemia microenvironment that allows for the recreation of a human marrow niche in mice. One such model is carried out by adding MSC and endothelial colony forming cells (ECFC) to matrigel and injecting subcutaneously into the flanks of mice (33). After an extended period of time, humanized bone and bone marrow forms and engrafts AML cell lines as well as primary patient samples. The model allows for the microenvironment to be genetically modified prior to injection and bone formation (33). Inclusion of ECFC appears to be optional, and vascular formation may arise from both MSC and ECFC (34). Such models are aimed at creating a better representation of the human microenvironment in the context of leukemia and LSC xenografts. Knockdown of HIF-1α, CTGF, or NFkB in MSC all affected AML engraftment (33, 35, 36).

A previous pilot study used the addition of cancellous bone chips implanted in the flanks of mice with MSC and ECFC (1 x 10^6 cells each) in matrigel and showed appropriate niche formation within 4 weeks (Figure 2A-B). To further test whether a scaffolding nidus for bone formation could be used to improve bone and niche formation with concomitant addition of human leukemia, we used 2-3mm cubiform human cancellous bone chips (Medtronic Cat#P00035) combined with 200 μL matrigel containing MSC ($2x10^5$ cells) and ECFC ($2x10^4$ cells). In addition, we added bulk leukemia cells or FACS-sorted subpopulations (including CD34+CD38-CD123+ LSC) from 2 primary AML patient samples. Five NOD-*scid IL2rγ*^{null} (NSG) mice were implanted with single bone chips, along with $8x10^3$ to $1.3x10^6$ AML cells per mouse. Mice were monitored daily for signs of illness, and retro-orbital peripheral blood sampling was performed at regular intervals to detect engraftment of human leukemia by flow cytometry of human CD45 and CD33.

Results

All mice tolerated procedure and implantation. Peripheral blood evaluation showed no engraftment of human AML up to 16 weeks. Interim analysis for leukemia of one mouse at 16 weeks was carried out due to a small area of redness at the site of bone chip implantation. Pathologic evaluation of bone marrow, spleen, and liver demonstrated no leukemia cells. Evaluation of the bone chip after EDTA decalcification demonstrated no engraftment of human leukemia at the interim time point. The cancellous bone chip showed adipose tissue, and fibrous connective tissue with an inflammatory multinucleated giant cell reaction (**Fig**2C-D). A hematopoietic niche was not identified in this chip. The remaining four mice are under continued observation.

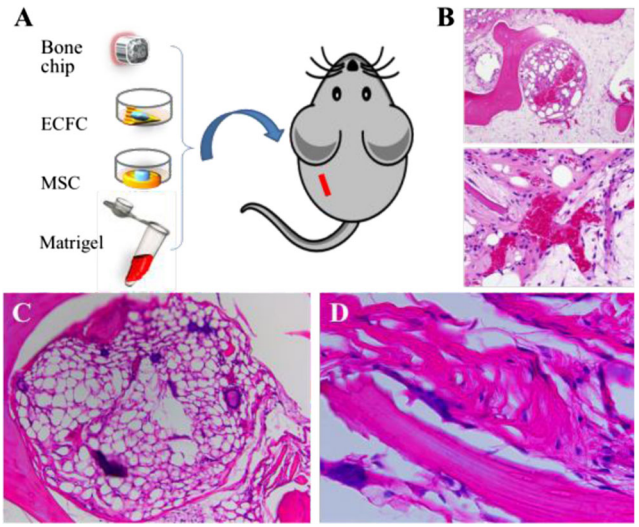

Fig2. Humanized bone marrow model in mice using cancellous bone chip. A) Method for creating humanized bone marrow using cancellous bone chip in mice (courtesy of Ye Chen). B) 10X (upper panel) and 40X (lower panel) of hematopoietic niche formation. C) 10X photomicrograph

showing adipose tissue in the bone chip spaces for one mouse implanted with chip + MSC + ECFC + AML. D) 40X photo-micrograph showing fibrous connective tissue and multinucleate giant cell reaction.

Summary

The use of human cancellous bone chip implanted with MSC, ECFC, and leukemia cells as adapted from previously described humanized bone marrow model in mice did not so far result in improved or faster marrow niche formation or AML engraftment. Monitoring of xeno-transplanted mice is ongoing. The previously described and established model of humanized heterotopic bone marrow model in mice continues as the gold standard, and may be used to genetically modify the AML micro-environment (22, 33-36).

Conclusions

As our understanding of the LSC evolves, so has our recognition that these cells function as a product of their surrounding microenvironment. Recent data suggests that clonal remnants in the form of pre-leukemic cells often remain after therapy, and may be responsible for detection of AML mutations by deep sequencing in remission (37). These pre-leukemic stem cells may occupy the bone marrow niche. Eradication of these cells could potentially take place with selective targeting of the niche where they reside. The optimal way to eliminate these cells within their niche is not yet clear, and niche-directed therapies, such as CXCR4 or E-selectin antagonists, may prove instrumental to prevent relapse. Enhanced testing of emerging therapies can be carried out using in vivo models that replicate structural and functional components of the human bone marrow microenvironment.

Conflict of interest: The authors declare that they have no conflict of interest

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Targeting *HOX* Gene Expression in Normal and Malignant Hematopoiesis

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Homeotic (*Hox)* genes are important regulators of development in multiple cell lineages including hematopoietic cells. High-level *Hox* gene expression is a recurrent feature of several different malignancies including diverse subtypes of acute myeloid leukemia (AML). We have previously shown that that H3K79 dimethylation (H3K79me2) by the enzyme Dot1L is required to maintain *Hox* gene expression and proliferation of *MLL*-rearranged leukemias. This has prompted the development of small molecules that are now in clinical trails. More recently we have studied the regulation of the Dot1L complex and identified the role of Dot1L in the control of gene expression. We find that H3K79me2 is converted to mono-methylation (H3K79me1) at critical *Hox* loci as cells transition from hematopoietic stem cell-enriched populations to myeloid progenitor cells thus coinciding with a decrease in *Hox* gene expression. Conversion of H3K79me1 to H3K79me2 is critical for continued expression of *Hox genes* in leukemia cells and H3K79me2 but not H3K79me1 is dependent on Af10, a core constituent of the Dot1l complex. Af10 loss of function reverses aberrant epigenetic profiles found in leukemia cells and dramatically impairs the transforming ability of MLL-AF9, MLL-AF6 or NUP98-NSD1 fusions – diverse, mechanistically distinct oncogenes that drive abnormal *Hox* gene expression. Furthermore, NUP98-NSD1 transformed cells, much like those transformed by MLL-fusion proteins, are highly sensitive to small-molecule inhibition of Dot1l. Also, we have performed a genome scale RNAi screen and found that suppression of the histone deacetylase Sirt1 and histone methyltransferase Suv39h1 rescued *MLL*-rearranged leukemia cells from their dependence on Dot1L. We find that Sirt1 and Suv39h1 are required for the acquisition of a repressive chromatin state consistent with facultative heterochromatin around MLL-fusion target genes after Dot1L inhibition. Epigenomic profiling revealed that Dot1L-dependent genes demonstrate an $H3K9ac^{high}/me2^{low}$ signature that is induced at MLL-AF9 bound loci. This signature is also normally found on a distinct set of Dot1L dependent genes in hematopoietic stem and progenitor cells. Finally, pharmacological activation of Sirt1 enhances the efficacy of Dot1L inhibitors against *MLL*-rearranged leukemia via more rapid and complete silencing of leukemogenic gene expression. These data show that the Dot1L complex is highly regulated in hematopoiesis and in leukemia. A major function of Dot1L is to inhibit Sirt1-mediated acquisition of a heterochromatic state to maintain leukemogenic gene expression, and to maintain specific gene expression programs during normal hematopoietic development. These mechanisms are being exploited therapeutically in ongoing clinical trials.

Conflict of Interest: The author declares that he has no conflict of interest

New Molecular Diagnostic Characterization of Acute Myeloid Leukemia in the 2015 World Health Organization Classification

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The World Health Organization (WHO) classification of acute myeloid leukemia (AML) is in the process of undergoing revision. The goal is a revised version to be published in 2015. From March 30, 2014 through April 1, 2014 the Clinical Advisory Committee (CAC) met to consider a series of proposed changes from the current version published in 2008 [1, 2]. It is expected that by the end of 2014 the WHO will have agreed upon which of the recommended changes from the CAC meeting will be incorporated into the 2015 WHO classification. These will be discussed.

Conflict of Interest: The author declares that she has no conflict of interest

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Prognostic Markers and Treatment of T-ALL

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Abstract
In adults, T-cell acute lymphoblastic leukemias (T-ALL) account for around 25% of all cases. In both pediatric and adult populations, T-ALLs are more frequently associated with a high white-blood cell count (WBC), a mediastinal tumor mass and a cerebrospinal fluid involvement. Contrarily to what is observed in BCP-ALL, T-ALL are more frequently observed in adolescents and young adults, making patients with T-ALL younger in the whole adult population. Moreover, less heterogeneity in disease characteristics was reported between children and adults, even if unfavorable immature T-ALL seems to be more frequent in adults.[1] Even more than in BCP-ALL, patients with T-ALL benefit from pediatric-inspired approaches with complete remission (CR) rates >90%, relapse incidence around 30%, and approximately 55-60% event-free survival and 65% overall survival at 5 year.

In most cooperative group, prognostication in T-ALL relies on initial WBC, phenotype, and response to induction therapy. A dismal prognosis has been reported for pro/pre-T (EGIL T-I and T-II) and mature T (EGIL T-IV) ALL compared to cortical T-ALL. The lack of CD1a and CD8 expression and the weak expression of CD5 define a subset of T-ALL, named "early T-cell precursor" ALL, that was associated with myeloid-related molecular features and a higher risk of treatment failure.[2] In contrary to BCP-ALL, conventional cytogenetic analysis is uncommonly used for risk stratification in T-ALL. The presence of t(10;14)(q24;q11)/*TLX1* has been associated with a better prognosis, while the negative impact of t(10;11)(p12;q14)/*PICALM-MLLT10* (formerly *CALM-AF10*) has been discussed. Complex karyotype (≥5 abnormalities) found in both BCP- and T-ALL has also been reported as an unfavorable feature.[3] In the last decade, molecular biology, has helped to refine the risk in T-ALL patients. Low expression of *ERG* and *BAALC* genes have been associated with a lower relapse risk and a better survival.[4] More recently, mutations of the NOTCH pathway involving *NOTCH1* or *FBXW7* genes have been shown to confer a favorable prognosis in T-ALL but also T-lymphoblastic lymphoma.[5] This feature may be modulated by the presence of *RAS/PTEN* alterations, so that patients with *NOTCH1*/*FBXW7* mutations w/o *RAS/PTEN* alterations have a particularly favorable outcome.[6] Finally, early response to therapy and quantitative minimal residual disease (MRD) monitoring during and after induction, either by clone-specific *IgH*/*TCR* gene rearrangement detection or by flow cytometry, may be used to summarize the risk at the individual patient level. As in many other diseases, these prognosis factors remain often dependent on the protocol used.

Open questions concerning this rare disease diagnosed in relatively young patients remain to be addressed: 1) What is the optimal way to combine disease-related prognostic factors and response evaluation to assess the risk of treatment failure?,[7] 2) Which patients are good candidates for allogeneic SCT, in other words which of these prognostic factors are good predictors of survival improvement after SCT? 3) Is there any subgroup of patients that may benefit from new therapeutic approaches? Among evaluated drugs, the nucleoside analogue nelarabine, already approved in relapsed patients, is currently being investigated front-line as post-remission therapy. Targeting NOTCH1, IL7-R and especially PI3K/AKT/mTOR pathways might be promising therapeutic approaches in this disease.

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MRD Monitoring in ALL

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Abstract
The analysis of minimal residual disease (MRD) has become an important tool for treatment tailoring in acute lymphoblastic leukemia (ALL). Methods to quantify MRD are PCR analysis of clonal immunoglobuline (IG) /T-cell receptor (TR) gene rearrangements, the molecular detection of recurrent genetic aberrations and immunophenotyping using multicolor flow cytometry. To ensure comparable MRD results between different MRD-laboratories, standardization and quality control are essential. Multiple reports have shown that detection of MRD in childhood and adult ALL is a powerful predictor of long term outcome and supersedes other widely used and accepted prognostic markers, both in de novo and relapsed ALL as well as in ALL patients undergoing stem cell transplantation. Consequently, MRD diagnostics has been incorporated into the treatment stratification within several ALL protocols. Within the German Multicenter ALL (GMALL) study patients with MRD persistence after consolidation exhibit a new high risk group. These patients display resistance to conventional drugs and are candidates for treatment with targeted, experimental drugs and allogeneic SCT.

Methods

Reliable techniques for MRD quantification must exhibit certain performance characteristics concerning sensitivity, specificity, standardization and quality control.[1] Currently, mainly multicolor flow cytometry (MFC) and molecular methods targeting clonal IG/TR gene rearrangements or recurrent genetic aberrations are used to monitor MRD. Within different

international collaborations validation and standardization of methods is performed: The EuroFlow network established fully standardized 8-color antibody panels as well as tools for bioinformatic assisted expert independent automated analysis of the data acquired.[2;3] Within the EuroMRD network guidelines for standardized real-time quantitative (RQ)-PCR of IG/TR gene rearrangements have been published already several years ago.[4] The network organizes periodic ring trials for external quality control of analysis. For BCR-ABL PCR standardization efforts were initially performed within the European Working Group for Adult Acute Lymphoblastic Leukemia (EWALL), and these efforts were pooled with the EuroMRD experience in 2008, when joined EWALL/EuroMRD meetings started. To date several issues regarding methodology and interpretation guidelines are addressed for generating reliable quantitative data for BCR-ABL transcripts in Ph+ ALL.

With the availability of high throughput technologies also next generation sequencing (NGS) emerged as molecular tool for MRD quantification. IG/TR NGS relies on amplification and high throughput sequencing of IG or TR gene rearrangements using multiplex PCR approaches. One major advantage of this workflow compared to ASO PCR is the applicability of a universal PCR assay without the need of patient-specific reagents. Recent developments offered the proof of principle that NGS-based MRD assessment is feasible and potentially even more sensitive than the standard options.[5-9] However, several aspects and potential pitfalls have to be adequately considered when MRD is analyzed and reported using NGS approaches. Therefore, also for IG/TR NGS MRD assessment a European consortium formed for setting standards in IG/TR NGS methodology and its applications in hemato-oncology. This EuroClonality-NGS consortium consists of several EuroClonality laboratories (formerly BIOMED-2; euroclonality.org) experienced in design of assays for detecting IG/TR rearrangements, supplemented by laboratories with an expertise in MRD measurement by IG/ TR gene analysis (EuroMRD; euromrd.org), IG/TR repertoire studies and immunoinformatics (European Research Initiative on CLL, ERIC; ericll.org).

Results

Within the German Multicenter ALL study Group (GMALL) we analyzed MRD in a total of 580 Ph-negative patients with standard risk (SR, n = 434) and high risk (HR, n = 146) features.[10;11] A complete MRD response after induction 2 and/or consolidation 1 was associated with a comparable clinical benefit irrespective of pretherapeutic risk factors. MRD was the only parameter with significant prognostic impact in multivariate analysis. The biologic differences between SR and HR patients were reflected by the significant different proportion of patients reaching a complete MRD response between SR and HR patients with about 20% point lower rates of MRD negativity in HR patients. MRD persistence after consolidation I identified patients with molecular failure as a new high-risk group.[11] Within the GMALL 07/03 trial, patients with persistent MRD $>10^{-4}$ after induction and/or first consolidation were therefore allocated to the MRD-HR group and qualified for allo-SCT. In 47% out of 120 MRD-HR patients, SCT was realized in first CR, with the SCT rate being significantly higher in HR compared with SR patients (71% vs 39%, P < .002). The probability of CCR after 5 years was significantly higher for patients receiving an MRD-directed SCT in first CR compared with those without SCT in first CR (66% vs 12% P < .0001). This also translated into a better OS at 5 years (54% vs 33%, P = .06).[11]

Within a phase II trial Blinatumomab was administered to GMALL B-cell precursor ALL patients with MRD persistence or MRD relapse. Sixteen out of 20 evaluable patients (80%) became MRD negative after one cycle of treatment. This translated into a relapse free survival of 66% after a median follow up time of 33 months for those 9 patients who received an allogeneic stem cell transplantation (SCT) after blinatumomab treatment.[12;13] Of the 11 patients, who received no subsequent allo SCT RFS was 60% at a median follow-up time of 31 months. Currently, the confirmatory single-arm, phase 2 BLAST trial evaluates blinatumomab in patients with MRD+ ALL in a larger population.

Conclusions

MRD has emerged as powerful and independent predictor of outcome in ALL and has surpassed other widely used and accepted prognostic indicators. Several international consortia aim for optimization and standardization of MRD methodology which is of utmost importance when MRD is used for treatment tailoring.

Conflict of interest: The authors declare that they have no conflict of interest

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UK Trials in Acute Myeloid Leukaemia

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Introduction

Trials for AML have been continuously available to investigators for nearly 50 years, Since 1988 there has been a protocol for patients generally younger than 60 years, frequently including children, and a protocol for older patients. In general questions in relation to induction and post induction and by risk group have been set. Both tested intensive therapy approaches. Each protocol poses several question which is facilitated by factorial design. Since 1994 a new approach was made available for older patients who were not considered fit for intensive therapy. Similarly, apart from pilot trials all interventions are subject to randomisation.

Trials for Younger Patients

Mylotarg*:* Recent studies for younger patients were AML15 and 17. For induction AML15 assessed the value of the addition of gemtuzumab ozogamicin (GO: Mylotarg) to induction therapy. This delivered an improvement in overall survival. When assessed by risk group it was clear that at a single dose of 3mg/m^2 did not benefit high risk patients. Since that observation a single dose of 3mg/m^2 was compared to a single dose of 6mg/m^2 in AML17. There was no benefit for 6mg overall, or in the favourable or intermediate risk patients. However 6mg improved survival in high risk patients (n=133) from 18 to 32% at 3 years. Since closure of the 3mg vs 6mg randomisation all favourable risk patients have received GO with induction which has delivered a survival >85%. Where a single dose is better than a fractionated schedule will be tested in the AML19 trial.

FLAG-Ida: Flag-Ida is a popular schedule for treatment of relapse, but has never been tested in a randomised trial as initial therapy. This was undertaken in AML15. Although there was no difference in in remission rate when compared with DA or DAE, 90% of the remission were achieved with one course. All patients received a second induction course. It was clear that FLAG-Ida resulted in more myelosuppression in later courses, and more deaths in CR. However the relapse risk was significantly reduced so the disease free survival was significantly improved but overall survival was not. Of interest was the retrospective, non-randomised, observation that 2 courses of FLAG-Ida was the same as 2 DA or DAE inductions plus 2 Ara-C consolidation. The value of the second FLAG-Ida course (which for 90% of patients was a consolidation course) is not clear, and will be a randomised in the AML19 trial.

Daunorubicin Dose: A daunorubicin dose of 90mg/m^2 has been suggested as a standard of care, however it has never been compared with the widely used dose of 60mg/m^2 X 3 in the first induction course. In AML17 >1200 patients were randomised. At a median to 2 years follow up, there was no survival difference between the doses, overall, or in any subgroup. The day 60 mortality was higher in the 90mg arm, but thereafter the survival was identical.

Consolidation Randomisation: The traditional consolidation in MRC trials was MACE (amsacrine/Ara-C/ etoposide) MidAC (Mitoxantrone/ Ara-C). When compared with conventional high dose Ara-C there was no overall difference. However for high risk patients MACE/MidAC was superior.

Ara-C Dose in Consolidation: 3g/m^2 has been the traditional dose in consolidation. However we found that 1.5g/m^2 for 2 consolidation courses was as effective with reduced myelosuppression.

Four vs Five Courses: Few studies has defined precisely how many sources are needed in total. We tested a total of 5 courses vs 4 courses. There was no survival difference.

Additional of Targeted Treatments: In AML15 the effect of **m-TOR inhibition** was tested by the addition of everolimus or not. This produced no benefit. In AML15 and 17 patients with a *FLT3* mutation were randomised to the **FLT3 inhibitor**, lestautinib, but this did not improve overall survival, although there was an observed improvement in patients receiving azole anti-fungal prophylaxis.

Transplantation in CR1: We developed and validated a risk group which added age/secondary disease/response to course 1/ WBC to cytogenetics. This refined the traditional cytogenetic risk grouping by moving about 20% of intermediate risk to high risk. We found no benefit for the residual intermediate risk of a myeloablative transplant, except for the 8% who had the *FLT3+/NPM1-* genotypes/ High risk patients had benefit. For patients over 40 years with a matched sibling (but not a MUD) with intermediate risk had a survival benefit after a reduced intensity trandplant. Any benefit in those with high risk is uncertain.

Trials for older patients suitable for intensive treatment

Mylotarg: In a similar approach to younger patients, the addition of mylotarg to induction in older patients was tested. A small but significant survival benefit was confirmed. The benefit was not limited to any recognised subgroup.

Induction Therapy: In an effort to improve remission DA was compared with Daunorubicin/ Clofarabine (n=806), and the addition of ATRA to DA or ADE (n=616), without showing any benefit.

Two Courses vs Three Courses: For patients who had at least a PR following course 1 and were in CR after the second induction course could be randomised to have a third course or not. No benefit was seen by the addition of course 3. We found the immunophenotypic assessment of MRD was the most important independent prognostic factor after entering remission.

Maintenance with Azacitidine: Patients in remission after 2 or 3 chemotherapy were randomised to receive 5 days of azacitidine 6-weekly for 9 courses. This was well tolerated. There was some improvement in DFS, but overall survival was not improved. However in patients who had only 2 induction courses or were MRD-ve there was benefit in adding demethylation maintenance.

Trials for older patients not fit for intensive treatment

These patients represent an increasing and important subgroup who collaborative group have, until recently, ignored. We developed our "Pick a Winner" program which is a rolling randomised phase 2 design with a futility analysis after approximately 87 events. Low dose Ara-C 20mg b.d. for 10 days is the standard of care control arm. To date the following treatments have been compared: 1)LDAC + tipifarnib/ 2)LDAC + Arsenic trioxide/ 3)low dose Clofarabine/ 4)LDAC + mylotarg/ 5)Sapactabine/ 6)Vosaroxin/ 7)LDAC+ Vosaroxin. /8) LDAC + ganetespib /9) LDAC + tosedostat /10) LDAC + Selinexor /11) LDAC + Lenolidomide /LDAC+ AC220. The aim was to double the remission rate as a surrogate for improving survival. Options 1,2,5,6,and 7 failed at the initial assessment. Options 3, 4and 8 qualified for the full randomisation, having improved CR in the first assessment. Option 3 and 4 however did not improve overall survival, and option 8 is on-going. Options 9, 10, 11 and 12 are recruiting to the first stage.

This design is efficient and aims to screen for novel approaches in this difficult patient group.

Conclusions

AML is a heterogeneous disease which is relatively uncommon. This presents considerable challenges in developing therapy. The will be no "one size fits all" solution. So factorial design with large recruitment is necessary. Since new treatments are likely to be expensive it is reasonable to assume that large differences will be require to justify use, particularly in older patients. This enable novel designs such as "pick a winner" to

rapidly screen novel approaches which may offer benefit or indeed no benefit.

Conflict of interest: Consultant to Pfizer (Gentuzumab Ozogamicin)

Deciphering the Role of Large Chromosome Deletions in Hematological Malignancies

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Abstract

Chromosome abnormalities are a common feature of cancer. It is generally believed that there are proto-oncogenes and tumor suppressors in chromosome amplifications and deletions, respectively. However, the role of these alternated regions in leukemiagenesis, is still unclear.
Recurring deletions of chromosome 7 and 7q [-7/del(7q)] occur in myelodysplastic syndromes and acute myeloid leukemia (AML) and are associated with poor prognosis. Using RNAi and CRISPR/Cas9 approaches, we show that a ~50% reduction in gene dosage of the mixed lineage leukemia 3 (MLL3) gene, located on 7q36.1, cooperates with other events occurring in -7/del(7q) AML to promote leukemogenesis. Mll3 suppression impairs the differentiation of HSPC. Interestingly, restoring the expression of Mll3 releases the differentiation block and impairs the progression of established AML. Further, shRNA library screening identifies additional tumor suppressors in -7/del(7q), whose loss might cooperate with Mll3 deficiency to promote AML. Thus, our in vivo functional analysis identifies tumor suppressors in -7/del(7q) in AML.
Besides identifying the individual tumor suppressors, we also studied the functions of large chromosome deletions as a whole. Chromosome 17p12 (11B3 in mice), containing the well-known tumor suppressor TP53, is a common deleted region in human cancer, including lymphoma and leukemia. Here, by mouse genetic engineering, we report that 11B3 heterozygous knockout promotes lymphoma and leukemia genesis in mice. Tumors started with 11B3 one-allele-loss have their p53 mutated or silenced on the second allele, resulting in one allele with large deletion while the other allele with p53 mutation or silenced, which precisely mimics the alterations on Chromosome 17p in the majority of human cancer. However, tumors started with p53 one-allele-loss or mutations only lose the second allele of p53 by UPD. Moreover, 11B3 deficient tumors had significantly shorter latency than those with p53 loss only, indicating there are additional tumor suppression functions in this common deleted region beyond p53 only. Further, we show that another tumor suppressor eif5a in Chromosome 17p cooperates with p53 to suppress tumorigenesis, possibly through promoting apoptosis. Thus, large chromosome deletions promote tumorigenesis more than single tumor suppressor, suggesting synergy between multiple tumor suppressors from the same regions.
Taken together, our studies conferred chromosome deletions as driver mutations in tumorigenesis. Our murine AML models recapitulate the pathology and drug response of human disease and therefore would provide a unique tool to understand the pathogenesis and therapeutic susceptibility of human AML with these chromosome alterations.

Molecular Breakdown of inv(3)/t(3;3) AML with Aberrant *EVI1* Expression

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Abstract

Acute myeloid leukemia (AML) is the most common form of acute leukemia. Understanding of the molecular- and biological abnormalities is essential for the development of hypothesis driven therapy of AML. Based on large scale gene expression (1) and gene methylation profile studies (2) we identified AML subtypes with unique genetic and epigenetic aberrations and study the biological consequences of these abnormalities. In particular, we discovered a unique mechanism of transformation of AMLs with either an inv(3)(q21q26.2) or a translocation t(3;3)(q21;q26.2) [inv(3)/t(3;3], malignancies associated with a dismal clinical outcome. Applying functional genomics (4C-Sequencing and ChIP-Sequencing) and genome-engineering (TALENS and CRISPR/CAS9), we that a distal enhancer of *GATA2* located at chromosome 3q21 had translocated to the leukemia disease gene *EVI1,* located at 3q26 (3). This unique translocation of the *GATA2* enhancer causes ectopic activation of *EVI1* in those leukemia cells. This novel mechanism of transactivation of *EVI1* in myeloid disorders was also reported by Yamazaki and colleagues in an elegant humanized mouse model. Besides *EVI1* overexpression, we also demonstrated that as the result of the elimination of the enhancer from the 3q21 site, *GATA2* at the affected allele is not expressed. Functional haplo-insufficiency arising from inactivating mutations in *GATA2* zinc finger DNA binding domains or in *GATA2* promoter sequences has been well established as an underlying cause of syndromes characterized by predisposition to myeloid leukemia. We therefore hypothesize that mono-allelic expression of *GATA2* is an additional oncogenic event in these AMLs with aberrant EVI1 expression. Thus, our data show that structural rearrangements involving single chromosomal repositioning of enhancers can cause deregulation of two unrelated distal genes, with cancer as the outcome.

Importantly, we found that the rearranged enhancer near *EVI1* had turned into a BET-inhibitor hypersensitive "super-enhancer". Inv(3)/t(3;3) AML cells appear to be highly sensitive to these BET-inhibitors, which cause strong downregulation of EVI1 expression accompanied by a cell cycle block and induction of differentiation and apoptosis. Our studies emphasize that molecular understanding of defective transcriptional control may provide leads for hypothesis driven targeting of cancer. Therefore, Although BET-inhibitors are promising new agents which are included in clinical trials in AML, we propose that understanding of the molecular defects that drive disease development may predict the success of BET-inhibitor response in AML.

We recently demonstrated many more aberrations in myeloid malignancies with inv(3)/t(3;3) rearrangements (5). We showed that 98% of those patients harbor mutations in genes activating RAS/receptor tyrosine kinase (RTK) signaling pathways. Moreover, in 15% of the patients mutations in *GATA2* were found. These mutations always occurred in the gene located on non-rearranged chromosome 3q21. Thus, in these patients no wild-type *GATA2* was expressed, since the *GATA2* gene at the rearranged allele was not expressed as the result of the enhancer-rearrangement. We also found frequent mutations in the gene encoding the transcription factor RUNX1 (12%). In addition, we identified mutations in genes encoding splice factors as well as in epigenetic modifier genes in the inv(3)/t(3;3) cases. The high incidence of activating RAS/RTK signaling mutations may provide a target for a rational treatment strategy in this high-risk patient group.

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Interference of Co-Inhibitory Molecules in Acute Myeloid Leukemia

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Abstract

The immune system can be a potent defense mechanism against cancer. Especially $CD8^+$ cytotoxic T cells have great killing capacity towards tumor cells. However, their potential is often dampened by immune suppressive mechanisms in the tumor microenvironment. Co-inhibitory molecules (CIM) expressed by tumor cells, immune cells and stromal cells can severely hamper $CD8^+$ T cell responses. Today, a variety of CIM, including PD-1, CTLA-4, BTLA, TIM-3, LAG3 and CD200R, have been implicated in tumor escape from T cell attack. Sustained signaling via these CIM can result in functional exhaustion of T cells, a process in which the ability to proliferate, secrete cytokines and mediate lysis of tumor cells is sequentially lost. Here, we discuss the influence of co-inhibitory pathways in autologous and allogeneic T cell mediated immunity against myeloid malignancies. In addition, we discuss data of immune-therapeutic approaches interfering with negative co-signaling, either as monotherapy or in conjunction with vaccination strategies. Numerous studies indicate that co-inhibitory signaling limits the clinical benefit of current T cell therapies. Therefore, interference with CIM is an attractive immunotherapeutic intervention for cancer therapy.

Introduction

Despite the powerful aspects of immune reactions, most often tumor cells are able to evade immune recognition and destruction. Mechanisms exploited by tumor cells to escape T cell immunity include disruption of antigen presentation, down-regulation of HLA molecules, secretion of immune suppressive cytokines, as well as recruitment of regulatory T cells (T_{REG}) and myeloid-derived suppressor cells. In the last decade, another powerful immune suppressive mechanism gained much attention: the repressive action of co-inhibitory molecules (CIM)[1]. Activation of T cells is predominantly dependent on both co-stimulatory and co-inhibitory members, including members of the CD28:B7 and TNFR family. The balance between positive and negative co-signals determines the functionality of T cells during immunity and tolerance. In addition to the native role of co-signaling, tumor cells can evade immune control by down-regulating co-stimulatory molecules such as CD80 and CD86, and up-regulating various co-inhibitory ligands such as PD-L1, thereby limiting the therapeutic potential of current immunotherapy against cancer.
Standard treatment for hematological cancers includes chemotherapy and radiotherapy, which reduce tumor burden and can induce long-term remission. Moreover, in the past years new therapeutics, including Dasatinib, Rituximab, Bortezomib, Lenalidomide and DNMT inhibitors, have been developed that target tumor cells. However, drug resistance and relapse remain major problems. In addition, cellular immunotherapy is an attractive treatment option to cure hematological malignancies. Such immune cell-based immunotherapies include allogeneic stem cell transplantation (alloSCT), T cell and NK cell adoptive transfer, and vaccination-based approaches using various antigen formulations or dendritic cells (DC). AlloSCT can be regarded as the most powerful immune cell-based immunotherapy. Donor-derived alloreactive T cell responses eradicate the malignant cells upon recognition of polymorphic HLA-presented peptides, known as minor histocompatibility antigens (MiHA). AlloSCT greatly enhanced the cure rate for aggressive hematological cancers, although many patients fail to launch productive immune responses and develop relapses. Moreover, a major drawback of alloSCT is the occurrence of graft-versus-host disease (GVHD), a potentially life-threatening side effect predominantly caused by alloreactive T cells recognizing healthy tissues, notably the skin, liver and gastrointestinal tract. Because hematopoietic restricted MiHA are solely expressed by the redundant patient hematopoietic system and the hematological malignancy, they hold the key to separate GVT from GVHD. Studies by us and others demonstrated that the cellular immunotherapies described above are often hampered by the action of CIM that attenuate tumor-reactive T cell responses, resulting in sub-optimal clinical results. Here, we address the role of CIM in immune evasion by myeloid malignancies, and discuss options to circumvent T cell inhibition without severe adverse effects, which was reviewed in more detail by us previously[2].

Co-inhibitory molecules in cancer

Today a variety of CIM, including PD-1 and CTLA-4, have been implicated in suppressing anti-tumor immunity against myeloid malignancies.
PD-1: Programmed death 1 (PD-1; CD279) is a member of the B7/CD28 family[3]. It is inducibly expressed on stimulated $CD8^+$ T-cells, $CD4^+$ T-cells, B cells and monocytes, and binds two ligands, PD-L1 (B7-H1; CD274) and PD-L2 (B7-DC; CD273). While PD-L1 is expressed on various non-lymphoid tissues, PD-L2 expression is mainly restricted to APC, like DC and macrophages. Furthermore, multiple tumor types express PD-L1 and its expression is elevated upon IFN-γ exposure. PD-L1 molecules on tumor cells can deliver negative signals towards PD-1-expressing tumor-reactive T cells, thereby inhibiting anti-tumor immunity. Indeed, PD-L1 expression has been associated with poor prognosis in solid tumors[4].
In addition to solid tumors, PD-1/PD-L interactions were shown to be of importance in myeloid malignancies[2]. For instance, long-term persistent murine leukemia cells were shown to sequentially up-regulate PD-L1 and CD80, thereby impairing immune destruction[5]. Upon PD-L1 or CTLA-4 blockade, CTL-mediated lysis of these persistent AML cells was improved. In addition to PD-L1 expression on APCs, PD-1 expression on $CD8^+$ T cells and T_{REG} was also important for tumor persistence of murine AML. Combining PD-L1 blockade with T_{REG} depletion, showed superior efficacy in clearance of AML, due to alleviation of PD-1-dependent T_{REG}-mediated suppression[6].
The involvement of PD-1 in alloSCT has been investigated both in mouse models and in the human setting. In a study using a retrovirus-induced

CML model, it was demonstrated that tumor-specific T cells can become exhausted[7]. In this model, consisting of PD-1$^+$ tumor-reactive T-cells and PD-L1$^+$ CML cells, exhaustion could be overcome by administration of either PD-L1 antagonistic antibody or PD-1 deficient T cells. In accordance, it was shown that the bulk T cell population from CML patients exhibited increased expression of PD-1[7]. Also, in the alloSCT setting, we observed high PD-1 expression on alloreactive CD8+ T cells that specifically recognize hemato-restricted MiHA in myeloid leukemia patients[8]. Furthermore, proliferation of these PD-1$^+$ MiHA-specific T cells by stimulation with MiHA-loaded DC *ex vivo* was suboptimal, indicating their dysfunction due to PD-1 signaling. Importantly, upon treatment with anti-PD-1 or anti-PD-L1 blocking antibodies *ex vivo* proliferation of the MiHA-specific CD8+ T cells was reinvigorated.

These and many more studies have led to clinical trials exploring the potency of PD-1 blocking antibodies, putting the PD-1 pathway in the forefront of antitumor therapy. Three antagonistic anti-PD-1 antibodies are currently in advanced clinical trials, *i.e.* pidilizumab (CT-011), nivolumab (BMS-936,558) and pembrolizumab (MK-3475). Furthermore, several anti-PD-L1 antibodies, BMS-936,559, MEDI4736, MPDL3280A and MSB0010718C, are being investigated in clinical trials. In 2012 exciting reports on the use of the anti-PD-1 nivolumab and the anti-PD-L1 blocking antibody BMS-936,559 in patients with advanced malignancies were published[9,10]. Response rates upon administration of anti-PD-1 to patients with solid tumors ranged from 18-28%, depending on tumor type. Notably, the therapy was relatively well tolerated[11]. Interestingly, also blocking PD-L1, could induce durable tumor regression with an objective response rate of 6-17%, with prolonged responses of over a year. In 2013, the results were reported for the anti-PD-1 antibody pembroluzimab in melanoma[12]. In this more homogenous patient group, a response rate of 38%, and even 52% in the highest dose, were obtained. As in the previous studies, most responses were durable. These exciting results have encouraged registration of these PD-1 blockers.

Currently, studies involving hematological cancers are being initiated. A phase I clinical trial performed with CT-011 in patients with varying hematological malignancies showed clinical responses in 6 out of 17 patients, with few adverse events[13]. Furthermore, several trials are underway either exploring PD-1 blockade as monotherapy or in combination with antitumor vaccines in AML and high-risk MDS.

CTLA-4: Cytotoxic T lymphocyte associated antigen-4 (CTLA-4; CD152), was the first identified CIM, and is partly similar to the co-signaling molecule CD28. While CD28 is constitutively expressed on the membrane of naïve T cells, CTLA-4 is primarily localized in intracellular compartments and quickly translocates to the cell membrane upon T cell activation. The inhibitory function of CTLA-4 was revealed in knockout mice, which showed multi-organ T cell infiltration leading to lethal lymphoproliferative disease. Like CD28, CTLA-4 has an extracellular domain containing the MYPPPY binding motif, enabling both receptors to interact with CD80 and CD86 expressed by APC. However, the binding affinity of CTLA-4 for these ligands is higher by a factor 10-100, thus outcompeting CD28 and thereby promoting immune inhibition[14]. In addition, CTLA-4 signaling in immunosuppressive T_{REG} mediates the control of auto-reactive T cells[15]. Therefore, the effect of CTLA-4 interference could either be due to depletion and/or inhibition of T_{REG}.

CTLA-4 signaling can attenuate adaptive immune responses in chronic viral infections and cancer. CTLA-4, like PD-1, as such is not a marker of exhausted cells, but elevated levels on tumor-reactive T cells correlated with their dysfunction in patients with metastatic melanoma[16]. Moreover, in various CD80 and CD86-positive tumor models, monotherapy with CTLA-4 blocking antibody resulted in elimination of established tumors and long-lasting antitumor immunity. Interestingly, CTLA-4 also has an influence on the motility of T cells. After addition of a CTLA-4 antagonist in a mouse model, it was shown that T cells exhibited increased motility, indicating that CIM blockade does not only restore cytolytic activity, cytokine secretion and proliferation, but could also enhance T cell migration[17]. Although anti-CTLA-4 treatment works *in vivo,* CTLA-4 blockade *in vitro* has not been successful in reversing T cell dysfunction. This can be due to limitations of the *in vitro* models, as CTLA-4 blockade may exert it's *in vivo* action via multiple immune mediators (*e.g.* effector T cells, antibody responses, T_{REG}).

All these preclinical findings have stimulated clinical exploration of anti-CTLA-4 blocking antibodies. At the moment, two blocking antibodies exist, ipilimumab (MDX-010) and tremelimumab (CP-675,206). Most studies have been performed with ipilimumab in melanoma, and in these patients the median overall survival almost doubled[18]. In follow-up studies, the effects of CTLA-4 blockade were consistent[19] and in 2011, the FDA and EMA approved ipilimumab treatment for advanced melanoma, thereby paving the way for further exploration of therapies targeting CIM in cancer. For both blocking antibodies, not all patients gained clinical benefit, and the occurrence of severe adverse toxic effects upon CTLA-4 blockade remains an issue[20].

Experimental and clinical studies have demonstrated that CTLA-4 can also hamper T cell immunity against hematologic cancers in both the autologous and allogeneic setting. This might be due to native expression of CD80 and/or CD86 on hematologic tumor cells, interacting with the CTLA-4 expressing T cells. For instance, in multiple myeloma (MM) patients, CD86 but not CD80 was expressed by tumor cells, while CTLA-4 was up-regulated on T cells, which led to anergy of the tumor-reactive T cells[21]. Similar to these results, T cells from chronic lymphocytic leukemia (CLL) patients responded to anti-CD3 activation by a decrease in CD28 and an increase in CTLA-4 expression, resulting in an inhibitory phenotype[22]. Also AML cells heterogeneously express CD86, but CD80 levels were generally low or absent[8,23].

In addition to autologous antitumor T cell responses, alloreactive T cell function after alloSCT is also strongly influenced by CIM[2]. The importance of CTLA-4 in modulating allogeneic immune responses has been observed by association of certain CTLA-4 genotypes with overall survival and the incidence of leukemia relapse after alloSCT. Furthermore, it was demonstrated in mice that CTLA-4 blockade shortly after alloSCT increased GVHD[25]. However, when anti-CTLA-4 was administered at later time-points after alloSCT, the GVT effect was boosted without signs of GVHD. In patients, ipilimumab administration at late time-points after alloSCT has been explored in one phase I trial[26]. Following a single infusion of ipilimumab in 29 alloSCT patients with a recurrent or progressive hematological malignancy, 3 clinical responses were observed. Importantly, no induction or exacerbation of clinical GVHD was reported, although similar to other CTLA-4 blockade trials 14% of the patients showed organ-specific immune-related adverse events. The lack of GVHD induction is likely attributed to the median interval of one year between last DLI and ipilimumab administration. This provides a window for antitumor immunotherapy in the post-alloSCT setting and emphasizes the importance of appropriate timing of CIM blockade.

Combining PD-1 and CTLA-4 blockade

It has been recognized that CTLA-4 and PD-1 exert their role in attenuating T cell activation at different physiological locations and moments of the immune response. CTLA-4 is mostly involved in the inhibition of CTL priming in the lymph node, while PD-1 seems to limit T-cell proliferation and function in lymphoid tissues as well as in the periphery, *i.e.* at the tumor site. Therefore, the effects of concurrent PD-1 and CTLA-4 blockade are of great interest. In a mouse tumor model, it was demonstrated that double-positive CD8$^+$ TIL were more dysfunctional than either single PD-1 or CTLA-4 positive CD8$^+$ T-cells. In addition, double PD-1/CTLA-4 blockade led to reversal of TIL dysfunction and subsequent tumor rejection in the majority of mice[27]. These distinct roles of PD-1 and CTLA-4 warranted combined clinical trials to investigate whether administration of blocking both CIM would have an additive or synergistic clinical effect. Recently, very impressive clinical responses have been reported with combined PD-1 and CTLA-4 blockade in

advanced melanoma[28]. These striking clinical responses were associated with grade 3/4 adverse events in 53% of the patients, which generally were reversible and were not more severe than observed with monotherapy.

Other co-inhibitory players

In addition to PD-1 and CTLA-4, other co-inhibitory players were shown to contribute to T cell inhibition and/or exhaustion in hematologic cancers. These include BTLA, TIM-3, LAG-3, CD200R, and PD-1H/VISTA, which are reviewed by us in reference 5. Several pre-clinical studies indicate that these CIM are co-expressed on tumor-reactive T cells, but can have non-redundant functions from PD-1. For instance, co-expression of PD-1, BTLA and TIM-3 rendered melanoma-specific $CD8^+$ T cells highly dysfunctional, which could be reversed by combined blockade of all three CIM[29]. Therefore, combinatorial CIM blockade holds great promise in T cell-based cancer immunotherapies.

Future prospects

Several therapeutic strategies are being developed to dampen the inhibitory signaling by CIM in order to enhance antitumor T cell immunity. The challenge of interference with immune checkpoints is to boost antitumor reactivity while avoiding systemic toxicity. This can potentially be achieved by combining the alleviation of co-inhibition with other therapeutic options or optimal dosage and timing of antibody administration. Appealing combinations are the simultaneous targeting of multiple CIM, use of co-stimulatory agonists in parallel with CIM antagonist, or incorporation of CIM interference in existing cellular therapies. For example, DC vaccination may be applied together with blocking antibodies against CIM to boost T cell-mediated antitumor immunity. In addition, siRNA-mediated silencing of PD-1 ligands on tumor antigen-loaded DC vaccines or *in vivo* in the tumor micro environment may be attractive way to exploit CIM interference to boost antitumor immunity[30]. Altogether, CIM play a pivotal role in natural and therapeutic T cell-mediated immunity against hematological cancers. With increasing knowledge of a growing number of CIM, novel mono- and combinatorial treatment options are becoming available. In the end, this can lead to optimized immunotherapy against cancer.

Conflict of interest: The authors declare that they have no conflict of interest

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Targeting of Ubiquitin Ligases for the Treatment of Hematologic Malignancies

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Lenalidomide is a highly effective drug for the treatment of myelodysplastic syndrome (MDS) with del(5q), multiple myeloma, and some additional B cell lymphomas. Lenalidomide binds directly to the CRBN-DDB1 E3 ubiquitin ligase. We have used a combination of quantitative proteomic approaches to demonstrate that lenalidomide acts by a novel mechanism of action for a therapeutic agent: in multiple myeloma cells, lenalidomide increases the binding of two substrates, IKZF1 and IKZF3, to the CRBN substrate adapter; increases the ubiquitination of these substrates; and causes the targeted degradation of IKZF1 and IKZF3, transcription factors that are essential for the differentiation and survival of plasma cells including multiple myeloma cells. In del(5q) MDS, lenalidomide targets CSNK1A1 for destruction. Haploinsufficiency for *CSNK1A1*, a gene located within the common deleted region on chromosome 5q, results in selective targeting of the del(5q) clone. Using deletion mutants of IKZF3 we identified a 75 amino acid degron in the N-terminal zinc finger domain that is sufficient for lenalidomide-induced degradation. Based on sequence alignment of that region between lenalidomide responding Ikaros proteins IKZF1 and IZKF3 vs. non-responding IKZF2, IKZF4 and IKZF5 we substituted a single amino acid ($IKZF3^{Q147H}$) that prevented binding of IKZF3 to CRBN and conferred resistance to lenalidomide induced degradation. IKZF1 and IKZF3 are essential transcription factors for terminal B cell differentiation, and we found that genetic inactivation of IKZF1 or IKZF3 in lenalidomide-sensitive multiple myeloma cells causes severe growth inhibition.

Conflict of interest: None

Lung Infections Affecting the Relevance of Minimal Residual Disease in Acute Myeloid Leukaemia

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Abstract

Acute myeloid leukaemia (AML) is highly heterogeneous at the molecular level. Nevertheless, risk stratification used to guide transplant practice in younger adults remains largely based on pre-treatment assessment of cytogenetics, an extremely limited panel of molecular genetic markers and morphological assessment of bone marrow blast percentage following induction therapy. However, application of more objective methodology such as multiparameter flow cytometry (MFC) has highlighted the limitations of morphology for reliable determination of remission status. Moreover, there is now overwhelming evidence that detection of submicroscopic levels of leukaemia (i.e. Minimal Residual Disease, MRD) using MFC or molecular-based approaches provides powerful independent prognostic information. Implementation of MRD assessment into clinical practice remains a major challenge, hampered by differences in the assays and preferred analytical methods employed by different laboratories. While this should be addressed through adoption of standardized assays with external quality control, it is clear that the molecular heterogeneity of AML coupled with increasing understanding of its clonal architecture dictates that a "one size fits all" approach to MRD detection in this disease is not feasible. However, with the range of platforms now available there is considerable scope to realistically track treatment response in every patient.

Introduction

The seminal TCGA consortium study that involved sequencing 200 AML genomes, has highlighted the molecular complexity of the disease, with the average case harbouring over 10 mutations[1]. Amongst the 200 AMLs analysed, ~200 genes were identified as recurrent mutation targets and over 1000 genes found to be mutated in at least 1 case[1]. The availability of high throughput sequencing technologies has fostered interest in the development of targeted sequencing panels to inform patient management[2,3]; however, the degree of complexity presents a major challenge to define the individual genetic abnormalities or combinations of markers that provide significant independent prognostic information. Indeed, in children and younger adults decisions regarding allogeneic stem cell transplantation (SCT) still remain largely based on cytogenetic risk group and very few molecular markers (FLT3-ITD, CEBPA and NPM1). There is however increasing evidence that assessment of minimal residual disease (MRD) status using flow cytometry or molecular approaches can provide powerful independent prognostic information and is increasingly being incorporated into algorithms to guide consolidation therapy. Acute promyelocytic leukaemia (APL) has provided a paradigm for MRD-directed therapy (reviewed [4]), with achievement of molecular remission (CRm) in the bone marrow (BM) incorporated into the standard response criteria in this subset of AML[5]. MRD monitoring used to guide early intervention with arsenic trioxide (ATO) has been reported to improve outcome[6], particularly in high risk patients and is now considered as a standard of care, as recognised in international disease guidelines[7].

The role of MRD monitoring in other subsets of AML is less well established. Having provided proof of principle in APL, we have investigated the prognostic impact of MRD in the commonest molecular subtype of AML, i.e. characterised by mutations involving the nucleophosmin (NPM1) gene. The mutations involve carboxy-terminal tryptophan residues required for nucleolar localisation and generate a nuclear export signal inducing delocalisation of the protein to the cytoplasm (designated NPM1c mutations)[8]. NPM1c leukaemias are molecularly heterogeneous, with outcome influenced by the pattern of cooperating mutations. This has generated interest in molecular profiling to guide treatment approach, particularly concerning allogeneic SCT in first complete remission (CR1). Patients with NPM1c AML with FLT3-ITD and/or DNMT3A mutations have been associated with poorer outcome and are widely considered candidates for SCT. Conversely, those with the NPM1c/FLT3-ITDneg genotype are no longer routinely transplanted in CR1, due to their overall relatively favourable outcome[9-11]. Given the molecular heterogeneity, we hypothesised that detection of MRD using RNA-based real time quantitative PCR (RT-qPCR) assays could improve outcome prediction. However, its value has been questioned by recent studies providing evidence that relapse can arise from pre-leukaemic clones[12,13].

Patients and Methods

Serial MRD samples were analysed in a cohort of 346 NPM1c patients (median age 50 years) treated on the UK National Cancer Research Institute (NCRI) AML17 trial, with a median follow-up of 35 months[14]. Patients were recruited between April 2009 and June 2012, during which time clinicians were blinded to the PCR results so that their prognostic significance could be reliably established. Diagnostic samples were subject to PCR and sequence analysis to define mutation type. Samples were analysed by RT-qPCR assays using the common forward primer and probe published by Gorello et al[15], in conjunction with mutation-specific reverse primers, allowing MRD detection in all patients, covering 27 different *NPM1* mutations. Assays were confirmed to be mutant specific, with a median sensitivity of 1 in 10^5. Overall 2,569 follow-up samples (902 BM, 1667 PB) were analysed (median 6 samples/patient). To determine if MRD assessment provided independent prognostic information, targeted sequencing (Haloplex, Agilent Technologies) of 51 genes was undertaken in 223 cases with available diagnostic DNA. The

panel included established recurrent mutation targets in NPM1c AML and 14 genes found to be mutated in whole exome sequencing of 27 cases with differing kinetics of relapse or sustained molecular remission.

Results and Discussion

For patients in documented morphological complete remission (CR), early MRD assessment distinguished those at markedly differing risk of relapse and overall survival (OS). For patients with *NPM1* mutant transcripts still detectable in peripheral blood (PB) following chemotherapy course 2, cumulative incidence of relapse was 77% at 3 years, compared to 28% in those testing PCR negative ($p<0.0001$), associated with poorer OS (25% vs 77%, $p<0.0001$). In multivariable analysis MRD provided the most powerful predictor of disease outcome ($p<0.0001$), far stronger than the mutational profile as determined by the 51 gene panel, including FLT3-ITD and DNMT3A mutation status.

Serial monitoring of paired BM and PB samples showed that analysis of marrow increases MRD detection rate, affording a median 1-log increment in sensitivity, associated with a longer time from molecular conversion to relapse. Detection of MRD beyond consolidation predicted progression in 53 of 64 patients, preceded by a median 0.7-log rise in transcripts per month; 8 patients remain in CR following pre-emptive therapy including SCT or epigenetic therapy and only 3 have not yet relapsed at 2-10 months follow-up. Targeted sequencing analysis at molecular or clinical relapse, showed mutant allele frequencies of genes including *DNMT3A* and *TET2* exceeding the *NPM1* mutation in ~40% of cases, consistent with presence of pre-leukaemic clones. Moreover, using digital PCR we have shown that the *DNMT3A* mutation persists following chemotherapy, based on analysis of long-term follow-up samples from patients in ongoing molecular remission of NPM1c AML. Irrespective of the clonal architecture, the *NPM1* mutation was found to be a stable marker of AML status, detected at relapse in 69 of 70 cases analysed. In this remaining patient the diagnosis of "relapse" was based purely on morphological grounds with ~9% blasts in the bone marrow. However, retrospective targeted sequencing of this sample using the 51 gene panel did not show any disease-associated mutations calling into question the clinical diagnosis of relapse.

In conclusion, while there is evidence that NPM1 mutant AML frequently arises on a background of pre-leukaemia, *NPM1c* RT-qPCR assays track the leukaemic clone with high fidelity, providing the most powerful predictor of relapse risk and therefore merit adoption into routine practice to assess response and inform patient management.

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Functional Characterization of Acute Leukemias

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Abstract

Acute leukemias display functional heterogeneity during the clinical course of patients, both between different patients, but also within the cells of a single patient. E.g., while most cells respond to first line treatment, residual tumor cells might persist and induce disease relapse.

The purpose of our work is to functionally characterize tumor cells from patients using suitable preclinical in vivo models, focusing on subpopulations within individual patient's samples and on challenging subpopulations requiring novel therapies.
Towards this aim, we used the individualized xenograft mouse model of acute leukemia. Primary patients' acute leukemia cells were transplanted into severely immuno-compromised mice to generate patient-derived xenograft cells (PDX cells). Genetic engineering was performed in PDX cells by lentiviral transduction. Stable expression of transgenes allowed molecular marking of tumor cells for isolation of phenotypic and functional distinct cell subpopulations as well as bioluminescence in vivo imaging.
Using these techniques, we could show that tumor cells within a single sample consist of functionally diverse cells in vivo. All samples consisted of a mixture of rapidly and slowly growing cells as well as of drug resistant and drug sensitive tumor cells. Slowly growing tumor cells tended to be more drug resistant in vivo than rapidly growing tumor cells.
We conclude that acute leukemia cells from a single patient inherit functionally diverse subclones. Favorable subclones are eliminated by standard polychemotherapy, while challenging subclones require research effort to develop effective subclone-directed treatment options in the future in order to prevent disease relapse.

Introduction

Patients with acute leukemia (AL) show highly different rates of tumor cell clearance upon first line polychemotherapy and AL appear as functionally heterogeneous diseases. Within cells obtained from a single patient, most tumor cells might respond to anti-tumor therapy, while a minority might persist in residual disease and might display major treatment resistance. Functional heterogeneity is a hallmark of AL and the subpopulation with the most dismal characteristics ultimately determines patient's prognosis.
The purpose of the present study was to functionally characterize tumor cells from patients with AL in the xenograft mouse model in vivo, focusing on subpopulations within individual patients' samples and on challenging subpopulations requiring novel therapies.

Methods

Towards this aim, primary tumor cells from children and adults suffering AL, either acute lymphoblastic leukemia or acute myeloid leukemia, were transplanted into severely immuno-compromised NSG mice (lacking B-, T- and functional NK-cells) according to published protocols (1). As primary AL cells are mainly reluctant towards in vitro growth, mice were used for cell amplification to generate patient-derived xenograft cells (PDX cells). In both acute lymphoblastic leukemia and acute myeloid leukemia, PDX cells were transduced with lentiviruses to stably introduce transgenes (2, 3). Positively transduced cells were enriched by flow cytometry using a recombinant fluorochrome as marker (**Fig1**).

Fig1 Construct for stable overexpression of transgenes

Expression of fluorochromes and surface tags enabled re-isolation of rare numbers of leukemia cells from bone marrow. Expression of luciferase enabled bioluminescence in vivo imaging for highly sensitive and reliable monitoring of disease development and treatment effects (**Fig2**) (2).

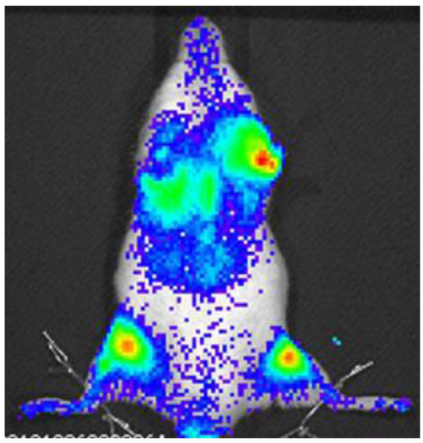

Fig2 Bioluminescence in vivo imaging in a mouse harboring transgenic cells from an adult with acute myeloid leukemia

Results

We studied the two functional characteristics, spontaneous proliferation and drug sensitivity in vivo.
In line with clinical experiences, in vitro data and published in vivo data (4, 5), AL samples from different patients varied widely regarding the rate of spontaneous proliferation in mice in vivo. In addition, we found that within a sample of individual patients, not all cells equally participated in leukemic growth and a mixture was present consisting in rapidly and slowly growing cells.

Major differences were also detectable regarding in vivo drug sensitivity. Differences were present not only between different samples, but also within single samples which displayed major heterogeneity regarding drug sensitivity. In addition, we found that within a single sample, slowly proliferating cells tended to be more resistant compared to rapidly proliferating cells.
Taken together, genetic engineering of PDX cells in the individualized xenograft mouse model of AL enabled their detailed functional characterization in vivo. Our preclinical in vivo studies revealed a marked functional heterogeneity not only between samples, but also within each sample. Thus, within the same sample of a single patient, functionally heterogeneous cells exist regarding both spontaneous proliferation in vivo and drug sensitivity in vivo.

Conclusion

We conclude that novel therapeutic approaches are required targeting important subpopulations present within samples from patients with AL. Our in vivo techniques enable isolating, characterizing and targeting challenging subclones from patients' AL samples for preclinical studies aiming to prevent disease relapse and to increase the prognosis of patients suffering AL.

Conflict of interest: The authors declare that they have no conflict of interest

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Flow Cytometric Analysis of Minimal Residual Disease in Acute Myeloid Leukemia

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Abstract

The relevance of minimal residual disease (MRD) assessment in acute myeloid leukemia (AML) is currently being studied in a number of clinical trials. The most widespread techniques used are multiparameter flow cytometry (MFC) as well as molecular-based approaches. While MFC has limited sensitivity, it is applicable in most patients, whereas molecular MRD assessment is confined to those cases with detectable molecular aberrations. Essentially, the assessment of MRD is thought to result in optimizing treatment strategies and ultimately, improving the outcome of patients. To achieve this, it is important to optimize the current assays and maybe more importantly, to gain further insight into the biology of those cells detected by MRD assays. Here, we highlight some of the current developments in the usage and improvement of MRD detection by flow cytometry.

Introduction

The assessment of minimal residual disease (MRD) has transformed the risk assessment and treatment of acute lymphoblastic leukemia (ALL). In acute myeloid leukemia (AML), there also is a large body of evidence correlating the presence of MRD with outcome [1]. This is true for MRD detection by molecular assays as well as by multiparameter flow cytometry (MFC). We and others have demonstrated that the detection of residual leukemic cells by MFC below the threshold of cytomorphology has adverse prognostic implications on relapse free as well as overall survival beyond current pre-treatment risk classifications, including cytogenetic and molecular aberrations [2, 3].
However, more than other techniques of MRD assessment, MFC relies on manual gating and is therefor prone to variations between laboratories. The most widespread gating strategy is based on the definition of leukemia-associated immunophenotypes (LAIP) at primary diagnosis. During follow-up, cells expressing this phenotype are defined as MRD. However, there is still variation on whether the final MRD value refers to the percentage of CD45+ cells, total nucleated cells or mononuclear cells. Furthermore, some studies include corrections for the degree of dilution by peripheral blood [4].
Additionally, from a more clinical perspective, it is still subject of investigation whether (and even more importantly which) treatment of MRD, i.e. achieving a MRD-conversion from positive into negative will impact patients' prognosis [5]. Studies investigating the prognostic impact of MRD show relapse rates in the MRD negative group of around 40% for younger patients and up to 70% for older patients, highlighting that additional factors play a role [3, 6]. Clinical trials prospectively assessing the value of MRD detection as well as treatments based on the detection of MRD are underway.

Methods

Bone marrow samples from patients with AML (excluding patients with acute promyelocytic leukemia) were analyzed by flow cytometry after Ficoll density-gradient centrifugation. From 2000 to 2011, MFC analysis was performed using a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA) and from 2011 onwards using a NAVIOS flow cytometer (Beckman Coulter, Brea, CA, USA). To be eligible for further analysis, patients needed to receive intensive induction chemotherapy within the German AMLCG trials AMLCG99, AMLCG2004 and AMLCG2008 or analogous to these study protocols. RFS was calculated from the time patients achieved complete remission (CR) to the time of hematological or extramedullary relapse or death from any cause. OS was calculated from primary diagnosis of AML to the time of death from any cause. SPADE as well as viSNE algorithms were performed as previously described [7, 8]. The Kaplan–Meier estimator and log-rank test were used to analyze survival data.

Results

In our first cohort (data obtained on the FACS Calibur flow cytometer), 178 bone marrow samples from patients subsequently achieving CR were available for MRD analysis by MFC during aplasia (typically on day 16-18 during induction therapy). 68 patients (38%) were MRD positive and 110 patients (62%) MRD negative. The 5-year relapse free survival (RFS) rate was 16% for MRD-positive patients and 43% for MRD-negative patients (log-rank test p=0.001). Five-year overall survival (OS) rates were 32% and 55%, respectively (p=0.019).
Of note, these analyses were performed by defining the cutoff for MRD positivity at 0.15%. Lowering the cutoff value to 0.1% did not change the observed shorter RFS rate (p=0.005). Additionally, if analyzed as a continuous variable, MRD levels were associated with a significant influence on RFS (p=.004) as well as OS (p=0.028).
As expected, age (<60 vs. ≥60 years) and cytogenetic risk group showed a high degree of correlation with survival. In multivariate analysis, MRD status during aplasia remained an independent determinant of RFS (p=0.009). Concerning OS, flow MRD status during aplasia was not an independent predictor of OS in this cohort (p=0.3). Concerning a lowered cutoff of 0.1%, multivariate analysis showed a significant association of MRD positivity during aplasia with RFS (p=0.03).
We are in the process of analyzing data obtained from a more comprehensive antigen panel obtained on the NAVIOS flow cytometer. This second cohort was analyzed not only utilizing "traditional" gating strategies, but also by determining the malignant fraction of the total primitive (stem) cell compartment as proposed by Terwijn et al.[9] as well as automated analysis utilizing SPADE[7] and viSNE[8] algorithms. First results of these efforts will be presented at the symposium.

Conclusion

Unlike ALL, MRD assays in AML are only slowly making their way into clinical trials and consequently only have little impact on clinical practice to date. This is most likely due to two reasons: (1) AML already is a highly heterogenous disease and there is no clear consensus on the

optimal application of the – albeit limited – treatment options. This is especially true for patients considered to have intermediate risk, where there is considerable debate over the optimal management (allogeneic transplant vs. chemotherapy). Despite numerous studies showing the additional prognostic impact of MRD detection in AML, it is very unlikely that this issue will be resolved easily. More likely, MRD status is going to be an additional variable to be considered when determining post-remission treatment regiments. (2) False-negative and false-positive rates for MRD detection are higher than those seen in ALL. In standard-risk adult ALL, early response with continued MRD-negativity starting on day 11 of induction has been associated with extremely low relapse rates approaching 0%, whereas those patients with persistent MRD almost all experience relapse [10]. For AML, even MRD negative patients show relapse rates that are unsatisfactory and some patients deemed MRD positive will not relapse despite receiving no additional therapy [1, 5]. Some of this could be due to limited sensitivity of the assays used. Indeed, increasing sensitivity of improved assays including next-generation sequencing has the inherent appeal of detecting residual disease well below the threshold of current assays. For ALL, high sensitivity of the MRD assay seems to be of clinical benefit, since it enables the treating physician to clearly identify patients at high risk of relapse. For AML, this could be true as well, however formal proof of this concept is lacking to date. Rather it is at least questionable whether there is any clinical benefit in lowering the threshold of MRD positivity to those used in ALL [11, 12]. Therefor, when considering the clinical implications of MRD assessment in acute leukemia, it has become increasingly clear that there are fundamental differences between ALL and AML[11].

In conclusion, since the main obstacle in AML is finding improved treatment approaches as well as optimizing selection of existing therapies, we believe MRD detection can offer significant insight in disease biology (as a starting point to evaluate novel approaches as well as an assay on *in vivo* chemosensitivity). However, the results from MRD-guided clinical trials are awaited urgently to define the role of MRD in clinical practice.

Conflict of interest: The authors declare that they have no conflict of interest

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The Distinct Role of the Bone Marrow Microenvironment in Regulating Myeloid Leukaemias

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Abstract

In analogy to normal haematopoietic stem cells leukaemic stem cells (LSC), the origin of disease progression and relapse, are thought to reside in specific niches in the bone marrow microenvironment (BMM), where they are protected from therapy. Targeting the BMM is a novel approach for eradicating LSC. Using a retroviral transduction/transplantation model of BCR-ABL1-induced chronic myelogenous leukaemia (CML) we showed that increased bone remodeling due to parathyroid hormone (PTH), the most potent regulator of bone, led to a 15fold reduction of LSC in CML via release of transforming growth factor (TGF)- β1 from the bone, but the same did not apply to acute myeloid leukaemia (AML). PTH treatment also led to reduced engraftment of human CML cells in immunedeficient mice. In summary, these data suggest that the BMM differs between CML and AML and that targeting of the BMM can lead to reduction of LSC, a requirement for cure of CML.(1)

Introduction

Haematopoietic stem cells, the normal counterparts to LSC, are situated in an HSC niche in the BM which consists of osteolineage cells, sinusoidal endothelial cells, mesenchymal stem cells, adipocytes and sympathetic neurons. Osteoblastic cells support haematopoiesis, have been shown to regulate HSC quiescence (2) and to influence the HSC pool size (3). Specifically, activation of osteoblastic cells in the bone marrow by PTH led to an increased number of HSC (3).

Consequently, we tested whether PTH-induced bone remodelling and -signaling could, similarly, affect leukaemo-genesis.

Methods

We used the well-described retroviral transduction/ transplantation model of *BCR-ABL1*$^{+}$ CML(4) and *MLL-AF9*$^{+}$ AML (5) to induce leukaemia in mice with osteoblastic cell-specific activation of the receptor for PTH and PTH-related peptide (Col1-caPPR mice, promoter: 2.3 kb fragment of the collagen type I α1 promoter) (6). Secondary transplantation assays were used to assess LSC frequency and Southern blots were employed to test

engraftment of transplanted cells. We used short hairpin-mediated knockdown (in the CML model) or overexpression (in the AML model) of the TGF-β receptor (TGFBR1), as well as immunofluoresence of LSC in AML and CML, in order to assess TGFBR1 signaling via pSMAD2/3.

Results

Transplantation of *BCR-ABL1*$^+$ bone marrow from 5-fluorouracil-pretreated syngeneic donor mice into Col1-caPPR mice recipients led to a significant delay in the latency and incidence of CML-like myeloproliferative neoplasia (MPN) compared to wildtype (WT) controls. This was not due to decreased engraftment in Col1-caPPR, as assessed by Southern blotting for retrovirally transduced clones. In fact, the prolonged latency of CML-like MPN in Col1-caPPR mice was due to a reduced number of LSC and a reduced frequency of cycling LSC compared to WT recipients, as tested by flow cytometry and secondary transplantation assays. In contrast, transplantation of bone marrow transduced with the oncogene *MLL-AF9*$^+$, known to be associated with AML in humans and mice, into Col1-caPPR recipients led to accelerated AML-like disease and increased disease clonality compared to WT recipients. Administration of an Fc fusion protein of osteoprotegerin, a decoy receptor for the receptor activator of nuclear factor kappa B ligand (RANKL) and, thereby, a potent inhibitor of the bone phenotype in Col1-caPPR mice, led to restoration of the CML-like MPN in Col1-caPPR mice.

We hypothesized that increased levels of TGF-β1, known to be released by PTH-stimulated and rapidly turning over bone, may suppress CML cells, which express TGFBR1 at greater levels than *MLL-AF9*$^+$ AML cells. Indeed, TGF-β1 protein and biologically active TGF-β1 was increased in the rapidly remodeling bones of Col1-caPPR mice. Further, in-vitro treatment of the *BCR-ABL1*$^+$ cell line K562, but not the *MLL-AF9*$^+$ cell line THP-1 with TGF-β1 led to suppression of the CML, but not the AML cell line. In order to test, if modulation of TGF-β1 signaling could restore or 'rescue' CML and decelerate AML, we knocked down TGFBR1 by shRNA-expressing lentivirus in the CML model and overexpressed TGFBR1 in AML on the donor bone marrow cotransduced with *BCR-ABL1* or *MLL-AF9*, respectively. Knockdown of TGFBR1 on the CML cells restored the CML like disease and overexpression of TGFBR1 decelerated AML in Col1-caPPR mice. Consistently, in-vitro treatment with TGF-β1 led to a significant increase of pSMAD2/3 (downstream of TGFBR1) in CML LSC, but to no difference in staining intensity in AML LSC. This suggested that *MLL-AF9*–induced AML is insensitive to TGF-β1 derived from the BMM due to lack of TGFBR1 expression or higher constitutive pSMAD2/3 signaling, whereas TGF-β1 suppresses CML-like MPN.

In order to enumerate LSC in a PTH-treated BMM, we transplanted the bone marrow from saline- or PTH-treated WT mice with CML into irradiated, untreated recipients in limiting dilution. This revealed that modification of the BMM by PTH led to a 15fold reduction of LSC in CML. Furthermore, cotreatment of WT mice with CML with imatinib, considered standard of care in CML, plus PTH led to longterm survival in 33% of mice compared to 6% in mice treated with imatinib alone and significantly reduced the number of cycling LSC in CML. Finally, in experiments to extend these studies to clinical samples, we demonstrated that PTH-treatment of immunedeficient NOD-SCID interleukin-2 receptor γc–deficient (NSG) recipients led to significant reduction of human CML cells without affecting the engraftment of normal human cells. These data suggest that modulation of the niche may also be deleterious to human CML progenitors.

Conclusions

This study suggests that

- a myeloid leukaemia is influenced by the BMM
- there is differential regulation of CML versus AML by the BMM
- modification of the BMM leads to reduction of LSC.

Therapeutic targeting of the BMM may be a complementary approach to conventional therapies.

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Is Maintenance Therapy Necessary for APL?

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Abstract

A clinical benefit from routine maintenance therapy with ATRA (tretinoin) and/or low-dose chemotherapy for APL patients in first remission has not been established. Maintenance therapy involves additional toxicity and costs. Recent data suggest that maintenance therapy offers little or no benefit when ATRA and ATO (arsenic trioxide) are included in initial therapy. However, patients without access to ATO may benefit from a lower incidence of relapse if maintenance is given.

Introduction

The introduction of ATRA (all-trans retinoic acid; tretinoin) and ATO (arsenic trioxide) into routine remission induction and post-remission consolidation therapy has dramatically improved the outcomes for patients with acute promyelocytic leukemia (APL) with typical t(15;17) and *PML/RARA* rearrangements. Following treatment, most patients achieve complete molecular remissions (CRm) with undetectable disease at levels of 10^{-5}. Whether there is any clinical benefit from prolonged maintenance therapy for these patients, and if so, whether a high-risk subset can be identified, remains controversial. Patients who fail to achieve a CRm due to drug resistance are not appropriate candidates for maintenance or observation, due to the high likelihood of relapse; these patients require additional rescue chemotherapy and/or stem cell transplantation.

The benefit of maintenance therapy is inversely proportional to the anti-leukemia effect of the preceding induction/consolidation therapy. As initial treatment with ATRA plus anthracycline (with or without cytarabine) or ATO has become more effective, the need for maintenance

therapy appears to have diminished. Thus, access to using ATO for frontline treatment regimens may in large part determine the need for maintenance.

Methods

Large randomized trials evaluating the role of maintenance therapy have been confounded by marked variability in induction and consolidation regimens, as well as by the pretreatment risk characteristics of the cohorts under study.[1] Several smaller studies have included maintenance with an observation only arm.

Results

Early randomized trials showed that maintenance therapy with ATRA +/- chemotherapy decreased the incidence of relapse and improved the disease-free survival (DFS) when only ATRA or ATRA plus chemotherapy had been used for remission induction and consolidation.[2,3] The European APL93 trial evaluated 4 different maintenance arms after induction therapy containing ATRA.[3] Compared to observation, ATRA maintenance provided improved DFS at 5 years (74% vs 55%) and lower rates of relapse at 10 years (33% vs 43%).[2,3]

However, other randomized trials failed to show benefit. In the APL97 study by the Japan Adult Leukemia Study Group (JALSG), the addition of 6 courses of intensive maintenance chemotherapy compared to observation only gave significantly worse 6-year DFS (63% vs 80%) and overall survival (OS; 86 vs 99%).[4] The AIDA 0493 trial also did not show a benefit for maintenance after achieving CRm.[5] These studies have generally used ATRA (45 mg/m2 daily) on an intermittent schedule (15 days every 3 months, or 7 days every 2 weeks) with or without mercaptopurine (60 mg/m2 daily) and methotrexate (20 mg/m2 weekly) or low-dose chemotherapy alone.

More recent studies have called into question the need for maintenance therapy when ATO is used early in frontline treatment of APL. In a European cooperative group trial, 77 patients with low-intermediate risk APL were randomized to receive 6 cycles of ATRA and ATO, followed by observation only.[6] Their 2-year event-free survival(EFS) was 97% compared to 88% for a control group randomized to induction and consolidation with ATRA plus chemotherapy, followed by ATRA plus low-dose chemotherapy maintenance. There was less hematologic toxicity and fewer infections but more hepatic toxicity observed in the ATRA/ATO arm, and their OS was superior (p=0.02)..

In the North American Intergroup noninferiority study (SWOG 0521), 105 patients with low-intermediate risk APL received ATRA plus chemotherapy induction followed by consolidation with 2 courses of ATO and 2 courses of ATRA plus anthracycline.[7] Among 68 CRm patients who agreed to be randomized to observation only or to maintenance with ATRA plus mercaptopurine and methotrexate, no relapses were observed on either arm after a median follow up of 36 months. An earlier North American Intergroup trial (CALGB 9710) that used the same remission induction and consolidation regimen had randomized an initial cohort of 50 patients, including all risk groups, to either observation alone or to maintenance with ATRA alone.[8] There were no significant differences in DFS or OS between these two arms although the power to detect a difference was low because of small numbers of patients.

The CALGB 9710 trial had enrolled 481 evaluable adults (15-79 years old) and showed a significant advantage for APL patients who received 2 cycles of ATO as their initial post-remission consolidation after induction with ATRA plus daunorubicin (50 mg/m2 for 4 days) and cytarabine (200 mg/m2 for 7 days).[9] After consolidation, 331 patients still in hematologic CR agreed to be randomized to maintenance therapy for one year either with ATRA alone (45 mg/m2 for 7 days every 2 weeks) or to the same dose and schedule of ATRA plus mercaptopurine (60mg/m2 daily) and methotrexate (20 mg/m2 weekly). After a median follow up time of >6 years, there were no significant differences in DFS or OS between the two maintenance arms. There was a suggestion of improvement in DFS with the 3-drug combination in patients who had not previously received ATO, but there was no improvement in OS. Stratified analyses by risk group showed no significant differences in DFS between the 2 maintenance regimens, either for the 258 patients with low-intermediate risk APL or for the 69 high-risk APL patients. Age, gender, CD56 expression and *FLT3*-ITD or TKD mutations at diagnosis did not have an impact on outcome by maintenance arm.

Although no treatment-related deaths were reported during maintenance therapy in the CALGB 9710 study, hematologic adverse events were more common in the combination ATRA plus chemotherapy arm (maximum grade 3 or 4, 18% vs 4%; p<0.0001), as were non-hematologic adverse events (maximum grade 3 or 4, 36% vs 25%; p=0.03). In the Japanese APL97 study, toxicity was significantly greater during the intensive maintenance chemotherapy, and 2 cases of therapy-related leukemia were reported compared with none on the observation arm.

Patients with high-risk APL, defined by initial white blood cell count >10,000/ul, have a higher incidence of both early death and relapse. Maintenance therapy with tamibarotene, a synthetic retinoid, showed significantly improved relapse-free survival compared with ATRA (both given orally for 14 days every 3 months) in a subset analysis of high-risk APL patients although no benefit was observed overall in a randomized trial by the JALSG.[10] However, no ATRA had been given during consolidation courses in this trial.

Conclusion

The role of maintenance therapy for APL patients who achieve CRm after initial therapy that includes both ATRA and ATO is controversial. Relapse is uncommon in patients who received ATO during induction or consolidation. Prospective studies with uniform induction and consolidation in which patients in CRm are randomly assigned to maintenance (with ATRA and/or chemotherapy) vs observation only would be required. Routine maintenance for 1-2 years involves additional toxicities and costs. Patients who received ATO as part of frontline treatment probably do not need maintenance, whereas those who do not have access to ATO for initial treatment may benefit from maintenance with a lower incidence of relapse.

Conflict of interest: The author declares that he has no conflict of interest

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Acute Promyelocytic Leukemia: High Risk and Relapsed APL

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Introduction

During the last decades, acute promyelocytic leukemia (APL) has developed from a highly fatal to the best curable type of acute myeloid leukemia. Approximately 80% of patients can currently be cured [1]. However important issues remain: (1) There is still need of further improvement, in particular in patients at high risk for early death (ED) or relapse. (2) The commonly used relapse risk score based on initial white blood cell (WBC) and platelet counts (Sanz score) was developed on the basis of treatment protocols consisting of all-trans retinoic acid (ATRA) and anthracyclines [2]. The applicability of this score to patients treated with other regimens including high dose ara-C or arsenic trioxide (ATO) has not been proven so far. (3) It is of high interest, whether the profile of molecular changes detected in APL blasts provides prognostic parameters, which may reliably identify patients with a high risk of relapse and which are independent of the administered therapy. (4) Up to now, the therapeutic management of patients with relapsed APL has not been standardized. ATO is presently regarded as the treatment of choice for APL relapses after frontline therapy with ATRA and chemotherapy. However, the optimal therapy to sustain ATO-induced 2nd remission is still unknown. Results of international studies for newly diagnosed APL and registry data may contribute to answer these open questions.

Results

As shown by registry results and by data of the German AML Cooperative Group (AMLCG), patients with initially high white blood cell (WBC) counts, poor performance status or older age have an increased risk to die early, either before the start of treatment or during the early phase of the therapy. Most frequent causes of ED were bleeding complications, mainly in the CNS [3-6]. The ED rate of patients older than 60 years registered by the AMLCG was 48% in non-eligible and 19% in study patients, whereas the ED rate of the study patients below 60 years was only 8% [6,7]. In both age groups, the early death rate was significantly higher in patients with pretreatment WBC counts over 10,000/μl. Bleeding complications were the cause of ED in 64% of the younger and in 30% of the elderly patients, respectively [6,7].
Concerning the widely used Sanz relapse risk score, the results of the German AMLCG in newly diagnosed APL could not reproduce the score for relapse free survival (RFS) and cumulative incidence of relapse, when an intensified therapy with double induction including high dose ara-C was administered [7]. Similarly, in a study from the USA reported by Powell et al, additional treatment with ATO consolidation therapy reduced the difference in RFS between patients with initially high or low WBC counts, in comparison to patients treated with conventional ATRA and chemotherapy without ATO [8].

Results from our center showed that the expression profile of several genes including *ERG (Ets' Related Gene)*, *BAALC* (B*rain And Acute Leukemia Cytoplasmatic*) and *WT1 (Wilms' Tumor 1)*, which were associated with prognostic importance in other types of leukemia, had prognostic impact in APL patients, who received uniform treatment according to the protocols of the German AMLCG. In particular, low *BAALC* expression had a favorable prognostic impact, whereas high *ERG* expression had an independently negative influence on overall survival (OS), RFS and remission duration [9,10]. In patients, who achieved a CR, low or high *WT1* expression was significantly associated with an inferior OS compared to intermediate *WT1* expression [11]. Remarkably, an optimized relapse risk stratification model combining the different expression levels of these three markers identified patients with an exceptionally good prognosis regarding the cumulative incidence of relapse [12]. Furthermore, SNP array analysis of APL blasts showed that patients with a higher number of copy number alterations (CNA) had an inferior prognosis [13]. Deletions of the chromosomal subband 1q31.3 were associated with a higher number of CNAs and were a strong independent prognostic factor for an increased risk of relapse [13].
In 2008, a European registry of relapsed APL was established and coordinated at the University Hospital of Mannheim. Of 237 patients registered in eight European countries, documented outcomes of 155 patients in first relapse of APL treated with arsenic trioxide (ATO) are available. Ninety-one percent of patients in hematological relapse (n=104) achieved complete hematological remission (CR), 7% had induction death and 2% resistance; 27% developed differentiation syndrome and 39% leukocytosis, whereas no death or side effects occurred in patients with molecular relapse (n=40). All patients with extramedullary relapse (n=11) entered clinical and molecular CR. Ninety-five patients underwent transplantation (60 autologous, 35 allogeneic). The remaining patients received heterogeneous post-consolidation therapy, most frequently ATO or chemotherapy of variable intensities. The overall survival of the 155 patients at three years was 70% and the event free survival 54% [14].

Conclusions

Guidelines and continuous education to raise the clinical awareness of the immediate actions needed for diagnosis and therapy of APL and rigorous supportive care are important to reduce the rate of ED. Data of the German AMLCG and of other clinical trials indicate that biological factors and the modification of APL therapy have influence on the relapse risk and that scores solely based on blood counts cannot be applied without taking into account the treatment regimen. Therefore, molecular markers may contribute to an improved risk stratification of APL. The results in relapsed APL suggest that at least 50% of the patients in first relapse have a chance to be cured by salvage therapy with ATO followed by individually adapted consolidation therapy. These results indicate the need of further improvement of relapse therapy.

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Significance of Novel Gene Mutations in AML

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Clinical, cytogenetic, and gene-based studies have been used to inform biology and improve prognostication for patients with acute myeloid leukemia (AML), myelodysplasia (MDS), and myeloroliferative neoplasms (MPN). Most recently, a series of candidate gene and whole genome studies have identified recurrent somatic mutations in AML patients including TET2, ASXL1, DNMT3A, and EZH2 mutations. We and others have shown these mutations are of prognostic relevance, and can be used to improve risk stratification in AML.

We identified genetic predictors of outcome that improved risk stratification in AML independent of age, WBC count, induction dose, and post-remission therapy and validated their significance in an independent cohort. Importantly, these mutational predictors involved complex genotypes, suggesting combinations of mutations mark prognostically relevant groups and segregate AML into distinct, biologically significant subsets. Integrating mutational data with dose-intensity revealed that high-dose daunorubicin improved survival in patients with *DNMT3A/NPM1* mutations or *MLL* translocations relative to treatment with standard dose daunorubicin, but not in patients wild-type for these alterations.

These data provide important clinical implications of genetic alterations in AML by delineating mutation combination genotypes that predict outcome in AML and improve AML risk stratification. Of biologic importance, the TET family of proteins have been shown to place a hydroxyl mark on methylated DNA and lead to DNA demethylation. We and others have found that TET2 mutations leads to loss of DNA hydroxymethylation and a hypermethylation phenotype in leukemia patients. In addition, in vitro and in vivo studies show that TET2 loss leads to impaired hematopoietic differentiation, increased stem cell self-renewal, and myeloid transformation in vivo. These data demonstrate that novel mutations coopt the epigenetic state of hematopoietic stem/progenitor cells in order to contribute to transformation and that these mutations have biologic and prognostic relevance.

Conflict of interest: The authors declare that they have no conflict of interest

Novel FLT3 Inhibitors and Targeted Therapies in AML

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Acute myeloid leukemia (AML) patients who harbor an internal tandem duplication of the *FLT3* gene (*FLT3/ITD* mutation) have a generally worse outcome than their counterparts with wild type *FLT3*, although this difference can be mitigated somewhat if they undergo allogeneic transplant in first remission [1]. Given that the ITD mutation leads to constitutive activation of the receptor and contribute to the transformed phenotype [2], clinically useful small molecule tyrosine kinase inhibitors (TKIs) of FLT3 have been a goal of numerous researchers around the world for more than a decade [3].

Initial efforts to develop a FLT3 inhibitor began with re-purposing multi-targeted kinase inhibitors, lestaurtinib (CEP-701) and midostaurin (PKC-412), as FLT3 inhibitors [4,5]. These agents were clinically disappointing, largely because the plasma levels of these drugs required to achieve complete FLT3 inhibition resulted in unacceptable off-target effects [6, 7]. Another re-purposed TKI, sorafenib, is somewhat more selective than previous agents, has in vitro and in vivo activity against FLT3 [8-10], and is the agent that has thus far been used to demonstrate a clear clinical benefit of FLT3 inhibition for FLT3/ITD AML patients, particularly in the post-transplant setting [11-13]. However, because of its multi-targeted nature, sorafenib use in patients is clearly associated with hand-foot syndrome, gastro-intestinal disturbance, and vascular events, rendering it somewhat difficult to use even in the best of circumstances.

Quizartinib (AC220) is the first FLT3 TKI to be developed specifically for FLT3-ITD AML. The drug is more potent in vitro and in vivo than any previously developed FLT3 inhibitor [14-16], and it's in vivo potency is probably directly attributable to its high degree of selectivity- cKit is the only other major target receptor affected. As monotherapy in patients with relapsed, refractory FLT3-ITD AML, quizartinib induced clearance of peripheral blasts and terminal differentiation of marrow blasts in roughly half of treated patients [17]. Importantly, most of these responses were classified as "CRi" (complete remission with incomplete count recovery) because of the persistent red cell and platelet transfusion dependence in these patients, despite their having a marrow blast percentage of well below 5% [16]. The failure to recover normal hematopoiesis in most patients could have been in part due to inhibition of c-Kit [18], but more likely was due to the marrow suppressive effects of minimal residual disease. The clinical benefit of these responses, however, was manifest by a large number of patients being successfully bridged to an allogeneic transplant [19]. The importance of proceeding relatively quickly to a transplant was underscored by the emergence of resistance-conferring

point mutations within the *FLT3* coding sequence in many patients while on quizartinib monotherapy [20]. Quizartinib has now advanced to a pivotal trial in which patients with relapsed/refractory FLT3-ITD AML are randomized to receive either salvage chemotherapy or monotherapy with quizartinib (NCT02039726).

These clinical studies have provided additional proof of the benefit of FLT3 inhibition, and the development of resistance mutations offer the best evidence yet of the relative importance of this oncogene in leukemogenesis. However, they highlighted two areas in which the approach could possibly be improved, namely by addressing the resistance mutations, and by avoiding inhibition of c-Kit. Two inhibitors have recently emerged into clinical development with the potential to address these issues.

Crenolanib is a TKI originally developed as a PDGFR inhibitor [21]. It is a potent FLT3 inhibitor both in vitro and in vivo, but with relatively less activity against the other type III RTK, c-Kit [22]. In addition, it has activity against the most common type of resistance-conferring mutation, namely that found at residue D835 in the kinase domain. Early clinical studies have confirmed a meaningful response rate induced by crenolanib as monotherapy [23], and the drug is now being studied in combination with salvage chemotherapy in a randomized trial (Vinay Jain, Arog Pharmaceuticals, *personal communication*).

ASP2215 is a novel small molecule TKI originally developed as an inhibitor of Axl. It has potent activity in vivo against FLT3, including both the kinase domain mutations as well as the so-called gatekeeper mutation at residue F691 [24]. A number of trials with this agent are underway (NCT02014558) or are in active planning, including those in combination with chemotherapy.

It seems inevitable that one or more of these agents will eventually make it through the drug approval process. The challenge going forward will be to find the best way to incorporate these TKIs into current AML treatment regimens to maximize the survival for these patients. While using these agents to improve remission rates and to maintain remission after allogeneic transplant seems likely to be the initial approach, combinations with additional targeted agents, specifically those that can overcome the protective effect of the bone marrow microenvironment, may ultimately be the optimal approach.

Conflict of interest: Clinical Advisory Board – Ambit, Arog, Astellas

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Clonal Hierarchy and Diagnostic and Prognostic Impact of Founder Mutations in Myeloid Neoplasms

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Abstract

The clinical heterogeneity of myelodysplastic syndromes (MDS) and secondary acute myelogenous leukemia (AML) is a reflection of molecular diversity at multiple levels, including karyotypic defects and combinations of diverse mutations. While only a few of the newly discovered somatic mutations showed impact on clinical outcomes, most of them did not improve diagnostic or prognostic resolution.
Apart of discovery of new somatic mutations, and germ line alterations, introduction of next generation sequencing (NGS) has led to recognition of intra-tumor variability and complex clonal architecture of myeloid neoplasms. As a result, ancestral events initiating malignant evolution can be identified. Founding lesions may shape the biology, subsequent clinical course such as succession of secondary events, and be more impactful in terms of the molecular definition of disease subtypes and prognostic utility.
In addition, subgrouping of cases according to the ancestral mutations greatly reduces the molecular complexity and allows for clinical comparison between individual defects. While some somatic mutations are exclusively of secondary nature, for others specialized analysis of clonal hierarchy is needed as they can occur as primary or secondary lesions. Using various methods, including serial exome sequencing, analysis of allelic frequencies and averaging of clonal sizes, we have identified the ancestral events and performed comprehensive genotype/phenotype association studies and analyzed clinical correlations.

Conflict of interest: The authors declare that they have no conflict of interest

Pre-Leukemic Hematopoietic Stem Cells in Human Acute Myeloid Leukemia

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Abstract

Molecular genotyping and analysis of acute myeloid leukemia (AML), including recent large-scale studies using next generation DNA sequencing, has identified recurrent mutations in many genes. These studies have demonstrated that on average there are 5 mutated genes in an individual case of AML, raising the question of how these multiple mutations accumulate in a single lineage of cells. We proposed a model in which mutations are serially acquired in clones of self-renewing hematopoietic stem cells (HSC), and set out to investigate this model through the analysis of residual HSC present in patient samples taken at the time of AML diagnosis. Using a combination of exome sequencing, targeted re-sequencing, and single cell analysis, we showed that leukemic mutations are serially acquired in clones of HSC, and that such pre-leukemic mutations are enriched in genes that regulate the epigenome. Finally, through the analysis of paired remission and relapsed samples, we found that pre-leukemic HSC persist in clinical remission and may give rise to relapsed disease through the acquisition of new mutations. Thus, pre-leukemic HSC may constitute a key cellular subset that must be eliminated for long-term cures.

Introduction

Acute myeloid leukemia (AML) is an aggressive cancer of myeloid precursors in the bone marrow that leads to the accumulation of immature blasts that disrupt normal hematopoiesis [1]. The development of massively parallel next generation DNA sequencing has been extensively applied to the investigation of AML genomes leading to the identification of recurrently mutated genes in AML, most notably by The Cancer Genome Atlas (TCGA) AML group, who reported the analysis of 200 cases of human AML [2]. This study, along with prior molecular genotyping studies of AML, demonstrated that an individual case of AML contains an average of 5 mutations in recurrently mutated genes. This observation raises the key question of how do these multiple mutations accumulate in a single lineage of cells, particularly in the myeloid lineage where the mature cells and their precursors have short lifespans measured in days to weeks.

We hypothesized that since most cells in the myeloid development hierarchy are short-lived, then leukemic mutations must be serially acquired in clones of self-renewing, long-lived HSC [3]. Based on this model, the residual HSC population at the time of diagnosis should contain some cells retaining normal function, but harboring mutations in some, but not all, of the genes mutated in the patient-matched AML cells. We would term these cells pre-leukemic HSC that harbor pre-leukemic mutations. In this study, we investigated this hypothesis through the analysis of residual HSC in cases of de novo AML.

Methods and Results

We took advantage of cell surface markers (TIM3 and CD99) that we identified to be differentially expressed on leukemic cells compared to normal HSC [4, 5]. Our general approach was to: (1) isolate residual HSC from AML samples taken at diagnosis using fluorescence-activated cell sorting (FACS); (2) conduct exome sequencing of each individual AML case using purified patient-matched T cells as the reference; (3) conduct targeted deep re-sequencing of each mutation in the residual HSC to

determine which are pre-leukemic mutations; and (4) genotype single clones of HSC using SNP-specific assays to determine the order of mutation acquisition. This approach allowed us to determine the frequency and genotype of pre-leukemic HSC in de novo AML.

We analyzed 19 cases of de novo AML from which we could isolate residual HSC based on differential expression of TIM3 and CD99 [6, 7]. Exome sequencing of these 19 cases identified mutations in many of the common recurrently mutated genes in AML including a mix from most of the major categories including: activated signal transduction mediators, DNA methylation regulators, chromatin modifiers, the cohesin complex, and transcription factors. Targeted deep re-sequencing of these leukemia mutations in the patient-matched residual HSC identified many, but not all, of these mutations in the residual HSC. Specifically, we identified pre-leukemic mutations in *TET2, IDH1, IDH2, DNMT3A, RAD21, SMC1A, CTCF, ASXL1,* and *NPM1*. Thus, consistent with our hypothesis, the residual HSC population consisted of some pre-leukemic HSC with some pre-leukemic mutations.

From these cases, we explored whether there was enrichment or common patterns of pre-leukemic mutations. We found that pre-leukemic mutations were more commonly identified in genes regulating the epigenome through DNA methylation, chromatin modifications, or the cohesin complex. Notably, genes involved in activated signal transduction, such as *FLT3* and *RAS*, were absent among the pre-leukemic mutations. Thus, it appears that the development of AML is initiated by mutations in genes that regulate the epigenome in HSC, followed by proliferative mutations that likely occur in downstream progenitors.

These observations suggest that the development of AML occurs from pre-leukemic HSC that persist in patients at the time of their diagnosis, a finding that has clinical implications. In particular, clinical experience with AML has demonstrated that most patients are able to enter remission after treatment with induction chemotherapy. However, the majority of such patients will eventually relapse and die from this relapsed disease. Thus, key questions are how do pre-leukemic HSC respond to induction chemotherapy, and is it possible that they can contribute to relapsed disease through the acquisition of novel mutations?

To explore these questions, we next investigated the persistence of pre-leukemic HSC in clinical remission after high dose induction chemotherapy. Out of 4 patients with detectable pre-leukemic HSC at diagnosis, all 4 showed persistence of these pre-leukemic HSC in remission where they gave rise to both lymphoid and myeloid cells, consistent with a normal function. Lastly, we investigated several cases of paired diagnosis and relapsed disease to determine the clonal basis of relapse. From these 3 cases, we detected 3 patterns. In the first, the relapsed disease was clonally identical to the disease at diagnosis indicating a failure to eradicate this dominant diagnostic clone. In the second, a minor subclone from the time of diagnosis was present as the dominant clone at relapse. In the third, a new mutation occurred at relapse suggesting further clonal evolution of a leukemic, or possibly, pre-leukemic clone. Thus, there may be multiple clonal paths to relapse in AML, and even potentially multiple paths in an individual patient.

In summary, we have shown that AML develops from the serial acquisition of mutations in self-renewing HSC that we term pre-leukemic HSC. Moreover, mutation acquisition is not random with patterns indicating that mutations in genes affecting the epigenome occur first, followed by mutations in genes that affect activated signal transduction and other pathways. It remains to be seen if this order is recapitulated universally in AML, or if there are clinical and biological differences if the order is reversed. In addition, we showed that pre-leukemic HSC persist in clinical remission, and that relapsed disease may arise from multiple mechanisms, including the possibility of new mutations occurring in pre-leukemic HSC. Thus, pre-leukemic HSC may constitute a cellular reservoir that must be eradicated in order to cure AML.

Conflict of interest: The author declares that he has no conflict of interest

Niche Dependency and Clonal Heterogeneity in Human Myelodysplastic Syndromes

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Abstract

Sequencing of MDS genomes recently revealed a complex genetic make-up, with specific patterns of mutational co-occurrence and mutual exclusion. These findings further reinforced the idea that no single genetic mouse model is likely to recapitulate the tremendous genetic and phenotypic heterogeneity observed in MDS patients. Therefore, we decided to develop patient-specific mouse models as tools to study the biology of this disease and to provide a highly relevant system by which to evaluate new therapeutic strategies and perform pre-clinical drug testing. Our work revealed a strong dependency of diseased MDS stem cells on their mesenchymal stromal niche cells, thus highlighting the existence of a "disease hematopoietic niche unit" that we efficiently exploited to develop robust patient-derived xenografts (PDX).

Introduction

MDS are a group of heterogeneous clonal disorders that mainly affect the elderly (45/100,000 in >70 years old). They are characterized by ineffective production of mature blood cells and have a propensity to evolve to acute myeloid leukemia or bone marrow failure. Patients are classified according to risk-score systems, such as the World Health Organization (WHO) or the International Prognostic Scoring Systems (IPSS and IPSS-R), which are used to adapt therapeutic options to individual patients. It is important to note that, so far, no treatment has been able to alter the natural course of this disease, except for hematopoietic stem cell transplantation, which is not an option for over 90% of MDS patients (due to advanced age and/or lack of a suitable donor). Therefore, there is an urgent need to better understand MDS biology and develop relevant model systems by which we can evaluate the effect of new therapeutic strategies.

Growing evidence in mice indicates that a large spectrum of hematological malignancies, including pre-malignant diseases such as myeloproliferative neoplasms (MPN) [1, 7] and myelodysplastic syndromes (MDS) [5], can be triggered by abnormal functional properties of the bone marrow niche cells. However, the contribution of the niche to human MDS is much less well established. In this report we investigated the complex interplay between hematopoietic cells and their mesenchymal stromal niche cells in lower risk human MDS. Our data revealed a striking dependency of MDS-propagating stem cells on disease-associated MSCs, which is likely mediated by a number of deregulated niche factors involved in intercellular cross-talk. Finally, we could show that MDS hematopoietic cells appear to "educate" their niche environment into a self-supporting one, thus highlighting the existence of a bidirectional cross talk that likely contributes to MDS pathogenesis.

Methods

Our study is based on xenotransplantation of material from 24 MDS patients classified as follows:

WHO	IPSS
RCMD (n=9) RAEB1 (n=5) MDS-U (n=2) Del(5q) (n=7) RARS (N=1)	Low Risk (n=5) Intermediate-1 Risk (n=19)

Xenotransplantation procedure: MDS CD34+ cells were co-injected with patient-derived bone marrow stromal cells (MSCs) directly into the bone marrow cavity (i.f) of NOD.Cg-PrkdscidIl2rgtm1Wjl/Szj (NSG) or NSGS mice (NSG mice expressing human SCF, IL3 and GM-CSF). The mice were analyzed between 14 and 28 weeks post transplantation.

Molecular tracking of primary MDS bone marrow was carried out by whole exome sequencing or targeted next generation sequencing interrogating up to 17 recurrently mutated genes. Mesenchymal stromal cells (MSCs) were used as a germline control. Mutational allelic burdens in xenografted cells were quantified with ultra-deep sequencing or custom pyrosequencing. Large scale lesions were quantified using interphase-FISH or a newly developed PCR assay that quantifies allelic loss at heterozygous short tandem repeat (STR) loci within deleted regions (JC. Jann et al., manuscript in preparation).

Results

Using a large number of primary lower risk MDS cases, we demonstrated that co-injection of MDS CD34+ cells with their corresponding MSCs in NSG mice leads to significant and long-term engraftment in over 70% of the cases analyzed (14/20 patients, range hCD45+= 1-22%). Most importantly, this effect was much less prominent when using MSCs derived from healthy age-matched donors. Likewise, and in line with previous reports [2, 3, 4, 6], injection of CD34+ cells alone only gave rise to very limited engraftment (1/7 patients; hCD45+=3%).

Transplanted samples exhibited a clear myeloid bias and significant engraftment of cells with progenitor (CD34+CD38+) and stem cell phenotype (CD34+CD38-) that could be serially transplanted. When compared to NSG recipients, NSGS mice exhibited an augmented human chimerism with readily detectable morphologically dysplastic cells, one of the main clinical features of MDS. Furthermore, molecular analysis of the engrafted cells (in NSG and NSGS strains) confirmed their "diseased" origin as they carried identical lesions to the ones present in the primary bone marrow, and recapitulated the clonal heterogeneity seen in the patients.

Using this PDX model, we were further able to demonstrate that disease-propagating stem cells in lower risk MDS exclusively reside within the lin-CD34+CD38- stem cell fraction. In order to investigate the molecular mechanisms underlying the enhanced ability of MDS MSCs to support MDS propagation, we performed RNA sequencing comparing healthy age-matched MSCs with MDS MSCs. Our data revealed that MDS MSCs displayed an altered differentiation program and deregulated expression of a number of niche factors involved in intercellular communication (secreted factors, extracellular matrix proteins, adhesion molecules, etc). More strikingly, in co-culture experiments using primary patient material, MDS hematopoietic cells, but not their healthy counterparts, were able to instruct healthy MSCs to adopt "MDS-like" features, thus highlighting the existence of a bi-directional cross-talk between hematopoietic MDS cells and their mesenchymal niche counterparts. This may well contribute to the establishment of the "MDS stem cell-niche unit" that promotes disease propagation *in vivo*.

Conclusion

In this study we have identified patient-derived MSCs as a critical functional component of lower risk MDS. Together with MDS stem cells, these patient MSCs form a functional stem cell-niche unit that allows for the propagation of the disease in a xenograft recipient. The striking changed expression in diseased MSCs of genes involved in processes like cytokine-cytokine receptor interaction, cellular adhesion, and ECM remodeling, further suggests that diseased MDS cells alter the function of the normal HSC niche into one that can support the requirement of MDS cells. This unique niche-dependent PDX model of lower risk MDS will now allow us to study the biology of human MDS in *vivo* and provide a platform for unraveling the molecular basis and putative role for niche cells in the establishment of the clonal dominance observed in this disease. More importantly, our model constitutes a robust platform for preclinical drug testing.

Conflict of interest: The authors declare that they have no conflict of interest

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Genetic Characterization of AML Patients by Targeted, Deep Sequencing Reveals Patterns of Cooperating Gene Mutations and Detects Subclonal Driver Mutations: Data from the AMLCG-2008 Cohort

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Abstract

We studied 275 adult patients with acute myeloid leukemia (AML) enrolled on the AMLCG-2008 multicenter trial by targeted amplicon sequencing, focusing on a panel of 70 leukemia driver genes. We found a median of 4 mutations per patient and identified patterns of co-occurring and mutually exclusive alterations. Based on the observed variant allele frequencies, we were able to discriminate clonal founder mutations and subclonal variants. Analyses of clinical outcomes are currently ongoing.

Background

Recurrent mutations in more than 100 different genes have been identified in adult AML patients by large-scale exome and genome sequencing efforts,[1] but the clinical relevance of most of these alterations has not been defined. Moreover, "deep sequencing" studies revealed that AML patients frequently harbor multiple, genetically related disease subclones, and that the neoplastic cell population may undergo clonal evolution during treatment.[2] It is unclear whether clonal heterogeneity at diagnosis associates with clinical characteristics or outcomes. In order to address these questions, we set out to characterize a relatively large, uniformly treated patient cohort for mutations in known and putative AML driver genes.

Patients and Methods

We studied adult AML patients who received high-dose cytarabine-based induction chemotherapy within the German multicenter AMLCG-2008 trial. The AMLCG-2008 protocol was a randomized phase III trial comparing two different induction chemotherapy strategies: a high-dose cytarabine-containing double induction regimen (standard arm) versus the dose-dense, high-dose cytarabine-containing sequential HAM (sHAM) regimen. From 2009 unteil 2012, 396 patients were enrolled, and 387 patients are evaluable for outcomes.

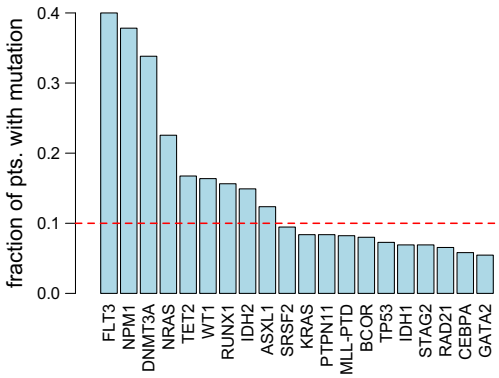
Genomic DNA was isolated from pretreatment blood or bone marrow specimens. Sequence alterations in 70 genes known to be recurrently mutated in AML or other hematologic neoplasms were analyzed by multiplexed amplicon sequencing (Agilent Haloplex; target region, approx. 321 kilobases). Sequencing was performed on an Illumina MiSeq instrument using 2x250bp paired-end reads. Single nucleotide variants and insertions/deletions (InDels) up to approx. 150bp were identified using an in-house custom analysis pipeline that included the VarScan and Pindel tools as variant callers. A variant allele frequency (VAF) threshold of 2% was set for single-nucleotide variant detection, corresponding to heterozygous mutations present in 4% of cells in a specimen. Variants were classified as known/putative driver mutations, variants of unknown significance, or known germline polymorphisms based on published data (including dbSNP, the Catalogue of Somatic Mutations In Cancer [COSMIC] and The Cancer Genome Atlas [TCGA]). In patients with more than one single nucleotide variant, the chi square test was used assess if the observed VAFs, adjusted for ploidy, were compatible with the presence of a single clone.

Results

Material for genetic analyses was available for 275 of the 387 participants (71%) enrolled on the AMLC-2008 trial (140 male, 135 female; median age, 56 years; range 18-86 years). Mean sequencing coverage of target regions was approximately 600-fold, and on average, 98.2% of target bases were covered >30-fold.

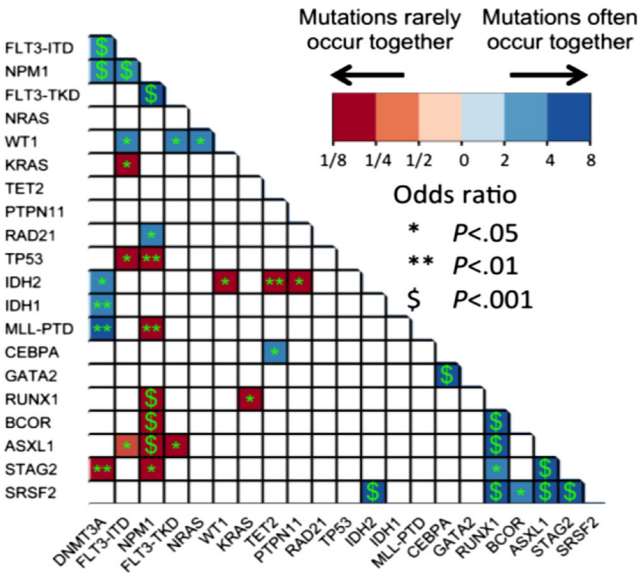
We detected a total of 1043 mutations in 47 genes, including 39 genes mutated in >1 patient. Nine genes (*NPM1, FLT3, DNMT3A, NRAS, WT1, IDH2, RUNX1, TET2* and *ASXL1*) were mutated in >10% of patients (**Fig1**, dashed line).

Fig1



We found a median of 4 mutations per patient (range: 0-10). Of note, only 1 patient had no detectable mutation and no abnormality on cytogenetic analysis. Patients with Intermediate-risk cytogenetics according to the MRC classification[3] harbored a higher number of driver gene mutations (median, 4) compared to patients with MRC Favorable (median, 2 mutations) or Unfavorable (median, 3 mutations) cytogenetics (P<.001).
When analyzing patterns of co-occurring and mutually exclusive mutations, we confirmed well-known associations (e.g., between *CEBPA* and *GATA2* mutations) and identified novel pairs of mutations that frequently occur in combination and, to our knowledge, have not yet been reported in AML (e.g., *ASXL1*/*STAG2, SRSF2/STAG2*). These findings may guide functional studies on the molecular mechanisms of leukemogenesis.

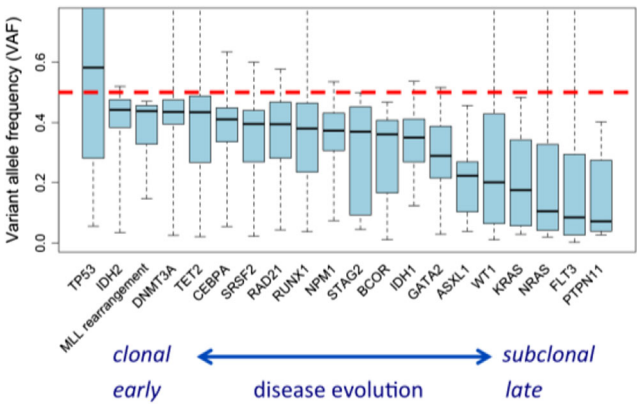
Fig2



We found evidence for clonal heterogeneity in 129 (52%) of 248 pts, based on the presence of mutations with significantly (P<.001) different VAFs within the same sample. Our analyses reveal differences in allele frequencies between different AML driver genes. Mutations can be

grouped into "early" events that often are present in the founding clone, and "late" events that frequently appear to be restricted to subclones (**Fig3**).

Fig3



In the entire patient cohort including all cytogenetic risk groups, mutations in three genes were associated with event-free survival (EFS): *NPM1*-mutated patients had favorable EFS compared to *NPM1*-wildtype patients, while *RUNX1* and *TP53* mutations associated with shorter EFS (P=.002 and P<.001, respectively).

Conclusion

Targeted sequencing allowed detection of mutations affecting a panel of known and putative AML driver genes in clinical specimens with high sensitivity. Our data from the AMLCG-2008 patient cohort reveal patterns of cooperating gene mutations, and show that the presence of subclonal driver mutations is a frequent event in AML patients.
Differentiating between "founding clone" mutations and subclonal mutations that typically occur later in the disease process has implications for choosing targeted therapies aimed at disease eradication.

Conflict of interest: The authors declare that they have no conflict of interest

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Genomic Characterization of Acute Lymphoblastic Leukemia

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Abstract

Genomic profiling has transformed our understanding of acute lymphoblastic leukemia (ALL), by (1) revising the classification of ALL; (2) characterizing the constellations of genetic mutations that define each subtype of ALL; (3) identifying genetic alterations that predict treatment success or failure; and (4) identifying new targets for therapeutic intervention. These advances are summarized in this review, with an emphasis on advances that promise to improve treatment outcomes for this disease.

Introduction

ALL is the commonest childhood malignancy and a leading cause of cancer related death in young people[1]. While less common in adults, the outcome is poor and improved treatment approaches required in this age group[2]. ALL is comprised of multiple subtypes that historically have been defined by genetic alterations identified by cytogenetic analysis, including aneuploidy (hyperdiploidy and hypodiploidy) and translocations that disrupt key hematopoietic transcription factors, tyrosine kinases and epigenetic regulators (e.g. ETV6-RUNX1, TCF3-PBX1, BCR-ABL1 and rearrangement of MLL)[3]. Such alterations are important determinants of treatment outcomes, but do not explain the full spectrum of genetic alterations or disease behavior. Moreover, with the exception of BCR-ABL1 and MLL-rearranged ALL, these subtypes are less common in adults, and the genetic basis of ALL in this age group has until recently been poorly understood.[2]

Genetic subtypes of ALL

Genomic profiling, initially using microarray analysis of gene expression and DNA copy number alterations, and more recently, next generation sequencing, have identified multiple new subtypes of ALL of clinical importance, and have shown that in the majority of patients, ALL is a polygenic disease, with mutations in at least 4 key cellular pathways observed in the majority of cases[4]. While the genes involved, and the mechanism of alteration (deletion/amplification, mutation, or translocation) varies between subtypes, these are important common themes. These include lymphoid development; cytokine receptor, kinase and Ras signaling; cell cycle regulation and tumor suppression; and epigenetic regulation.

Ph-like ALL is characterized by a gene expression profile similar to BCR-ABL1 (Ph+) ALL, absence of the BCR-ABL1 oncogene, genetic alteration of lymphoid transcription factors such as *IKZF1* (IKAROS), and poor outcome[5-7]. The frequency of Ph-like ALL rises with increasing age, from 10% in children to over 25% in young adults, with the prevalence in older adults to be defined[8]. Genome sequencing has shown that Ph-like ALL cases harbor alterations activating several cytokine receptor and tyrosine kinase signaling pathways. These include rearrangements of "ABL1-class" kinases ABL1, ABL2, CSF1R and PDGFRB; rearrangements of CRLF2 (often with mutations of JAK1/JAK2)[9,10], rearrangements of JAK2/EPOR, mutations of JAK-STAT signaling genes (IL7R, SH2B3, and others) and less common alterations targeting Ras signaling and tyrosine kinases[8,11]. Importantly, the majority of alterations result in activation of signaling pathways sensitive to ABL1 inhibitors (imatinib, dasatinib et al) and JAK2 inhibitors (e.g. ruxolitinib), and anecdotal data has shown efficacy of tyrosine kinase inhibitors (TKI) in otherwise treatment resistant Ph-like ALL[8,12,13]. The activity of TKIs will soon be tested in prospective clinical trials of Ph-like ALL in children, and is a priority in adults where this entity is more common.

CRLF2-rearranged ALL. CRLF2 encodes cytokine receptor-like factor 2, which with the interleukin 7 receptor alpha chain (IL7RA) forms the receptor for the cytokine thymic stromal lymphopoietin (TSLP). CRLF2 is nor normally expressed in B lymphoid cells, but is deregulated by

rearrangement to *IGH* or by a deletion upstream of the CRLF2 locus that results in *P2RY8-CRLF2* fusion. Both result in overexpression of CRLF2 detectable by flow cytometry. *CRLF2* rearrangement is present in approximately half of Ph-like ALL cases but is also present in non-Ph-like B-ALL, particularly Down syndrome-associated B-ALL.[9,10] The majority of *CRLF2*-rearranged cases have concomitant genetic changes activating JAK-STAT signaling, particularly JAK1/2 mutations, but also JAK3, IL7R and FLT3 mutations. CRLF2-rearranged leukemic cells typically exhibit activated JAK-STAT signaling that is inhibited by JAK inhibitors[14,15], but the efficacy of these agents in vivo is presently unknown.

ERG deregulated ALL comprises 5-7% of childhood and adult ALL and is characterized by genomic alterations perturbing expression of ERG, a transcription factor. Identification of this subtype is important as such cases commonly have alteration of *IKZF1* (IKAROS), which in the context of Ph+ or Ph-like ALL is a poor prognostic feature, but here is not associated with adverse outcome[16,17].

Hypodiploid ALL comprises up to 5% of ALL and is characterize by marked aneuploidy. Genome sequencing has defined the genomic basis of this entity with several clinical implications[18]. Near haploid ALL cases have 24-31 chromosomes, an exceptionally high frequency of activating Ras pathway mutations, particularly *NF1* (otherwise rare in ALL) and mutations of *IKZF3* (AIOLOS). Low hypodiploid cases have less severe aneuploidy (32-39 chromosomes), alteration of *IKZF2* (HELIOS), and near universal alteration of *TP53* which is inherited in over half of low hypodiploid cases. Thus, *TP53* mutational testing is warranted in hypodiploid ALL, particularly low hypodiploid cases in children. The majority of cases show activation of PI3K and Ras-Raf-MEK-ERK signaling pathways, and sensitivity to PI3K inhibitors and BCL-2 inhibitors[18].

Genomic predictors of outcome in ALL

A key goal of genomic profiling studies is the identification of genomic features that predict treatment success or failure in ALL. These can be grouped into three categories: (1) subtypes, (2) genomic alterations identified at diagnosis; and (3) acquired alterations that facilitate resistance to specific therapeutic agents and promote relapse.

Key subtypes associated with poor outcome in ALL include Ph+, MLL-rearranged and hypodiploid ALL have long been recognized to be associated with poor outcome. In addition, Ph-like ALL and ALL with intrachromosomal amplification of chromosome 21 (iAMP21) are also associated with poor outcome. Detection of these entities is thus important at diagnosis, although the biologic basis of poor treatment outcome remains poorly understood (with the exception of Ph+ ALL, which is in part due to alteration of *IKZF1*.

Specific genetic alterations transcend these key subtypes and influence outcome in ALL. The most important of these is *IKZF1* alterations. *IKZF1* encodes IKAROS, a transcription factor required for the development of all lymphoid lineages. Genetic alterations of *IKZF1* are present in up to 25% of B-ALL cases, and include deletions that result in loss of IKAROS activity, and focal deletions and sequence mutations that result in the expression of dominant negative alleles. These alterations result in the acquisition of a primitive, stem cell like phenotype and abnormal bone marrow stromal adhesion and resistance to therapy.

Genetic alterations and relapse in ALL

Several classes of genetic alteration are acquired and promote resistance to therapy in ALL. These include deletions or mutations of *CREBBP* (encoding CREB-binding protein), *NT5C2* and epigenetic regulators[19]. CREBBP is a histone and non-histone acetyl transferase that in part mediates the transcriptional response to glucocorticoids[20], a mainstay of ALL therapy. CREBBP mutations result in resistance in glucocorticoids, thus strategies to inhibit histone deacetylation are being explored to reverse this phenotype.

NT5C2 encodes a 5' nucleotidase that regulates metabolism of nucleosides and mutations in this gene confer resistance to thiopurines used in ALL therapy. *NT5C2* mutations are rarely, if ever observed at diagnosis, but in contrast are acquired during the course of therapy[21,22]. Thus, the detection of these mutations may be a harbinger of relapse and may prompt implementation of alternative treatment strategies.

Additional pathways targeted by genetic alteration at relapse include Ras and JAK-STAT signaling and epigenetic regulation. Mutation of epigenetic regulators is increasingly recognized in ALL. Such mutations include a diverse range of histone writers, readers and erasers[23,24]. Although there are currently limited data regarding the manner in which such mutations contribute to leukemogenesis and treatment resistance, this is an active area of research and a key area of potential therapeutic intervention.

An important observation is that there is substantial clonal evolution and mutation turnover during therapy in ALL, and that relapse commonly arises from a minor clone at diagnosis that shares only partial identity with the predominant clone at diagnosis, but that acquires mutations that facilitate resistance to therapy and relapse. Identification of these low level clones, and the mutations that facilitate relapse, represents an important challenge and opportunity to identify patients at high risk of relapse who may benefit from alternative therapeutic approaches.

Conflict of Interest: The author declares that he has no conflict of interest

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Alliance Trials for AML

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Introduction

The Alliance for Clinical Trials in Oncology has an extensive portfolio of clinical and translational trials in acute myeloid leukemia. In the last few years, Alliance clinical trials have been focused on novel induction strategies based on cytogenetic or molecular risk groups, induction strategies for the elderly, acute promyelocytic leukemia and post-remission maintenance. The leukemia correlative sciences team has utilized next-generation sequencing and other novel technologies to molecularly characterize thousands of bone marrow and peripheral blood specimens obtained in conjunction with Alliance trials. These efforts have lead to the development of highly enriched prognostic stratification systems and provide significant insights into the biology of AML. The objective of this abstract is to briefly review the most important clinical and basic science publications during the last year based on Alliance trials, as well as to summarize recently completed and ongoing trials for all phases of AML treatment.

Methods

The Alliance has been a world leader in the molecular characterization and sub-classification of cytogenetically normal AML, the largest and most heterogeneous subgroup of the disease. Marcucci et al. have identified novel, prognostically relevant AML subgroups using a seven-gene score based on epigenetic and genetic information.[1] Using next-generation sequencing analysis of methylated DNA, differentially methylated regions (DMRs) associated with prognostic mutations were identified in 134 older (≥60 years) adults with cytogenetically normal AML. Seven genes were identified which had promoter DMRs and expression level associated with overall survival, including *CD34, RHOC, SCRn1, F2RL1, FAM92A1, MIR155HG, and VWA8*. For all of these genes, lower expression and higher DNA methylation were associated with longer overall survival. The authors determined that patients who had high expression of only zero or one of these genes had excellent rates of complete remission (CR, 91% and 100%, respectively) and overall survival (82% and 76%), compared to very poor outcomes in patients with six or seven highly expressed genes (CR 71%, 53%; OS 24%, 5%; for 6 and 7 genes, respectively). This seven-gene score performed very favorably compared to other published models and it is hoped that a practical quantitative assay can be developed to facilitate rapid integration into clinical practice.[1]

Data from Alliance AML trials were used as a validation cohort for a combined molecular and clinical prognostic model for relapse and survival in cytogenetically normal AML patients of all ages.[2] Pastore et al. used data from 572 cytogenetically normal AML patients treated on the German AML Cooperative Group 99 (AMLCG99) study comparing thioguanine, cytarabine, and daunorubicin plus high-dose cytarabine and mitoxantrone (HAM) versus HAMHAM as induction therapy were used for. Validated prognostic indices for relapse and survival were developed based on age, performance status, white blood cell count and mutation status of NPM1, CEBPA and FLT3-internal tandem duplication (ITD). These data are readily available to clinicians and application of this schema should be straightforward. The authors conclude that theirs is the first prognostic system to combine clinical and molecular features specifically for cytogenetically normal AML and irrespective of age.[2]
Metzeler et al. studied 364 patients (ages 18-83) with cytogenetically normal AML treated on Alliance trials in an effort to determine whether a previously identified stem cell-like gene expression signature is independently associated with inferior outcomes.[3] It is believed that AML is derived from a distinct, quiescent, chemotherapy-resistant population of leukemia stem cells (LSCs). Eppert et al. compared gene expression profiles between functionally defined LSCs and leukemic cell populations without stem cell activity and identified a "core enriched (CE)" signature with 44 stem-cell associated genes that are highly expressed in LSCs. In this work, patients with cytogenetically normal AML who had an increased CE signature had worse overall outcomes.[4,3] These data were confirmed using the Alliance cohort. Furthermore, it was shown that a high CE score was an independent predictor of decreased rates of complete remission, disease-free survival and overall survival even after adjusting for standardly accepted clinical and molecular prognostic indicators. Future studies to identify the functional role of LSC signature genes and to investigate the relationships between CE signature and other molecular abnormalities in AML are required.[3]

Niederwieser et al. investigated the clinical significance of the DNA methyltransferase *DNMT3B* using samples from 210 older cytogenetically normal AML patients (age 60-83) treated with intensive chemotherapy on Alliance trials.[5] Patients with high expression of *DNMT3B* had significantly inferior rates of complete remission, disease-free survival and overall survival. Also, *DNMT3B* expression was an independent predictor or poor outcomes in multivariate analyses. Further studies are ongoing to investigate the clinical and therapeutic relevance of this finding.[5] The prognostic significance of *GAS6* expression was also

investigated in 270 older AML patients with cytogenetically normal AML treated with intensive chemotherapy on Alliance clinical trials.[6] GAS6 is the common ligand for all three TAM receptor tyrosine kinases (TYRO3, AXL and MERTK) and binds AXL with high affinity. *AXL* expression has been reported as a poor prognostic indicator in cytogenetically normal AML and it is possible that aberrant autocrine expression of *GAS6* may result in activation of AXL.[7] Whitman et al. demonstrated that GAS6 expression is an adverse prognostic indicator in cytogenetically normal AML and suggest development of targeted treatments against GAS6/AXL signaling.[6]

Although patients with normal cytogenetics comprise the largest and best characterized subgroup in AML, chromosomal abnormalities are common and extremely important for prognosis. Alliance investigators reviewed the cytogenetics database of 4,701 patients with AML and MDS and identified 7 new balanced translocations, including t(7;11)(q22;p15.5), t(10;11)(q23;p15), t(2;12)(p13;p13), t(12;17)(p13;q12), t(2;3)(p21;p21), t(5;21)(q31;q22), and t(8;14)(q24.1;q32.2). Fifty-two unique balanced translocations were also reported.[8] Trisomy 8 is the most frequent chromosome abnormality in AML and occurs in approximately 9% of adult patients. Becker et al. reported the molecular and clinical characterization of 80 adults with de novo AML and sole +8 enrolled onto Alliance trials.[9] Outcomes were poor, with 64% CR and 15% overall survival at 5 years. There were no significant differences in CR, DFS or OS between younger and older patients with sole +8. The abnormality was found to be molecularly heterogeneous, with frequent mutations in *RUNX1, ASXL1, IDH2, DNMT3A, NPM1 and FLT3-ITD*. High *BAALC* expression and *FLT3-ITD* were associated with inferior outcomes in younger patients and *TET2* mutations and wild-type *RAS* resulted in worse outcomes for older patients with isolated +8.[9]

Patients with acute promyelocytic leukemia (APL) enjoy excellent outcomes, made even better by the use of arsenic trioxide as part of up-front treatment of newly diagnosed patients. In previously untreated patients with low or intermediate risk APL, it is possible that treatment with arsenic trioxide during consolidation (CALGB 9710) may eliminate the need for maintenance chemotherapy.[10] A non-inferiority trial, terminated due to poor accrual, demonstrated no relapses in either group of 105 APL patients randomized to maintenance or observation. In another follow-up study of Alliance 9710, 48% of 245 newly diagnosed adult patients with APL were found to have mutations in FLT3, 31% FLT3-ITD and 14% with a point mutation (FLT3-D835).[11] Neither type of mutation affected rates of CR, induction death, DFS or overall survival and treatment with arsenic trioxide during consolidation improved outcomes regardless of mutational status. However, even after treatment with arsenic trioxide, APL patients with complex karyotype had significantly decreased overall survival.[11]

Treatment outcomes for older patients with AML remain dismal worldwide and novel strategies are clearly needed. Alliance study 10502 investigated the addition of the proteasome-inhibitor bortezomib to standard daunorubicin/cytarabine induction and to intermediate-dose cytarabine consolidation in 95 newly diagnosed patients with AML age 60-75 years.[12] Bortezomib inhibits the master transcription factor nuclear factor kappa-B (NFκB), which is increased in AML cells and LSCs.[13] Although bortezomib has virtually no activity as a single agent in AML, it showed preliminary efficacy when combined with chemotherapy in patients with relapsed disease.[14] In newly diagnosed patients, the combination of bortezomib and standard chemotherapy was feasible, had an acceptable toxicity profile and resulted in an encouraging rate of complete remission (65%). Further investigation of this regimen in older adults with AML is warranted.

The role of bortezomib in AML induction was explored in Alliance trial 11002, in which newly diagnosed older AML patients were randomized between ten days of decitabine with and without the addition of bortezomib. The trial was designed based on favorable clinical outcomes of the ten-day induction schedule from three single-center studies and previously demonstrated clinical and pharmacodynamics activity for the combination.[15-18] CALGB 11002 also included comprehensive geriatric assessments and extensive correlative scientific studies. Accrual has been completed and data are anticipated imminently. CALGB 11002 ran concurrently with trial 11001, a phase II trial of conventional daunorubicin and cytarabine combined with sorafenib for newly diagnosed AML patients ≥60 years with FLT3 mutations. Preliminary results from this single-arm, multicenter phase II trial were presented at the 2013 American Society of Hematology Annual Meeting and showed CR or complete remission with incomplete platelet recovery (CRi) in 69% of 52 evaluable patients. There was no excess toxicity attributed to sorafenib.[19] Interestingly, a similar trial conducted in Europe failed to show a benefit for this regimen in older AML patients.[20]

Conventional intensive chemotherapy induction is often withheld from older patients due to well-justified morbidity and mortality concerns. However, although the median age at diagnosis of AML is over 70 years, it is well-known that clinical trials include few patients with very advanced age. Investigators from the Alliance, German AML Cooperative Group (AMLCG), German-Austrian AML Study Group (AMLSG), and the Acute Leukemia French Association (ALFA) pooled data from several large studies of older patients with AML treated with intensive induction and identified 138 octagenarians.[21] The majority of the patients had a performance status of zero or one (69%) and normal cytogenetics (47.1%). Complex karyotype was identified in 14.5% of patients and isolated trisomy 8 in 9.4%. The complete response rate for patients surviving beyond 60 days was 39%. Overall survival at 1 year was 24%, with only 8% surviving for 3 years. FLT-3 internal tandem duplication was not associated with overall survival, while NPM1 mutations were associated with significantly longer survival.[21]

AML is a highly molecularly heterogeneous disease and efforts are underway to adapt induction strategies to specific molecular subtypes. CALGB 10801 was a phase II study of induction (daunorubicin/cytarabine) and consolidation (high-dose cytarabine) chemotherapy plus the tyrosine kinase inhibitor dasatinib, followed by continuation therapy with dasatinib alone in newly diagnosed patients with core binding factor AML. Diagnosis of core binding factor AML was defined by the presence of t(8;21)(q22;q22) or the molecular equivalent *RUNX1/RUNX1T1* fusion transcript or inv(16)(p13q22) or t(16;16)(p13;q22) or the molecular equivalent *CBFB/MYH11* fusion transcript. Preliminary data are highly anticipated and will be presented at the 2014 ASH meeting. Data are also expected from CALGB 10603, an international, randomized phase III study of induction (daunorubicin/cytarabine) and consolidation (high-dose cytarabine) chemotherapy combined with midostaurin or placebo in treatment-naive patients with FLT3 mutated AML.

The role of maintenance therapy in AML remains unclear. Although it is well-known that substantial residual disease remains after AML therapy and it is intuitive that ongoing treatment could eliminate or suppress this population of residual cells over time, clinical trials of chemotherapy and immunotherapy have failed, to date, to demonstrate an improvement in overall survival. Several trials have shown improvements in relapse-free survival, but the regimens have not been adopted into standard practice.[22-24] CALGB 10503 was a phase II study of maintenance therapy with decitabine following standard induction and cytogenetic risk-adapted intensification in previously untreated patients with AML < 60 years. The goals were to determine the efficacy, feasibility, and toxicities associated with one year of maintenance therapy with decitabine administered to patients (ages ≥ 15 and < 60 years) with untreated AML and to assess the 1-year disease free survival rate after decitabine maintenance. The trial has been difficult to evaluate due to patient refusals and noncompliance. Similarly, the efficacy of immunotherapy with

IL-2 as a post-remission maintenance therapy for younger patients with newly diagnosed AML (CALGB 19808) could not be assessed due to patient and physician refusals to proceed with the planned protocol-directed therapy.[25]

Conclusion

Despite the enormous efforts of AML patients and investigators, it is painfully clear that therapeutic strategies in AML still lag far behind the substantial breakthroughs in understanding disease pathobiology. Major goals for the next decade should include creative incentives to encourage patients and physicians to participate in AML clinical trials; faster incorporation of novel agents into clinical trials of newly diagnosed AML patients; streamlining arcane and bureaucratic processes in academic medicine, industry and government that impede new trials**;** and the rapid development of real-time, feasible assays to allow direct application of novel biology-based prognostic scoring systems into clinical practice. Perhaps most importantly, we should strive for increased cooperation among and collaboration between national and international cooperative groups to limit clinical trials in unselected AML patient populations and facilitate rapid accrual of distinct, biologically defined subgroups of AML patients onto rationally designed protocols.

Conflict of interest: The author declares that she has no conflict of interest

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Sorafenib versus Placebo in Addition to Standard Therapy in Younger Patients with Newly Diagnosed Acute Myeloid Leukemia: Results from 267 Patients Treated in the Randomized Placebo-Controlled SORAML Trial

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Background

Sorafenib is a multi-kinase inhibitor with activity against several oncogenic kinases that may play a role in the pathogenesis of acute myeloid leukemia (AML). We present the results of the randomized placebo-controlled SORAML trial testing sorafenib versus placebo as add-on to standard induction and consolidation treatment in AML patients ≤60 years.

Patients and methods

276 patients from 25 centers were enrolled in the SORAML trial (NCT00893373). The main eligibility criteria were newly diagnosed AML, age from 18 to 60 years and suitability for intensive therapy. The treatment plan for all patients included two cycles of induction with DA, followed by three cycles of high-dose cytarabine. At study inclusion, patients were randomized to receive either sorafenib (800 mg/day) or placebo as add-on to standard treatment in a double blinded fashion. Study medication was given on days 10-19 of DA I+II or HAM, from day 8 of each consolidation until 3 days before the start of the next consolidation and as maintenance for 12 months after the end of consolidation. The primary endpoint of the trial was event-free survival (EFS).

Results

267 patients received study treatment, 134 in the sorafenib arm and 133 in the placebo arm. Demographic and disease characteristics were equally distributed between the two arms; the incidence of FLT3-ITD was 17%. After a median observation time of 36 months, the median EFS was 9.2 months in the placebo arm and 20.5 months in the sorafenib arm, corresponding to a 3-year EFS of 22% versus 40% (p=0.013). Median RFS after standard treatment plus placebo was 23 months and not yet reached after sorafenib treatment, corresponding to a 3-year RFS of 38% and 56%, respectively (p=0.017). The median OS had not been reached in either arm; the 3-year OS was 56% with placebo versus 63% with sorafenib (p=0.382). in favor of sorafenib was observed. The most common reported AEs Grade ≥3 were fever (40%), infections (22%) and bleeding events (2%). The risk for fever, bleeding events and hand-foot syndrome was significantly higher in the sorafenib arm while the incidence of all other AEs showed no significant differences.

Conclusions

In younger AML patients, the addition of sorafenib to standard chemotherapy in a sequential manner is feasible and associated with antileukemic efficacy. We observed a higher incidence of infections and bleeding events under sorafenib. Whereas OS in both treatment arms was similar, sorafenib resulted in a significantly prolonged EFS and RFS.

Conflict of interest: The authors declare that they have no conflict of interest

Management of Childhood Acute Lymphoblastic Leukemia: The Current BFM Strategy

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Abstract

The detection of minimal residual disease (MRD) has become part of the state-of-the-art diagnostics to guide treatment in pediatric acute lymphoblastic leukemia (ALL). This applies to the treatment of *de novo* and recurrent ALL. In high risk ALL, MRD detection is considered an important tool to adjust therapy, in some patients to adjust the optimal time for hematopoetic stem cell transplantation. Precise quantification and quality control is instrumental to avoid false treatment assignment. MRD detection may be used to modulate treatment intensity once it has been demonstrated at well-defined informative checkpoints that certain levels of MRD can reliably predict the risk of relapse. In addition, MRD is used as endpoint to determine the activity of a given agent or treatment protocol. If activity translates into antileukemic efficacy, MRD may be considered a surrogate clinical endpoint.

More recently, the prognostic power of MRD detection at given time points was demonstrated in novel genetic subtypes of ALL which may comprise a significant proportion of disease recurrences. This observation illustrates again that so far no genetic subtype with a uniform response pattern to contemporary treatment schedules has been described. In contrast, all subtypes display heterogeneity in treatment response and outcome.

Introduction

In vivo sensitivity of acute lymphoblastic leukemia (ALL) as measured by the early blast cell reduction in peripheral blood (PB) or bone marrow (BM) after exposure to one or several antileukemic agents is used to risk-stratify patients with ALL because response is of high prognostic relevance.[24, 35] Lack of adequate response, in particular at the end of remission induction, indicates poor prognosis even though this may vary significantly according to individual patient characteristics.[34] The choice of technique for MRD detection mainly depends on the aims of the clinical trial and on the availability of resources. [6, 15, 39] [3, 5, 11] It is now widely acknowledged that MRD detection is part of state-of-the-art diagnostics and needed in the management of ALL. More importantly, MRD detection may even replace other prognostic factors. [4, 8, 18, 33]

Prognostic information based on response to treatment

In the largest study for *de novo* ALL published so far, the BFM (Germany, Austria, Switzerland) and AIEOP (Italy) study groups utilized MRD (measured by RQ-PCR) for risk stratification in a total of 3184 pcB-ALL and 464 T-ALL patients. All patients were treated by identical chemotherapy in the first 9 weeks of therapy. Large differences in pEFS between MRD defined subgroups were found.[8, 33] MRD was analyzed in the BM at end of induction (day 33), and at end of induction consolidation (day 78). Importantly, within pcB-ALL, the prognostic impact of MRD was maintained even in the two large subgroups of *TEL/AML1* positive and hyperdiploid ALL.[8]
The AIEOP-BFM ALL 2000 trial confirmed the strong prognostic impact of MRD at end of induction when combined with a subsequent timepoint. This trial also demonstrated that in pcB-ALL the MRD-intermediate risk group (MRD-IR) comprises the largest group of patients (51.7%), but also the majority of relapses: 69% of all relapses were found in this group which showed an overall relapse rate of 21% at five years.[8] The situation in T-cell ALL was similar: 62.9% of all patients were defined as IR, comprising 55% of all relapses (cumulative incidence of relapse 17.6% at 5 years).[33] This observation illustrates the need for more refined approaches to identify the patient at high risk to relapse. Recent work of several study groups has identified intriguing properties in subsets of pcB-ALL which in part appear to show prognostic impact independent of MRD. This may be clinically quite relevant as the distribution of relapses as shown before requires further refinement in risk assessment and treatment adaptation.[7, 9, 10, 23, 25, 26, 28, 31, 40]

Differential response in ALL subgroups

Two major differences between pcB- and T-ALL can be found: i) MRD at end of induction (day 33) is more informative in pcB-ALL, while MRD at end of consolidation (day 78) is more informative in T-ALL. ii) MRD levels in pcB-ALL correlate with risk of systemic relapse whereas MRD in T-ALL is predictive of both systemic and extramedullary relapse. The results obtained here in combination with the results obtained by FCM in the same trial has changed the risk group definition for HR patients as used by AIEOP-BFM: Any patient who has more than 10% leukemic blasts by FCM on day 15 is enrolled into the HR group; any patient with pcB-ALL who has MRD $\geq 10^{-3}$ (0.1%) at day 33, and is still MRD positive at day 78, is stratified into the HR group; any pcB-ALL patient with MRD <0.1% at day 15 is eligible for SR if PCR results (with at least one highly sensitive molecular target, quantitative range up to 10^{-4}) at day 33 and day 78 are also negative.[3, 17]

Post-remission MRD surveillance: Should MRD before and after hSCT be monitored?

Additional post-remission MRD assessment is performed in several clinical trials. In AIEOP-BFM ALL 2000, all patients with MRD at a level of $\geq 10^{-3}$ at day 78 were stratified into the HR group, and then monitored after each reconsolidation element. This strategy is currently used to adjust further chemotherapy and prepare for allogeneic stem cell transplantation (SCT). While post-induction MRD was also found to have significant prognostic impact in relapsed ALL[12], MRD

monitoring of pre-SCT response demonstrated the necessity to optimize the quality of remission before transplant to prevent post-SCT relapse.[1, 2, 22]

Philadelphia-chromosome positive ALL

The long-term follow-up of Ph+ patients treated in COG with imatinib in addition to intensive chemotherapy provides interesting insights into the efficacy of a tyrosine-kinase inhibitor when combined with chemotherapy.[20, 36] In those patients treated most intensively with imatinib, the prognostic value of MRD was nearly abrogated. At the same time, the authors were able to demonstrate that additional genetic abnormalities had a significant impact on event-free survival.[36] In adult Ph+ ALL, the picture was similar, demonstrating a limited prognostic impact of persisting MRD when patients were treated with a tyrosine kinase inhibitor (TKI) in addition to chemotherapy.[30] Very intriguingly, Foa et al. demonstrated that a TKI (dasatinib) combined only with steroids and intrathecal chemotherapy can induce complete remission in patients with Ph+ ALL, even though a significant number relapsed later on.[16] This indicates a potentially significant progress as such approaches may contribute to future treatment regimen that carry less toxicity due to the replacement of (some) chemotherapy by more targeted agents.

Clonal evolution

MRD monitoring in relapsed patients carries some potential pitfalls mainly due to clonal evolution.[13] Detailed analysis of all molecular markers at first diagnosis and at time of relapse may reveal a different origin of the predominant clone.[37] Regular monitoring of MRD by those markers defined at first diagnosis may fail if clonal evolution occurs, also when occurring after the first relapse.[19] Ongoing research will determine if next generation sequencing (NGS) is the appropriate tool to prevent such diagnostic “failures”.[14, 21]
In adult patients, a striking difference was found between *de novo* and relapsed Ph+ ALL with regard to the levels of *bcr-abl* kinase domain mutations. This may not only explain the lack of treatment efficacy when treating with TKI but also impact the utility of RT-PCR monitoring of relapsed Ph+ ALL patients.[29]

Endpoint in clinical trials

To define antileukemic activity, MRD response can also be utilized as primary endpoint in clinical trials. It needs to be taken into account, however, that MRD response is only a more sensitive tool to assess response when compared to the cytological response. It may completely depend on the scenario, if activity translates into improved efficacy of a given intervention, thus improving the relevant clinical endpoints such as event-free survival and overall survival. This said, it is noteworthy that recent novel immunotherapeutic strategies e.g. with an anti-CD3, anti-CD19 bispecific monoclonal antibody showing strong antileukemic activity (as measured by MRD response) may contribute to long-term remission as such agents may improve the quality of remission needed to perform subsequent allogeneic SCT successfully.[38] [18, 32] By contrast, a British study comparing reinduction therapy with mitoxantrone versus idarubicin in relapsed pediatric ALL demonstrated that MRD response (activity) was not predictive of treatment efficacy.[27] This observation indicates that MRD response may be misleading in drug evaluation as long as activity is considered to be equal to efficacy.

Conclusions

Essentially all subtypes of ALL display heterogeneity in response. MRD detection has evolved as one of the most powerful diagnostic tools in the prognostic assessment and also for the clinical management of ALL. In its prognostic power, MRD cannot be replaced by upfront diagnostic markers. New techniques such as whole genome sequencing may overcome some of the shortcomings of current MRD technologies if validation proves successful. Clinical intervention based on MRD results must rely on robust prospective data indicating the precise prognostic impact of a given MRD level at predefined timepoints. In well-defined circumstances, MRD detection can be a very useful guide for modulating therapy.

Conflict of interest: The author declares that he has no conflict of interest

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Flow Cytometric MRD and Leukemia Stem Cell Frequency Assessment to Develop Risk-Stratified Approaches in AML

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Abstract

Minimal residual disease (MRD) has been found to offer an independent post-remission prognostic marker in acute myeloid leukaemia (AML). We have confirmed this in a large blinded study in which MRD was assessed in parallel to a HOVON therapy protocol. Subsequently, MRD is now included as part of a new risk classification in a HOVON/SAKK treatment protocol where intermediate risk patients, previously all scheduled for allogeneic stem cell transplantation, are scheduled for autologous transplantation in case of MRD-negativity. In parallel, leukaemia stem cell load at clinical follow up was identified as a factor that adds significantly to the prognostic value of MRD load. Especially relapse in MRD-negative patients can now be better predicted based on leukaemia stem cell (LSC) frequency. A single tube assay was therefore developed which facilitates stem cell load assessment and allows this in the majority of patients. Lastly, false-negative MRD and false-negative LSC cases may be identified by monitoring upcoming aberrant populations that, due to low frequencies, were not properly identified at diagnosis.

Introduction

Treatment of AML patients usually is performed according to classification in risk groups defined by molecular, cytogenetic

and clinical parameters, which thereby identify patients with favourable, intermediate and poor/very poor risk.[1] Although very useful, this classification is still far from the ideal situation in which patients should have individualized risk assessment, potentially allowing individualized therapy: all risk groups still harbour patients with good and poor outcome, even the most favourable patient group, while the poor risk patient group still harbours good performing patients. Parameters that would integrate all known and unknown factors contributing to individual patient's clinical outcome were therefore thought to be urgently needed. A post-remission parameter like MRD has been proposed to represent such a factor and is meanwhile extensively studied. [2] Our recent study in the HOVON/SAKK consortium (http://www.HOVON.nl) has shown for young patients (HO42A, for details see website) that MRD assessed by flowcytometry in an unbiased way (prospective sampling and MRD quantitation in a multi-institutional way, with complete lack of knowledge on clinical outcome until final analysis) is an independent prognostic factor in all individual risk groups.

However, despite these improvements, still a considerable portion of MRD-negative group (defined as having MRD below a threshold level of 0.1% of WBC) relapse. [3] Also, the terms MRD-negative and MRD-positive (above the threshold level of 0.1%) have a different meaning in the different risk groups: MRD-positive patients in the favourable risk group still have relatively good prognosis, while MRD-negative patients in the poor risk group still have relatively poor prognosis. Intrinsic properties of MRD cells may underlie this phenomenon and one of the factors may be the LSC load. LSC are hypothesized to initiate leukaemia, to be therapy resistant, and at the basis of re-growth of the leukaemia after treatment. [4] As a single independent parameter it has been shown that stem cell load at diagnosis is a prognostic marker[5,6,7,8] and, more recently in the HO42A study, we also showed prognostic impact after therapy. [7] The stem cell load, when taken into account in a risk assessment that also includes MRD, may further improve MRD based risk assessment.

Methods

Flowcytometric MRD was performed using the approach in which leukaemia associated immunophenotypes (LAIP) were defined at diagnosis.[3] In the HOVON42A study risk was based on the combination of cytogenetics, molecular aberrations and clinical parameters.

LSC can be defined as CD34+CD38-. Strategies to define such population at diagnosis and at follow up have been described elsewhere. [7] LSC specific markers (not expressed on CD34+ CD38- normal hematopoietic stem cells, HSC), included lineage markers like CD7, CD19, CD56[9]and, in addition, CLL- 1 (CLEC12A)[10] , were originally included in the LSC panel. [7] In a new protocol, tested in more than 500 AML samples, additional markers have been included (e.g CD123, CD33, CD96, TIM-3, CD44).

The combination of MRD and LSC frequencies after induction therapy was tested for relapse free survival in a small (n=91) patient group. Threshold levels used for LSC were much lower than for MRD: 1 in 100,000 cells to one in a million cells.[7]

Results

MRD: Based on the prognostic impact of MRD in the HO42A clinical study, the present HO132 protocol contains a risk group previously designated as intermediate risk, but now, based on MRD positivity (MRD % >0.1) after induction courses, is part of the poor risk group. These patients, whenever possible, are scheduled for allogeneic transplantation (Table 1).

Classical risk group	New risk group
Good risk	Goog risk/Irrespective of MRD− or MRD+
Intermediate risk	Intermediate risk/MRD−
Poor risk	Intermediate risk/MRD+ Poor risk/MRD-
Very poor risk	Poor risk/MRD+ Very poor risk/Irrespective Irrespective of MRD− or MRD+

Table 1: Global risk stratification in present (HO 132) and previous (HOVON 102)

Since MRD had the strongest prognostic impact after the second induction course, for logistical and financial reasons it was decided to restrict the protocol to MRD assessment after the second induction course. MRD status after consolidation, although informative [3,11] is not considered in decision making, since it does not comply with the timely finding of an allogeneic donor for part of the patients.

Based on previous validation study [12], it was decided to perform MRD assessment in a largely centralized way. Accrual of patients has started (target number 800; 83 patients already included).

LSC ± MRD: When combining MRD load and stem cell load (Figure 1) it was found that: 1. The MRD negative group consists of two different groups with different prognosis based on LSC load; 2. The LSC negative group can be divided in a MRD-positive and a MRD-negative group with different prognosis; 3. Double negativity goes along with good prognosis, while double positivity goes along with very poor prognosis.

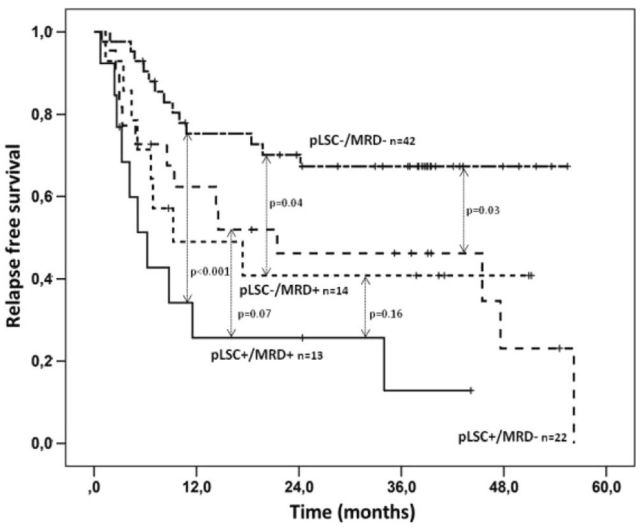

Fig1: Combination of MRD and LSC in prognosis of patients. For MRD a threshold value of ≤ 0.1% (of WBC) defines MRD-negative group and > 0.1% defines MRD positive group. For LSC similarly ≤ 0.0002% (of WBC) defines LSC negative group and > 0.0002 % defines LSC positive group. Data for another threshold value have been published.[7]

LSC could be accurately determined in part of the patients only.[7] Therefore, additional markers were studied with the aim to increase that part. Using the markers indicated in Methods, it is now possible to assess LSC in >90% of the patients. Based on extensive study of the redundancy of markers, we are now able to assess total LSC load in a given AML both at diagnosis and after therapy in a one tube assay that includes 13 different markers. This approach is included as a side study in the HO132 protocol,

where the possible contribution of LSC load assessment to prognosis will be studied.

Immunophenotype shifts in MRD and LSC assessment: Although LSC markedly improves risk stratification based on well-known risk factors and now also including MRD load, still the double negative patient group (MRD- LSC-) contains relapsing patients while the two single positive groups still contain both relapsing and non-relapsing patients (Figure 1). We and others found immunophenotype shifts in around 60% (median in different studies[13]) of patients, and these contribute to MRD- and LSC false negatives. With the knowledge of immunophenotype and molecular shifts in mind, the present HO132 protocol, apart from the inclusion of LSC load assessment, also assesses the contribution of immunophenotype shifts to MRD (and LSC) negativity.

Conclusions

We conclude that, based on many studies, and especially on our recent unbiased MRD study, MRD contributes significantly to the existing risk group stratification that is based on cytogenetic and molecular factors as well as on clinical factors. The decision-making process whether a patient is allocated to an allogeneic stem cell transplantation can now be guided by MRD assessment. Moreover, the inclusion of other factors like stem cell load and immunophenotype changes, as seen for MRD and LSC, will contribute to the final goal of individualized therapy.

Conflict of interest: G.J. Schuurhuis has received a grant from Becton Dickinson to develop the flowcytometric single stem cell tube

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Genetic Evolution in Relapsed AML

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Introduction

The evolution of acute myeloid leukemia (AML) has been previously described either in studies of large patient cohorts with focus on only a restricted number of AML-associated genes or in smaller series of relapsed patients studied by genome-wide techniques [1]. We set out to comprehensively characterize the genetic evolution in a large AML cohort in order to understand molecular mechanisms of relapse and therapy-resistance.

Methods

We performed exome-sequencing of matched bone marrow or peripheral blood samples taken at diagnosis, complete remission and relapse from 47 patients with cytogenetically normal AML (CN-AML). Samples were collected within the German Cancer Consortium (DKTK) at the partner sites in Berlin and Munich. The median age at diagnosis was 65y (range: 21-89y). *FLT3* internal tandem duplication (ITD) and *NPM1* mutation status at diagnosis was available for all but one patient (*FLT3*-ITD$^-$/*NPM1*$^-$, n=5; *FLT3*-ITD$^+$/*NPM1*$^-$, n=9; *FLT3*-ITD$^-$/*NPM1*$^+$, n=16; *FLT3*-ITD$^+$/*NPM1*$^+$, n=16).
On average, 96% of the target sequence was covered at least 10-fold (minimum coverage defined for variant calling). The following criteria were applied for identification of somatic mutations: Variant allele frequency (VAF) ≥20% either at diagnosis or at relapse and VAF<5% at remission. We filtered for mutations with translational consequences, excluded known error-prone genes and dismissed common germline polymorphisms (dbSNP 138; MAF≥1%).

Results

We identified a total of 777 genes to be somatically mutated, of which 104 were recurrently affected. Mutation frequencies of 12 genes found mutated both in our cohort and in 86 CN-AML patients reported by The Cancer Genome Atlas [2] are shown in Figure 1 A. Seven genes were recurrently altered only at diagnosis (e.g. *CBL*) and 16 genes were recurrently altered only at relapse in our cohort (e.g. *KDM6A, SF3B1 and SRSF2*). At diagnosis, the number of somatic mutations per patient varied between 5 and 34 (median: 17). At relapse, the number of mutations ranged from 2 to 57 (median: 17). Mutations in several AML-associated genes (e.g. *DNMT3A, RUNX1, IDH1* and *IDH2*) showed similar VAFs at diagnosis and relapse in the vast majority of cases. In contrast, *WT1* mutations were gained at relapse in 4/6 (67%) patients and *FLT3* point mutations were below 5% VAF at relapse in 7/12 (58%) patients initially positive for these variants. In total, 92 mutations present at diagnosis were lost at relapse (VAF <5%) while 116 mutations were acquired during disease progression.

Fig. A: Recurrently mutated genes at diagnosis and relapse

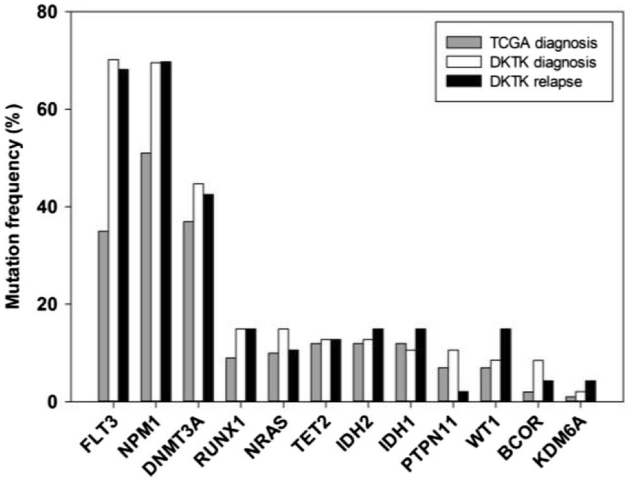
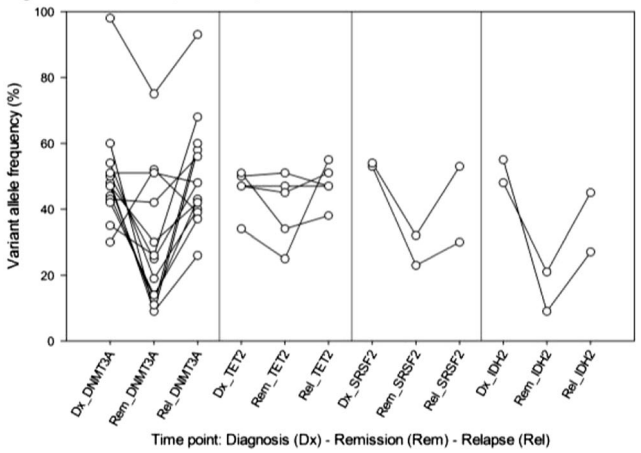


Fig. B: Patterns of persisting mutations



Clonal evolution, i.e. gain or loss of mutations at the time point of relapse, was observed in 42/47 (89%) of patients. We evaluated clonal heterogeneity based on data from targeted re-sequencing. So far, analysis is complete for 32 patients. At diagnosis, 31/32 samples showed evidence for the presence of multiple subclones. In 19 patients (59%), the major clone at diagnosis remained predominant at relapse whereas expansion of a subclone during disease progression was observed in 13 patients (41%). Based on cytogenetics and copy number alteration (CNA) analysis of exome data, we detected partial or complete gain/loss of chromosomes. Five patients (11%) acquired chromosomal alterations during disease progression. Trisomy 8 was the only recurrent chromosomal abnormality gained in 3 patients (6%) at relapse.

To detect pre-leukemic lesions [3, 4], we evaluated our exome data for the persistence of mutations in 40 AML-associated driver genes during remission. We limited our analysis to mutations previously reported as confirmed somatic (COSMIC annotation) to avoid confounding with private germline variants. Strikingly, 22/47 (47%) of patients carried non-silent mutations in these genes with VAF>5% (median: 31%, range: 9-75%) at remission (27 mutations in total). In contrast, other mutations (e.g. in *FLT3* or *NRAS*) found in these patients could not be detected at remission, consistent with therapy response. Based on VAF, 22/27 81%) persistent mutations showed a dynamic pattern over the course of disease with a relative change of >20%, likely due to partial eradication/expansion of leukemic or pre-leukemic clones. Persistent mutations in *DNMT3A, TET2, SRSF2 and IDH2* were observed in 28%, 11%, 4% and 4% of patients in our cohort, respectively (Figure 1 B). Among patients with *DNMT3A* mutation at diagnosis, those with persistent mutations tended to relapse earlier (n=13; median time to relapse 270 days; range: 81-586) than patients without detectable *DNMT3A* mutations at remission (n=7; median time to relapse 508 days; range: 235-1697; p=0.111).

Conclusion

Our findings provide insights into the genetic evolution during the course of disease in a large cohort of relapsed CN-AML. Information about the dynamics of genetic lesions (e.g. persistent or relapse-specific mutations) may have prognostic significance and allow for tailored approaches to treat or to prevent relapse of AML.

Conflict of interest: The authors declare that they have no conflict of interest

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Epigenetic (De)Regulation in Stem Cells in AML and MDS

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Introduction

Acute myeloid leukemia (AML) is characterized by disruption of hematopoietic stem and progenitor cell differentiation. Disruption of function of stem cell and differentiation stage-specific transcription factors is frequently observed in AML patients, and recent work has shown that AML is associated with mutations in genes encoding epigenetic modifiers. Furthermore, newer data suggest that aberrations at the transcriptional and epigenetic level are founding events in the multistep pathogenesis of AML.

Methods

We studied DNA cytosine methylation during physiological hematopoietic stem cell (HSC) differentiation hypothesized, reasoning that analysis of alterations in DNA methylation patterns during healthy HSC commitment and differentiation would yield epigenetic signatures that could be used to identify stage-specific prognostic subgroups of AML. We performed a nano HpaII-tiny-fragment-enrichment-by-ligation-mediated-PCR (nanoHELP) assay to compare genome-wide cytosine methylation profiles between highly purified human long-term HSC, short-term HSC, common myeloid progenitors, and megakaryocyte-erythrocyte progenitors.
We observed that the most striking epigenetic changes occurred during the commitment of short-term HSC to common myeloid progenitors and these alterations were predominantly characterized by loss of methylation. Analysis of changes at the level of individual loci showed that between the progression of LTHSC to STHSC, 40% of the 509 significantly differentially methylated loci were demethylated and 60% were more methylated in the STHSC cells. Similarly, transition from CMP to MEP identified 127 loci (52%) with decreased methylation levels and 116 (48%) with increased methylation levels, which indicates predominantly balanced changes in the numbers of hypomethylated and hypermethylated loci. However, the transition between STHSC to CMP showed that, of the 793 differentially methylated loci, 95% were more methylated in STHSCs than in CMPs.
We developed a metric of this HSC commitment–associated methylation pattern, and then applied this epigenetic signature to analyze published gene expression data and clinical outcome data from patients with AML. The HSC commitment-associated signature proved to be highly prognostic for overall survival in 3 independent large AML patient cohorts, regardless of patient treatment and epigenetic mutations. Interestingly, mutations in genes that are known to regulate DNA cytosine methylation, such as isocitrate dehydrogenase 1 (*IDH1*), *IDH2* and methylcytosine dioxygenase 2 (*TET2*), were not enriched in either the 'signature score high' or the low-scoring patients. Application of the epigenetic signature metric for AML prognosis was superior to evaluation of previously published commitment-based gene expression signatures.

Conclusion

Together, our study has delineated a human stem cell commitment–associated methylome, and shows that that assessing the status of HSC commitment using an epigenetic signature is an independent tool for prognostication of overall survival in patients with AML.

Conflict of interest: A provisional patent application for the diagnostic use of the described stem cell commitment-associated methylation signature in AML has been filed by the Albert-Einstein-College of Medicine.

Next-Generation Dendritic Cells for Immunotherapy of Acute Myeloid Leukemia

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Abstract

Postremission therapy of patients with acute myeloid leukemia (AML) is critical for the elimination of minimal residual disease (MRD) and a prerequisite for achieving cure. Cellular immunotherapy is a highly effective treatment option as demonstrated by the low relapse rate after allogeneic stem cell transplantation (SCT). However, many patients are not eligible for this treatment. Therapeutic vaccination with autologous dendritic cells (DCs) is a promising strategy to induce anti-cancer immune responses. We have developed a GMP-compliant protocol for the generation of *next-generation* DCs. A short 3-day differentiation period is combined with a novel maturation cocktail including a TLR7/8 agonist, resulting in DCs characterized by a positive costimulatory profile, high production of IL-12p70, polarization of T helper cells into Th1 and efficient stimulation of cytotoxic T lymphocytes and NK cells.

Material and methods

In a current proof-of-concept phase I/II clinical trial we are evaluating *next-generation* DCs as postremission therapy for AML patients with a non-favorable risk profile (NCT01734304). Standard exclusion criteria apply, and patients have to be ineligible for allogeneic SCT. DCs are generated from patients´ monocytes and loaded with RNA encoding the leukemia-associated antigens WT1, PRAME or CMVpp65 as an adjuvant and surrogate antigen. Patients are vaccinated intradermally with $5x10^6$ DCs of each of the three different batches up to 10 times within 26 weeks. Primary endpoints are feasibility and safety, and secondary endpoints include immune responses and disease control with a particular focus on MRD conversion. Phase I will include 6 patients, and phase II another 14 patients.

Results

So far, six patients have been enrolled and three of them have been vaccinated for at least six times each. DCs fulfilled all quality criteria (phenotype, viability, sterility, cell count, purity), and after thawing maintained their positive costimulatory profile as well as their capacity to secrete high amounts of IL-12p70. DCs expressed all three antigens and were able to induce a selective T cell response *in vitro*, suggesting proper antigen processing and presentation. In vaccinated patients a delayed type hypersensitivity reaction developed. Apart from erythema and itching at the injection site, no higher grade adverse events have been observed. As of yet, all vaccinated patients are relapse-free.

Conflict of interest: The authors declare that they have no conflict of interest

DH2 Inhibitors in Acute Myeloid Leukemia

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Abstract

Isocitrate dehydrogenase (IDH) mutations were first identified in acute myeloid leukemia (AML) in 2009, and by 2013, IDH inhibitors were brought into the clinic for patients with relapsed/refractory AML. IDH2 inhibitors have shown unprecedented activity in a notoriously difficult to treat disease, demonstrating the power of bench-to-bedside research and the translation of genomic findings into the clinic.

Introduction

IDH is an enzyme that interconverts isocitrate and a-ketoglutarate (aKG) as part of the citric acid cycle. Mardis and colleagues first

identified an IDH mutation in AML after performing massively parallel sequencing on 188 AML samples, noting the presence of IDH1 mutations in 8% of patients[1]. Cancer-associated point mutations in IDH1 lead to the acquisition of aneomorphic activity that allows for the NADPH-dependent reduction of aKG to the oncometabolite (R)-2 hydroxyglutarate (2HG) which is present in vast excess in patients with IDH1 mutations[2]. The demonstration of elevated 2HG levels in IDH1-wildtype AML patients ultimately led to the discovery of IDH2-mutant AML, which occurs in 8-12% cases[3-5]. IDH mutations affect one of three active-site arginine residues of the enzyme, IDH1/R132, IDH2/R140, or IDH2/R172. The expression of either the IDH1 or IDH2 mutant in AML induces an increase in global DNA hypermethylation, and leads to a block in cell differentiation[6,7]. IDH1 and IDH2 mutations are mutually exclusive in the great majority of cases, and the IDH2/R172 mutation appears to be mutually exclusive with mutations in NPM1 and FLT3-ITD[4,8-11].

There have been inconsistent findings regarding the prognostic impact of IDH mutations in AML. One study showed a lack of a prognostic association with IDH1 or IDH2 mutations[9]. Two studies reported that IDH2 mutations are associated with a favorable prognosis when IDH-2 mutations co-occur with NPM1 mutations, although the favorable effect was restricted to patients with the IDH2/R140 mutations[10,12]. One study reported a negative prognostic association to IDH1 mutation alone[8], yet others report both IDH1 and IDH2 mutations as associated with an unfavorable prognosis[4,11]. Needless to say, this is an area that warrants further investigation prior to drawing final conclusions regarding the prognostic impact of these mutations.

Pre-clinical data with IDH2 inhibitors

Wang and colleagues developed a small molecule IDH2 inhibitor, AGI-6780, which was demonstrated to potently and selectively inhibit the IDH2/R140Q mutant. The investigators treated ex vivo human AML cells (IDH2/R140Q mutant cells and IDH2/wild-type cells) with AGI-6780. The IDH2 mutant cells demonstrated a dose-dependent reduction in the amount of both intracellular and extracellular 2HG. Further, a burst of cell proliferation was observed in the IDH2 mutant cells treated with AGI-6780, with an increase in the number of viable, more mature CD45-positive cells. After 6 days of treatment, there was a dose-dependent decrease in the percentage of blast cells. These effects did not occur in the IDH2/wild-type cells. The authors concluded that AGI-6780 is able to promote outgrowth and differentiation of the cells harboring the IDH2 mutation, and should be investigated clinically[13]. AG-221 is a potent, selective inhibitor of the IDH2 mutant enzyme, and it has been demonstrated to reduce 2HG levels by >90%, to reverse histone and DNA hypermethylation *in vitro*, and to induce differentiation in leukemia cell models. Yen et al. evaluated the efficacy of AG-221 in an IDH2/R140Q-mutant AML xenograft model and found that AG-221 reduced 2HG in bone marrow, plasma, and urine of the engrafted mice. This conferred a survival benefit to treated mice, and survival correlated with the onset of cellular differentiation[14].

Clinical experience with IDH2 inhibitors

The pre-clinical data for IDH2 inhibition led to the development of a phase I, multi-center, open-label, dose-escalation study, examining AG-221, a first-in-class, potent, reversible, selective inhibitor of the mutant IDH2 enzyme. The data from the phase I trial (NCT01915498) were presented at the 56th American Society of Hematology (ASH) meeting in December 2014. Eligibility criteria included presence of an IDH2 mutation in advanced hematologic malignancies. The medication was continuously administered orally as a single agent either once daily or twice daily in 28-day cycles. The first cohort received a dose of 30 mg twice daily, and sequentially higher doses are ongoing (currently at 150 mg twice daily or 200 mg once daily)[15].

To date, 48 patients have been dosed and 27 remain on study drug as of July 2014. AG-221 has been well tolerated with the majority of adverse effects being grade 1 and 2, and maximum tolerated dose has not yet been reached. Of the nine deaths, eight occurred within the first cycle of therapy, with only one death being reported as "possibly" related to study drug in a patient with severe pneumonia[15].

At time of the ASH presentation, 32 patients had undergone day 28 bone marrow biopsies and were evaluable for efficacy. Investigator-assessed objective responses have been seen in 20 patients, with 8 complete responses (CR), 1 CR with incomplete platelet recovery, 3 CR with incomplete blood count recovery, and 8 partial responses. Five patients have stable disease and continue on study drug, and seven patients have had progressive disease (**Table 1**). Responses seem durable with a CR lasting up to 4.5 months and ongoing among current subjects. Five patients who achieved CR proceeded to allogeneic stem cell transplantation[15].

Response	N (%)
CR	8 (25%)
CRp	1 (3%)
CRi	3 (9%)
PR	8 (25%)
Stable disease	5 (16%)
Progression	7 (22%)

Table 1: Objective responses of the 32 evaluable patients

Conclusion

IDH2 inhibition has shown unprecedented activity in relapsed/refractory AML patients whose leukemia cells express IDH2 mutations in this phase I study. These findings warrant further investigation in the phase II setting. Additional considerations for the future include whether IDH2 inhibition can be moved into the front-line setting in combination with conventional induction therapy and whether IDH2 inhibition should be continued post-transplant for maintenance. IDH1 inhibitors are now in clinical trials for patients with IDH1 mutant AML. Ultimately, IDH2 inhibitors have served as a powerful model for the translation of genomic findings into the clinic.

Conflict of interest: The author declares that he has no conflict of interest

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Chimeric Antigen Receptors for Specific Targeting of Acute Myeloid Leukemia

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Abstract

Despite the progress in the treatment of acute myeloid leukemia (AML), a significant number of patients are still refractory or relapse after conventional chemotherapy. Immunotherapy with T cells genetically modified to express chimeric antigen receptors (CARs) represents a novel alternative approach option. CARs are artificial T-cell receptors constituted by a specific antigen-binding domain, and a signaling region, that, upon antigen recognition, leads to T-cell activation and lysis of the target cells. AML is a potential optimal target because of over-expression of a number of surface antigens like CD33, CD123. In the present report we will describe the *in vitro* and *in vivo* efficacy and safety of the anti-AML CAR approach based on Cytokine-Induced-Killers (CIK) cells genetically modified to express CAR molecules specific for the CD33 or CD123 antigens.

Introduction

Acute myeloid leukemia (AML) is a heterogeneous clonal disorder characterized by the accumulation of abnormal immature blasts, still associated with high rate of recurrence when treated with conventional regimens (Estey and Dohner 2006). In the last years, great interest has been focused on the identification of surface molecules that are preferentially expressed by AML cells and leukemia stem cells (LSCs), in order to selectively target the tumor population, whilst sparing the normal counterpart of hematopoietic stem/progenitor cells (HSPCs) (Majeti 2011), and possibly impeding disease recurrence. To this aim, the genetic engineering of T cells by the use of chimeric antigen receptor (CAR) has changed the therapeutic landscape of immunotherapy approaches, and very promising results have been reached also in the clinical setting (Davila 2014). CARs are artificial T cell-receptors constituted by an extracellular-antigen-binding domain (consisting of the variable chains of a monoclonal antibody linked together as a single chain Fragment variable-scFv-) and an intracellular-signaling region (usually the ζ chain of the TCR/CD3 complex) that is triggered after antigen recognition, leading to T-cell activation, with consequent killing of target cells and cytokine release (Gross *et al,* 1989).
A proper effector population to be redirected by CARs is represented by Cytokine-induced killer (CIK) cells, a heterogeneous population, enriched in CD3+CD56+ compartment, sharing some functional properties with NK cells (Baker 2001). CIK cells can lyse different tumor targets in a non-MHC restricted manner, and display anti-tumor activity in vivo (Nishimura 2008). Additionally, they show negligible allo-reactivity and thus induce minimal graft versus host disease (Introna 2007; Sangiolo 2011). Thus, arming CIK cells with a CAR molecule could represent a suitable strategy to target AML. To address this issue, we compared *in vitro* and *in vivo* the efficacy and safety of CIK cells genetically modified to express CAR molecules specific for two AML-overexpressed antigens, CD33 and CD123.

Methods

CIK cells were transduced with SFG-retroviral vectors encoding anti-CD33.CAR and anti-CD123.CAR. For the *in vitro* studies first generation CAR have been used, while for the *in vivo* studies third generation CARs have been designed, carrying CD28 and 0X40 co-stimulatory molecules within the endodomain of the CAR. The efficacy and safety profiles of transduced cells were characterized *in vitro* by short and long-term cytotoxicity assays. Their potential reactivity against HSPCs was evaluated by colony-forming unit assay after 4-hours co-incubation. *In vivo* tests were performed using a xenograft model of immunocompetent mice (NSG), that were treated with CAR-redirected CIK cells, upon injection of primary AML cells to test the efficacy profile, or CD34+ cord-blood-derived cells to the safety profile against HSPCs.

Results

Anti-CD123.CAR$^+$CIK cells were able to strongly kill CD123$^+$ THP-1 cell line (60%±5.4%, Effector:Target –E:T- *ratio* of 5:1, n=3), as well as primary AML blasts (59%±5.4%, E:T *ratio* of 3:1, n=4) in 4-hours citotoxicity assays. The same results were observed with anti-CD33.CAR$^+$CIK cells, as expected according to the overexpression of both target antigens. In long-term cytotoxicity assay we used as targets the

THP-1 cell line and primary AML blasts, observing that anti-CD123.CAR-transduced CIK cells were lytic against the target populations, equally to CD33.CAR$^+$CIK cells. Indeed, we observed at an E:T *ratio* of 1:100 an average THP-1 survival of 3.5%±1.5 (n=5) and a primary AML blasts survival of 2.4±1.4 (n=3), for co-cultures with CIK cells expressing anti-CD123.CAR, compared to an average survival of 82.7%± 7.4% (THP-1) and of 72.8±6.6 (AML blasts) when co-cultured with unmanipulated (NT) CIK cells. Interestingly, secondary colonies experiments after co-culture of cord blood-derived HSPCs with anti-CD123.CAR$^+$CIK cells demonstrated that anti-CD123.CAR better preserved the normal hematopoietic reconstitution in contrast to anti-CD33.CAR (total number of colonies of 146.8 ±6.6, 66.4±5.1, 117.6±4.6, after co-culture with NT CIK cells, anti-CD33.CAR$^+$CIK cells, anti-CD123.CAR$^+$CIK cells respectively, n=4). Furthermore, a limited killing of normal CD123$^+$monocytes and CD123-low expressing endothelial cells was measured.

Once injected into low-level AML engrafted NSG mice (median of hCD45$^+$CD33$^+$ 0.6% before treatment), genetically modified T cells had a potent antitumor effect. Indeed, the bone marrow of control untreated animals or mice treated with un-manipulated CIK cells, was infiltrated by leukemic cells (86% and 81% leukemic engraftment), while in 7/8 anti-CD33-CD28-OX40-ζ and in 8/10 anti-CD123-CD28-OX40-ζ treated mice AML cells were not detectable. Similar results were obtained when T cells were injected in mice with an established high AML burden (median of hCD45$^+$CD33$^+$ 70% before treatment). One week after the last CIK injection the level of AML engraftment was 96%, 87%, 0.35% and 0.34% for untreated mice, mice treated with un-manipulated CIK cells and with anti-CD33-CD28-OX40-ζ and anti-CD123-CD28-OX40-ζ transduced CIK-cells, respectively. We performed secondary transplantation on residual AML cells and mice were treated again with transduced CIK cells. Residual AML cells were still sensitive to CARs approach, leading once again to an almost complete eradication of the disease (median level of hCD45$^+$CD33$^+$ engraftment was 98%, 0.02% and 0.04% respectively for untreated mice, anti-CD33-CD28-OX40-ζ and anti-CD123-CD28-OX40-ζ transduced CIK-cells).

Furthermore, a fundamental issue was to determine the safety profile of such approach against normal haematopoietic precursors. In untreated mice injected with primary cord blood-derived CD34$^+$ cells the level of engraftment of hCD45 compartment was 42%, whilst in mice treated with un-manipulated, anti-CD33-CD28-OX40-ζ or anti-CD123-CD28-OX40-ζ transduced CIK-cells the levels of human compartment was 40%, 11.7% and 26.3% respectively. Moreover, when we considered specifically the CD34$^+$CD38$^-$ compartment, enriched in HSC, the level of engraftment was 1.92%, 1.02%, 0.55% and 0.83%. Secondary transplantations using remaining HSC repopulating cells confirmed these findings.

Conclusion

Taken together, our results indicate that both anti-AML CARs strongly enhances anti-leukemic CIK functions towards AML, having anti.-CD123 CAR with an improved safety profile on normal HSPCs compared to anti-CD33.CAR, paving the way for the development of novel immunotherapy approaches for the treatment of resistant forms of AML.

Conflict of interest: The authors declare that they have no conflict of interest

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NGS Based MRD Detection in Leukemia

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Abstract

The detection of minimal residual disease (MRD) has become one of the most important aspects in the treatment of patients with leukemia, especially in CML, ALL and AML. Several studies have shown that detection of MRD is able to identify patients at risk for early disease recurrence. While translocations, like in CML and small subgroups of patients with AML or the IgV_H or TCRB rearrangements are very suitable targets for sensitive and quantitative detection of MRD, the predominant genetic lesion in AML and MDS are single base pair point mutations or small insertion or deletions, which can be much more difficult to detect at a comparable level of sensitivity.

Next generation sequencing (NGS) is a novel technology, which is able to generate massive amounts of sequencing data (up to 10^{12} bases). Several groups have shown that this technology can be used not only to resequence genetic material but also to identify variant alleles at very low frequency (1, 2). We aimed to use NGS for the sensitive detection of MRD, especially in AML. However, pilot experiments using established protocols clearly showed insufficient sensitivity due to an increased background, predominantly induced by DNA-polymerase base misincorporation.

In order to overcome these inherent limitations and artifacts induced during data analysis due to subsampling and barcode allocation, we performed extensive optimization of the reaction parameters and developed novel algorithms for data analysis. Using the optimized conditions, we were able to detect variant alleles in genes like DNMT3A, JAK2, IDH1, IDH2, BRAF, KIT, KRAS, NRAS with a sensitivity down to 1/50.000 cells, making the method applicable to detect MRD.

A major advantage of this procedure compared to other currently available methods, e.g. digital-PCR, is the quantitative, scalable and flexible mode of detection allowing for MRD assessment based on genetic alterations present in DNA-stretches up to 100 bp.

Conflict of interest: The author declares that he has no conflict of interest

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HLA-Haploidentical Transplantation in High-Risk Acute Leukemia

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Introduction

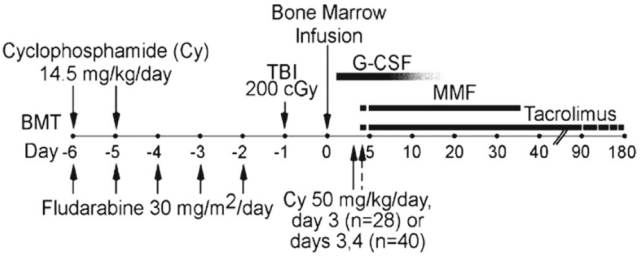
HLA-haploidentical hematopoietic stem cell transplantation (haplo-HSCT) is a valuable treatment option for patients with various hematologic disorders who lack an HLA-matched donor. The high and ready availability of a one HLA-haplotype-mismatched family donor is an important advantage of this alternative strategy, especially in patients who do not have an HLA-sibling donor, but need an urgent transplant due to aggressive, active disease. Historically, the main risks limiting the benefit of haplo-HSCT were graft rejection, severe graft-versus-host disease (GvHD) and early death due to toxicity and infections. To allow for crossing of the HLA-barrier,
HLA-haploidentical transplantation strategies were developed involving intensive preparative regimens and in-vivo and ex-vivo T cell depletion (TCD). Unwanted consequences of these approaches were reduction of cellular immunity, and delayed immune reconstitution resulting in high rates of mortality due to infections, toxicity and relapse. The use of reduced intensity conditioning [1,2], infusion of mega doses of CD34+ cells [3], and graft manipulations such as selective T cell depletion [3] were helpful to achieve engraftment with lower rates of GvHD, but relapse and infectious complications remained a problem, in particular in patients with high-risk, non-remission diseases. Thus, the development and employment of strategies that allow a safe engraftment with low rates of GvHD, toxicity and infection while providing a sufficient disease control have been an important task in haplo-HSCT.

T-cell-replete HLA-haploidentical transplantation

Recently, novel strategies using T-cell-replete grafts (TCR) and high-dose cyclophosphamide (HD CY) post-transplantation have made haplo-HSCT markedly safer (TCR/HD CY approach; Figure 1). Encouraging results inducing remarkable tolerance were achieved showing low rates of graft failure (13%), GvHD (acute grade II-IV: 6%; chronic: 5%) and non-relapse mortality (NRM at one-year: 15%)[4], as well as improved immune reconstitution and less opportunistic infections in comparison to a TCD strategy using CD 34+ selected grafts and in-vivo T cell depletion with anti-thymocyteglobulin (ATG) [5].

So far, over a number of years the Baltimore/Seattle group was able to successfully translate the experimental hypothesis of a bi-directional immunologic tolerance which is induced by the use of HD CY applied in a narrow time window post-transplantation into a clinical protocol. They hypothesized, that HD CY applied after transplantation results in a selective depletion of proliferating, allo-reactive T cells which are responsible for graft rejection and GvHD, while resting cells such as memory T cells which provide post-transplant immunity to infections were spared. It was been shown that resistance of hematopoietic cells in the graft to the toxic effects of CY can be mediated through the enzyme aldehyde dehydrogenase (ALDH) which is contained in those cells on a high expression level [6]. In addition, regulatory T cells have also the ability to up-regulate the cytoplasmatic enzyme ALDH which is responsible for the detoxification of activated CY in an inactivated form [7]. Thus, acute and chronic GvHD was effective prevented by this TCR/HDCY approach with no impact of the higher degree of the HLA-disparity on the rate of GvHD or NRM while a trend to a lower relapse risk and improved event-free survival was observed [8].

Fig1



(Luznik L et al., Biol Blood Marrow Transplant 2008

T-cell-replete HLA-haploidentical transplantation in high-risk acute leukemia

However, disease control achieved by those non-myeloablative TCR/HD CY haplo-HSCT protocols was disappointing with more than one half of the patients relapsing after one year, and relapse being the predominant cause of treatment failure. One attempt to improve disease control in patients with high-risk disease (refractory, relapse) is to itensify conditioning, the other is to use peripheral blood stem cells (PBSCs) instead of bone marrow. Accordingly, the Atlanta group treated 20 patients with hematologic malignancies (high-risk relapsed/refractory n=11) using the TCR/HD CY platform for myeloablative conditioning with fludarabine and busulfan [9]. All patients engrafted. While the incidence of acute GvHD grade II-IV was 46% in patients with relapsed and refractory diseases, and although they observed a rather high rate of CMV reactivation (81%) and BK-virus associated hemorrhagic cystitis (75%), NRM (10% at one year) was unexpected low. Control of disease was favorable for the 20 patients of the entire cohort (one-year DFS 50%), but survival data for the high-risk group with relapsed and refractory disease were not shown in particular. They conclude that myeloablative T-cell-replete haplo-HSCT is a valid option in patients with high-risk disease who lack the access to a conventional donor in a reasonable timeframe.

The Genua group used the TCR/HD CY platform and performed a myeloablative conditioning consisting of thiotepa, busulfan and fludarbine or fludarabine and total body irradiation (TBI) in 50 patients with hematologic malignancies [10]. In contrast to the Baltimore protocol, the calcineurin inhibitor and MMF were started before the initiation of HD CY to better control GvHD, and the second dose of HDCy was applied on day +5 rather than of day +4 to reduce toxicity. While the impact of these alterations in immunosuppression remains unclear, this protocol allowed for a successful engraftment (90%) with low rates of GvHD (acute grade II-IV: 12%; chronic 10%) and acceptable NRM for relapsed patients (26%). However, with a short follow up (in median 8 months) DFS at 22 months was 68% for patients in remission, while it was only 37% in patients with active disease. They observed – consistent with the findings of the Atlanta group - a rather high rate of hemorrhagic cystitis (40%) with predominance after the busulfan-based conditioning.

In contrast to the Baltimore group, the TCR haplo-approach of the Peking group utilized an intensive preparative regimen including in-vivo T cell depletion with ATG and a combined graft (peripheral blood stem cells and bone marrow, both G-CSF mobilized), while post-grafting immunosuppression consisted of cyclosporine A, methotrexate and MMF [11]. The leukemic control for the patients with high-risk disease was acceptable (3-year DFS 59% for AML 59%, and 24 % for ALL), but NRM was markedly high in high-risk patients, reaching 30% in AML and exceeding 50% in ALL patients mostly due to severe infections. Rates of acute (46%) and chronic (54%) were also markedly higher as observed after the TCR approach of the Baltimore group using HD CY post-transplantation.

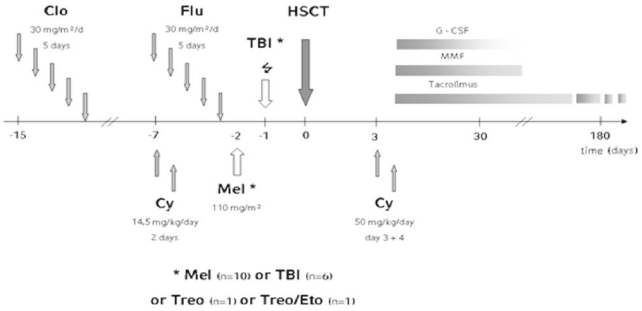
Results of sequential T-cell-replete HLA-haploidentical transplantation in the treatment of patients with high-risk acute leukemia and relapse after a first allogeneic transplantation

To enhance the disease control in patients with advanced, non-remission acute leukemia we adopted the TCR/HD CY haplo-HSCT approach of the Baltimore group and introduced sequential therapy utilizing clofarabine as cytoreduction [12]. Clofarabine has shown anti-leukemic acitivity, both in myeloid and lymphoid blasts. Over the past years the concept of a sequential therapy consisting of a cytoreductive chemotherapy followed by RIC in preparation for allogeneic hematopoietic transplantation (allo-HSCT) has been demonstrated to be an efficient and tolerable treatment strategy for patients with relapsed, refractory or high-risk acute myeloid leukemia undergoing HLA-matched transplantation at our center and elsewhere [13].

Sequential therapy addresses one major concern in non-remission patients: the reduction of the leukemic burden and the enhancement of the disease control by administration of a highly effective anti-leukemic chemotherapy before allo-HSCT in order to provide more time for the presumed graft-versus-leukemia (GvL) effect after allo-HSCT. We treated 18 patients (median age 39 years; nine male) with advanced, acute leukemia (AML n=15; ALL=3) who lacked a suitable HLA-matched donor. No patient was in remission prior to transplant. All patients received cytoreduction with clofarabine (5 x 30 mg/m2 IV over 5 days), followed after three days of rest by conditioning for T-cell-replete haplo-HSCT. HD CY (50 mg IV on day +3 and 4), tacrolimus (targeted levels 8-12 μg/l) and MMF (4 x 500 mg IV) were used for post-grafting immunosuppression. In absence of GvHD, MMF was stopped on day +35 and tacrolimus was tapered and discontinued until day +180.

In contrast to the Baltimore protocol disease-specific and individual modifications were undertaken in the last part of conditioning in most patients due to the high variability given among patients with relapsed and refractory non-remission acute leukemia. Hence, conditioning consisted of fludarabine, cyclophosphamide plus either melphalan, treosulfan/etoposide or TBI. The treatment schedule in detail is shown in Figure 2. Bi-directional tolerance was induced: while allowing successful engraftment, rates of acute GvHD II-IV (22%) und chronic GvHD (28%; no severe) were low. Cumulative incidence of NRM was 6% at day + 30, but increased to23 % at one-year with infectious complications being the main cause of NRM. Most commonly seen severe toxicities (grade III-IV) were transient elevation of liver enzymes (44%) and mucositis (40%), while the incidence of hemorrhagic cystitis was low (11%). Given the high-risk profile of our patients results were encouraging. This is reflected by a CR rate of 78% on day + 30, while OS was 56% and RFS was 39% one-year after sequential haplo-HSCT. In particular, outcome was favourable for relapsed-ALL patients with 2 out of 3 patient still alive two-years after haplo-HSCT. We conclude that sequential therapy is feasible in the context of the TCR-haplo HSCT approach and provides an acceptable toxicity profile and anti-leukemic activity in high-risk acute leukemia patients with active disease at time of transplant, while rates of GvHD and NRM were comparable to results of HLA-matched transplantation.

Fig2



(Tischer J et al. Ann Hematol 2013)

Using HLA-haploidentical donors and the TCR/HD CY haplo-HSCT platform proved also to be feasible and showed encouraging results in patients with acute leukemia relapse after a first allogeneic transplantation [14] . We retrospectively analyzed the courses of 20 consecutive adults (median age 37 years; 12 male) with AML (n=14), ALL (n=5) and acute bi-phenotypic leukemia (n=1). Conditioning consisted of fludarabine, CY and either melphalan or TBI or tresosulfan+/-etoposide.

While engraftment was achieved in 85%, the rate of grade II-IV acute GvHD was 35% and chronic GvHD occurred in five patients. Toxicity was moderate. Most commonly observed severe toxicities (grade III-IV) were mucositis (30%), hyperbilirubinemia (20%), elevation of transaminases (20%) and creatinine (20%), while invasive fungal infection affected 30%. One-year NRM was 36%. At a median follow-up of 17 months, one-year OS was 45% and RFS was 33%. We conclude that early outcome and NRM are at least comparable with results after a second allo-HSCT from HLA-matched donors without donor change at HSCT2. In addition, we observed that this TCR/HD CY approach may allow a fast immune reconstitution, even after a second allogeneic transplantation, which is comparable to that of HLA-matched transplantation. Accordingly, CD4+ T cells already reached median counts >200/μl on day +100 and achieved an almost normal level (median: 430/μl) on day +360 after a second haplo-HSCT.

T-cell-replete HLA-haploidentical transplantation in view of HLA-matched and alternative donor transplantation

Recently retrospective studies have shown that the TCR/HD CY haplo-HSCT approach is as safe and effective as transplantation from an HLA-matched related and unrelated donor [15], and is comparable to use of two umbilical cord blood transplantation using two units in adults [16]. In a single center study the Genua group has compared outcomes of patients undergoing their TCR/HD CY haplo-approach (as above described) with results after HLA-matched sibling donor, matched unrelated donor, mismatched unrelated donor and umbilical cord blood transplantation. They suggest that TCR/HD CY haplo-HSCT is comparable to that of sibling transplantation, while UCB transplantation provides an inferior survival [17].

Conclusions

Introduction of sequential therapy in the TCR/HD CY haplo-HSCT concept by our group proved to be feasible, safe and effective in treatment of patients with advanced, high-risk malignancies. Moreover, the TCR/HD Cy approach can provide a safe platform for a second allogeneic HSCT in patients with relapse of acute leukemia after a first HLA-

matched transplantation resulting in encouraging outcomes. Since the almost universal and ready availability of an HLA-haploidentical family donor and since the TCR/HD CY approach is un-expensive and simple requiring no expertise in graft manipulations and avoiding complicated, cost and time intensive GMP procedures, this approach can challenge strategies of donor choice in patients lacking an HLA-matched donor. In patients with active and aggressive hematological disease lacking an HLA-matched sibling donor sequential therapy utilizing HLA-haploidentical family donors and TCR/HD Cy platform might be our first choice in future. Further, in patients with advanced, non-remission disease at time of transplant the utility of this TCR/HD CY haplo-HSCT in particular regarding myeloablative conditioning regimens remains to be determined; comparative and prospective trials are needed.

Conflict of interest: The authors declare that they have no conflict of interest

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Targeting Eradication of BCR-ABL-Rearranged Acute Lymphoblastic Leukemia

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Introduction

Approximately one-third of B-cell acute lymphoblastic leukemias (B-ALLs) among adults harbor BCR-ABL rearrangements [1]. These cases have a particularly poor prognosis with conventional cytotoxic chemotherapy. However, the introduction of the ABL tyrosine kinase inhibitors (TKIs) imatinib, nilotinib and dasatinib into up-front regimens against BCR-ABL ALL has markedly improved rates of complete remission and disease-free survival [2]. Single-agent treatment of BCR-ABL ALL with a TKI results in complete hematologic remission (equivalent to >99.9% reduction in leukemia burden) in a significant fraction but is commonly followed by relapse [3]. The relapsed clones typically harbor mutations that affect TKI binding, most notably ABL T315I. The TKI ponatinib has activity against clones harboring T315I, but leads to hematologic remission in only one-third of patients after relapse [4].
Allosteric inhibitors of ABL kinase activity that bind the myristoylation site in ABL have recently been developed. We aimed to determine the therapeutic potential of a newer-generation myristate mimetic (NVP-ABL001), both alone and in combination with type II ABL inhibitors, in human BCR-ABL ALL. At the same time, we utilized these compounds to define the curative potential of prolonged and sustained inhibition of BCR-ABL in cases of B-ALL with this rearrangement.

Methods

We have established a panel of primary murine BCR-ABL B-ALLs that propagate in secondary transplant recipients. In addition, we established 7 BCR-ABL B-ALL patient-derived xenografts (PDXs) that propagate in NOD.Scid.*IL2R*$^{-/-}$ (NSG) mice. The latter include two PDXs with ABL T315I mutations, one PDX with a GNB1 K89E mutation [5] and 3 PDXs with BCR-ABL p210.

Upon engraftment, mice were randomized to treatment with vehicle, single-agent nilotinib, single-agent ABL001, single-agent ponatinib, or combinations of ABL001 with nilotinib or ponatinib (n=8 per group/line). Sentinel mice (n=2 per group/line) were sacrificed on treatment day 7 for pharmacokinetic and pharmacodynamic evaluation. This included peripheral blood flow cytometry for hCD19+/hCD45+ cells, BCR-ABL qRT-PCR, and spleen and bone marrow immunohistochemistry for leukemic burden, immunoblotting for phosphorylated BCR-ABL and downstream signal transduction molecules for on-target drug effect.
Mice were monitored on treatment by weekly peripheral blood flow cytometry until relapse (defined as >5% peripheral blood human CD45/human CD19-positive cells), at which point circulating leukemia cells were collected for evaluation of resistance mechanisms. Relapsed mice from single-agent nilotinib or ponatinib arms then crossed-over from monotherapy to combination therapy with ABL001. When mice progressed on combinations, they were sacrificed and leukemia cells were isolated from bone marrow and spleen.
We tracked the clonal architecture of BCR-ABL resistance mutations in the PDX mice by PCR amplification of the ABL kinase domain followed by next-generation sequencing (NGS). By sequencing to depth ~500,000, we can easily identify variants present at 10^{-3}-10^{-4} and distinguish these from background sequencing errors [6]. Mice in which acquired BCR-ABL mutations did not explain relapse (*i.e.,* clonal composition of the relapsed leukemia was primarily wild-type) were further subjected to unbiased genetic evaluation to identify BCR-ABL-independent mechanisms of resistance.
Continuous variables were compared using the t-test while the Fisher's exact test was performed to compare categorical variables. Overall survival was analyzed by the method of Kaplan and Meier and compared using the log-rank test.

Results

Results from these studies will be outlined at the Acute Leukemias XV meeting.

Conflict of interest: Dr. Weinstock is a consultant and receives research funding from Novartis

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Role of *TP53* Mutations in the Origin and Evolution of Therapy-Related Acute Myeloid Leukemia

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Abstract

Therapy-related acute myeloid leukemia (t-AML) and therapy-related myelodysplastic syndrome (t-MDS) are defined as AML or MDS arising after a previous exposure to cytotoxic chemotherapy or radiotherapy[1]. Compared to their *de novo* counterparts, these disorders are associated with a higher incidence of cytogenetic abnormalities including deletions in chromosomes 5 and 7, a poorer response to chemotherapy, and a worsened overall survival[2]. The key difference between the pathogenesis of t-AML and *de novo* AML is the exposure of the former to cytotoxic therapy during its evolution. However, the exact mechanism by which this exposure to cytotoxic therapy contributes to leukemogenesis remains unclear. In this presentation, we will discuss how recent findings by our research group on the genetic heterogeneity of AML and normal hematopoietic cells may shed light on the pathogenesis of t-AML and t-MDS.

Methods

Recent genomic studies performed by our research group have revealed AML to be a clonally heterogeneous disease[3]. Although somatic mutations arising early in the evolution of AML are present in every leukemic cell (i.e. in the founding clone), we are also often able to identify multiple subclonal leukemic populations at the time of diagnosis. Each subclone harbors its own set of unique somatic mutations in addition to the mutations present in the founding clone. These additional mutations have presumably provided the tumor cell acquiring them with a competitive advantage, allowing it to clonally expand. The selective pressure of cytotoxic therapy can dramatically alter the clonal architecture of AML, with certain (often minor or even undetectable) subclones at diagnosis becoming the dominant leukemic population at the time of relapse after induction chemotherapy[4]. The clonal evolution of tumor cells under the selective pressure of cytotoxic therapy is increasingly being recognized as a common phenomenon in a variety of cancers[5]. This is important clinically as curative therapy requires the elimination of both the founding clone and all of its subsequently arising subclones.

Our research group has revealed the hematopoietic progenitors from healthy individuals to be also genetically heterogeneous[6]. By performing exome sequencing on the progeny of sorted hematopoietic stem and progenitor cells (HSPCs) obtained from healthy donors, we discovered that these cells acquire somatic mutations as a function of age. HSPCs obtained from cord blood samples possessed a low number of validated somatic mutations. On the other hand, HSPCs from adult donors possessed an increased number of mutations, with the total number of mutations strongly correlating with the age of the donor. Thus, each individual possesses a genetically heterogeneous HPSC population with each HSPC having its own unique set of somatic mutations and with the total number of mutations increasing as the individual ages.

The majority of these age-related mutations are likely biologically and clinically insignificant. However, multiple studies have shown that, in elderly individuals without a known hematologic disorder, oligoclonal hematopoiesis often is identifiable[7-13]. This suggests that, in these individuals, a genetically distinct hematopoietic cell has gained a competitive fitness advantage over its counterparts and clonally expanded. Of particular note, our research group recently analyzed blood leukocyte sequence data from 2,728 patients enrolled in The Cancer Genome Atlas without a known hematologic malignancy[14]. Somatic mutations were identified in over 2% of the subjects and in over 5% of the subjects greater than 70 years of age. Many of these mutations were in genes with well-described roles in hematologic malignancies including *DNMT3A*, *TET2*, *ASXL1*, and *TP53*. This data suggests that mutations in these classic AML and MDS "driver" genes may provide the HSPCs acquiring them with a competitive advantage. Whether the presence of a clonally expanded HSPC population carrying a mutation in these genes predisposes an individual to the future development of AML or MDS remains to be determined.

Conclusion

In the case of t-AML and t-MDS, this selection is occurring under the influence of cytotoxic chemotherapy and/or radiotherapy. As with relapsed AML, cytotoxic therapy may potentially alter the hematopoietic clonal architecture of an individual with "normal" hematopoiesis, providing certain hematopoietic progenitors with a competitive fitness advantage. In this presentation, we will discuss how the influence of cytotoxic therapy on a genetically heterogeneous hematopoietic progenitor population may contribute to the pathogenesis of t-AML and t-MDS, resulting in diseases that are both clinically and biologically distinct from AML and MDS arising in the absence of cytotoxic therapy exposure.

Conflict of interest: The authors declare that they have no conflict of interest

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AGIHO – EDUCATIONAL SYMPOSIUM

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Conventional and Non-Culture Based Microbiological Diagnostics

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Introduction

Diagnosing the infectious causes of pneumonia and lung infiltrates that affect patients with leukaemia and recipients of a stem cell transplant presents several challenges to the clinician and to the laboratory. Firstly, there is no such thing as the optimal sample other than obtaining tissue directly from the lung which is not only hazardous but is also seldom possible given the patient population. Hence the aim is to obtain the best possible specimen that fits the purpose best. Sputum is seldom, if ever, useful. not least because the lack of neutrophils means it is not produced during neutropenia. Rather the respiratory secretions that are expectorated are just that but often very mucoid and viscous and may only represent the slime frequently associated with mucositis. Moreover bacteria recovered from culture typically represent the oral microbial flora unless, of course, a professional pathogen such as *Streptococcus pneumoniae* is recovered. Most often pulmonary infectious diseases are caused by opportunistic pathogens making any distinction between colonization and infection all but impossible.

Representative sampling

Many years ago it was assumed that aetiology of pulmonary infectious diseases could be deduced from blood cultures when bacteraemia occurred at the same time. Hence the aetiology was ascribed to bacteria and certain yeasts such as *Candida albicans* on that basis alone. However this notion has been rightly discarded as being wrong. With the advent of better radiology and fibre optic bronchoscopy it has become more the norm to sample the lung by instilling fluid typically saline into the lungs, washing the site and attempting to recover most of it for microbiological investigation. The technique is widely known as bronchoalveolar lavage and requires a skilled and experienced respiratory physician to perform it reliably and safely but only if indicated by radiography. It is also true to say that not all bronchoscopies are the same and neither are all BAL fluids. None the less the specimen obtained at least has the merit of giving a more representative sample of the affected area of the lung. Hence it is recommended in almost every guideline for diagnosing the cause of pulmonary infection of immunocompromised patients
The real challenge appears once the specimen has been obtained. What should be done with it and who should get what? Clearly there has to be an agreement if the right people are to get the right specimen to perform the right tests in a timely fashion.

Likelihood and prevalence

Before exploring specific microbiological investigations it is worth considering the question that is being asked in terms of the likelihood of any given infectious disease. For instance, if the prevalence of a given infection is very low then the test should lean towards excluding the diagnosis rather than confirming it. By contrast, if the prevalence is relatively high the test would be better employed to confirm the diagnosis reliably. Detecting galactomannan to diagnosis aspergillosis provides a useful example. Firstly, a bronchoscopy is only done when certain abnormalities are seen and there is least a real possibility of an infectious aetiology. Moreover nodules with or without a halo sign already meet the definition of possible disease thereby increasing the likelihood of aspergillosis markedly to perhaps as high as 30%. It is often forgotten that positive and negative predictive values of a given test are the product of test sensitivity or specificity and prevalence, and that one has usually to sacrifice sensitivity to obtain the highest specificity and vice versa. In the example of galactomannan it can be shown that although the analytical threshold is set at an optical index of 0.5 the clinical threshold should be set at a higher value least 1.0 to obtain the highest positive predictive value so that a positive test result is credible and confirms the diagnosis of aspergillosis. In most cases, a so-called typical radiographic appearance as described by the EORTC/MSG definitions during neutropenia is strongly associated with *Aspergillus* infection rather than one due to any other mould. The question remains what should be looked for in the BAL fluid? For instance should the presence of *Pneumocystis jerovecii* be detected as well in this case? If so, one would expect the result to prove negative irrespective of the test used given the low likelihood and would be surprised indeed if the fungus was detected. So should test for both fungi be done? If both testes yielded positive results what store should be placed in detecting *Pneumocystis* since the chances would be high that this would be a falsely positive result. Similarly, should BAL fluid be examined for the presence of bacteria, yeasts and viruses? Should a range of 20 plus tests be conducted "just in case"? If so not only is this costly but the value of the information will be difficult to ascertain as the result may be wholly inconsistent with the clinical signs and radiographic appearance. Moreover, the information may only add confusion rather than any real value. Hence the choice of tests needs to be guided by the clinical context and the population epidemiology to provide at least an estimate of likelihood.

Nucleic acid detection assays

Far from solving the problem, the adoption of an increasing number nucleic acid detection assays, typically PCR tests, may have aggravated it since detection of nucleic acid from a potentially pathogenic pathogen does not of itself help distinguish colonization from infection. There is also the additional problem of contamination to contend with and the lack of approved standards. The European *Aspergillus* PCR initiative (www.eapcri.eu) is making progress towards establishing a standard for *Aspergillus* PCR but, apart from CMV detection [http://www.nibsc.org/documents/ifu/09-162.pdf], no other PCR as been formally standardised and validated for this purpose. Quantitative techniques may help but there have been few, if any, studies done that help define the clinical as opposed to the analytical threshold.

Selection of tests

Hence, two things must drive the selection of tests. Firstly, the likelihood of a given opportunistic pathogen causing infection in a particular setting and secondly, whether or not the test chosen confirms or excludes the aetiology reliable. This aspect will be explored in full with the aim to proposing a core set of diagnostic tests and guidance on when to consider supplementary tests.
Conflict of interest: The author declares that he has no conflict of interest

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Immunocompromised Host: Typical Findings for Specific Lesions

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Aims of the presentation

Combination of pattern recognition with knowledge of the clinical setting is the best approach to pulmonary infectious processes. A specific pattern of involvement can help suggest a likely diagnosis in many instances. Different combinations of parenchymal and pleural abnormalities may be suggestive for additional diagnoses. When pulmonary infection is suspected, knowledge of the varied radiographic manifestations will narrow the differential diagnosis, helping to direct additional diagnostic measures, and serving as an ideal tool for follow-up examinations.
The aim of this presentation is to summarize the radiologic and CT findings of some of the most common infectious processes affecting the immunocompromised hosts.

Summary of the presentation

Despite advances in diagnosis and treatment, pulmonary infections are a major cause of morbidity and mortality in adult patients. Pneumonia is the sixth most common cause of death in the United States and more than 6 millions cases of bacterial pneumonia occurs each year in the immunocompetent population (1). Pulmonary infection is also a major cause of morbidity and mortality in patients with impaired immune function. In the last several decades, AIDS epidemic, advances in the treatment of cancer, organ transplantation, and immunossupresive therapy has resulted in large numbers of patients who develop abnormalities in their immune system (2-3). Mildy impaired host immunity as it occurs in chronic debilitating illness, diabetes mellitus, malnutrition, alcoholism, advanced age, prolonged corticosteroid administration and chronic obstructive lung disease have also been regarded as predisposing factors of pulmonary infections (4).
Diagnosis of pneumonia calls for a combination of clinical awareness, appropriate microbiologic tests, and radiographic studies.

Conventional Chest Radiography

Plain chest x-ray is an inexpensive test that can rapidly demonstrate the presence of pulmonary abnormalities. According to American Thoracic Society guidelines, posteroanterior (PA) (and lateral when possible) chest radiography should be obtained whenever pneumonia is suspected in adults (5).
In most cases the plain film findings may be diagnostic for pneumonia and may eliminate the need for additional radiographic procedures.
The more common radiographic findings include segmental or lobar consolidations and interstitial lung disease. Other less common radiographic findings include mediastinal lymphadenopathy, pleural effusion, cavitation, and chest wall invasion. Despite that, the nonspecificity of radiographic findings as well as the wide range of potential causes often lead to frustration when evaluating the imaging findings of a patient with suspected pneumonia.

High-resolution Computed Tomography

Computed tomography (CT) is a useful adjunct to conventional radiography in selected cases (6-9). There is a large literature indicating that CT is a sensistive method capable of imaging the lung with excellent spatial resolution providing anatomical detail similar to that seen by gross pathological examination. Differences in tissue attenuation and parenchymal changes caused by an acute inflammatory process can be seen readily by CT (8,9). Unlike chest radiography, CT provides cross sectional images and the pattern and distribution of pulmonary processes are therefore much more readily appreciated than on conventional examinations.
High-resolution CT can be helpful in the detection, differential diagnosis, and management of immunocompromised patients with pulmonary complications (9-11). Although accurate clinical information is essential to narrow the differential diagnosis, it is often still imposible to determine the cause of parenchymal abnormalities in this group of patients. Combination of pattern recognition with knowledge of the clinical setting is the best approach to pulmonary infectious processes. A specific pattern of involvement can help suggest a likely diagnosis in many instances.
The most common patterns seen at HRCT in acute pulmonary infections include nodules, tree-in-bud appearance, ground-glass attenuation, consolidation and airway disease.

Nodules

Angioinvasive aspergillosis occurs almost exclusively in immunocompromised patients with a severe neutropenia (12). The characteristic CT findings consist of nodules surrounded by a halo of ground-glass attenuation (“halo sign”) or pleural based wedge-shaped areas of consolidation. In severely neutropenic patients the halo sign is highly suggestive of angioinvasive aspergillosis (Fig.1). The ground-glass halo reflects the presence of hemorrhage surrounding the nodule. A similar appearance

has been described in a number of other conditions including infection by herpes simplex and cytomegalovirus.

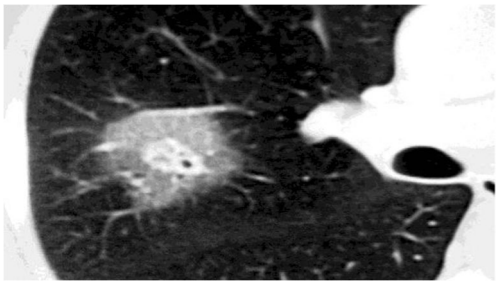

Fig1: Angioinvasive Aspergillosis

Close-up view of a CT scan shows the classical CT appearance of an angioinvasive pulmonary aspergillosis. A nodular opacity representing a lung infarction is surrounded by a ground-glass pattern of attenuation ("halo"). Although non-specific, this particular CT finding, in the appropriate clinical setting is highly diagnostic of angioinvasive aspergillosis. Nontuberculous mycobacteria infection may present as nodular lesions on the chest radiograph. The nodules may be single or multiple and are frequently associated with centrilobular nodules and branching opacities resulting in a "tree-in-bud" appearance (13).

"Tree-in-bud" pattern

The "tree-in-bud" pattern, first described in diffuse panbronchiolitis and endobronchial spread of tuberculosis, represent bronchioles filled with mucus or inflammatory material resulting in centrilobular tubular, branching, or nodular structures (13). A variety of bacterial, mycobacterial, fungal, and viral pathogens may cause bronchogenic dissemination and bronchiolar impaction by mucus or pus, resulting in typical "tree-in-bud" appearance. The centrilobular nodules have a patchy distribution in the lung and are similar to those seen in a number of different infectious conditions, including endobronchial spread of pulmonary tuberculosis, M. Avium-intracellulare, viral and mycoplasma pneumonia.

Ground-glass opacity

Ground-glass opacity is defined as a localized increase in lung attenuation that allows visualization of vascular structures coursing through the affected region. Ground glass attenuation is a common but nonspecific HRCT finding in the non-HIV immunocompromised patients. This finding may represent either alveolar or interstitial disease and may also be associated with different entities. In immunocompromised patients the differential diagnosis includes infections such as PCP (Fig3), CMV (Fig2) and Mycoplasma, drug induced lung disease, pulmonary hemorrhage and cryptogenic organizing pneumonia (10-11,14).

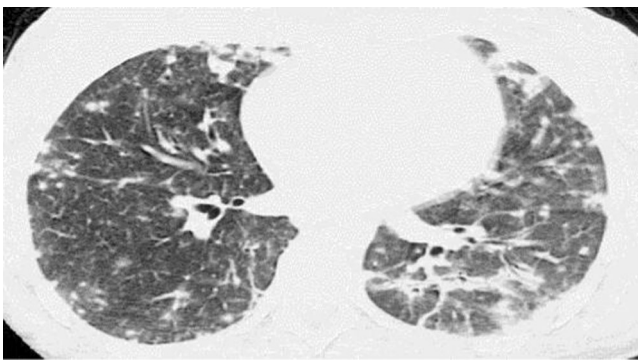

Fig2: CMV Infection

Thin-section CT scan shows bilateral and multiple patchy areas of ground-glass opacities in a 28-year-old woman 48 days after allogeneic BMT. Concurrent small nodules surrounded with a "halo" of ground-glass attenuation are also visible throughout the lungs. Transbronchial biopsy was diagnostic for CMV infection.

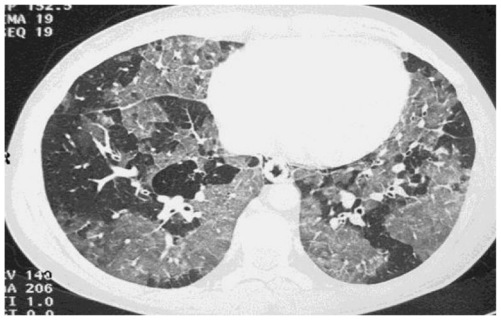

Fig3: PCP Infection

Pneumocystis jirovecii pneumonia in a 46-year-old inmunossupressed man. High-resolution CT image at the level of lung bases shows extensive ground-glass attenuation in both lungs, in a geographical appearance, with only a few focal areas of sparing

Consolidation

Focal or multifocal areas of consolidation may be seen with various infections. Bacterial infections are more frequent than fungal infections in non- HIV immunocompromised patients. Infections caused by fungi are most commonly seen in neutopenic patients with hematological diseases. A distinct form of aspergillus infection called semi-invasive or chronic necrotizing aspergillosis, may be seen in patients with chronic debilitating illness, diabetes mellitus, malnutrition, alcoholism, advanced age, prolonged corticosteroid administration, and chronic obstructive lung disease. Radiologically, unilateral or bilateral segmental areas of consolidation with or without cavitation and/or adjacent pleural thickening, and multiple nodular opacities are demonstrated (5). Mycobacterial infection may also cause focal areas of consolidation, with or without cavitation.

Airway disease

Infectious causes for airway disease include bacterial bronchitis, tuberculous involvement of the airway, and necrotizing tracheobronchial aspergillosis. Pyogenic infectious airways diseases have been increasingly recognized in recent years. Necrotizing bronchial aspergillosis is a rare form of invasive aspergillosis that may be seen on CT as an endobronchial mass, an obstructive pneumonitis and/or collapse, or as a hilar mass.. The diagnosis of this form of aspergillus infection is usually based on the presence of abnormal chest radiograph and bronchoscopic biopsy specimen consistent with tissue invasion (15).

Conflict of interest: The author declares that he has no conflict of interest

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Pneumonia and Lung Infiltrates in Leukemia and Stem Cell Transplant Patients: Risk-Adapted Antimicrobial Intervention in Patients with Hematological Malignancies

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Abstract

Up to 25% of patients with profound neutropenia lasting for >10 days develop lung infiltrates, but pathogens isolated from blood cultures, bronchoalveolar lavage or respiratory secretions may not be etiologically relevant. Testing for *Aspergillus* galactomannan, beta-D-glucan or DNA in blood, bronchoalveolar lavage or tissue samples may facilitate the diagnosis. Pulmonary toxicity from cytotoxic drugs or radiotherapy as well as pulmonary involvement by the underlying malignancy should be included into differential diagnosis and eventually give reason for invasive diagnostic procedures. In high-risk patients, pre-emptive treatment with mold-active systemic antifungal agents improves clinical outcome, while other microorganisms are preferably treated only when documented. High-dose trimethoprim-sulfamethoxazole is first choice for treatment of *Pneumocystis* pneumonia, while cytomegalovirus pneumonia is treated primarily with ganciclovir or foscarnet.

Introduction

Cancer patients with high-risk neutropenia developing lung infiltrates carry a high risk of treatment failure. Microbial etiology frequently remains undetermined, and differential diagnoses include lung involvement by the underlying malignancy, capillary leak syndrome, cryptogenic organizing pneumonia, immune reconstitution syndrome and lesions caused by chemotherapy or radiation [1-3]. It has not yet been demonstrated that diagnostic procedures such as bronchoscopy and bronchoalveolar lavage improve clinical outcome in these patients [4]. It has been reported that response to broad-spectrum antibacterial treatment is less than 30%, whereas mold-active systemic antifungals added to first-line antimicrobial treatment increases the response rate to up to 78%[5].

However, inadequate overuse of broad-spectrum antifungal agents must be avoided. In patients currently receiving mold-active antifungal prophylaxis, invasive diagnostic intervention is considered in order to detect rare pathogens such as *Nocardia* and non-microbial causes of LI. In contrast, patients who have been treated with nucleoside analogs such as fludarabine or cladribine or with long-term T-cell depleting agents such as alemtuzumab or antithymocyte globulin may be affected by microorganisms typical for severe T-cell immunosuppression such as Cytomegalovirus, *Pjirovecii,* mycobacteria, yeasts, pneumococci, *Staphylococcus aureus, Pseudomonas aeruginosa* or *Aspergillus* spp. Respiratory viruses such as *Influenza*, *Parainfluenza*, Coronavirus, Rhinovirus, *Human Metapneumovirus* or *Respiratory Syncytial Virus* may be relevant pathogens for respiratory tract infections [6] , particularly during the winter seasons, however, there are almost no effective antiviral drugs for the treatment of these infections available.

Methods

An updated guideline [7] was prepared by a multi-disciplinary group of experts in a stepwise consensus process. Systematic literature search was conducted by all co-authors, data were extracted and tabulated, preliminary recommendations were proposed for discussion and sent to all co-authors. Tables were revised after email-based discussion and presented for final discussion at the AGIHO guideline conference. If no clear consensus was achieved, majority voting of the conference was adopted. The 2001 criteria for strength of recommendation and quality of evidence published by the Infectious Diseases Society of America were used. For microbiologic diagnostics, only criteria for the strength of recommendation were used [8].

Treatment recommendations

Pre-emptive antimicrobial therapy in patients with severe neutropenia

These patients with should be treated with a broad-spectrum beta-lactam with anti-pseudomonal activity, as used for empirical treatment of unexplained fever *PLUS* (if lung infiltrates are not typical for PcP or lobar bacterial pneumonia) mold-active systemic antifungal therapy with voriconazole or liposomal amphotericin B. There is a significant benefit from prompt as compared to delayed mold-active antifungal therapy. In patients pretreated with voriconazole or posaconazole for systemic antifungal prophylaxis and in whom a breakthrough filamentous fungal pneumonia is suspected, measurement of antifungal drug levels and invasive diagnostic procedures should be discussed, and treatment should be switched to liposomal amphotericin B. Systemic antifungal treatment should be continued until hematopoietic recovery and regression of clinical and radiological signs of infection. In patients who had not received cotrimoxazole for prophylaxis and have a CT scan suggesting PcP, and who have a rapid rise of serum lactate dehydrogenase, prompt start of high-dose trimethoprim-sulfamethoxazole therapy should be started, even before bronchoscopy is performed. Antiviral agents such as ganciclovir are not recommended for early pre-emptive therapy, and glycopeptides, fluoroquinolones or macrolide antibiotics without a

specific pathogen documented from clinically significant samples should not be used as well.

Pre-emptive antimicrobial therapy in other subgroups of febrile patients with hematological malignancies

In individual patients undergoing high-dose chemotherapy and autologous hematopoietic stem cell transplantation with febrile neutropenia and LI of unknown origin, whose conditioning regimen included total body irradiation or who have been treated with alemtuzumab, anti-thymocyte globulin or fludarabine, cytomegalovirus should be considered. If a rapid culture or "immediate early antigen" is positive, prompt ganciclovir treatment is recommended, while antifungal therapy should not be given pre-emptively in this patient cohort.

Antimicrobial treatment in patients with documented pathogens

The interpretation of microbiological findings, typically originating from blood cultures or bronchial lavage samples, may be difficult. If etiologically significant pathogens are detected, particularly multidrug-resistant bacteria, critical revision of antimicrobial treatment therapy is recommended. For *Pseudomonas aeruginosa* pneumonia, primary combination antibacterial therapy including an antipseudomonal beta-lactam plus an aminoglycoside or ciprofloxacin is preferred, however, adequate beta-lactam monotherapy may also be appropriate [9]. Depending on their in-vitro susceptibility pattern, multi-resistant gram-negative aerobes such as Extended-Spectrum-Beta-Lactamase-producing *E.coli*, *Enterobacter* spp. or *Klebsiella* spp. as well as *Acinetobacter* spp. or *P.aeruginosa* require antimicrobial treatment selected appropriately according to this pattern. In individual patients, aerosolized colistin has been successfully used as a part of the antimicrobial strategy. For *Stenotrophomonas maltophilia* pneumonia, high-dose trimethoprim-sulfamethoxazole (15 to 20 mg/kg/day of trimethoprim) is recommended. In individual patients, tigecycline-based treatment may be an appropriate alternative [10]. Pneumonia caused by methicillin-susceptible *Staphylococcus aureus* should be treated with oxacillin or flucloxacillin, while methicillin-resistant *Staphylococcus aureus* (MRSA) should preferably be treated with vancomycin. Linezolid is a possible alternative for first-line treatment.

First-choice antiviral treatments for cytomegalovirus pneumonia are foscarnet or ganciclovir.

For treatment of possible, probable or proven pulmonary aspergillosis, intravenous voriconazole or liposomal amphotericin B are recommended, while for mucormycosis (zygomycosis), liposomal amphotericin B is preferred. Combination antifungal first-line treatment in patients with invasive mould infections is controversial.

If *Pneumocystis* pneumonia is suspected, treatment with trimethoprim-sulfamethoxazole (TMP/SMX; co-trimoxazole) at a dosage of TMP 15-20 mg/kg plus SMX 75-100 mg/kg daily should be initiated immediately after asservation of representive samples (induced sputum or BAL). Delay of treatment onset may increase the mortality rate. Therapy should be continued for at least 2 weeks. In patients with respiratory failure, systemic corticosteroids are controversially discussed. Recent studies could not show a clinical benefit [11].

Conflict of interest: Consultations: Gilead; Sponsored research: Pfizer; Honoraria: Astellas, Gilead, MSD, Pfizer

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Pulmonary Failure in Hematological and Stem Cell Transplant Patients: Indications and Limitations for Intensive Care

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Purpose

This article highlights the importance of the acute respiratory failure (ARF) in patients with hematologic malignancies (HM) and stem cell transplant (SCT) recipients in terms of prevalence, prognostic impact, indications and limitations for intensive care unit (ICU) therapies, early recognition of patients at risk, as well as therapeutic management with focus on ICU specific issues.

Background

The ARF depicts the most common reason for ICU admissions in patients with HM and SCT recipients.[1-3] In fact, the development of critical illness without respiratory involvement is rather uncommon in these patients.[4] Most cases of ARF are caused by pulmonary infections or extrapulmonary sepsis.[5] However, in newly diagnosed acute leukemia, ARF is predominantly caused by disease-related mechanisms, such as

hyperleukocytosis, pulmonary leukemic infiltration, or acute lysis pneumopathy.[6] Patients with undetermined causes of ARF have worse outcomes. Thus, identifying the underlying aetiology in order to establish the proper causative treatment is of upmost interest. Today, several prospectively evaluated algorithms are available, which assist physicians to choose the most appropriate diagnostic strategy in individual cancer patients with ARF.[7,8]

Prognostic impact

The ARF is one of the most important prognostic factors in patients with HM and SCT recipients and independently associated with adverse outcome.[1,3] Trivial clinical signs like cough, sputum, rales, or requirement of only 1 L/min oxygen already correlate with the development of ARF and, therefore, mortality.[4,9] Over the last decades ARF mortality rates have markedly dropped in patients with HM, but stay above 50% if mechanical ventilation (MV) becomes necessary.[1,5] However, mortality rates of SCT recipients with ARF and MV remain unacceptably high, especially in the presence of uncontrolled or refractory graft-versus-host disease (GVHD).[2,3,10]

Indications and limitations

In principal, ICU treatment should be considered if 1) the critical condition may be reversible from the perspective of an intensivist, 2) the hematologic or long-term prognosis and underlying comorbidities allow improvement of survival or quality of life, e.g. by intensive therapies, and 3) the patient does not refuse ICU treatment.

Full code

Accordingly, an evidence-based expert consensus recommends offering a "full code" management with unrestricted allocation of ICU resources including MV to critically ill cancer patients in case of: complete remission of the malignant disease, availability of curative therapeutic options (e.g. patients with induction or consolidation chemotherapy), newly diagnosed malignancy with life expectancy >1 year, complications of autologous SCT, myeloma with partial remission, and selected patients with low-grade hematologic malignancies (e.g. chronic lymphatic or myeloid leukemia and low-grade lymphoma). In patients after allogeneic SCT, full code management including MV is recommended in 1) the earliest phase of SCT, 2) in patients with no or controlled GVHD, and 3) in patients with status epilepticus due to posterior reversible encephalopathy syndrome.[11]

No ICU

ICU admission cannot be recommended on a routinely basis if no lifespan-extending therapies exist for the malignant disease, life-expectancy with ongoing anti-cancer therapy is <1 year (which, of course, can be adapted according to the individual situation of the respective patient), the patients has been mainly bedridden within the last 3 months, or in case of a patients refusal. In SCT patients, no aggressive ICU treatment such as MV can be recommended in patients with uncontrolled or refractory GVHD.[11]

ICU trial

In patients with intermediate hematologic prognosis (not fitting in any of the above-described categories), unrestricted ICU therapy can be offered for a limited period of time with a successive re-evaluation of the goals of therapy. Importantly, the development of the clinical courses of these patients cannot be estimated within the first 3 to 5 days after the ICU admission. Those, who progress to multi-organ failure after the 3rd ICU day have markedly reduced chances of survival. However, end-of-life decisions should not be taken before the 3rd ICU day.[12]

Physiologic triggers

Patients with dyspnoea or any respiratory events should be evaluated thoroughly concerning the presence or onset of ARF. Especially patients with neutropenia should be managed rigorously concerning diagnostic procedures and (empiric) antimicrobial treatment.[13] Early recognition of patients at risk and prompt transfer to the ICU at an early phase of ARF is recommended. Although no definite cut-off parameters have ever been evaluated prospectively, PaO_2/FiO_2 ratios < 200, respiratory rates >30/min, or any combination with ≥1 organ dysfunction seem to be agreeable triggers for an ICU referral. However, decisions for an even earlier ICU admission should be evaluated regularly on an individual basis.

Management

Besides the need for aggressive diagnostic measures and specific treatment of the underlying ARF trigger, choosing the appropriate mode of MV deserves special considerations. According to the landmark-trial of Hilbert et al, immunocompromised, mainly HM patients with pulmonary infiltrates and ARF (PaO_2/FiO_2 ratio < 200) profit from non-invasive ventilation (NIV) via a face mask in the ICU setting in terms of reduced intubation and mortality rates.[14] However, a trial in SCT recipients could not reproduce these findings in the setting of a standard care hematology ward.[15] Furthermore, several case control studies and observational data have shown conflicting results on the impact of NIV in HM and SCT patients.[16-19] However, patients with secondary intubation after NIV failure have consistently been shown to have markedly reduced survival rates. Taking the existing evidence in HM and SCT patients into account, NIV can be recommended early in the course of mild to moderate ARF without additional extra-pulmonary organ failure and without risk factors for NIV failure.[20] Further detailed evidence on the impact of NIV in immunosuppressed patients shall be derived from the currently ongoing large prospective IVNIctus-trial.[21]

A series of investigations documented higher mortality rates in patients with delayed admissions to the ICU.[1,22,23]Although not proven in prospective trials, early admissions are thought to be capable of preventing evolution to multi-organ failure. In fact direct admission to the ICU in patients with high-risk acute myeloid leukemia and physiologic disturbances but no organ dysfunction seems to be associated with improved outcome regarding rate of intubation and survival.[24] As of today there is no compelling evidence to perform ICU measures in HM or SCT patients in a standard hematology ward. However, early consultation of an intensivist may be useful not only with regard to developing an appropriate strategy concerning MV, but also concerning general ICU management issues, such as fluid balance.[25]

Interestingly, the use of NIV with curative intention concerning the ARF in cancer patients who have declined tracheal intubation is associated with considerable 90-day survival rates. In addition the ICU burden of survivors and their relatives is in favour of offering curative NIV as ceiling therapy to selected patients.[26]

The use of extracorporeal membrane oxygenation (ECMO) has been described in a very limited number of HM patients and SCT recipients in therapy-resistant acute respiratory distress syndrome or as "awake ECMO" to avoid intubation.[27,28] Although this approach may be a therapeutic alternative for selected patients in the future, it has to be regarded experimental at this point.

Conclusion

The acute respiratory failure is a frequent and serious complication of the underlying disease or its therapy in patients with HM and SCT recipients. Due to its prognostic impact and tendency for rapid progression, the ARF can be considered as a medical emergency. Awareness, early recognition,

rigorous assessment of the underlying causes and initiation of the appropriate treatment must be instituted promptly. In patients eligible for a full code management or an ICU trial, early referral to the ICU and close collaboration between hematologists and intensivists should be warranted on short notice. In the absence of additional extra-pulmonary organ dysfunctions, an early NIV trial may be performed.

Conflict of interest: The author declares that he has no conflict of interest

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SATELLITE SYMPOSIA

SATELLITE SYMPOSIUM I

(Pfizer Pharma GmbH)

How Can We Improve Induction Strategies in AML?

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Introduction

Optimal induction pre-supposes that achieving complete remission is an essential requirement for durable survival. This is generally accepted, but perhaps may be different in older more frail patients. Improving the rate of CR is desirable and measureable by microscopy, but optimising induction, even if it does not change the CR rate is still important because it can reduce subsequent risk of relapse.

An anthraclycline and nucleoside analogue

For nearly 3 decades "3 + 7" comprising an anthracycline and Cytarabine has been the standard of care, with a strange reluctance to try alternatives. Other anthracyclines than daunorubicin or idarubicin have failed to make a consistent difference to survival based on an extensive meta-analysis. Dose intensification of daunorubicin to 90mg/m^2 X 3 has been shown to be superior to a 45mg/m^2 dose, but 60mg/m^2 is widely used and has until recently never been compared with 90mg/m^2. In a recent large randomised study with 1200 patients randomised there was no evidence that 90mg was better overall or in any subgroup.

Escalation of the Ara-C daily dose has similarly not resulted in a consistent survival benefit, and there seems to be little difference between a continuous infusion or twice daily dosing. Adding a third drug such as thioguanine or etoposide is unconvincing. There has been recent interest in newer nucleoside analogues. Substitution of cytarabine with clofarabine in combination with daunorubicin did not improve outcome in older patients, but the addition of cladrabine to DA has recently delivered interesting results, particularly in patients with adverse cytogenetics where it showed a survival benefit. An alternative mechanism of delivering the D and A combination is in a fixed ration, stabilised in a liposome (CPX571). A large randomised trial of this approach, based on a successful randomised phase 2 experience is currently underway.

Flag-Ida

FLAG-Ida (fludarabine/Ara-C/ G-CSF and Idarubicin) in various schedules has been widely used for relapsed disease but seldom compared as frontline therapy. The MRC AML 15 trial compared it with DA or ADE. It did not improve the overall remission rate, but 90% of those who achieved CR, did so with one course. It is associated with more myelosuppression, so fewer patients progressed to consolidation, but it was of interest that a non-randomised retrospective comparison of patients only receiving two induction (FLAG-Ida) courses with four courses of DA or ADE + HD Ara-C showed no difference. For those FLAG-Ida patients who did receive FLAG-Ida and consolidation the OS was 66%, which provides an incentive to learn how to deliver this schedule. Flag-Ida had a significantly reduced relapse rate.

Gemtuzumab Ozogamicin

There has been much interest for more than 10 years in the immunoconjugate, gemtuzumab ozogamicin (GO). One strategy that has been addressed has been to augment remission induction by adding it to conventional induction chemotherapy. Dose is crucial. A single or fractionated dose of 3mg/m^2 is well tolerated. The alternative of 6mg/m^2 is generally well tolerated, but some degree of hepatotoxicity is expected. A recent meta-analysis of the 5 completed trials in adult using individual patient data, showed and overall survival benefit which was present in favourable and intermediate risk patients but not those with adverse risk. The UK group randomised over 800 patients to a comparison of a single dose of 3mg/m^2 vs 6mg/m^2 and found no benefit using the higher dose, with the possible caveat that there may be a benefit for adverse risk using the 6mg dose level. Similar data as seen in the 5 adult trials has emerged in children. It remains to be established whether a repeated 3mg dose is superior to a single 3mg dose schedule. Such studies are planned in both adults and children.

Assessment of "remission"

What is remission anyway? The accepted conventional definition requires marrow blasts to be <5% as well as recovery of neutrophils and platelets to 1.0 x 10^6/L and 10 x 10^9/L respectively. During the early experience with GO, the observation was that several patients achieved the marrow criteria without full peripheral blood count recovery. This appeared to have some survival benefit, but usually less than that of a true CR. So the definition of CRi and CRp was born. There is a certain imprecision in this definition, depending on when the marrow and count recovery were assessed. For example if a marrow is done and shows <5% blasts but on that day the counts are not fully recovered, but a week later without more therapy they have, is that CR or CRi? There is a trend to define CR based on minimal residual disease status in the response marrow. A fair proportion (perhaps 50%) of "remission" remission marrows are MRD+ve, and this has important implications for survival. Recent studies have suggested that patients with CRi are more likely to be MRD +ve. So far MRD has not been accepted as a surrogate for survival benefit, and it is unclear that the +ve patients are the ones who will benefit from intensification treatment. It maybe that the negative patients are the most curable with intensification. This issue needs to be carefully resolved by randomised comparisons.

Conclusions

There are several alternative induction therapies to the traditional "3+7" approach. Some of these require prospective confirmation in further trials, and some options will be delayed due to due unavailability (e.g.GO). Although in young patients the remission rate is already high, and therefore difficult to improve upon, a better "quality" of remission may be achieved, which manifests itself, not in a changed remission rate, but in a reduction of relapse risk. It has always been accepted that the more sensitive patients will get into CR. There is now the opportunity to corroborate this with objective MRD techniques. But it should not be assumed that the negative patients do not need intensification. Trials to test this will need to be done.

Conflict of interest: Consultant to Pfizer (Gentuzumab Ozogamicin)

SATELLITE SYMPOSIUM II

(Janssen Pharmaceutical Companies of Johnson & Johnson)

The Role of Allogeneic Stem Cell Transplantation in Older Patients with Acute Myeloid Leukemia

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Abstract

The advent of reduced intensity conditioning regimens has permitted the extension of a potentially curative graft-versus-leukaemia (GVL) effect to older patients with haematological malignancies in whom allogeneic transplant was previously precluded because of excess toxicity. Nowhere has this advance been of more clinical significance than in the management of AML where allografting is now a central component of the treatment algorithm in selected fit older patients. A number of substantial issues remain to be resolved however if allogeneic transplantation is to be deployed to maximal effect in this population. Chief amongst these is the identification of patients most likely to benefit both on grounds of tolerability of the attendant transplant related toxicities. Beyond this the optimal conditioning regimen for older patients with AML remains to be resolved both with regard to anti-tumour activity and transplant toxicity, particularly graft-versus-host disease (GVHD). Separately the development of post-transplant pharmacological or immunotherapeutic interventions raise the possibility of uncoupling GVHD from GVL with the aim of improving relapse free survival.

Introduction

Abundant evidence attests to the potency of the graft-versus-leukemia (GVL) effect in adults allografted for acute myeloid leukemia (AML)(1). The decreased toxicity of reduced intensity conditioning (RIC) regimens now permits the exploitation of an alloimmune anti-tumor response in the large number of older patients with AML whose outcome with myelosuppressive chemotherapy can be predicted to be poor(2). Recent cooperative group and registry studies confirm the promise of RIC allografts in delivering long term survival older patients with high risk AML but organ

toxicity, graft-versus-host disease (GVHD) and disease relapse remain major causes of treatment failure. Importantly all of these transplant complications demonstrate increased frequency and different characteristics in older patients and therefore require specific attention if transplant outcomes are to be optimized in this particular population. At the same time challenges remain in both deciding optimal treatment options in patients with primary refractory or relapsed AML and the identification of older patients fit enough to safely proceed to allograft.

Results

Considerable progress has been made in the identification of older patients in whom allogeneic SCT can be predicted to be associated with an acceptable toxicity. Central to this process has been the development of the Hematopoietic Cell Transplant Comorbidity Index (HCT-CI)(3). This has recently been shown to accurately predict both early transplant related mortality and overall survival in patients over the age of 60 years transplanted using either a T replete stem cell inoculum or *in vivo* T cell depletion**(4, 5)**. Additional precision is however required in terms of appropriately weighting individual co-morbidities aswell as prospectively validating this scoring system in the setting of individual conditioning regimens with particular reference to both stem cell source and the degree of patient: donor HLA disparity. Alternative pre-transplant scoring systems such as the pretransplantation assessment of mortality (PAM) will provide valuable data in this regard(6).

Acute and chronic GVHD remain major challenges in older patients transplanted using T replete stem cell inoculums making the use of *in vivo* T cell depletion an attractive form of GVHD prophylaxis in this population. Both the use of ATG and alemtuzumab have been shown to reduce the incidence and severity of GVHD in RIC allografts without necessarily increasing the risk of disease relapse. There have been no prospective comparisons of ATG and alemtuzumab in RIC allografts but one registry study has suggested a higher risk of relapse in patients with AML allografted using an alemtuzumab based regimen. Alternative strategies aimed at reducing the risk of GVHD such as the infusion of regulatory T cells or more effective treatment of acute and chronic extensive GVHD using extracorporeal photapharesis are of particular interest in the elderly given their increased risk of GVHD(7).

Disease relapse remains the major cause of treatment failure in patients allografted for AML. The increased frequency of adverse risk cytogenetics in older patients with AML accordingly increases the risk of disease relapse in this patient population. The great majority of patients destined to relapse after an allograft for AML do so within the first year post-transplant and consequently strategies with the potential to reduce the risk of disease relapse focus around either intensification of the conditioning regimen, without a concomitant increase in transplant toxicity, or optimization of a GVL effect early post-transplant. The development of sequential RIC regimens incorporating a prior course of myelosuppressive chemotherapy followed by a fludarabine/ low does TBI (FLAMSA) based RIC allograft appear to have the capacity to reduce the risk of disease relapse in patients allografted for high risk AML(8). There remain questions concerning their toxicity in older patients and this has led to the substitution of intravenous busulphan for low dose TBI. The activity and tolerability of this modified FLAMSA-Bu regimen compared with standard RIC regimens is currently being examined in a prospective randomized trial in the U.K. An alternative approach towards intensification of the conditioning regimen without increasing toxicity is the use of BCNU combined with fludarabine and melphalan which also shows encouraging Phase II data in older patients(9).

A number of strategies aimed at optimization of a GVL effect in patients allografted for AML are currently under investigation. Prosaically it is important to remember that the intensity of post-transplant cyclosporine (CsA) exposure, particularly in the first 21 days post-transplant, is an important determinant of disease relapse, particularly in recipients of T cell depleted allografts (10). Consequently meticulous attention should be paid to the degree and duration of immunosuppression delivered post-transplant with adjustments made according to donor stem cell source, degree of donor:patient HLA disparity, the use of T cell depletion and crucially the predicted risk of disease relapse. The administration of donor lymphocyte infusions (DLI) either prophylactically in patients deemed to be at high risk of relapse or pre-emptively in the setting of molecular or immunophenotypic evidence consistent with relapse is of considerable interest. The main challenges associated with the use of DLI in this setting include the attendant risk of GVHD, particularly in recipients of alternative donor transplants or when DLI is administered in the first six post post-transplant. An additional important limitation of this approach is that it perforce can only be implemented a number of months post-transplant when a significant number of patients have already relapsed. The post-transplant administration of targeted therapies with the capacity to manipulate either the kinetics of disease relapse or agents with the potential to manipulate the alloreactive response therefore has the potential to overcome this limitation. One area of active interest is the post-transplant administration of epigenetic therapies, such as the DNA methyltransferase inhibitor azacitidine, which both up-regulates the expression of epigenetically silenced putative tumour antigens and hastens T regulatory cell reconstitution post allograft(11).

Discussion

RIC allografts represent one of the most important recent therapeutic advances in the management of AML in older adults. It is becoming increasingly apparent that the complications of transplant have particular features in this patient population requiring the development of specific peri- and post transplant strategies. At the same time the challenge of how best to identify older patients with AML in whom the toxicity of allogeneic transplantation is acceptable will become even more acute as we move towards the prospect of universal donor availability.

Conflict of interest: The author declares that he has no conflict of interest

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SATELLITE SYMPOSIUM III

(Amgen GmbH)

The Potential of Bispecific T-Cell Engager (BiTE®) in Cancer: Blinatumomab and More

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Abstract

Bispecific T-cell engager (BiTE®) antibody constructs represent a promising therapeutic modality for the treatment of cancers. Blinatumomab, which targets CD19-expressing B-cell malignancies, has demonstrated high response rates and durable remissions in patients with non-Hodgkin's lymphoma (NHL) and acute lymphoblastic leukemia (ALL). AMG 330, which targets CD33-expressing leukemic cells, has demonstrated promising preclinical activity in acute myeloid leukemia (AML). This article introduces the mechanism of action of BiTE® antibody constructs, and briefly summarizes the preclinical and clinical development of blinatumomab and AMG 330.

Introduction

Immuno-oncology is a rapidly evolving field, with recent therapeutic advances seen in the development of medicines that enhance the ability of the immune system to eradicate tumors. Stimulating the anti-tumor activity of T cells has emerged as an effective therapeutic strategy, using methodologies such as chimeric antigen receptor-expressing T cells, vaccines, immune checkpoint inhibitors, and BiTE® antibody constructs. BiTE® molecules are single-chain Fv antibody constructs designed to bind with one arm to a tumor-specific cell-surface antigen and with the second arm to CD3, which is part of the T-cell receptor complex. Redirecting T cells to tumor cells results in T-cell activation, proliferation, and serial tumor-cell lysis.[1]

BiTE® antibody constructs

BiTE® antibody constructs bring the T cell and tumor cell in close proximity to one another, thereby inducing the formation of a cytolytic synapse between the effector and target cell and triggering the T-cell receptor signaling cascade.[2] Granules containing granzyme and the pore-forming protein perforin fuse with the T-cell membrane and ultimately induce apoptosis of the tumor cell.[3] Expression of MHC class I receptors is not required for BiTE®-mediated tumor cell lysis, indicating that BiTE® activity does not depend on a specific T-cell receptor or peptide antigen presentation. The BiTE® mechanism of action is therefore therapeutically advantageous in that it may circumvent escape mechanisms frequently employed by tumors to evade T-cell–mediated immune responses.
This article focuses on two BiTE® antibody constructs that have been developed for the treatment of hematologic malignancies: blinatumomab (bispecific for CD3 and CD19) and AMG 330 (bispecific for CD3 and CD33).

Blinatumomab

CD19 is a highly specific B-cell marker that is expressed throughout B-cell development and on >90% of B-cell lineage cancers.[4] Blinatumomab is designed to redirect CD3+ cytotoxic T cells to CD19+ normal and malignant B cells.[5] Blinatumomab was first tested in clinical studies in patients with NHL and subsequently in patients with ALL. The serum half-life is 2–3 hours, and so it is administered by continuous IV infusion via a portable mini-pump to maintain constant steady-state serum concentrations. Continuous IV infusion of blinatumomab induces predictable pharmacodynamics according to its mode of action. Complete depletion of peripheral B cells occurs rapidly after initial administration, resulting in the sustained absence of circulating B cells throughout treatment. T-cell activation and redistribution also occur rapidly, followed by T-cell expansion due to the proliferation of memory T-cell subsets (CD8+ T_{EM} and T_{EMRA} cells, and CD4+ T_{CM} and T_{EM} cells). A transient release of cytokines occurs within 48 hours after initial administration (mainly IL-6, IL-10, and IFN-γ), predominantly in the first cycle of treatment.[6] Cytokine release appears to correlate with dose of blinatumomab and with B-cell counts. Cytokine release syndrome (CRS) as a clinically significant adverse event has been observed in patients with ALL,[7] but can be mitigated by step-wise dosing of blinatumomab in the first cycle (i.e., 9 μg/day on days 1–7 and 28 μg/day on days 8–29) and premedication with dexamethasone. No differences have been detected in the timeframe and hierarchy of cytokine release between clinical responders and nonresponders, but slightly lower mean cytokine concentrations were observed in nonresponders.
Blinatumomab has been shown to induce rapid and long-term responses in patients with relapsed or refractory NHL and ALL, both representing difficult-to-treat populations with historically poor rates of response and overall survival. In patients with relapsed/refractory diffuse large B-cell lymphoma, the most recent analysis of 21 evaluable patients from a phase 2 study demonstrated an overall response rate of 43%, with a median response duration of 11.6 months.[8] The activity of blinatumomab was examined in a pivotal single-arm phase 2 study in a population of adult patients with relapsed/refractory ALL with particularly high risk of poor outcome.[9] Of 189 patients treated, 43% achieved a CR/CRh (CRh = complete response with partial recovery of peripheral blood counts). The median relapse-free survival was 5.9 months and the median overall survival was 6.1 months. A molecular response (MRD negativity) was achieved by 82% of the evaluable patients with a complete response. Three patients had Grade 3 CRS. Neurologic events, which were typically reversible, occurred in 11% (Grade 3) and 2% (Grade 4) of patients. Based on these data, blinatumomab was recently approved by the FDA

for the treatment of Philadelphia chromosome-negative relapsed or refractory B-cell precursor ALL.

AMG 330 and other BiTE® antibody constructs

Overall survival of patients with AML is poor, and standard treatment with intensive chemotherapy regimens followed by stem cell transplant is associated with significant morbidity and mortality. The CD33 antigen is expressed at varying levels on >99% of AML patient samples and correlates with known cytogenetic and phenotypic abnormalities, providing a rationale for its potential as a therapeutic target. AMG 330 is a novel investigational BiTE® antibody construct directed at CD33 and CD3, developed for the treatment of AML.[10] In long-term co-cultures of primary AML samples, incubation with AMG 330 led to expansion of T cells from within the patient sample and caused significant AMG 330-mediated T-cell lysis of leukemic blasts, even at low effector to target ratios. Evaluation of AMG 330 in patients with AML is warranted.
Other BiTE® antibody constructs are also under development targeting different antigens with specificity for various types of solid tumors (Table 1).

Table 1. BiTE® Antibody Constructs in Development

BiTE®	Target antigen	Tumor	Clinical status
AMG 103 blinatumomab	CD19	ALL, NHL	Approved in R/R ALL Phase 2 in NHL
AMG 330	CD33	AML	FIH planned
AMG 110 solitomab	EpCAM	Advanced solid tumors	Phase 1 completed
AMG 212 BAY2010112	PSMA	Prostate cancer, Pan-carcinoma	Phase 1 ongoing
AMG 211 MEDI-565	CEA	Advanced gastrointestinal cancers	Phase 1 ongoing

FIH = first in human, EpCAM = epithelial cell adhesion molecule, PSMA = prostate specific membrane antigen, CEA = carcinoembryonic antigen

Conclusions
BiTE® antibody therapy has therapeutic potential against a wide range of target antigens and tumor types. The proof-of-concept for BiTE® therapy has been demonstrated by blinatumomab, which was recently approved in the US for the treatment of Philadelphia chromosome-negative relapsed or refractory B-cell precursor ALL.
Conflict of interest: Employee of Amgen Research (Munich) GmbH; Shareholder in Amgen Inc.

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The Anti-CD33 BiTE (AMG 330): New Option for AML

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AMG 330 is a novel investigational anti-CD33 BiTE® antibody construct with promising activity on primary AML cells. The target antigen CD33 is expressed in over 99% of AML samples although highly variable. CD33 expression level has been shown to correlate with defined cytogenetic and molecular aberrations in AML. Leukemic stem cells within the $CD34^{+}/CD38^{-}$ compartment displayed CD33 at a higher level than healthy donor stem cells. Targeting CD33 ex vivo using AMG 330 in primary AML samples led to T-cell expansion and remarkable BiTE®-mediated cytotoxicity, suggesting efficient therapeutic potential in vivo. Target antigen density and effector-to-target cell ratio were identified to be critical determinants for AMG 330-mediated lysis kinetics. Evaluation of AMG 330 in an early-phase trial in relapsed/refractory AML is urgently warranted.

Conflict of interest: Research collaboration with Amgen

SATELLITE SYMPOSIUM V

(TEVA GmbH)

New Therapies for Higher-Risk APL

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Background of APL

Acute promyelocytic leukemia (APL) is considered a rare disease with estimated 200 to 300 newly-diagnosed cases per year in Germany. According to the FAB classification APL is designated as "M3 leukemia" and assigned to the WHO defined type of AML with recurrent cytogenetic abnormalities, "acute promyelocytic leukemia with t(15;17)(q22;q12), (PML/RARα) and variants". Detection of PML-RARα fusion is carried out by conventional cytogenetics including fluorescence in situ hybridization (FISH) and/or RT-PCR. Alternative fusion partners are the zinc finger (PLZF), the nucleophosmin gene (NPM), the nuclear mitotic apparatus (NUMA) or the STAT5b gene. These fusion partners may be of therapeutic relevance, e.g. for the sensitivity to all-trans-retinoic-acid (ATRA), which does not exist with the involvement of the PZLF gene [t(11;17)(q23;q21)].

In addition to age, the most important prognostic factor is the leucocyte count at diagnosis which divides patients into a high risk (>10 Gpt / l) and a non-high-risk group (? 10 Gpt / l). Through use of a risk-guided therapeutic approach the outcome of patients within both groups including risk of relapse has become almost comparable[1-3].

The need for trials in high-risk disease

After a common initial induction treatment (AIDA), subsequent risk-guided (high-risk: WBC ≥ 10, non high-risk: WBC <10) consolidation cycles have been shown to equalize the risk of relapse between both APL risk groups. Current efforts focus on the reduction of toxicity associated with standard therapy including the implementation of less toxic and highly effective agents such as ATO. ATO is the most effective single agent in APL[4;5]. ATO acts via complex mechanism in APL that is not yet fully explained. At a high concentration (0.5 to 2.0 μmol/L) ATO induces apoptosis *in vitro*, while at lower concentrations (0.1 to 0.5 μmol/l) it induces partial differentiation of leukemic promyelocytes through PML/RARα degradation and inhibits angiogenesis[6-9].

ATO is licensed for the treatment of relapsed and refractory APL in the USA and Europe[10-18] and can achieve remission rates in up to 90 % of patients. Concerning its toxicity profile, ATO is usually well tolerated and its use is associated with a series of manageable adverse events (hyperleucocytosis, increase of liver enzymes, APL differentiation syndrome, prolongation of the QT interval[19]). Most of the adverse events mentioned above are usually mild and manageable[10;18].

The antileukemic efficiency of ATO is increased when combined with ATRA. ATO as single agent is able to induce durable molecular remission after two cycles in the majority of patients treated for disease recurrence[17]. Results of various studies conducted with ATO as single agent or combined with ATRA for newly diagnosed APL patients reported CR rates of 86-95 %, molecular remission rates after two cycles of 76-100 % and survival rates of 86-88 %, with significantly better responses being obtained in patients with non-high risk disease as compared to high-risk patients[20-24]. Recent data of the APL0406 – Intergroup Study (GIMEMA/AML-SG/SAL) showed that ATO plus ATRA is at least as effective as AIDA-based therapy as first-line treatment in non-high risk APL patients[1;24]. In particular, early mortality was almost absent in the experimental treatment arm combining ATO and ATRA. In addition, the results of a recent published randomized trial evaluating ATO in first-line therapy during consolidation demonstrated that ATO further reduced the risk of recurrence and improved survival[25]. Thus available data strongly suggest that at least non-high risk APL patients may be cured without chemotherapy (with ATO/ATRA only), which is a pivotal step in the treatment of APL[26]. Based on this APL0406 study in non-high-risk a randomized study (APOLLO) in high-risk APL is planned which will compare standard treatment with an ATO/ATRA combination supplemented with low-doses of conventional chemotherapy only during induction.

Conflict of interest: The author declares that he has no conflict of interest

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ABSTRACTS - POSTER SESSION

I. LEUKEMIC STEM CELLS

1

Clonal Variegation and Dynamic Competition of Leukemia-Initiating Cells in Infant Acute Lymphoblastic Leukemia with MLL Rearrangement

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Aim: In this study we aim to dissect the clonality of the leukemia-initiating cell (L-IC) population in MLL+ infant ALL, a very aggressive form of leukemia occurring within the first year of life, with a poor outcome.
Method: To achieve this goal we used a xenotrasplantation assay into immune-deficient mice, and performed Ig/TCR rearrangement analysis and genome-wide SNP array screening of diagnostic samples from patients and xenograft leukemias from transplanted mice
Results: We have observed that MLL-AF4+ infant ALL is composed of a branching subclonal architecture already at diagnosis, frequently driven by an Ig/TCR-rearranged founder clone. Each clone is potentially able to contribute to the diverse phenotypic pool of MLL-AF4+ L-ICs. Interestingly, some clones which were minor at diagnosis can reactivate and become dominant upon serially transplantation in mice, whereas other major dominant clones at diagnosis can become quiescent and disappear. Investigation of paired diagnostic and relapse samples suggested that relapses often occur from subclones already present (but quiescent) at diagnosis. Finally, Copy-number alterations arising at relapse might contribute to the activation and expansion of previously quiescent subclones.
Conclusions: Our results point out the existence of a dynamic competition between multiple leukemia-initiating subclones. Unraveling the subclonal architecture and dynamics in MLL+ infant ALL may provide a possible explanation for the therapy resistance and the high incidence of relapse frequently observed in infant patients.
Conflict of Interest: NONE

2

Towards New Clinical Hypothesis Using A Mathematical Model of Acute Leukemias

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Background: Mathematical modelling can give valuable insights into the characteristic behavior of biological systems. In the research of acute leukemias a growing body of literature describes modelling approaches to explain the mechanistics of hematopoietic cells with respect to the emergence of leukemic diseases.
Methods: We analyzed a mathematical model, based on ordinary differential equations, published by Stiehl et al. (J.R.S. Interface, 2014), to characterize acute leukemias. In the model we primarily focused on the dependence of the duration from the first mutation to the diagnosis of the leukemic disease (time-to-diagnosis) on leukemic stem cell's (LSC) selfrenewal and proliferation rates. We simulated a healthy hematopoietic system and added a small amount of mutated stem cells with predefined proliferation (range: 0-2) and selfrenewal (range: 0-1) to the system. The development of a leukemia is observed for 2000 days and the time-to-diagnosis (days) is calculated for each parameter combination.
Results: The model shows that even slowly proliferating LSC can become a leukemia when selfrenewal is high enough. The analysis of the time-to-diagnosis showed that LSC with high proliferation and high selfrenewal are responsible for "fast" leukemias, i.e., lead to a short time between initial mutation and diagnosis, while decreased proliferation or decreased selfrenewal leads to "slower" leukemias. We could also show that selfrenewal is the more sensitive parameter with regards to "leukemic pace". In particular, there seems to exist a lower threshold for selfrenewal. Below this threshold no leukemia occurs any more, at least in the model.
Conclusion: Mechanistic mathematical models are useful tools to analyze hematological diseases. In particular, we analyzed the emergence of leukemia before therapy starts to generate an individualized model based risk classification, i.e. the mechanistic model can be used as basis for a statistical model to fit longitudinal patient data to determine a patient's "leukemic pace type". The next step will be to incorporate chemotherapy into the model and generate hypotheses about optimal therapy schemata.
Conflict of interest: None

3

The Impact of Chromosome 17p Deletion on Tumorigenesis and Therapy Response

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Chromosome 17p deletions are among the most frequent genetic abnormalities in various cancers and associated with a dismal prognosis. However, they have long been simply considered as the inactivation of tumor suppressor gene TP53. Previous work in our laboratory identified additional tumor suppressor genes next to TP53, indicating that large deletions on 17p may contribute to

cancer biology beyond their impact on eliminating wildtype TP53 allele. To explore this further, we generated a novel genetically-engineered mouse model that incorporates conditional deletion of chromosome 11B3, which is syntenic to the common deletion region on human 17p13. We found that besides p53, heterozygous deletion of linked genes on 11B3 not only promotes Myc-driven lymphomagenesis or Nf1; Mll3-defective acute myeloid leukemogenesis as seen by shorter tumor latency and overall survival than controls with only p53 loss, but also contributes to the poor outcome of chemotherapy treatments as shown by additional resistance to cyclophosphamide, vincristine and methotrexate. Furthermore, most tumor cells generated from heterozygous deletion of 11B3 have spontaneously missense or frame-shift mutations on the other wildtype p53 allele during the loss of p53 heterozygosity, which represents the major p53 configurations in human cancers. We further identified key players in 17p13 region that is capable of promoting Eμ-Myc lymphoma development by its own or cooperating with p53 suppression in tumorigenesis. Together, our results provide the new aspects of chromosome 17p deletions in cancer biology and may shed light on developing new therapeutic methods.
Conflict of interest: None

4

Leukemia Initiating Cells in Acute Lymphoblastic Leukemia are Characterized by Low Cellular Energy Metabolism

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Introduction: The nature of leukemia initiating cells (LIC) in acute lymphoblastic leukemia (ALL) remains elusive, although being of key clinical relevance since LICs need to be eradicated in order to cure the disease. The hierarchical concept with few LIC being able to propagate leukemia upon transplantation onto immunodeficient mice has been challenged by data showing LIC-activity also in committed cells supporting a stochastic model. However, as a common strategy, these studies are based on defining sub-populations by distinct surface marker profiles.
Material and methods: Aberrant energy metabolism was reported in cancer including altered redox states in cells with stem cell properties. In this study, we analyzed redox states of primary ALL cells and analyzed whether cellular energy metabolism determines LIC activity. Patient derived xenograft ALL samples were investigated for reactive oxygen species (ROS) in combination with cell cycle analysis. Cellular sub-fractions sorted according to high or low ROS levels were analyzed for repopulating activities in our NOD/SCID/huALL xenograft model. Interestingly, we identified a low oxidative state in cells of early G0/G1 cell cycle phases, which also show increased NOD/SCID repopulation. In contrast, ROS^{high} cells originate from later cell cycle phases (G2/M) suggesting that the ALL cells' oxidative state is indicative for its leukemia initiating activity. To functionally address this hypothesis, sorted ROS^{high} and ROS^{low} patient derived leukemia cells were transplanted onto NOD/SCID mice. Both sub-fractions led to leukemia engraftment with ROS^{low} cells showing higher repopulating activity compared to ROS^{high} ALL cells.
Conclusion: In conclusion, our data indicate that all cells in ALL show LIC activity, with cells of low energy metabolism representing the driving leukemia initiating cell compartment, thus pointing to redox modulation as a potential therapeutic target in ALL.
Conflict of Interest: None

5

GPR56 Identifies the Leukemia Stem Cell Compartment in Acute Myeloid Leukemia

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Purpose: The purpose of this study was to identify genes associated with leukemic stem cell (LSC) activity in primary human acute myeloid leukemia (AML) specimens to gain novel insights into the molecular networks controlling LSC self-renewal.
Methods: We combined next-generation sequencing technologies with functional *in vivo* assays to identify LSC associated genes and used combinatorial cell sorting strategies to determine the enrichment for LSCs in sorted fractions.
Results: We identified the G-protein coupled receptor 56 (*GPR56*) as a novel LSC marker in AML. Sorting experiments revealed a hierarchical architecture, in which GPR56 identified more primitive cells compared to GPR56 negative cells, both *in vitro* and *in vivo*. $GPR56^{+}CD34^{+}$ double positive cells were most highly enriched for engrafting LSCs not only in fresh samples, but also after *in vitro* culture in optimized conditions that support LSC activity *ex vivo*. AML samples with high *GPR56* gene expression were significantly enriched for adverse genetic aberrations such as *FLT3*-ITD, *EVI1* rearrangements, and mutations in *TP53* and *RUNX1*. Furthermore, high *GPR56* gene expression on its own was associated with poor prognosis in independent patient cohorts.
Conclusion: Here we established GPR56 as a novel LSC marker that reveals a leukemic hierarchy and as a factor of poor prognosis in AML. Our data provide evidence that LSC phenotypes are heterogeneous and remain to be defined in GPR56 negative samples.
Conflict of Interest: none

6

Cell of Arrest of Cancer: Functionnal Impact and Genetic Determinism in Acute Myeloid Leukemia

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The hematopoietic stem cell differentiates in progenitors and then in mature blood cells. Mechanisms of leukemogenesis block this differentiation. We defined the Cell of Arrest of Cancer (CAC) as the stage of hematopoietic differenciation in which leukemic cells are blocked. We thought that the cell of arrest of acute myeloid leukemias (AML) dictated functionnal properties of AML and was induced by specific genetic anomalies.
Patients and methods: We extensively phenotyped normal hematopoietic progenitors to create signatures of CAC of AML. These signatures allowed us to classify 932 AML patients seen in our Leukemia Unit between 2000 and 2012 as well as 142 AML

patients published by the Cancer Genome Atlas Research Network project (NEJM, 2013). This study thus relies on our own data and databases publicly available.
Results: Our phenotypes of HSC, MPP, CMP, GMP and GP/MP allowed us to classify AML as HSC-like, MPP-like, CMP-like, GMP-like or GP/MP-like. We validate the phenotypic classification of AML with a transcriptomic signature of normal stem cells and hematopoietic progenitors (HSCP) and gene set enrichment analysis. Based on normal HSCP properties, we show that CAC influences functions of AML as proliferation, chemoresistance and metabolism. Earlier the blockage occurs in the differentiation process and poorer the prognosis of patients is. Identification of the CAC allows to highlight the role of CEBPA in stage –specific blockage. This method gives insight of leukemogenesis mechanisms and defines unexpected stage of blockage.
Discussion: We propose a new concept in cancer that we called Cell of Arrest of Cancer and used AML to characterise its validity. The identification of the CAC will permit to study in details leukemic blockage mechanisms in a heterogenous disease as AML. CAC classification will allow to consider new therapeutic approaches including terminal differentiation induction in specific subtypes of AML.
Conflict of interest: None

7

Whole Exome Sequencing in Single Cells from Pediatric Patients with Acute Myeloid Leukemia and NPM1/Flt3 Mutations

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Introduction: The detection of several mutations in a blast population of pediatric acute myeloid leukemia (pAML) is supposed to be caused by a clonal evolution from a leukemic stem cell (LSC) to leukemic blasts. Since LSC and potential leukemic subclones are only present in small subpopulations, it has been a major technical challenge to particular analyse only the specific population. To acquire a better understanding of the underlying mechanisms of mutagenesis, clonal evolution and leukemogenesis, the aim of this study was to establish methods that allow the analysis and detection of mutations in single cells of a subpopulation known to contain HSC as well as LSC (CD34+CD38-).
Methods and results: The mutational status of sorted single cells from three patients suffering from pAML was analysed and compared to mutations detected at initial diagnosis in DNA from a bulk of bone marrow (BM) cells. Single CD34+CD38- cells were sorted, applied to Whole Genome Amplification (WGA) and whole exome sequencing. The mutational status was compared to mutations initially detected. WGA from single cells resulted in an amount of 29 to 31.7μg DNA from each of five single cells. A 4bp insertion in exon 12 of NPM1, (MutA) initially detected from a bulk of cells, was identified in amplified DNA from single cells in 2/3 patients. Internal tandem duplications in Flt3, indicated by mismatches in the alignment, could be detected in amplified DNA from single cells of two patients. The detected ITD resemble those initially detected in DNA from a bulk of BM cells.
Conclusion: Single cell sequencing provides a useful tool to amend the detection of genetic aberrations from a bulk of cells and to confirm the presence of specific mutations in single cells from small subpopulations. It therefore helps to get further insights into the clonal evolution in pAML.
Conflict of interest: None

II. AML – Biology

8

IDH Mutation Deregulates Lyn Kinase and Uncovers a Synergistic Clinically Achievable Response with Atra and Dasatinib

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Background: Acute myeloid leukemia (AML) is characterized by the accumulation of malignant blasts with impaired differentiation due to recurrent mutations, amongst which IDH mutations occur in 15% of AML patients.
Methods and results: We showed both in vitro and in a xenografted mice model, that clinically achievable doses of ATRA are sufficient to achieve a terminal granulocytic differentiation, on primary AML samples and AML cell lines harboring IDH1-R132H mutation. There is no effect at this concentration on the WT controls. This is associated with reduction of both proliferation and colony formation, and further lead to apoptosis, improving then overall survival of mutant xenografted mice. We further showed that ATRA sensitivity is due to a constitutive reduced Lyn activation in the presence of the mutation. This will also sensitizes to clinically achievable doses of dasatinib, a Lyn inhibitor. Moreover, as ATRA induces a brief Lyn activation that transiently reduces ATRA activity, its association with dasatinib synergistically increases differentiation. This association might also be considered for others IDH mutations producing 2-hydroxyglutarate, since treatment with the mutant-specific oncometabolite (eg. 2-hydroxyglutarate) sensitizes AML cells to ATRA-induced differentiation.
Summary: Constitutive reduction of Lyn in IDH1-R132H blasts sensitizes this subgroup of AML to both dasatinib and ATRA and results in a highly synergistic effect between these two drugs.
Conclusion: As ATRA and dasatinib are already used in the clinic, and because IDH mutations are systematically conserved at relapse, combining ATRA to dasatinib should be promising to achieve a long-term remission for this AML patient subgroup.
Conflict of interest: None

9

Overweight is An Independent Adverse Prognostic Factor in Acute Myeloid Leukemia (AML) in Younger Patients

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Background: In recent years, the impact of overweight has moved into the focus of medical research, as various types of cancer are positively correlated with overweight. The results of such investigations concerning leukemia, in particular acute myeloid leukemia (AML), are unequivocal.
Methods: The AMLCG1999 trial was analyzed for the impact of body mass index (BMI) on response to treatment and survival parameters (overall survival [OS] and relapse free survival [RFS]). Weight categories were classified according to WHO (with overweight defined as a BMI ≥ 25). RNA expression data for 17389 genes from microarray analyses were available in 474 patients.
Results: 2.748 patients with AML (*de novo* or secondary), which had received intensive induction and postremission chemotherapy as previously published (Büchner et al., J Clin Oncol 2006) were analyzed. Median age was 61 years (16-86). Median BMI was 26 (15-55) at the time of enrollment into the trial. In 68% of patients, BMI was ≥ 25, and

this cut-off level was chosen for further analysis. Both groups were well balanced concerning genetic risk groups but more male patients were overweight. There was no significant difference in CR rates between both groups (60% vs. 58%). However, in younger patients (<60 years), BMI ≥ 25 was significantly associated with reduced OS and RFS as compared to patients with BMI < 25 (OS: 10.0 vs. 4.9 years, p<0.02, RFS: 2.7 vs. 1.8 years, p<0.02). This result was independent from the type of AML (*de novo* or secondary), karyotype and FLT3 mutation. Also, BMI conferred a worse prognosis in AML with intermediate and unfavorable cytogenetics and in patients with FLT3-ITD. Multivariate analysis confirmed the increased risk for younger overweight patients of 28% for OS. Interestingly, RNA expression analysis in 474 patients showed not one single differently expressed gene in AML blasts from overweight patients, making overweight a truly independent prognostic factor.

Discussion: This analysis establishes overweight as an independent negative prognostic factor in younger patients with AML. This is of particular importance since overweight is the only factor that could be altered in during the course of treatment. Further research is needed to clarify if overweight correction could improve prognosis.

Conflict of interest: None

10

In-Depth Global DNA Methylation Analysis Reveals An Extreme CPG Island Hypermethylator Pheno-Type

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Background: Besides genetic alterations, epigenetic changes are recognized as an additional mechanism contributing to AML. To identify marker genes that may provide a basis for improved patient outcome prediction, diagnosis and prognosis we performed a systematically genome-wide screening of DNA methylation profiles across 23.000 CpG islands of 28 AML samples using the Methyl-CpG-Immunoprecipitation assay combined to CpG island microarrays. These profiles were then used to select a subset of aberrantly methylated CpG islands (380 regions covering 15.000 CpGs) for quantitative DNA methylation profiling in a larger cohort of 196 AML patients using MALDI-TOF analysis of bisulfite-treated DNA. Meta-analysis clearly separated a subgroup of CpG island regions showing highly correlated DNA methylation changes that were also marked by H3K27me3 in normal hematopoietic progenitor cells (HPC). The residual group of CGIs not targeted by polycomb group repressors in HPC displayed heterogeneous methylation patterns across patients that clustered with genetic markers. A fraction of AML patients (5/196) displayed aberrant hypermethylation at almost all studied loci, representing a rare CpG island methylator phenotype (CIMP) in AML. These patients present immature leukemia with various chromosomal aberrations but very few mutations and show high resemblance with a recently reported CEBPA methylated subgroup displaying a similar strong methylation signature (Figueroa et al, 2009). DNA methylation analysis revealed that most of these CEBPA-silenced cases fall into our CIMP category.

Method: To explore the whole range of epigenetic alterations in this rare group of hypermethylated patients we performed in-depth global DNA methylation analyses. Interestingly, hyper-rmethylated gene promoters represent many transcriptional regulators that are involved in the differentiation of myeloid lineages (e.g. CEBPA, IRF8, GATA2). In addition, these patients frequently show hypermethylation of the TET2 promoter which could result in a loss of maintenance DNA demethylation and therefore successive hypermethylation at CGIs.

Conclusion: It is likely that the aberrant silencing of key lineage regulators stabilizes the differentiation arrest in these cells, and that these patients may particularly benefit from therapies that revert DNA methylation.

Conflict of interest: None

11

Myelodysplastic Syndromes in Ukrainian Chernobyl Clean-Up Workers

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Background: The association of radiation exposure not only with acute leukemias but also with myelodysplastic syndromes (MDS) has been suggested based on a vast array of epidemiologic data, in particular in LSS cohort study in Japan

Among 295 cases of leukemias and lymphomas diagnosed in 1996-2010 at the Reference Laboratory of the Immunocytochemistry and Oncohematology Department in Ukrainian Chernobyl clean-up workers of 1986-1987, various forms of MDS were detected in 16 patients (5.42%) while in patients with hematological malignancies of the general population of Ukraine in our study (2697 persons in total), 107 MDS cases were revealed (3.70%). Extra 4 MDS pts. were diagnosed among clean-up workers in 2011-2012. Among 20 MDS pts., refractory anemia (RA) was detected in 11, refractory anemia with ring sideroblasts – in 2, refractory anemia with excess of blasts (RAEB-1 and RAEB-2) – in 7 cases. RA and RAEB were also the predominant forms of MDS in Japanese cohorts of A-bomb survivors.

In our study, we have described 7 pts. among clean-up workers with various cytological variants of AML (AML with minimal differentiation, acute myelomonocytic leukemia and acute erythroid leukemia) in whom the overt leukemia was preceded by MDS (15.2% of all AML cases diagnosed in clean-up workers). At the same time, only six (1.5%) cases of preceding MDS were found upon examination of AML patients in general population of Ukraine in our study.

Conclusion: Study of the secondary MDS and its relation to AML in post-Chernobyl period seems to be relevant taking into account the data suggesting that MDS risk among A-bomb survivors remains to be significantly increased even in 55-60 years after radiation exposure.

Conflict of interest: None

12

The Histone Methyltransferase EZH2 Controls Drug Resistance in Acute Myeloid Leukemia (AML)

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Introduction: Resistance to chemotherapy and subsequent relapse is the most challenging issue in the treatment of patients with Acute Myeloid

Leukemia (AML). However, the underlying mechanisms still remain incompletely understood. Here we report that loss of the histone methyltransferase EZH2 and subsequent reduction of H3K27 trimethylation contribute to chemoresistance in AML.

Method: In Myelodysplastic Syndrome (MDS) and Myeloproliferative Neoplasms (MPN) EZH2 is often inactivated due to mutations which is associated with poor prognosis. By use of quantitative PCR and immunohistochemistry we show that a decrease of *EZH2* mRNA and protein also correlated with a poor prognosis of AML patients indicating a tumor suppressor role of EZH2 in AML. EZH2 is located on chromosome 7q36.1 and it is not yet fully clear whether EZH2 expression is affected in MDS and AML patients with del(7)/del(7q) who are largely refractory to chemotherapy and have a poor prognosis. We found EZH2 levels to be reduced in del(7)/del(7q) AML patients as determined by Western Blot. Notably, the reduction of EZH2 protein levels via treatment with H3K27 Methyltransferase inhibitors or lentiviral knockdown was sufficient to induce chemoresistance of Normal Karyotype (NK)- AML cells *in vitro* and in a xenograft mouse model. Furthermore, we observed that EZH2 loss occurred during the acquisition of drug resistance in a Tyrosine Kinase Inhibitor- and Cytarabine- resistant AML cell line. Functionally, the loss of EZH2 directly induced upregulation of *HOX* genes, which suggests a stem-cell-like signature to be associated with the resistance phenotype. Pharmacological inhibition of Cyclin-dependent kinase 1 (CDK1) and treatment with the proteasome inhibitor Bortezomib, respectively, increased EZH2 protein levels and restored drug sensitivity of the resistant cell line. To evaluate the effect of Bortezomib on EZH2 levels in patients` blasts we treated NK- AML blasts *ex vivo* with Bortezomib. Importantly, in those patient samples in which EZH2 protein levels were increased by Bortezomib we observed a significantly increased cell death.

Conclusion: Our data strongly indicate that restoration of EZH2 protein levels e.g. via Proteasome inhibitors and thereby restoration of EZH2 function might be a novel promising approach to increase therapy response in AML.

Conflict of Interest: None

13

DNMT3A Gene: Distribution of the Mutations and their Stability during Treatment in Patients with AML

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Introduction: Mutations in DNA methyl-transferase 3A gene (DNMT3A) are the third most common recurrent genetic alternation in AML patients; are identified in 20-35% of AML patients with normal karyotype (NK-AML). With regard to the size of the gene (23 exons) and distribution of mutation (60% mutations in codon R882 and other anywhere in coding region) a suitable method for the mutation analysis of the gene is next generation sequencing (NGS). The aims of our study were: i. characterize the distribution of mutation in the gene, ii. determine stability of mutations during AML treatment.

Patients and methods: DNA from bone marrows (BM) samples of 25 NK-AML patients, at the time of diagnosis, were analyzed for distribution of mutation in exons 9-23 by 454 technology, developed in the framework of the Inter-laboratory Robustness of Next-generation sequencing (IRON-II) consortium, on a GS Junior Instrument (Roche, Germany). Stability of mutations was tested in 7 patients (13 BM samples), initially carrying R882 mutation, at the time of complete remission (CR) and/or disease relapse by Sanger sequencing.

Results: Four different mutations were identified in 11/25 patients: 2/4 mutations are novel and not described yet (L347P and Y347S), other two mutations are common (R882C and R882H). 5/11 patients carried mutation R882C, 4/11 mutation R882H. In 4/7 patients, initially carrying mutation in R882 codon, mutations were detected also at the time of CR.

Conclusions: Our results confirm that mutations in DNMT3A gene are a frequent event in NK-AML patients. In some patients, DNMT3A mutations persist during CR. These results may indicate the presence of preleukemic AML stem cells with mutation in DNMT3A gene, which are resistant to chemotherapy.

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Conflict of interest: None

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Identification of Novel NUP98-NSD1 Fusion Transcripts in Adult *DE NOVO* Acute Myeloid Leukemia Patients with t(5;11)(q35;p15.4)

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Background: The cytogenetically cryptic t(5;11)(q35;p15.4) is a frequently observed chromosomal aberration in AML, which results in the translocation of the *NUP98* and *NSD1* genes and expression of a NUP98-NSD1 fusion protein. Due to its association with poor prognosis and dismal clinical outcome, there has been increasing interest to improve the identification, therapy and minimal residual disease monitoring of patients with NUP98-NSD1.

Method: In the present study, we applied next generation RNA-sequencing, aCGH and molecular cloning techniques together with capillary sequencing to study NUP98-NSD1 fusion transcripts in adult *de novo* AML patients with t(5;11)(q35;p15.4). By sequencing full-length NUP98-NSD1 cDNA transcripts cloned from the leukemic patient cells, we were able to identify two in-frame transcripts and one out-of-frame transcript. One of the in-frame transcripts joined *NUP98* exon 11 to *NSD1* exon 6, while the other joined *NUP98* exon 12 to *NSD1* exon 6. The latter had intra-exonic splice variation at the end of *NUP98* exon 7. The out-of-frame transcript joining *NUP98* exon 12 to *NSD1* exon 6 lacked *NSD1* exon 7. The relative expression levels of all transcripts were assessed from the RNA-seq data based on aligned read counts. The RNA-seq data indicated that the in-frame transcripts are expressed at a higher level than the out-of-frame transcript, while also indicating that the transcript joining *NUP98* exon 12 to *NSD1* exon 6 is the predominant transcript.

Results: The results of the present study demonstrate that the *NUP98-NSD1* fusion gene undergoes alternative splicing in AML, while additional studies are needed to determine the pathophysiological relevance of the novel transcripts.

Conflict of interest: None

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BCL2 Inhibitors Target Diagnosed and Relapsed/Refractory AML

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Background: BCL2 family proteins are key regulators of apoptosis. BCL2 and BCLXL promote survival by preventing mitochondrial

apoptotic pore formation. BH3 mimetic drugs such as venetoclax (ABT-199) and navitoclax (ABT-263) promote apoptosis by inhibiting BCL2 alone (venetoclax) or both BCL2 and BCLXL (navitoclax). In AML, the expression of anti-apoptotic proteins is highly variable. In a recent study venetoclax showed single-agent activity in 6/12 AML cell lines and 20/25 patient samples (Pan et al. Cancer Disc 2014). The samples with complex cytogenetics were largely resistant. Sensitivity correlated to expression level of *BCL2* and resistance to expression of *BCLXL* and *MCL1*.

Method: In this study, we assessed the *ex vivo* sensitivity of fresh BM MNCs from 11 diagnosed and 34 relapsed/refractory AML patients to venetoclax (22 samples) and navitoclax (45 samples). Exome seq was performed on 32 samples and RNA seq on 25 samples. Data from primary BM MNC of healthy individuals (n=11), and CMML (n=9) or CLL (n=2) patients were used as controls.

Compared to healthy controls, CMML (all non-responsive) and CLL (all sensitive), the AML samples exhibited heterogeneous responses. 12/22 (54%) of AML samples were sensitive to venetoclax and 30/45 (67%) to navitoclax. Majority of diagnostic samples showed sensitivity to navitoclax (9/11, 82%) and venetoclax (4/5, 80%). Of relapsed and/or refractory samples 21/34 (62%) showed sensitivity to navitoclax whereas 8/17 (47%) were sensitive to venetoclax. To determine if the responses remained constant throughout the disease, we examined navitoclax responses in 8 patients at several time points. In most (6/8) patients the response remained unchanged (responsive or non-responsive). We observed responses across all mutational groups (FLT3-ITD, NPM1, TP53, NRAS, IDH) and also in samples with complex or monosomal karyotype. Although *BCL2* transcript levels tended to correlate to navitoclax sensitivity, a better predictor for sensitivity was the gene expression of specific proapoptotic *BCL2* family members.

Conclusion: In conclusion, both navitoclax and venetoclax show responses across most common mutational groups in AML and at all disease stages. Expression of specific pro-apototic genes are a potential biomarker for predicting response.

Conflict of interest: None

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Microenvironmental Hypoxia Induces Prognostically Relevant IL-8 IN Blasts of Acute Myeloid Leukemia

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Background: The bone marrow micro-environment is generally hypoxic (mean 6% O_2), however for the stem cell niche concentrations as low as 1% O_2 have been reported. Blasts of acute myeloid leukemia (AML) reside in the bone marrow and the stem cell niche and may be protected by the microenvironment, causing relapse in the course of the disease. Various Cytokines could be involved in creating this microenvironment.

Methods: Experiments were conducted under standard laboratory conditions (21% O_2) and reduced oxygen environment (1% O_2) with cell lines as well as primary samples. Cytokine arrays, ELISA and flow cytometry were used. Survival parameters were analyzed according to RNA expression and clinical data from 533 patients with AML treated uniformly within the AMLCG1999 trial. Serum samples of patients with AML were also analyzed.

Results: Initial screening of 80 different cytokines showed induction of HGF and IL-8 at 6% O_2 and hence were further analyzed. In AML cell lines KG1a and OCI-AML3 we observed a significant dose and time dependent induction of IL-8 expression under hypoxia with significant increase of IL-8 expression only below 6% O_2 (1% O_2 2.6fold increase as compared to 21% O_2 [$p<0.01$]; 2.6fold increase of IL-8 after 2 days, 8.6fold after 10 days respectively, at 1% O_2, [$p<0.01$]). This findings could be confirmed in primary AML samples (n=23); subgroup analyses are pending and will be presented at the meeting. However, we failed to observe a similar effect of hypoxic induction of HGF by AML cell lines. The *in vivo* effects of IL-8 in AML are still elusive, as we observed no effects of IL-8 on proliferation. However, a proliferative effect *in vivo* can nevertheless be assumed, as serum levels of IL-8 in patients with AML were increased during aplasia and decreased after hematological recovery. Also, IL-8 RNA expression was associated with peripheral leukocyte count. RNA expression analyses revealed a significantly higher expression of IL-8 in AML blasts harboring the FLT3-ITD mutation; in addition, in FLT-ITD positive patients, higher IL-8 RNA expression was associated with inferior RFS (HR 1.495, $p<0.05$) and OS (1.344, $p<0.05$).

Conclusion: Physiological hypoxia as encountered in the bone marrow microenvironment specifically increases IL-8 expression of AML blasts (as opposed to HGF expression). While the exact function of IL-8 in AML remains unclear, IL-8 was shown to be prognostically unfavorable. Potential mechanisms include proliferative or microenvironmental issues. Further investigations are warranted.

Conflict of interest: None

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Development of Nested Multiplex PCR For Simultaneous Detection of Multiple Genetic Alterations In Acute Leukemia

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Introduction: Acute leukemia (AL) is the most widespread malignancy in childhood and frequent in adults. Detection of molecular genetic abnormaities in AL is of great significance as they define biological properties of malignant cells, associated with treatment response and can be markers for MRD monitoring and targets for therapy. Number of clinically relevant mutations is constantly increasing and even with introduction of new and effective high-throughput approaches for detection of molecular genetic abnormalities, development of simple and quick method for the simultaneous detection of multiple clinically significant molecular-genetic alterations (chimeric genes) can be useful.

Method: During the project execution multiplex nested PCR was developed for the detection of expression of main described splice-variants of clinically relevant chimeric genes: *TEL-AML1, E2A-PBX1, SIL-TAL1, E2A-HLF* in ALL, *PML-RARa, NPM1-RARa, PLZF-RARa, CBFB-MYH11, AML1-ETO, DEC-CAN* in AML, *BCR-ABL, CALM-AF10*, *MLL-AF4, MLL-AF6, MLL-AF9, MLL-AF10, MLL-ENL, MLL-ELL, MLL-MLLT11, MLL-EPS15, MLL-FOXO4, MLL-SEPT6, MLL-SEPT9* in AL. Protocol includes two-step coamplification of listed genes and internal control – housekeeping gene Abelson (ABL). combined in 8 reactions. Reaction conditions were optimized and results were confirmed by reference methods and/or by direct sequencing of PCR products. In some cases (rare MLL rearrangements) data from sequencing were used for design primers/probe for MRD monitoring.

Conclusion: In this study quick, sensitive and flexible method of simultaneous detection of multiple chimeric oncogenes in samples from acute leukemia patients was developed.

Conflict of interest: None

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Proteasome Inhibitors Induce FLT3-ITD Degradation through Autophagy in AML Cells

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Introduction: Internal tandem duplication of the Fms-like tyrosine kinase-3 receptor (FLT3-ITD) is found in 30% of acute myeloid leukemia (AML) and is associated with a poor outcome. We show that AML samples bearing FLT3-ITD mutations (n=14) are more sensitive to

proteasome inhibitors than wild type samples (n=13) (mean IC50 42nM vs 170nM) and this sensitivity is strongly correlated with a higher FLT3-ITD allelic burden (p=0.0084, r=-0.719).

Method: We uncovered by immunoblotting, electron microscopy and confocal analyses that proteasome inhibitors induce autophagy in AML cells which is responsible for the early degradation of FLT3-ITD leading to the inhibition of FLT3-ITD signaling and to cell death. Moreover, proteasome inhibitors overcome resistance to quizartinib (a potent second generation tyrosine kinase inhibitor) induced by mutations in the kinase domain of FLT3, suggesting that these compounds may prevent the emergence of mutant clones arising from tyrosine kinase inhibitor treatments. Finally, *in vivo* studies using a mouse xenograft model, confirmed that bortezomib was able to decrease FLT3-ITD protein level and to inhibit leukemic cell growth.
Conclusion: Therefore, selecting patients according to FLT3-ITD mutations could be a new way to detect a significant clinical activity of proteasome inhibitors in AML patients
Conflict of interest: None

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Activation of TRK Receptors in Murine Hematopoietic Stem / Progenitor Cells Induced Acute Leukemia and Mastocytosis

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Background: Recent data suggest that TRK (tropomyosin-related kinase) signalling might contribute to the pathogenesis of mastocytosis by autocrine and paracrine loops. We have recently demonstrated a potential role of neurotrophins including nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) and TRK signalling in leukemia development.
Material and methods: In the present study, 57 C57BL/6J mice were transplanted with retrovially gene-modified primary hematopoietic stem/progenitor cells (TRKB/BDNF=17, TRKB=8, BDNF=5, tCD34=7 in 3 independent experiments, and TRKA/NGF=8, TRKA=6, NGF=6 in 2 independent experiments). Interestingly, lymphoblastic leukemia was diagnosed in 5 animals with co-expression of TRKB/BDNF, whereas the other 12 animals unexpectedly developed systemic mastocytosis (SM), affecting mainly spleen, liver, and bone marrow with multifocal compact mast cell infiltrates. Mast cells demonstrated mainly features of mature hyper-granular mast cells. Most SM animals followed an indolent course, while leukemic mice survived <6 months after transplantation. In contrast, no animals with TRKB alone, BDNF alone or tCD34 showed SM or other hematological malignancies. These data strongly suggest that activation of TRKB by BDNF is important to promote mastocytosis. Interestingly, the frequency of SM disease in Kit D816V transgenic mice was lower than in our model (8/28=29% vs. 12/17=71%), whereas retroviral-mediated expression of Kit D816V even failed to induce SM in transplanted animals. Five out of 8 animals co-expression of TRKA/NGF developed acute leukemia (mainly mixed phenotype acute leukemia and erythroleukemia). It is interesting to know whether the remaining 3 animals from the TRKA/NGF group will also develop mastocytosis.
Conclusion: In summary, we provide the first direct evidence for induction of mastocytosis by activation of TRKB in hematopoietic stem/progenitor cells *in vivo*. Our data indicate an important role of TRK receptors in the pathogenesis of acute leukemia and mastocytosis.

Conflict of interest: None

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ASXL1 Mutations Associated with Cytogenetic Findings in *DE NOVO* and Secondary Acute Myeloid Leukemia

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Purpose: Mutations in the additional sex comb-like 1 (ASXL1) gene were recently shown in various myeloid malignancies including AML. The vast majority of these mutations which affect the twelfth exon of the gene, are frameshift and nonsense mutations and they are generally associated with an aggressive phenotype and poor clinical outcome. The encoded protein is involved in the epigenetic regulation of gene expression. This study aimed to identify the *ASXL1* mutations in de novo and secondary AML patients and associate them with the cytogenetic findings.
Methods: Our study included 200 AML patients (148 *de novo* AML and 52 *s*-AML) and 10 healthy donors. Conventional cytogenetic analysis was performed on unstimulated bone marrow cells. In order to detect A*SXL1* exon 12 mutations molecular analysis was performed by PCR and subsequent direct Sanger sequencing in all patients' and controls' samples.
Results: A successful karyotypic analysis was performed in all AML samples at diagnosis. Among them, 128 (64%) exhibited clonal karyotypic abnormalities while 72 (36%) showed a normal karyotype. *ASXL1 mutations* were detected in 38 out of 200 patients (19%). A significantly higher frequency of *ASXL1* mutations was observed in s-AML compared to *de novo* AML patients (28.8% *vs* 15.5%, respectively; p=0.035). None *ASXL1* mutation was observed in the control group. The most frequent *ASXL1* mutation was the c.1934dupG (31/38, 81.5%). *ASXL1 mutations* were more frequently found in males than in females (20.4% *vs* 18.8%, respectively), while stratification of patients according to age revealed a higher frequency of *ASXL1 mutations* in patients ≥60years than patients <60years (p=0.004). Among the 38 AML patients with *ASXL1* mutations, 9 patients had trisomy 8 (23.7%), 12 had a normal karyotype (31.5%), 2 had t(8;21)(5.3%), 2 had trisomy 13 (5.3%) and 13 patients other chromosomal abnormalities (34.2%).
Conclusions: These results suggest that *ASXL1* mutations are frequent in AML, especially in s-AML. *ASXL1* mutations are highly correlated with older age, male sex and specific karyotypic aberrations. The most common chromosomal abnormality associated with *ASXL1* mutations is trisomy 8.
Conflict of interest: None

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The Role Of Stat5 in FLT3-Mediated Leukemogenesis

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Introduction: Tandem duplication of the FLT3 juxtamembraneous domain (ITD) and point mutations of the tyrosine kinase domain (TKD) are among the most frequent alterations in AML. Although both lead to constitutive FLT3 signaling, only FLT3-ITD signals through the STAT5 pathway. In a murine bone marrow transplantation model, FLT3-ITD leads to MPN induction, whereas FLT3-TKD induces a lymphoid disease

with significantly longer latency. Recently, SRC was identified as a crucial interaction partner of FLT3-ITD for STAT5 activation.
Method: Here, we have investigated the role of STAT5 for the latency and phenotype of the FLT3-mutant induced diseases using a conditional *Stat5* knockout approach. Our studies proved that STAT5 is necessary for the induction of a MPN by FLT3-ITD, while FLT3-TKD is not dependent on activation of STAT5. Mice with deletion of the *Stat5* allele displayed expansion of lymphoid cells with an increased latency similar to the FLT3-TKD disease. In additional experiments, we were able to show differential activation of STAT5 in myeloid and lymphoid progenitors. Furthermore, *Oncostatin M,* a STAT5 target gene, is exclusively expressed in Flt3-ITD^{+} myeloid progenitor cells and in Flt3-ITD^{+} mice with intact *Stat5*. Strikingly, we could show that FLT3-TKD expression in combination with Oncostatin M is sufficient to induce a MPN, pointing to a pivotal role of STAT5 for induction of FLT3- induced myeloid neoplasms. Furthermore, significantly elevated *Src* expression levels could be detected in proliferating myeloid progenitor cells compared to lymphoid progenitors.
Conclusion: Taken together, STAT5 seems to be critical for the induction of a MPN by FLT3-ITD in a murine transplantation model. Interaction of SRC and FLT3-ITD leads to the activation of STAT5 in myeloid progenitors, but not in lymphoid progenitors, where ectopic expression of *Oncostatin M* can mimic the FLT3-ITD induced myeloid phenotype.
Conflict of interest: None

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Prevalence of PML/RARA, AML1/ETO, CBFB/MYH11 Translocations and Expression Level of Myeloid CD Markers amongst AML Patients of Assam, India

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Purpose: To document the prevalence of PML/RARα (bcr1 and bcr3), AML1/ETO and CBFβ/MYH11 translocations in AML patients from Assam, India. Expression level of CD markers was also recorded.
Methods and results: Blood/bone marrow samples were collected from 68 AML patients with informed consent at the time of daignosis. RNA was extracted & converted to c-DNA to Detect translocations. CD45/Side Scatter (SSC) gating strategy were used for immunophenotyping.
Out of 68 patients 14 patients (21%) were detected as PML/RARα positive, where bcr1 isoform was detected in 6 patients (43%) and bcr3 isoform was detected in 8 patients (57%), 12 patients (18%) were detecetd as AML1/ETO positive and in 1 patient (1.5%) CBFβ/MYH11 translocation was detecetd. The most common myeloid CD markers found in these patients were CD 13, CD 33, CD 45, CD117, CD 34 and CD 64.
Summary: Acute myeloid leukaemia (AML) is a type of cancer that affects the blood and bone marrow and one of the leading haematological malignancies in Assam, India. PML-RARα t(15;17)(q22;q21), AML1/ETO t(8;21)(q22;q22) and CBFβ/MYH11 t(16;16)(p13;q22) are reported to be the most frequent karyotypic abnormalities with diagnostic and prognostic value.
Conclusions: Our results conclude that PML/RARα is most prevalent translocation found in the study population followed by AML1/ETO. CD 13, CD 33, CD 45, CD117, CD 34 and CD 64 are most common in these patients.
Conflict of Interest: None

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The Mutatome of *CBFB*/*MYH11* Rearranged Acute Myeloid Leukemia (AML)

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Purpose: The fusion gene *CBFB*/*MYH11* results from inversion of chromosome 16 [inv(16)] and is found in 6-8% of adult de novo AML cases. Since expression of *CBFB*/*MYH11* is not sufficient to cause leukemia it is likely that additional mutations are essential for malignant transformation.
Methods: To systematically identify non-silent mutations which may collaborate with *CBFB*/*MYH11* during leukemogenesis we performed exome sequencing of 9 AML samples with inv(16) and matched remission samples. To test the recurrency of the candidate-genes found mutated by exome sequencing, we designed a custom gene panel.
Results: We found somatic mutations in *NRAS* (35%), *KRAS* (21%), *FLT3* (27%), *KIT* (22%), *WT1* (9%) and *PTPN11* (6%). In addition we found novel recurrent mutations in *FAT1* (6%), *NFE2* (6%) and *ZFHX4* (6%). In corresponding relapse samples from 7 patients with inv(16) all mutations in kinase genes (i.e. *NRAS*, *KIT*, *FLT3*) detected in 6 out of 7 cases at diagnosis were lost at relapse. Of note, one of these patients acquired mutations in *WT1* and *ZFHX4* at the time of relapse. Another patient gained a *CBL* mutation at relapse.
Conclusion: The mutatome of *CBFB*/*MYH11* rearranged AML is genetically complex with frequent secondary mutations in kinase genes such as *NRAS*, *KIT*, *FLT3*. These kinase mutations seem to be unstable during disease progression and the actual driver of relapse remains elusive in many cases.
Conflict of interest: None

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A Leukemia Associated CD34/CD123/ CD25/CD99-Positive Immunopheno-Type Identifies *FLT3*-Mutated Clones in Acute Myeloid Leukemia

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Background: Leukemia-associated-immuno-phenotypes (LAIP) defined by multiparameter flow cytometry (MPFC) are present at diagnosis in ~90% of Acute Myeloid Leukemias (AML) and are used to identify and measure MRD. We assessed in parallel LAIP profiles and gene mutational status in AML to better identify patients at high risk of relapse. Screening for *FLT3* mutations is relevant for patient risk stratification and recent studies suggest that relapse of *FLT3*-ITD AML may originate from very small subclones which are undetectable at diagnosis using conventional techniques.
Method: We have previously developed a patient-specific real-time quantitative PCR (ps-RQ-PCR) to implement *FLT3*-ITD detection, however this approach is not applicable at diagnosis to detect minor subclones. To improve detection of small populations harboring *FLT3*-ITD and potentially associated to chemoresistance, 9-color MPFC and

molecular analyses were used in parallel. Within the CD34+ve cell fraction, we identified a discrete population expressing high-levels of the IL-3 receptor alpha-chain (CD123) and MIC-2 (CD99) in combination with the IL-2 receptor alpha-chain (CD25). The presence of this population positively correlated with *FLT3* mutations. Receiver operating characteristics (ROC) showed that within the CD34+ve cell fraction, a percentage of CD123/CD99/CD25+ve cells≥11.7% predicted *FLT3* mutations with a specificity and sensitivity of >90%. CD34/CD123/CD99/CD25+ve clones were retrospectively evaluated and detected at presentation in 3 patients with *FLT3*wt/*NPM1*+ AML, who relapsed with *FLT3*-ITD/*NPM1*+ AML. The ps-RQ-PCR designed for each *FLT3*-ITD confirmed the presence of low copy numbers of the mutation in diagnostic samples in all three cases. Finally, MPFC revealed that the CD34/CD123/CD99/CD25+ve population correlated with absent or low CD38 expression, suggesting that this LAIP may identify leukemia initiating cells.

Results: Our data show that patients with *FLT3*-wild type/NPM1+ AML by routine PCR who carry at diagnosis a CD34/CD123/CD99/CD25+ subpopulation likely harbor small *FLT3*-ITD subclones potentially associated with chemoresistance and disease relapse. We propose that such patients are kept under "special surveillance" and stringently monitored for possible emergence of *FLT3*-ITD after remission induction and during follow-up.

Conflict of interest: None

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Leukemic Bone Marrow Cells Derived Microvesicles Increase microRNA21 Expression in Umbilical Cord Blood Hematopoietic Stem Cells (*IN VITRO*)

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Purpose: Cancer cells emit microvesicles to their microenvironment to represent an important mode of intercellular communication by serving as vehicles for transfer cytoplasmic proteins and RNAs. Since, microRNA21 is overexpressed in leukemia, this study considered the effect of microvesicles from leukemic microenvironment on microRNA21 expression in umbilical cord blood hematopoietic stem cells.

Methods: Umbilical cord blood hematopoietic stem cells sorted by MACS (CD34 antibody), were treated with 30 ug microvesicles isolated from bone marrow samples of patients with acute myeloid leukemia in diagnosis phase (written consent form was obtained) and were divided to three groups: 1) control group without microvesicles, 2) normal group treated with microvesicles from normal bone marrow samples and 3) leukemia group treated with microvesicles from leukemic bone marrow samples. Cell count, qPCR for microRNA21 expression and flowcytometry analysis for CD34 and viability test were performed after 7days.

Results: Number of cells in leukemia group showed a significant increase in comparison with other both groups ($P<0.05$). Also, 2.7 fold change of microRNA21 expression was observed in leukemia group ($P<0.01$). Surface CD34 results did not show any significant different among all studied groups in comparison with 97% CD34 in day0 sorted hematopoietic stem cells ($P>0.05$). Cell viability test done by 7AAD was more than 90% in all groups.

Conclusion: This study showed that microvesicles from leukemic microenvironment are able to deregulate microRNA21 expression in cord blood hematopoietic stem cells.

Conflict of interest: None

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Visualization of AML-Specific FLT3 Mutants and Differential Downstream Signaling

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Purpose: In acute myeloid leukemia (AML) patients *FLT3* is frequently mutated and clinical outcome varies depending on the type of mutation. Only internal tandem duplications (ITD) of *FLT3* are associated with unfavorable prognosis, whereas point mutations do not show a significant impact on patient outcome. Recent reports indicate this might be due to different subcellular localization and downstream signaling of the various FLT3 mutants. However, it remains unclear whether FLT3-ITD receptors are consistently retained within intracellular compartments or whether they are rapidly internalized after ligand stimulation. Therefore, we set out to study the differential localization of FLT3 mutants.

Methods: FLT3 mutants, namely N676K, D835Y, K644R and two different ITDs, were expressed in U2OS or BaF3 cells and analyzed by confocal and high resolution microscopy in combination with immunofluorescence (IF). To study the subcellular localization of FLT3 and to determine qualitative and quantitative differences upon ligand stimulation we used subdiffraction multicolor 3D structured illumination microscopy (3D-SIM). The FLT3 trafficking/activation kinetics are analyzed by live cell microscopy utilizing a spinning disc microscope. Imaging conditions are optimized for fluorescently tagged expression constructs (FLT3-GFP/FLT3-RFP), as well as tetracysteine motif tagged expression constructs allowing successive pulse labelling with two different colors. FLT3 inhibition is performed using PKC412 and AC220.

Results: Our IF staining confirmed a predominant ER-localization of FLT3-ITD, whereas FLT3 wildtype and point mutations localize to the cell membrane. While ITD activates STAT5 signaling, N676K and D835Y activate the MAPK pathway.

Conclusion: The type of FLT3 mutation impacts on the subcellular localization and downstream signaling.

Conflict of interest: None

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Hypoxia Regulates Flt3 Expression and Function in Patient Samples and in the BA/F3 Model

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Summary: Disease relapse remains a major cause of mortality for patients with acute myeloid leukaemia (AML). FLT3 is a receptor tyrosine kinase expressed by normal and leukemic myeloid progenitors and 25% of AML cases harbor a FLT3-ITD mutation, associated with an even inferior outcome due to increased relapse rate. AML relapse might be correlated to interactions between leukemic blasts and the bone marrow microenvironment (e.g. hypoxia), promoting leukaemia survival e.g. chemo-resistance. Hence, the effects of hypoxia on FLT3 expression could provide novel insight into relapse biology. **Material and methods:** Patient samples and the murine pro-B-cell line Ba/f3, stably transfected with FLT3-WT and with different FLT3-ITD mutants (W51, W78,

NPOS) were used. Experiments were carried out in parallel under standard laboratory conditions and reduced oxygen environment of 1% O_2. Applied methods comprise cell culture, flow cytometry (cell cycle analysis, apoptosis), real-time PCR and western blot. Survival analyses were done according to the method of Kaplan-Meier.

Results: AML patients samples were cultured under hypoxic conditions for 48 hours and 40% (n=6) of those expressing FLT3, showed a down-regulation of total FLT3 protein level independent on the mutational state. This finding could not be observed in any human AML cell line screened (n=14), however both Ba/f3 WT and all three ITD mutants checked showed FLT3 down-regulation after 72 hours at 1% O_2. This was accompanied by a significant decrease in cell growth as compared to 21% O_2, without significant increase in apoptosis. Real-Time PCR analysis showed no difference in the transcriptional regulation of FLT3 under 1% O_2 compared to 21% O_2. Susceptibility against FLT3-TKI was unaffected despite decreased FLT3 expression. Interestingly, half-life of FLT3 protein was shorter at 1% O_2 (1 hr) as compared to 21% O_2 (2 hrs). Degradation of FLT3-ITD is mediated by proteasome, which could reliably be inhibited under normoxic and hypoxic conditions. Re-oxygenation of hypoxic AML blasts with 21% O_2 revealed that down-regulation of FLT-3 is reversible within 24 hours. The mechanisms of down-regulation for FLT3 seem to be complex: degradation is certainly mediated by proteasome and seems not to be transcriptionally dependent. Clinically, FLT3 regulation status might be of prognostic importance (n=8, relapse free survival (RFS) for non-regulators 2.2 months versus 10.0 months for regulators, p=0.07).

Conclusion: This data shows that hypoxia influences FLT3 expression in primary patient samples and the Ba/f3 model. The functional consequences are still unknown, but patients that fail to down-regulate FLT3 tend to have a shorter RFS. The mechanisms involved in down-regulation comprise a proteasome-dependent degradation. Ongoing projects focus on molecular mechanisms responsible for oxygen-dependent regulation of FLT3 and on biological consequences.

Conflict of interest: none

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Systems Biology of Mixed Lineage Leukemia

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Purpose: This study comprehensively records the kinetics of global gene expression changes that accompany the transition from a normal hematopoietic precursor to a transformed pre-leukemic cell. Employing MLL-fusion transformed cells as a paradigma of hematopoietic transformation the hierarchy and wiring of control circuits governing the decision between self-renewal and differentiation are visualized.

Methods and results: With the help of a knock-in model introducing a conditional MLL-fusion oncogene into the mouse germ-line we are presenting a „reverse-engineering"strategy that allows kinetic sampling of the "transformed to normal" transition. By determination of global transcription rates through nascent RNA sequencing coupled with genome-wide ChIP-Seq probing for oncogene presence and function we identify the crucial downstream mediators of MLL-fusion induced transformation. Combining kinetics with occupancy data allows to discriminate drivers from bystanders. Thus novel players, pathways and principles determining the transforming activity of MLL fusion proteins could be identified. Biological verification experiments involving a select set of hitherto unknown MLL-fusion targets confirmed their role in MLL-induced transformation and revealed the importance of pathways including stable RNA-synthesis, splicing and control of metabolism that had not yet been associated with transformation by MLL-fusion proteins. This comprehensive knowledge of the MLL-triggered transforming network will allow for the rational design of novel inhibitors as potential therapeutic strategies.

Conclusion: MLL-fusions are multifunctional oncoproteins that control a discrete and highly redundant network of control circuits that cooperate to keep hematopoietic precursors in an undifferentiated state. The analysis strategy applied in this work can be universally adapted for other oncogenes facilitating the elucidation of different transforming principles.

Conflict of interest: None

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IN VIVO Imaging Facilitates Reliable and Sensitive Monitoring of Preclinical *IN VIVO* Treatment Trials in the Individualized Xenograft Mouse Model of AML

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Purpose: In the individualized xenograft mouse model of AML, primary patients' AML cells are transplanted into immunocompromised mice. We aimed at improving this model to enable reproducible, sensitive and reliable disease monitoring for preclinical treatment trials.

Methods: Primary AML patient cells were engrafted in immunocompromized NSG mice and serially retransplanted. Patient-derived xenografted (PDX) cells were lentivirally transduced in vitro to express a fluorochrome and enhanced firefly luciferase. Transgenic t-PDX cells were enriched by flow cytometry, re-injected into mice, and monitored over time using bioluminescence in vivo imaging (BLI). Mice were treated in preclinical treatment trials; effect of drugs on leukemia burden was monitored by BLI.

Results: About 30% of primary AML patient samples allow serial transplant-ation in NSG mice and thereby generation of stable PDX cells. PDX cells were derived from genetically distinct patient samples, mimicking the known heterogeneity of AML. Targeted resequencing of 43 genes important for AML leukemogenesis revealed identical mutations in primary and PDX cells after initial or serial transplantation, besides two losses of minor suclones.

Until now, five PDX samples were genetically engineering by lentiviral transduction. Recombinant expression of luciferase enabled BLI for reliable follow up of leukemia growth in mice. Imaging was highly sensitive and detected t-PDX cells on MRD level (10^{-4} leukemic cells within mouse BM). Furthermore, imaging facilitated reliable analysis of preclinical treatment trials, visualizing drug effects in single mice over time.

Conclusion: We advanced the individualized xenograft mouse model of AML by introducing serial transplantation, lentiviral transduction and *in vivo* imaging. These improvements allow sensitive preclinical treatment trials and molecular *in vivo* studies.

Conflict of interest. None

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Detection Of Chromosome 9q Deletion in Acute Myeloid Leukemia (AML) Patients Using Targeted Sequencing Data

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Introduction: Whole exome sequencing (WES) or custom gene panel sequencing (GPS) allows to detect gain or loss of genomic material in tumor cells (i.e. copy number alteration; CNA) and thereby whole chromosome aneuploidies and submicroscopic amplifications or deletions of genomic regions.

We performed WES at diagnosis and complete remission of AML patients with partial deletions of chromosome 9 (AML del(9q), n=5) as well as GPS of known mutational targets in AML and candidate genes located on 9q of 26 diagnostic AML del(9q) samples and 21 AML patients without any detectable cytogenetic aberration on chromosome 9.

Method: CNA profiling based on WES showed somatically acquired stretches of significantly reduced read counts for genes located on 9q in 2 of the 5 patients, which is in line with corresponding SNP array results. CNA profiling based on GPS defined a common deleted region with a size of at least 8.4 Mb, detected in 18 out of 26 (70%) patients. Patient samples without detected 9q deletion in our CNA analysis have significantly fewer metaphases with 9q deletion (median 27%, range 8-77%) compared to samples tested positive for 9q deletion by our CNA analysis (median 95%, range 24-100%; p=0.001), as reported by routine cytogenetics. Furthermore, the varying clone size harboring the 9q deletion in the diagnostic samples is limiting for CNA detection.

Result: Beyond the identification of somatic mutations with single nucleotide resolution, sequencing data enables the efficient detection of recurring and/or somatic CNAs in AML.

Conflicts of interest: None

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Evaluation of DNA Repair Gene Polymorphisms In Acute Myeloid Leukemia

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Purpose: Deregulated DNA repair is one of the hallmarks of cancers including Acute Myeloid Leukemia (AML), as it results in genomic instability. ATM gene functions as a sensor, activates cascade of events leading to stimulation of multiple DNA damage- responsive signaling pathways. Principal DNA repair mechanism activated in the hematopoietic stem cells is the Non Homologous End Joining (NHEJ) pathway. However, this pathway was shown to be error prone. Functional SNPs in the genes involved in DNA repair might influence the gene expression leading to altered DNA repair which might confer the risk to AML.
Methods: This hospital-based case-control study included 225 AML patients and 326 cancer-free controls from South Indian population. Six polymorphisms of XRCC5, XRCC6, XRCC7 and ATM were genotyped using polymerase chain reaction (PCR)-Restriction Fragment Length Polymorphism (PCR- RFLP) method. Statistical analyses were performed by using SPSS (version 20.v) and SNPSTAT online tool. Protein-Protein Interaction (PPI) analysis was also done to see the relationship between these genes.
Results: We found that there was an elevated risk of AML associated with the XRCC5 VNTR 0R repeat and A allele of 2408G>A polymorphism (p-0.04 and p<0.0001 respectively). The frequencies of G allele (p-<0.0001) of XRCC6 -1310C>G and T allele (p-0.003) of ATM -5144A>T polymorphisms were also significantly increased in AML cases. Further, analyses of the variant genotypes with epidemiological and clinical variables revealed a significant association of the risk genotypes with development and progression of AML. However, XRCC6 -61C>G and XRCC7 6721G>>T polymorphisms did not show any association with AML
Conclusion: The XRCC5 VNTR (0R repeat), 2408G>A (A allele), XRCC6 -1310 C>G (G allele) and ATM-5144A>T (T allele) polymorphisms play an important role in the pathogenesis of AML.
Keywords: AML, PCR- RFLP and NHEJ pathway
Conflict of interest: None

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Acute Myeloid Leukemia Preceded by t-MDS After Succesful Treatment of Chronic Lymphocytic Leukemia with FC-R Regimen: Secondary Leukemia? Another Myeloid Neoplasm? - A Case Report

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Introduction: Chronic lymphocytic leukemia (CLL) is a high-risk disease for developing secondary neoplasms due to immunodeficiency associated with the disease and/or with the treatment. Acute myeloid leukemia(AML)or myelodysplastic syndrome (MDS) was a rarely entity, these were observed in 3.5% to 6.1% of patients treated with fludarabine alone or in combination. AML or MDS coexisting with untreated CLL were also reported.
Case report: We report here a case of 60-year female with the diagnosis of CLL Rai stage 1 with no chromosomal changes, who developed AML preceded by t-MDS, 3 years after completion of CLL therapy consisted with 6 courses with FC-R regimen. She achieved CR with incomplete recovery of platelet and WBC count. In August 2009 she was admitted to hospital with symptoms of pleuro-pneumonia and deep pancytopenia with 5% of blasts in peripheral blood (PB)and 15% in bone marrow aspirate-RAEB was diagnosed. A week later performed BM revealed 42% of blasts which displayed a myelomonocytic phenotype.Cytogenetic analysis showed complex chromosomal abnormalities; del 5 (13q33), trisomy 8, trisomy 20, monosomy 21. No abnormalities concerning chromosomes; 11, 12, 13 and 17 were seen. The diagnosis of AML/RAEB-t was made. No CLL cell clone was found in BM, no organomegaly was seen in CT examination. The patient was still in CR of CLL. The patient received induction chemotherapy with daunorubicine plus cytarabine(3+5)and obtained CR. Relapse occurred three months later. The patient died 8 months later due to leukemia with its complications (infections, haemorrhages from GI tract).
Conclusions: In presented case we can consider drug-related origin of AML in patient with complete remission of CLL or another myeloid neoplasm from unknown origin.
Conflict of interest: None

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Mimicking The Bone Marrow Niche *IN VITRO*: Establishment Of A 3D Perfusion Culture System for Mesenchymal Stromal Cells

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Introduction: Hematological disorders such as leukemia are associated with disruptions of the microenvironment within the bone marrow (BM) niche. The aim of this study was to establish a 3D perfusion culture system for leukemic cells and human mesenchymal stromal cells (MSC), which constitute an important supportive cellular component of the bone marrow niche.
Methods: We used a modular perfusion culture system (Will W. Minuth, Regensburg, Germany). MSC were cultured on porous membrane filters consisting of mixed cellulose which were permanently perfused with fresh culture medium. The viability and induction of apoptosis of the MSCs and leukemic cells in different culture conditions was tested.
Results: A perfusion rate of 20.8 μl per minute and a concentration of 50 mM HEPES buffer were observed to be optimal. We could show that the amount of viable MSCs cultured in perfusion culture was considerably higher (OD_{450}: 0.37 ± 0.03 vs. 0.2 ± 0.01) than in conventional cell culture. The basal level of apoptotic cells in a co-cultivation of MSC and a leukemic cell line is 1.7x increased in conventional cell culture compared to perfusion culture. MSC showed no differences in the expression of exemplary chosen genes relevant for the BM niche (CXCL12, Jag1) due to the different culture conditions. Additionally, MSC cultured on membranes formed 3D-like networks. No differences in morphology were observed with respect to the different culture conditions.
Discussion and conclusion: The 3D perfusion culture system is sufficient to increase the viability and proliferation of MSC without changing the gene expression profile of exemplary chosen genes. The system can be used for further experiments including co-culturing experiments with MSCs and leukemic blasts and might be an important option to mimic the hematopoietic stem cell niche *in vitro*.
Conflict of interest: None

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The DNA-Repair Machinery as a Novel Target in a Humanized T(8;21)+ Pre-Leukemia Model with Activated C-KIT

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Introduction: The RUNX1/ETO fusion protein, which originates from the t(8;21) chromosomal rearrangement, is one of the most frequent translocation products found in *de novo* acute myeloid leukemia (AML). In RUNX1/ETO leukemias, activated forms of the c-KIT tyrosine kinase receptor are frequently found, thereby suggesting oncogenic cooperativity between these oncoproteins in the development and maintenance of t(8;21) malignancies.
Method and discussion: We show that activated c-KIT cooperates with RUNX1/ETO to expand human CD34+ hematopoietic progenitor/stem cells *ex vivo*. Morphologically and cytometrically, CD34+ cells expressing both oncogenes precisely resemble the AML-M2 myeloblastic leukemia cell phenotype. In contrast, RUNX1/ETO-expressing cells largely undergo granulocytic differentiation. Oncogenic c-KIT amplifies RUNX1/ETO-depended clonogenic growth and protects cells from exhaustion. Of note, activated c-KIT reverts the RUNX1/ETO-induced DNA-damage followed by apoptosis, a recently described RUNX1/ETO-dependent phenomenon. In the presence of activated c-KIT, RUNX1/ETO-downregulated DNA-repair genes including OGG1 and BRAC2 are re-expressed leading to an enhancement of DNA-repair efficiency via homologous recombination. Through this mechanism activated c-KIT protects from RUNX1/ETO-induced cellular stress. This points to a direct link between the high frequency of mutated c-KIT and the occurrence of t(8;21) and herewith provides a rational mechanism of oncogenic cooperativity and drug resistance.
Results: Together, our results provide new mechanistic insight into RUNX1/ETO and c-KIT oncogenic cooperativity in human primary CD34+ progenitors and suggest that augmented DNA-repair might account for the increased chemoresistance and decreased overall survival rates observed in t(8;21)-positive AML patients with activated c-KIT mutations. This cell protective mechanism represents a new therapeutic target, as RUNX1/ETO cells with activated c-KIT are highly sensitive to pharmacological inhibitors of DNA-repair.
Conflict of interest: None

III. AML – THERAPY

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Evaluation of New DNA Methyltransferases Inhibitors in AML

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Introduction: The discovery of new drugs and drug targets can help to improve the cure rates of Acute Myeloid Leukemia (AML). DNA methyltransferases (DNMTs) inhibitors represent a group of promising compounds for AML treatment because leukemic cells have altered genome methylation, as hypermethylation of tumor suppressor genes promoters.
Method: The present study aims the in vitro evaluation of two DNMTs inhibitors, called B and Q that are nanaomycin A analogs. Cytotoxic activity was carried out in K562 AML cell line by XTT reduction assay, performed 48 hours after addition of compounds increasing concentrations. Enzymatic activity inhibition of DNA methyltransferase DNMT1 and DNMT3B were accessed by Western Blot technique followed by quantitative RT-PCR. In addition, we investigated the methylation of CpG islands located in promoter regions of six tumor suppressor genes, by pyrosequencing, before and after treatment with both compounds.
Discussion: K562 showed an IC50 of 10,4uM and 6,6uM for compounds B and Q respectively. At protein level, the treatment with B compound resulted in decrease of both DNMT1 and DNMT3B levels while Q treatment resulted in decreased DNMT1 and increased DNMT3B isoforms, when compared to control sample (DMSO). At mRNA level, the treatment with B showed a decreased DNMT1 with no significant alteration on DNMT3B while the Q treatment showed a decreased DNMT1. We also verified an increased DNMT3B mRNA level when cells were treated with Q compound at 0,5μM. Regarding methylation of tumor suppressor genes promoters, we observed that no ESR1 and RASSF1 genes promoters were methylated in K562 cells since methylation levels of these promoters in control cells (DMSO) were also very low. CADM1, Mir141, MGMT and THBS1 promoter regions showed methylated patterns, however no difference in methylation of these regions was observed between treated and control cells.
Conclusion: We concluded that compounds B and Q were both cytotoxic for K562 cell line and decreased DNMT1 protein levels. Besides, compound B also decreased DNMT3b protein levels. Currently, we are working on DNMT1 and DNMT3B in vitro inhibition assay to elucidate the

compounds mechanisms of action and on K562 gene expression profiles to understand some of the genes pathways affected by these compounds.
Conflict of interest: None

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New Therapeutic Targets For MLL+ AML Identified via Mll-AF9 Depletion

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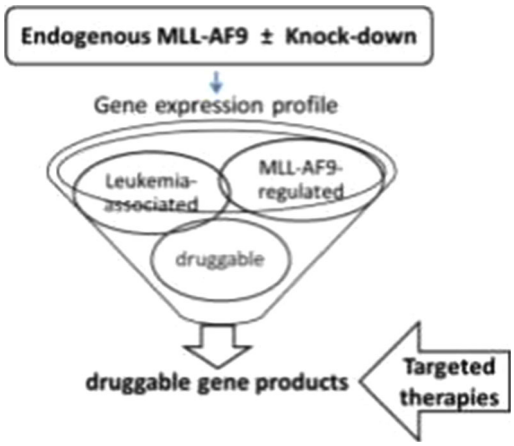
Background: MLL-translocations are frequent in infant acute myeloid leukemia (AML). Their leukemogenic potential is widely accepted, but unfortunately MLL-fusions are not amenable to therapeutic intervention. We thus set out to identify novel therapeutic targets downstream of MLL-AF9 with the prospect to improve the still unsatisfactory prognosis of MLL+ AML.
Methods: Gene expression profiles were generated and bioinformatically interpreted after efficient and specific silencing of endogenous MLL-AF9 in THP1 (AML) cells.
Results: A strategy to prioritize genes which mediate MLL-AF9 leukemogenic effects resulted in a set of 41 out of hundreds of differentially regulated genes. Seven of these qualified as druggable and thus could offer novel therapeutic targets for MLL+ AML. We show that targeting one of the encoded proteins, the dopamine receptor D5, was able to reduce leukemic cell characteristics of THP1 cells.



Conflict of interest: None

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Localized Biliar Myeloid Sarcoma Followed by Acute Myeloid Leukemia Treated with Chemotherapy and Consequent Allogeneic Stem Cell Transplantation: A Case Report

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Background: Myeloid sarcoma (MS) is an extramedullary localization of immature granulocytic precursor cells. Gastrointestinal tract involvement is a rare phenomenon. A 62 year old man was admitted to our department with fever, abdominal pain and jaundice due to a common bile duct mass constricting the choledochus duct.
Method: After endoscopic stent implantation, an explorative laparotomy with cholecystectomy lead to diagnosis of myeloid sarcoma with involvement of cholecystis and choledochus. Further tumor manifestations, in particular bone marrow infiltration, were excluded. Due to present febrile cholestasis subcutaneous low dose Cytarabine (LDAC, 20mg/bid) together with broad spectrum antibiotics were initiated. Staging after one cycle of LDAC documented a significant tumor mass reduction, thus two intensive chemotherapy cycles with Idarubicin, Cytarabine, and Etoposide were performed. During both aplasia periods a biliary drainage change and broad antimicrobical therapy were required because of febrile cholestasis. Consequent re-staging documented a complete remission (CR), therefore a consolidation radiotherapy with 24 Gy was added. After four months of follow-up (FU) fever and petechial skin due to piastrinopenia and leucocytosis with 74% of myeloid blast in peripheral blood appeared. Intensive chemotherapy induced a CR of the AML, thus a second consolidation cycle was performed, both complicated by febrile cholestasis requiring again biliary drainage replacement. Given the fact that a CR of AML and MS was obtained, a semi-intensive Daunorubicin/Citarabin cycle was given and after myeloablative chemotherapy hematopietic stem cell transplantation (HCT) with peripheral blood stem cells from a 10/10 matched unrelated donor realized. Graft versus host disease prophylaxis contained Ciclosporin, Methotrexate and antithymoglobulin. The aplasia period was complicated by fever without cholestasis. During the recovery the drainage was not opened and the patient could be discharged after 21 days. The patient is currently at 4 month FU maintaining a CR of AML with 100% chimerism and no MS or infection signs.
Conclusion: The present case highlights the complexity of an high risk disease with severe infective complications managed in an interdisciplinary approach including final HCT.
Conflict of interest: None

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A Fluorescence in-Situ Hybridization-Based Screen Allows Rapid Detection of Adverse Cytogenetics in Patients (PTS) with Acute Myeloid Leukemia (AML)

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Purpose: Conventional cytogenetic analysis is a mandatory component of AML workup. In older pts (>60 y), presence of adverse cytogenetic changes is associated with poor response to standard induction chemotherapy and short overall survival. Conventional cytogenetic studies are time-consuming, and therapeutic decisions are often delayed until the karyotyping result is available. Thus, we investigated whether a targeted FISH screening would allow the rapid identification of the most common unfavorable chromosomal changes in AML.
Patients and methods: We studied 318 newly diagnosed AML pts (263 pts>60 y). Conventional cytogenetic analyses were performed using standard techniques. For FISH analyses probes directed against 7 genomic loci were used: 5p15.2 (D5S721, D5S23), 5q31 (*EGR1*), 7cen (D7Z1), 7q31 (D7S486), 17p13 (*TP53*), 17cen (D17Z1) and 3q26.2 (EVI1). The result was considered positive if the signal pattern was consistent with one of the following abnormalities: del(5)(q31) or -5, -7, abn(17p13) or -17, or inv(3)/t(3;3).
Results: FISH screening was positive in 75 pts, and 73/75 belonged to the ELN adverse genetic group. Thus, a positive FISH result indicated the adverse karyotype in 97% of cases (pos. predictive value). Contrary, 21 out of 243 $FISH^{neg}$ pts belonged to the adverse group according to their

karyotype. These pts mostly carried complex structural changes without involvement of chromosome 5, 7, or 17.
Conclusion: The median time from receipt of the specimen until the FISH screening result became available was two days. Our targeted screening assay using a limited number of FISH probes could rapidly identify a large majority of older AML pts with unfavorable cytogenetics and represents a valuable addition to the standard genetic workup of AML.
Conflict of interest: None

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The Method of Risk-Adapted Therapy Adult Acute Myeloid Leukemia Based on the Monitoring Minimal Residual Disease by Multicolor Flow Cytometry

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Background: The main methods of monitoring minimal residual disease (MRD) in acute myeloid leukemia (AML) are currently the detection of specific gene mutations at the time of initial diagnosis and stages of therapy and identification of leukemic phenotype (LAF) using multicolor flow cytometry.
Method: Identified the following markers (options) LAF: 1. Expression linearly lymphoid cell specific markers: CD19, CD7, CD22, CD4, CD56; 2. The absence or reduction in the expression of specific markers linearly myeloid cells: CD13, CD33; 3. The absence or reduction in the expression of markers of nonlinear characteristic of a given stage of differentiation: HLA-DR CD38, 4. Hyper expression of nonlinear and linear-specific markers: SD15, CD11b, Tdt; 5. The expression 7.1.
As an additional warranty to minimize the probability of relapse accepted the need to intensify therapy in patients with the expression of the interleukin 2 receptor alpha (CD25) clonal blast cells. Algorithm for risk-adapted induction therapy is as follows. When the content of CD25 + cells before treatment and at least 10% in the absence (0%) of cells with LAF after the first course of remission induction therapy protocol "7 + 3" with escalated doses of anthracyclines (in 14 days from start of treatment) were limited to one course of induction remission, the patient is transferred to consolidation remission therapy with high-dose cytarabine protocol HiDAC. When the content of CD25 + cells before treatment and at least 10% of cells with LAF on day 14 of 0.01% or more was conducted two courses of remission induction protocol "7 + 3", and the first course with escalated, and the second with standard doses of anthracyclines. When the content of CD25 + cells prior to treatment is 10% or more and no (0%) cells at day 14 LAF performed two cycles of remission induction protocol "7 + 3", a first course with escalated, and a second standard doses of anthracycline . When the content of CD25 + cells before treatment to 10% or more, and cells LAF on day 14 of 0.01% or more, conducted two courses of induction of remission, the first course protocol "7 + 3" with escalated doses of anthracyclines, and the second course FLAG-Ida.
Result: As a result of applying this algorithm frequency cytomorphological complete remission was 89.5% complete remissions cytomorphological and immunophenotypic - 84%.
Conflict of interest: None

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The Hedgehog Pathway Mediator GLI Represents a Negative Prognostic Marker in Acute Myeloid Leukemia and its Inhibition Mediates Anti-Leukemic Effects *IN VITRO*

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Purpose: The Hedgehog pathway plays an important role in stem-cell biology and malignant transformation. Therefore we investigated expression and prognostic impact of Hedgehog pathway members in acute myeloid leukemia (AML).
Methods: Pre-treatment samples from 104 newly diagnosed AML patients (AMLSG 07-04 trial) were analyzed by qPCR and expression of Hedgehog family members was correlated to clinical outcome. Inhibition of *GLI* by GANT61 or shRNA was investigated in AML cells *in vitro* and *in vivo*.
Results: Expression of receptors *SMO* and *PTCH1* and their downstream mediators *GLI1, GLI2* and *GLI3* was detected in AML patients while no expression of Hedgehog ligands was found.*GLI2* expression had a significant negative influence on event-free (EFS), relapse-free (RFS) and overall survival (OS; p=0.037, p=0.026 and p=0.013, respectively) and was correlated to FLT3 mutational status (p<0.001). Analysis of a second, independent patient cohort confirmed the negative impact of *GLI2* on EFS and OS (p=0.007 and p=0.003, respectively; n=290). Within this cohort, also *GLI1* had a negative prognostic impact (p<0.001 for both EFS and OS). Although AML cells did not express Hedgehog ligands, AML patients had significantly increased Desert Hedgehog (DHH) plasma levels compared to healthy subjects (p=0.002). Provision of DHH could be ascribed to BM niche cells which showed elevated DHH expression levels compared to the whole cell BM compartment. Moreover, the *GLI* inhibitor GANT61 or knockdown of *GLI1/2* by shRNA caused anti-leukemic effects including induction of apoptosis, reduced proliferation and colony formation in AML cells and a survival benefit in a mouse model.
Conclusion: *GLI* expression is a negative prognostic marker that might represent a novel therapeutic target in AML.
Conflict of interest: None

IV. ALL – BIOLOGY

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Influence of Submicroscopic Genomic Rearrangements on Gene Expression in T-Cell Large Granular Lymphocyte Leukemia

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Objectives: We searched for genetic alterations in T-cell large granular lymphocyte leukemia (T-LGL) patients and MOTN-1 cell line (established from T-LGL patient) to better understand the molecular pathogenesis of T-LGL. We further analyzed their impact on gene expression and influence on cell biology.

Methods: mFISH analysis of the MOTN-1 cell line was performed as well as paired-end next generation sequencing (NGS; Illumina HiSeq2000) of MOTN-1 and one T-LGL sample. In addition, a distinct 6q region was characterized in three T-LGL samples using high resolution comparative genomic hybridization (FT-CGH) and LM-PCR. Gene expression was studied by RNA sequencing (RNAseq; SOLID5500).
Results: Rearrangements were detected within 1p and 2q in MOTN-1 affecting expression of *FGR, ZEB2* and *CASP8*, and within 6q in MOTN-1 and one T-LGL sample affecting *MAP3K5* and *IFNGR1*. Nineteen genes, among them *FON3, RIN3, AKT1, PPP2R5C,* were overexpressed as a result of an amplification in 14q in one T-LGL patient. Two novel fusion transcripts were identified: *CASP8-ERBB4* in MOTN-1 and *SBF1-PKHD1L1* in T-LGL sample.
Conclusions: Submicroscopic genomic rearrangements change gene expression in T-LGL. Several genes involved in rearrangements were previously linked to cancer and survival pattern that characterizes T-LGL cells. In further experiments the involvement of the genes detected will be analyzed in more samples to delineate affected pathways.
Conflict of interest: None

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Knockdown of XIAP Sensitizes B-ALL CELLS Towards Chemotherapy in an Innovative, Dual Luciferase *IN VIVO* Competition Assay

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Introduction: Acute lymphoblastic leukemia (ALL) is a frequent disease in children and adults which remains difficult to treat in many patients. Novel therapeutic options are intensively desired.
XIAP, the X-linked inhibitor of apoptosis, is a potent kinase inhibitor, highly expressed in hematological malignancies and contributing to chemoresistance in different cancer cells.
Study aim and method: Here we aimed at characterizing the role of XIAP for *in vivo* chemosensitivity of ALL cells using a novel *in vivo* competition assay. The preB-ALL cell line NALM6 was grown in immuno-compromised mice. Lentiviral transduction allowed expression of luciferase and *in vivo* imaging in ALL cells for highly sensitive, reliable and continuous follow up of leukemic growth *in vivo*, enabling the precise evaluation of novel therapeutic approaches preparing their translation into the clinic.
For *in vivo* competition assays, we expressed either Gaussia or enhanced Firefly luciferase for *in vivo* imaging which allowed simultaneous monitoring of two different cell populations in a single mouse by injecting their respective substrates, Coelenterazine and Luciferin. Thus, two different cell populations were comparatively monitored in real time in a single mouse, e.g., each containing a different genetic alteration. Furthermore, we expressed miRNA embedded shRNA coupled to a fluorescence marker using different color labeling for control and knockdown population. Finally, control and knockdown cells were grown in a single mouse and were continuously distinguished *in vivo* by dual luciferase imaging and post mortem by precise quantification of the fluorescent markers.
Results: Using this approach, we could show that down-regulation of XIAP expression on its own did not affect engraftment and development of NALM6 cells in NSG mice. However, knockdown of XIAP significantly enhanced the anti-tumor effect of chemotherapy, as measured both by dual luciferase imaging and by *ex vivo* quantification of leukemia cells. Taken together, we established a system allowing comparative *in vivo* assays of two cell subpopulations in real time *in vivo* within the same animal. Our results show that targeting XIAP in ALL improves the effectiveness of systemic chemotherapy *in vivo*.
Conflict of interest: None

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Clonality-Analysis in BCRABL-Induced Leukemia By Genetic Barcodes

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Purpose: Genetic barcodes (BCs) are short sequences that are introduced into the genome as an expression-independent method for stable and unique marking of cells and their progeny. We used a BcrAbl-BC-transduced cell line to investigate clonal competition in vitro as well as in the development and progression of BcrAbl-induced leukaemia in the mouse model.
Methods: We constructed a γ-retroviral vector encoding BcrAbl in conjunction with GFP. In this vector, our BC32-sequence was introduced to establish a γ-GFP-BC32-BcrAbl-vector plasmid library. γ-retroviral vectors were generated and used to transduce the murine, IL-3-dependent cell line Ba/F3. After IL-3 withdrawal, cells were cultured for 52 days; samples were taken every 2-3 days to follow the clonal dynamics *in vitro*. 10.000 GFP-positive cells were transplanted into female Balb/C mice without conditioning. Diseased mice were sacrificed about 20 days after transplantation and haematopoietic organs were analysed for BC32-content.
Results: The complexity of the plasmid library was $>8x10^4$ different BCs. Transduction of the initial Ba/F3 cell culture leads to 2.9% GFP-marked cells which corresponds to 2900 BCs. Six recipient mice developed leukaemia in about 20 days after transplantation of GFP-positive cells. Investigation of the BC-sequences revealed a monoclonal leukaemia in all animals, which was caused by only two different BcrAbl clones. The integration-site of each clone could have supported the clonal dominance of these clones.
Conclusion: Our γ-GFP-BC-BcrAbl vector is a suitable tool to follow-up clonal competition in vitro and in vivo. Marking with barcoded vectors can be expected to provide novel insights in the clonal development of normal tissue but also during progression of malignant diseases.
Conflict of interest: None

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Drug Sensitivity of Patient-Derived ALL Tumor Cells Is Impaired by Co-Culture with Feeder Cells Mimicking the *IN VIVO* Micro-Environment

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Background: The success of anti-leukemia treatment depends on the response of leukemia cells towards drug treatment; treatment resistance leads to persistence of tumor cells, post-therapeutic residual disease and potentially disease relapse.
Patients and methods: Here, we studied the influence of the feeder cells on drug sensitivity of patient-derived ALL cells in vitro. Towards this aim, primary ALL cells from two children with high-risk or relapsed BCP-ALL were amplified in immuno-compromised mice and cells freshly isolated from mice were subjected to in vitro experiments. Several cytotoxic drugs of routine ALL treatment were used to stimulate patient-derived ALL cells in vitro. Stimulations were performed in the presence

and absence of feeder cells mimicking the in vivo microenvironment.
Results: Feeder cells only slightly prevented spontaneous cells death of patient-derived ALL cells in vitro. While several cytotoxic drugs induced marked apoptosis when used in clinically relevant concentrations in vitro in the absence of feeder cells, the presence of feeder cells markedly reduced apoptosis induction. In certain drugs, reduction of apoptosis by feeders was up to 50 %.
Conclusion: From our data we conclude that the environment inherits major inhibitory effects on drug sensitivity of ALL cells and should be considered in novel treatment protocols for ALL.
Conflict of interest: None

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SINGLE CELL CLONES FROM A PATIENT WITH ALL SHOW DIFFERENT DRUG SENSITIVITY AND GROWTH BEHAVIOR IN VIVO

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Background: Acute leukemias consist of heterogeneous cell populations and the most aggressive subpopulation determines prognosis and outcome in each patient. Here, we aimed to functionally discriminate clonal subpopulations in order to link adverse characteristics. Towards this aim, we characterized several single cell clones regarding their functional properties in vitro and in vivo using tumor cells from a patient with ALL.
Method: We amplified the patient's ALL cells in immuno-compromised mice within the individualized xenograft mouse model. RGB marking enabled multicolor staining of patient-derived xenograft ALL cells based on lentiviral genetic engineering; RGB marking allowed isolation of individually colored viable single cell clones from the ALL patient.
Results: Single cell clones were characterized for their functional properties in vitro and in vivo as well as for phenotypic markers. When 2 differently RGB marked clones were mixed within the same recipient mice, one clone overgrew the other indicating different proliferation rates of both clones. In vitro, chemosensitivity clearly differed between several clones tested, especially regarding stimulation with glucocorticoids, indicating that resistant subclones existed before start of treatment.
Conclusion: Taken together, RGB marking allowed generation of viable single cell clones from primary tumor cells of an ALL patient for functional characterization in vivo and in vitro. Within the heterogeneous tumor bulk, aggressive subclones exist showing slow tumor growth or drug resistance. Our studies might help to eradicate challenging subclones to increase the prognosis of patients with ALL.
Conflict of interest: None

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PH-Like ALL is Associated with Homogenous Molecular Alterations and Inferior Outcome in Adults

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Background: Patients with Philadelphia-like B-cell precursor acute lymphoblastic leukemia (Ph-like ALL) are characterized by distinct genetic alterations and inferior prognosis in children and younger adults (AYA). The purpose of this study was the genetic and clinical characterization in adults. Affymetrix gene expression profiles of 207 adult B-cell precursor ALL patients were classified as Ph-like ALL according to published algorithms.
Patients and methods: Clinical and outcome analysis were restricted to patients treated within GMALL trials 06/99 and 07/03 (n=107). The molecular characterization included copy number variation analysis and targeted amplicon sequencing. We identified 26 patients (13%) with Ph-like gene expression profile. Among 105 B-cell precursor ALL patients that were negative for Ph- and MLL-translocations (B-other) the incidence of the Ph-like subtype was 25%. In contrast to the incidence of Ph-positive ALL, which is known to continually increase with age, Ph-like ALL shows the highest incidence in AYA (19-27%) with B-cell precursor ALL. The incidence of Ph-like ALL significantly decreases with more advanced age forming a bell-shaped-curve ($p=0.03$; AYA vs. adults and older adults). At 5 years, the Ph-like ALL subgroup had a lower probability of continuous complete remission (24% vs 62%; $p<0.001$) and overall survival (22% vs 64%; $p=0.006$) compared to B-other ALL patients. Deletions of *IKZF1* were significantly more common in Ph-like ALL (13/16, 81% vs. 7/21, 30%; $p=0.007$). Mutations in *JAK2* were exclusively found in the Ph-like subgroup (7/16, 44% vs. 0/53, 0%; $p<0.001$).
Result: Our study independently validates previous data regarding the incidence of Ph-like ALL in AYA and provides important evidence on the frequency of this subtype in patients with more advanced age. Furthermore, our study demonstrates that Ph-like ALL in adults is a molecular homogenous entity and associated with inferior survival in a homogenously treated group of patients.
Conflict of Interest: None

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Myelodysplasia in Adults with Near-Tetraploidy and Lymphoid (ALL) or Ambiguous Lineage (ALAL) Acute Leukemias

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Aims: Near-tetraploidy (NT, 81-104 chromosomes) in acute myeloid leukemias (NT-AML) in European adults (>18 years) is characterized by large blasts with CD34 expression, multi-lineage involvement (dysplasia), in 40% M0 type (Bene et al., Leukemia 20: 725, 2006). Our aim was to study myelodysplasia in NT-ALL/ALAL as a feature for lineage assignment and choice of therapy.
Patients and methods: The EGIL retrieved data on 16 NT-ALL and 6 NT-ALAL. Results. Blasts of 6 cases with NT-ALAL were unclassifiable by morphology and expressed CD34 and HLA-DR in 5/5 tested cases. Blasts from a 78-year old female were positive for cyCD79a, CD19, CD10, TdT, CD65 and classified as BCP-ALL. Genetic examinations showed 46,XX,del(5)(q14q34)[21] / 81~85,XXXX,-1,-2,-3,-4, del(5)(q14q34)x2,-7x2,-9x2,del(9) (p21p21)x2,+11x4,-16,-17,-19,-20,-22[cp3] and no rearrangements for IgH nor TCR genes. Bone marrow (BM) exhibited erythroid and megakaryocytic dysplasia (DysE, DysM) pointing to the diagnosis of AML, too. The patient opted for palliative treatment and survived 2 months. Blasts from a 67-year old male with AL post MDS were positive for cyCD22, TdT, CD13, CD33, CD117 only and exhibited two NT-clones (20%), one tetraploid and another with del(5)(q13q33), 80% normal metaphases. Therapy 3+7 did not induce CR, survival was 1 month. Blasts from a 74-year old man were positive

only for cyCD79a, TdT, CD13, CD117; BM revealed DysE and DysG. Treatment with ALL-directed protocol did not induce CR (OS 2.8 months) but induced CR (+10 mo.) in another 60-year old female with therapy-related AUL without dysplasia.
Results: No myelodysplasia was observed in 2 NT-MPAL cases, 8 cases with NT-BCP ALL, 2 NT-Burkitt ALL, and 6 cases with NT-T-ALL. Conclusion: Myelodysplasia may represent a useful diagnostic marker (with limitations) for AMLs in complex AL classification
Conflict of interest: None

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Overcoming Apoptosis Resistance in Acute Lymphoblastic Leukemia in a Preclinical ALL Xenograft Model

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Introduction: Treatment resistance and relapse in acute lymphoblastic leukemia (ALL) are considered to be due to apoptosis defects. Since inhibitor of apoptosis proteins (IAPs') negatively regulating apoptosis signaling are highly expressed in leukemias, small molecule inhibitor antagonists (SMAC mimetic, SM) provide a promising strategy to overcome apoptosis deficiency and effectively treat high risk ALL. In this study, we addressed and characterized effects and effectivity of SM in ALL.
Method: Exposure of ALL cells to SM at nanomolar concentrations led to clear cell death induction in ALL cell lines and 70% of primograft ALL samples (n=42). Interestingly, apoptosis induction could be inhibited by the soluble TNF-a receptor etanercept, thus indicating TNF-a dependency. Previously, we described that rapid NOD/SCID/huALL engraftment is associated with deficient apoptosis signaling and indicative for early patient relapse. Importantly, SM activated constitutive deficient apoptosis signaling in high risk/early relapse ALL. Next, we investigated the effectivity of SM on high risk ALL in a preclinical *in vivo* setting and treated leukemia bearing recipients either with vehicle, SM, multidrug chemotherapy, or a combination of both.
Results: Importantly, a profound reduction of tumor load and prolonged survival of animals was observed upon SM treatment alone, and even more pronounced in combination with multi-agent chemotherapy. Interestingly, concomitant *in vivo* application of etanercept revoked the SM effect, indicating that SM induced apoptosis sensitization involves signaling via TNF-a also *in vivo*, thereby pointing to a potential biomarker for the identification of patients who would benefit from SM treatment.
Conclusion: In conclusion, the small molecule SM induce cell death *ex vivo* and *in vivo* in primary patient-derived ALL overcoming apoptosis deficiency, pointing to a novel strategy for targeted therapy of high-risk ALL.
Conflict of interest: None

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Prognostic Importance of MN1-Transcript Levels in Patients with Acute Lymphoblastic Leukemia (ALL) Treated According to the Protocol ALL-MB-2008

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Background: Expression of meningioma 1 (MN1) gene can be associated with ATRA resistance. Purpose of the study was to investigate MN1 expression levels in patients (pts) with ALL treated by all-trans retinoic acid (ATRA) on the protocol ALL-MB-2008.

Patients and methods: MN1 expression was measured in 9 pts (2,9-27,3 years old) with primary precursor B-lineage MLL-AF4-positive ALL by semi-quantitative real-time reverse-transcriptase polymerase chain reaction. All pts were stratified to high-risk group according to the clinical trial (protocol ALL-MB-2008) and received ATRA treatment. 44,4% (4/9) pts had bone marrow transplantation (unrelated or related). 88,8% (8/9) pts achieved complete remission, 33,3% (3/9)pts developed relapse, 50% (4/8) pts – in CCR.
Results: Level of MN1 expression in pts with ALL was correlated with resistance to ATRA treatment. Patients with low MN1 expression (5/9, 55,6%) didn't have relapse. 3/4 (75%) relapsed pts had high MN1 expression. Patients with low MN1 expression had prolonged relapse-free survival (P=0.06) comparing with pts with high level of MN1.
Conclusion: Our results suggest that the increase of relapses in high risk MLL-AF4-positive ALL patients treated according to ALL-MB-2008 protocol was associated with high MN1 expression. MN1 expression independently predicts outcome in patients with ALL.
Conflict of interest: None

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Incidence of IKZF1 Deletions In Russian Cohort of Pediatric B-Precursor ALL. Results of the Russian Moscow-Berlin Study Group

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Purpose: *IKZF1* gene deletion is an independent adverse prognostic factor in BCP-ALL. We aimed to characterise *IKZF1* deletions in our cohort of pediatric B-ALL cases to identify the correlation with clinical features.
Patients and method: *IKZF1* deletion status was analyzed in bone marrow samples of 171 pediatric *BCR-ABL1*-negative B-ALL cases enrolled in the ALL-Moscow-Berlin 2008 treatment. We used P335 SALSA MLPA kit (MCR Holland, The Netherlands) to identify whole or intragenic *IKZF1* deletions.
Results: In total, 22 *IKZF1* deletions were identified in 171 cases (12.9 %). 162 pts were enrolled in final analysis. In detail, 4 patients (18 %) showed deletions of exon 4-7, 2 cases (9 %) - exon 2-7, 2 cases (9 %) - exon 4-8, 2 cases (9 %) - exon 1-7, 2 cases - exon 2 and 10 patients - other deletions (one case each). In cytogenetic subgroups *IKZF1* deletions were detected in 3 out of 5 iAMP21 cases, in two out of 66 cases with t(12;21), in one case of *IgH/CRLF2* translocation, in 3 out of 30 hyperdyploid cases, in case of monosomy 7 and in case of DS without known translocation. Patients with *IKZF1* deletions were older then patients without deletions (median 6.36 vs 4,31 year) with strong male predominance (m/f 2.67). According to ALL-MB2008 treatment protocol 14 pts were stratified to ImRG, 5 to SRG and 3 to HRG.
Conclusions: Our data on genetic *IKZF1* alteration is compatible with data published by others groups. However, the analyzed patient group is not sufficiently large and has a prominent imbalance in genetic variants (66 cases with t(12;21)). Moreover, the follow-up is too short to analyze survival and risk of relapse.
Conflict of interest: None

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AXITINIB Targets Gatekeeper-Mutant BCR-ABL1(T315I)-Driven Leukemia in a Distinct And Selective Fashion

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Introduction: The T315I gatekeeper mutation confers resistance to majority of approved ABL1 inhibitors, with only ponatinib demonstrating efficacy in BCR-ABL1(T315I)-driven disease. However, vascular adverse events and selection of resistant compound mutations limit its clinical utility. Hence, there is an unmet need for novel treatment strategies for patients with gatekeeper-mutated Ph+ leukemia.
Method: In this study we integrated comprehensive drug sensitivity testing with structural analysis to characterize the tyrosine kinase inhibitor axitinib as a potential novel therapy for BCR-ABL1(T315I)-driven leukemias.
Ex vivo drug sensitivity testing of primary cells derived from a Ph+ ALL patient revealed a cancer-selective response to the VEGF inhibitor axitinib (currently approved for renal cell carcinoma). Strikingly, axitinib exhibited higher sensitivity in T315I positive Ph+ patient samples in comparison to T315I negative patient samples. In accordance, axitinib inhibited the kinase activity of ABL1(T315I) with similar potency as its intended primary target VEGFR2, while showing >30-fold lower potency towards non-mutated ABL1. Analogously, in engineered Ba/F3 cells, axitinib showed a 10-fold higher potency to inhibit T315I than non-mutated ABL1-driven autophosphorylation and cell growth. The axitinib:ABL1(T315I) crystal structure revealed that axitinib bound to a T315I-mutant induced active conformation of ABL1(T315I), different than the binding mode in non-mutated ABL1, likely explaining the increased potency towards the gatekeeper mutated protein.
Result: Compassionate two week treatment of a CML patient harboring the T315I mutation with axitinib, resulted in a rapid 5-fold reduction of T315I transcript levels in the bone marrow, further suggesting that axitinib can produce specific and effective responses in patients with BCR-ABL1(T315I)-driven leukemia.
Conclusion: We demonstrate that axitinib potently inhibits BCR-ABL1(T315I) via a gatekeeper mutant-selective mechanism. Since axitinib is in clinical use for treatment of refractory renal cell carcinoma with a manageable safety profile, our data provide a sound basis for readily repurposing axitinib for BCR-ABL1(T315I)-driven leukemia. Finally, the distinct mechanism of inhibition by axitinib serves as an exemplar for development of even more effective gatekeeper-mutant selective inhibitors targeting ABL1 as well as other clinically important kinases, such as EGFR, FLT3 and KIT.
Conflict of interest: None

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The Metabolic Stress Sensor MLXIP Mediates Malignancy of cALL *IN VIVO*

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Background: The MAX like protein X interacting protein (MLXIP) is a metabolic stress sensor and a proglycolytic transcription factor involved in metabolic addiction of leukemia, i.e. the Warburg effect. MLXIP dimerizes with MLX within the MYC interactome and promotes longevity in *C. elegans* (*Johnson et al. 2014*). Key players of MYC interactome (MYC, MAD and MLXIP) regulate proliferation, differentiation, and metabolism by dimerization with MAX or MLX. **Study aim:** In contrast to the function of *MLXIP* as tumor suppressor in certain solid tumors (*O'Shea & Ayer, 2013*), we have extended our previous observation of up regulation of MLXIP in common B cell acute lymphoblastic leukemia (cALL, *Burdach & Richter 2007*) by demonstrating that MLXIP induces stemness, proliferation, and B cell receptor signaling pathway signatures in cALL (*Wernicke et al. 2012*).
Method: We now found MLXIP to be overexpressed in both cALL and AML. Here we report on the role of MLXIP in malignancy of cALL *in vivo* using a xenograft model in RAG2-/-gc-/- mice (*Richter et al. 2009*). NALM6 cALL line was retrovirally transduced with *MLXIP* short hairpin RNA, which reduced the *MLXIP* expression by 80%. Upon *MLXIP* knock down (kd), kd and control lines were injected iv; CD10+ blasts in blood, spleen and bone marrow were assessed after 30 days. Importantly, in vivo MLXIP maintained 90-99% of CD10+ leukemic blasts. Spleen size and weight became normal by MLXIP kd:
Results: While signs of leukemia engraftment were observed in all mice, the differences of CD10+ blasts in blood, bone marrow and spleens in the control vs. the MLXIP kd group were highly significant (p=0.008). The decrease of leukemic splenomegaly after MLXIP kd was most impressive: Median spleen weight was 0.22g vs. 0.08g in the control vs. the kd group. In conclusion, these findings demonstrate that MLXIP maintains leukemic burden and malignancy of cALL *in vivo*.
Conflict of interest: None

V. ALL – Therapy

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International Validation of a Harmonized NGS PGM ASSAY at Clinical Laboratories in the US and the EU

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Introduction: Personalized molecular medicine is gaining traction as more doctors are insisting on reliable biomarker testing to diagnose and stratify their patients for treatment or enrolment in clinical trials. Pharmaceutical companies seek to shorten drug development by being more selective (recruiting "the right patient for the right drug") before trial enrolment. Wider recruitment to access sufficient patient populations to power development of new therapeutic agents requires more laboratories with harmonized testing for reliable stratification and selection for trials. Standardized and harmonized international clinical testing is now required.
Analytical metrics were used to harmonize and validate a next generation sequencing (NGS) assay manufactured under ISO 13485 for clinical use in our US and EU clinical labs.
Methods: All reagents were manufactured under cGMP and QC tested under a QSR compliant regulatory system for use in validation studies. Site to site validation tested Limit of Detection (LOD), Blank (LOB), Linearity, Precision and Reproducibility (Repro) to pre-specified standards. The LymphoTrack® *IGH* PGM assay was used for this validation. LOD and LOB validation consisted of 1 operator using 1 instrument to run a dilution series of a cell line DNAs in tonsil DNAs in each lab. Samples were run in duplicate with 1 lot of master mix and a 1 lot of tonsil DNA control. The Repro study included 2 runs by each of 2 operators in each lab (8 runs total). JMP software assessed the LOD, LOB and Repro runs of the assay based on 96 data points.
Results: Concordance between the labs was excellent with %CVs <20% and both labs showing inter and intra repeatability and reproducibility between labs, demonstrating harmonization among labs that use cGMP reagents and assays.
Conclusion: We demonstrate excellent concordance in results when cGMP quality reagents are used with harmonized protocols in multiple labs.
Conflict of interest: None

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A Phase Iii Multicenter, Randomized, Study Evaluating Efficacy And Safety Of Erythrocyte Encapsulated L-Asparaginase (ERYASP) Versus Native L-Asparaginase (L-ASP) in Combination with Cooprall Regimen in Patients with First Relapse of Acute Lymphoblastic Leukemia (ALL)

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Introduction: Asparaginases are a cornerstone in the treatment of ALL, but their utility is limited by toxicities including hypersensitivity. ERYASP improves pharmacokinetics and tolerability, especially hypersensitivity reactions, while maintaining circulating asparaginase (ASPA) activity due to the protective barrier of the erythrocyte membrane.
Patients and methods: This phase III study enrolled patients with primary relapsed or refractory Ph- ALL. The endpoints for efficacy were comparison of the duration of ASPA activity >100IU/l and the incidence of ASPA hypersensitivity during induction. Patients, aged 1-55 years, without prior ASPA hypersensitivity were randomized to ERYASP (150 IU/kg) or L-ASP(10.000IU/m^2) in combination with COOPRALL. The patients with prior ASPA hypersensitivity received ERYASP.
Results: ERYASP significantly reduced the incidence of ASPA hypersensitivity (0/26 vs 12/28; $p<0.001$; 7/12 were grade≥3), while maintaining ASPA activity >100IU/l significantly longer than L-ASP (20.5±5.2 vs 9.4±7.4 days; $p<0.001$). CR and clotting parameters were also significantly improved in the ERYASP arm. No other adverse events were significantly different. 1-year OS was 77% in the ERYASP arm vs 68% in L-ASP arm. ASPA activity was not reduced in hypersensitive patients confirming the protective effect of the encapsulation.
Conclusion: These highly encouraging results show that ERYASP is a suitable option for patients with relapsed ALL maintaining ASPA efficacy with improved tolerability.
Conflict of interest: None

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***IN VIVO* Response to Remission Induction Poly-Chemotherapy Modeled in NOD/SCID Mice Reflects Patient Risk and Outcome**

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Background: In the treatment of acute lymphoblastic leukemia (ALL) response to initial remission induction (RI) therapy is a strong prognostic factor used for patient risk stratification. As 20% of ALL patients relapse, new anti-leukemic agents are essential, which most likely will be applied to already existing multi-agent chemotherapy regimens and will undergo *in vivo* preclinical testing.
Study aim: In this study we aimed to (i) establish an *in vivo* treatment strategy, which resembles RI therapy in our NOD/SCID/huALL mouse model and to (ii) analyse the *in vivo* response to RI treatment of distinct patient-derived xenograft (pdx) ALL samples in the context of pre-treatment leukemia features and patient outcome.
Patients and methods: We have treated 7 pdx samples with RI therapy combining vincristine, dexamethasone and asparaginase (VDA), or vehicle for 2 weeks. After treatment, the time to leukemia reoccurrence (TTR) was estimated as weeks from treatment start until onset of leukemia related morbidity. VDA significantly delayed TTR compared to vehicle treatment in all individual samples, even inducing long-term remission in 1 primograft sample with no ALL reoccurrence within 30 weeks of monitoring.
Resutls: Previously, we described a short time to leukemia engraftment (TTL^{short}) being associated with an early relapse in contrast to superior patient outcome of TTL^{long} leukemias. Interestingly, rapid TTR after VDA therapy is significantly associated with TTL^{short} phenotypes and TTL^{long} leukemias display a longer TTR indicating increased *in vivo* sensitivity for VDA treatment.
Conclusion: In summary, we have established an effective *in vivo* RI protocol allowing evaluating novel anti-leukemic agents in a preclinical setting for ALL. In addition, short TTR is associated with inferior relapse free survival and NOD/SCID engraftment suggesting that pre-treatment high risk features reflect leukemia-intrinsic characteristics such as post-treatment leukemia reoccurrence and relapse.
Conflict of interst: None

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Effective *IN VIVO* Targeting of BCP-ALL in a NOD/SCID/huALL Mouse Model by CD70 Directed Immunotherapy

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Introduction: Despite successful treatment of ALL, relapse of the disease remains a major problem associated with poor prognosis. This emphasizes the need for novel treatment strategies to be applied in addition to established chemotherapy regimens. Previously, we identified the CD70 transcript is significantly up-regulated in high risk/relapse ALL subgroup characterized by rapid onset of leukemia (TTL^{short}) in a NOD/SCID/huALL mouse model. CD70 is involved in T/B cell priming and generation of memory B cells and only expressed in activated immune cells.
Study aim: In this study, we addressed expression of CD70 in pediatric patient-derived primograft leukemia samples and further evaluated CD70 as a therapeutic target for directed immunotherapy *in vitro* and in our BCP-ALL xenograft system *in vivo*.
Method: Flowcytometric analyses of CD70 surface expression in 28 patient-derived xenograft samples (TTL^{short} n=8, TTL^{long} n=20) revealed a higher expression of CD70 on ALL cells with a TTL^{short}/early relapse phenotype compared to TTL^{long} samples. To take advantage of increased CD70 expression in BCP-ALL, the efficacy of monoclonal antibody based CD70-directed immunotherapy was evaluated in NOD/SCID mice.
Result: A marked reduction of leukemia load in peripheral blood, bone marrow and spleens of the animals was detected in anti-CD70 treated $CD70^{hi}$ primograft ALL cells. This effect could be abrogated both by NK-cell depletion in the NOD/SCID recipients and by using NK-cell deprived NSG mice as recipients, indicating that decreased *in vivo* leukemia growth upon anti-CD70 treatment is mediated by NK-cell induced cytotoxicity of CD70 positive ALL cells. Consistently, CD70 antibody-dependent cell-mediated cytotoxicity (ADCC) was observed in vitro.
Conclusion: Taken together, we identified significantly up-regulated CD70 expression in BCP-ALL that provides a novel target for directed immunotherapy.
Conflict of interest: None

VI. APL – THERAPY

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Proteasome Activity is Dispensible for the Degradation of PML-RARA: Efficacy of Bortezomib along with Arsenic Trioxide in the Treatment of ATO Sensitive and Resistant Acute Promyelocytic Leukemia

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Background: Degradation of *PML-RARA* upon treatment with ATO is predominantly mediated by the proteasome complex. Reports suggest

that in relapsed APL patients (treated upfront with ATO) mutations in the B2 domain of PML in *PML-RARA* gene are involved in resistance and poor clinical outcome (NEJM 2014).
We had previously reported that Bortezomib (Bo) was able to synergize with ATO by inducing apoptosis (*Blood.* 2012;120 ,3552). We further evaluated the mechanism of degradation of PML-RARA when ATO was combined with Bo.
Method and results: We generated in-house ATO resistant NB4 cell lines (NB4EV-ASR1 (with A216V mutation), ASR2 and ASR3). The combination of ATO and Bo induced a significant apoptosis in the resistant cells similar to naïve NB4 cells.
We observed a time dependent accumulation and degradation of ubiquitinated proteins and ubiquitin binding protein p62 in ATO+Bo treated naïve NB4 cells, correlating with induction of autophagy and degradation of PML-RARA. Similar results were seen in the resistant cells. Blocking autophagy by 3-methyl adenine or knock down of p62 showed an accumulation of PML-RARA in the combination treated cells. We also observed an interaction between P62,LC3II and PML-RARA suggesting that PML-RARA was cleared by autophagy via p62 protein. In a transplantable APL mice model, combination prolonged the life span of the mice. A reduction in the LIC was demonstrated by secondary transplantation experiments. A phase II clinical study combining Bo with ATO and chemotherapy has been initiated for relapsed APL (NCT01950611).
11 patients have been enrolled, all achieved complete molecular remission. The combination was well tolerated. Beyond induction the combination does not require in-patient care and there is no significant cytopenia.
Conclusion: In conclusion, PML-RARA degradation is mediated by up-regulation of autophagy and the synergy was further confirmed in a pre-clinical model and it is also effective in ATO resistant cell lines.
Conflict of interest: None

VIII. PEDIATRIC ACUTE LEUKEMIAS

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Single Cell Developmental Classification of B Cell Precursor Acute Lymphoblastic Leukemia (BCP ALL) Reveals Link between Phenotype, Signaling, and Drug Response

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Background: Mass cytometry allows for multi-parametric analysis of single cells to distinguish phenotypic and functional features of subpopulations from leukemia samples. Mass cytometric analysis of pediatric Ph+ BCP ALL constructs a novel model of ALL organized along the normal B cell developmental trajectory that may be used as a lens through which to examine signaling features, drug response, and clinical data.
Methods: Mononuclear cells from diagnostic bone marrow samples were obtained from pediatric patients with BCP-ALL (n=52) or healthy controls (n=6). Mass cytometry analysis of 40 proteins was performed at baseline state and perturbed state (cytokines and tyrosine kinase inhibitors). Healthy bone marrow samples were gated as previously described along the trajectory of developing B cells (Davis et al., 2014). These populations were used as the foundation for a classifier in which each leukemia cell was assigned to its nearest healthy population based on Mahalanobis distance in nine dimensions.
Results: ALL samples demonstrated phenotypic heterogeneity but maintained features consistent with stages of healthy B cell development. The single-cell classifier assigned each cell to its most related healthy B cell population. Across all samples, leukemic cells preferentially straddled the preproB cell to preB1 transition with expansion of these compartments at the expense of progenitor and immature B cell compartments. Each leukemia had a distinct occupancy of the developmental trajectory. Within the developmental compartments, blast cells retained functional features of their healthy counterparts including developmental enzymes TdT and RAG as well as transcription factors (Ikaros, PAX5). Activation of relevant signaling pathways including JAK-STAT and PI3K signaling was assessed in each compartment. Response to TKI's varied based on developmental compartment of the blasts. Finally, the association between state of developmental arrest and clinical features (age, MRD, relapse, genetics) will be presented.
Conclusions: Deep proteomic profiling of BCP ALL establishes a single-cell classification linking phenotype with functional attributes of leukemic cells. This data demonstrates that heterogeneity within leukemia may be related to developmental state of the leukemic cell.
Conflict of interest: None

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Primary Induction Failure and Early Relapses in Children with AML

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Introduction: Primary induction failure (PIF) and early relapses (ER) in children make the prognosis in AML worse.
Patients: 271 patients with AML were included in Moscow-Minsk 2000 - 2006 AML protocol (2000 – 2012 years). Of the 40 patients (18M / 22 F), 26 patients had PIF and 14 patients had ER. ADE-HAM induction chemotherapy regimen received 35 pts and ADE regimen – 5 pts. Median age was 3.7 (0.1-18) years. Median leukocytes was 15 x 10^9/л (0.9 - 300).
Results: Of the 26 pts with PIF, 8 reached I CR (complete remission) after second line therapy.
In an attempt to achieve remission patients received 1-7 courses of therapy (median was 2). 4 pts underwent HSCT in remission (1 pts is alive after auto-HSCT) and 6 pts – in active disease (1 pts is alive after haplo-HSCT).
Of the 14 pts with early AML relapse (before 6 month), 4 pts reached II CR. 4 pts underwent HSCT (all are alive).
5 year overall survival (pOS) all patient (n=40) was 39% in HSCT and 0% in CHEMO group (p=0,0001). In group PIF (n=26) pOS was 22% in HSCT and 0% in CHEMO (p=0,0003)
Conclusions: Cure of patients with refractory AML and early AML relapses is possible only by using HSCT. Attempts to achieve remission by reusing conventional chemotherapy agents are not effective. Long-term survival is not dependent on disease status at the moment HSCT.
Conflict of interest: None

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Gata1-Centered Genetic Network on Chromosome 21 Drives down Syndrome Acute Megakaryoblastic Leukemia

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Background: Children with trisomy 21 (Down syndrome, DS) are predisposed to develop acute megakaryoblastic leukemia (DS-AMKL) as well as the antecedent transient leukemia (DS-TL). Mutations in the transcription factor GATA1 (GATA1s) are present in nearly all children with DS-AMKL and DS-TL. GATA1s is both essential and sufficient to cause DS-TL in synergy with trisomy 21.
Study aim and methods: To elucidate how the presence of an extra copy of hsa21 perturbs fetal hematopoiesis to provide a GATA1s sensitive background during leukemogenesis, we integrated an RNAi viability screening (512 shRNAmirs against 210 genes on hsa21), a proteomics approach and a chromatin occupancy analysis. The RNA-screening followed by functional validation revealed 8 genes to specifically affect proliferation, cell viability, apoptosis or differentiation in DS-AMKL cell lines (CMK and CMY); whereas 9 genes were essential for all cell lines (K562, M07).
Gain- and loss-of-function studies of 12 selected candidates (8 DS-AMKL-specific oncogenes plus 4 global oncogenes) in $CD34^+$ hematopoietic stem and progenitor cells uncovered that six genes (C21orf33, CHAF1B, IFNGR2, WDR4, RUNX1 or GABPA) function as positive regulators of megakaryopoiesis. All 12 candidate genes acted synergistically to enhance the self-renewal efficiency of murine fetal liver cells *in vitro*.
Results: Using an *in vivo* biotinylation approach, we showed that ^{bio}GATA1 is associated with protein-complexes of 10 different hsa21-oncogenes, involved in splicing, deubiquitination and transcriptional regulation. Direct interactions as well as transcriptional regulation of several of these factors are perturbed in N-terminal truncated GATA1s.
Conclusion: Thus, we deciphered a complex interactive oncogenic network on hsa21 centered on GATA1, positively regulating megakaryopoiesis. Deregulation of this network results in synergistic effects on hematopoietic differentiation, which can promote transformation of GATA1s-mutated fetal hematopoietic progenitor cells.
Conflict of interest: None

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Early T Precursor (ETP) Phenotype Impact on Outcome of Pediatric Acute Lymphoblastic Leukemia (ALL)

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Study aim: We conducted a retrospective, single-center, study including all patients (pts) younger than 16 years diagnosed with T-ALL between Mar2007 and Jun2014 to determine differences in presenting features, response to remission-induction therapy, relapse rate (RR), overall survival (OS) and event-free survival (EFS), between ETP ALL, defined by immunophenotyping (CD5weak/- CD1a- CD8- and ≥1 myeloid or stem-cell related antigen), and non-ETP pediatric T-ALL.
Method and results: We included 35 pts, 9 (25.7%) had an ETP phenotype and 22 were male. Median age at diagnosis was 6y (11 months-14y); it was higher on the ETP group (10y[4-13] VS 5.5y[0-14]). At diagnosis, the median WBC/mm3 was 63300(3500-504900); this was inferior in the ETP group (34170[3500-318300] VS 89740[18100-504900]). No statistically significant (SS) differences were found in presenting features, as well as in the routinely analyzed genetic alterations (MLL-gene rearrangements, BCR-ABL or SIL-TAL1 fusion genes). 30 cases achieved complete remission (CR) after induction (88.2%). Non-CR was more frequent among the ETP pts (p=0.048). There were 5 relapses and all pts died. None of the ETP pts with CR after induction relapsed. The median follow-up time was 29 months. There were no SS differences in RR, OS or EFS between the 2 groups (OS ETP 75% VS non-ETP 74%, p=0.9; EFS ETP 67% VS non-ETP 71% p=0.59).
Conclusions: Our series showed a higher frequency of ETP-ALL than reported, and a similar outcome for ETP and non-ETP ALL. This could be, at least in part, explained by the inclusion of CD5- cases, not mentioned in the first reports. However, ETP- ALL showed a higher induction-failure rate: there may be some benefit in introducing new drugs in their induction regimen. To clarify these observations, we will extend the follow up time and the sample. The outcome for relapsed T-ALL, ETP and non-ETP, remains extremely poor: there is an urgent need for new therapeutic approaches.
Conflict of interest: None

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Monitoring of Asparagine in CSF after Administration of Pegylated E.COLI Asparaginase within the AIEOP-BFM ALL 2009 TRIAL

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Purpose: Depletion of asparagine (ASN) in serum and CSF is considered important for the antileukemic efficacy of asparaginase (ASE). In the AIEOP-ALL-BFM 2009 trial ASE frontline treatment switched from native to pegylated E.coli ASE (PEG-ASE). To evaluate treatment intensity of frontline PEG-ASE in CSF ASN levels in CSF were monitored along with serum ASE activities.
Methods: Between 02/2011 and 10/2012 1,135 CSF samples from 523 patients were analyzed for ASN content by HPLC with a limit of quantification of 0.2 μmol/L.
Results: Administration of 2,500 U/m^2 PEG-ASE significantly reduced ASN levels in CSF. In protocol I after administration of 2 doses at 2 weeks interval ASN reduction in CSF lasted for 40 days. At serum ASE activities between >0 and <100 U/L ASN levels >0.2 μmol/L were detectable in 90% of CSF samples. This rate declined to 76% at ASE activities between ≥100 and ≤1,000 U/L, but did not fall below 60% at serum ASE activities of 1,000 U/L or higher.
Conclusion: In contrast to native E.coli ASE, which resulted in CSF-ASN <0.2 μmol/L in almost all patients[1,2], a considerable number of patients treated with 2,500 U/m^2 PEG-ASE frontline displayed relevant ASN levels in CSF. Moreover, these observations suggest that intensifying PEG-ASE treatment will not further increase the rate of ASN depletion in CSF.
References: [1] Boos J, et al. Eur J Cancer. 1996;
[2] Ahlke E, et al. Br J Haematol. 1997
Conflict of interest: None

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Outcome of Pediatric Acute Myeloid Leukemia Treated with AML-BFM 98 PROTOCOL in a Developing Country: Lessons and Challenges

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Purpose: We undertook a retrospective study to evaluate the clinical outcome of pediatric patients (≤15yrs) with a diagnosis of AML referred to our tertiary care center between Jan 2008 and Dec 2013 and treated with a BFM AML-98 protocol.
Results: There were 83 newly diagnosed patients in this period. Median age was 9.8years (range: 1-15) and there were 52(62.7%) males. Cytogenetic data was available in 71, of which 14(19.7%) and 16(22.53%) were in the good risk and adverse risk groups respectively. Only

40(48.2%) opted for intensive chemotherapy, the rest cited financial constraints and opted out of standard therapy. 34(85%) received chemotherapy as per the AML-BFM 98 protocol. There were 8(23.5%) inductions deaths and of these 6 were due to gram-negative sepsis, with multidrug resistant (MDR)organisms in 4. Post induction chemotherapy, blood cultures were positive in 18(52.9%) patients on at least one occasion. Of these a Gram negative bacilli(GNB) was present in 13(38.2%) and Gram positive organisms in 5(14.7%). Of the GNB, in 5 patients a MDR isolated. Seven patients(20.6%) had evidence of fungal infection during induction. Of 26 patients who completed induction therapy, 16(61.5%) attained CR. Of the 10 that did not, 3 patients did not proceed with further therapy due to financial constraints; refractory disease was seen in 2 and 4 attained CR on subsequent cycles of chemotherapy. Out of the 19 patients who received subsequent cycles of consolidation chemotherapy there were 4 deaths (3 with bacterial sepsis and 1 due to fungal infection). The 5 year KM estimate of OS and DFS was 31.8 ± 9.2% and 49.1% ± 17.2% respectively.

Conclusion: The major reason for not proceeding with treatment is the lack of financial resources. Induction deaths are related to a high incidence of multi-drug resistant organisms and fungal infections, and these risks persist during consolidation therapy.

Conflict of interest: None

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Loss of Fusion Gene In Leukemia with MLL-Rearrangement Provides Insight to Development of 3-Way-Translocations

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Background: Leukemia is the most common childhood malignancy, translocation of the mixed-lineage leukemia (MLL) gene on chromosome (chr.) 11[1,2] is frequent.

Method and discussion: We present an infant, that was diagnosed with ALL and treated according to the INTERFANT 06-protocol. A MLL-rearrangement was detected by FISH. Long-distance-inverse (LDI)-PCR[3] identified one of the t(9;11) resulting fusion genes: AF9-MLL. This fusion gene was monitored for MRD detection via qPCR on gDNA and became negative during therapy. On day 226 during therapy an increase of blasts to 60% was observed. FACS proved a relapse with lineage switch from ALL to AML, but MRD remained negative. Surprisingly on expression level (cDNA) the fusion transcript of t(9;11), MLL-AF9, was found. This is the counterpart of AF9-MLL, which was found initially. New mFISH analysis showed a complex karyotype including t(5;9;11) (q21;p21;q21). Multiplex-PCR and sequencing (gDNA) confirmed a MLL-AF9 fusion gene. Retrospective measurement proved that the MLL-AF9 fusion gene was the same at initial diagnosis and in relapse. In contrast, the initially detected AF9-MLL fusion gene was only present in the initial sample. This loss of the AF9-MLL fusion gene is probably due to a second translocation between chr. 5 and chr. 9, resulting in the found 3-way translocation t(5;9;11). To our knowledge it has never been described that a 3-way translocation and the corresponding 2-way translocation were found in the same patient.

Conclusion: These findings confirm on molecular level, that a 3-way translocation is a multistep event and that the critical genetic event for leukemia is the translocation of 9p onto 11q, since this is present in both samples.

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2.)Schoch C, et al. *Blood*. 2003.
3.)Meyer C, et al. *Leukemia*. 2009.

Conflict of interest: None

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Therapy Adaption in the Genetically High Risk Group in Childhood Acute Myeloid Leukemia: A Report of AML-BFM-STUDY 2004

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Purpose: The aim was to evaluate the outcome of a genetically high risk group in a large cohort of uniformly treated patients to improve risk-adapted therapy.

Patients and methods: In study AML-BFM 2004 765 patients with de-novo AML (according to WHO classification, age <18 years, patients with Down syndrome excluded) were included. In 702 (92%) patients genetic data were conclusive for cytogenetics and/or FISH analyses and/or RT-PCR (initial bone marrow or blood samples). According to published data (Neuhoff et al. 2010) the following aberrations were defined as genetically high risk (HR) factors: -7, der12p, t(9;22)(q34;q11), t(7;12)(q36;p13), t(4;11)(q21;q23), t(6;11)(q27;q23), t(6;9)(p23,q24), t(8;16)(p11;q13).

Results: Patients with genetically HR factors (n=41) had a significantly worse outcome (pEFS 29%, SE 7%, p=<.0001, pOS(5years)=46%, SE9%, p=<.0001) compared to other patients. Complete Remission (CR) was achieved by 31 of these patients. Out of this group the patients which were transplanted in 1.CR (n=11, pEFS 73%, SE 13%) had a significantly better prognosis than those without HSCT in 1.CR and median survival of more then 4,3 months (the median time to allogeneic transplantation, HSCT) (n=19, pEFS 20%, SE 10%, p(Mantel-Byar)=0.04).

Conclusion: Our results show that allogeneic HSCT in 1.CR seems to be the therapeutic option in patients with genetically high risk factors. It is desirable to improve the CR rate in this group for example by introduction of new agents upfront in therapy as the achievement of complete remission seems to give these HR patients a realistic chance of cure.

Conflict of Interest: None

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Expression of MECOM Transcripts in Childhood Acute Leukemia

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Introduction: Overexpression of *MECOM* (*MDS1* and *EVI1* complex locus) gene correlates with adverse outcome in MDS and AML. In AML high expression of *MECOM* gene is associated with *MLL* rearrangements. In this study MECOM expression was analyzed in pediatric patients with acute leukemia (AL).

Methods: Expression of *MECOM* transcripts (*EVI1*, *MDS1*/*EVI1* and a region common to *EVI1* and *MDS1*/*EVI1* (*cEVI1*)) was investigated in bone marrow samples of 27 patients with childhood AML and 43 - with ALL (11 with T-ALL and 32 with B-ALL). Reverse transcriptase RQ-PCR was performed on CFX96 (Bio-Rad, USA). Expression level was calculated using $2^{-\Delta Ct(GUS-GOI)}$ equation and subdivided by median for each leukemia subtype (AML, T- and B-ALL).

Results: Overexpression of *MECOM* gene in AML, B- and T-lineage ALL is achieved mainly through the high expression *MDS/EVI1* transcript (p<0,01). AML patients characterized by highest level of *MDS1/EVI1* and *cEVI1* expression (p<0,01). In AML expression of c*EVI1* was higher in *MLL-AF9*-positive patients (p<0,05), including those with M7. Higher levels of *MDS1/EVI1* and *cEVI1* were found in AML with complex karyotype (p<0,01). In B-ALL with *MLL* rearrangements expression of *MDS1/EVI1* and *cEVI1* was lower comparing with group without rearrangements (p=0,014 and p=0,035). Correspondingly *MDS1/EVI1* expression was lower in *MLL-AF4-positive ALL* (p<0,05), while patients with prognostically favorable *TEL-AML1* had higher expression of MDS1/EVI1 (p<0,01). The association between expression of various gene MECOM transcripts and survival in studied groups were not revealed.
Conclusion: Patients with AL are characterized by high expression levels of *MDS1/EVI1* transcript of *MECOM* gene. Expression of *MDS1/EVI1* is associated with such prognostic molecular genetic markers as *TEL-AML1*, *MLL* rearrangements and complex karyotype. However, the mechanisms regulating expression of *MDS1/EVI1* vary depending on the subtype of AL.
Conflict of interest: None

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Tumor Cells' Immunophenotype Predicts the Presence of *MLL* Gene Rearrangements in Infant Acute Leukemia

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Aim: Description of immunophenotype in infant acute lymphoblastic and acute myeloid leukemia (ALL and AML respectively) due to presence of *MLL* gene rearrangements.
Methods: 113 patients (59 boys and 54 girls) aged from 5 days to 11 months were included in the study group. Their data was compared to 427 cases of acute leukemia in older children.
Results: ALL was found less frequently in infants than in older children (68.1% and 86.9% respectively, p<0.001) while percentage of acute myeloid leukemia cases was higher in infants (27.4% and 11.5% respectively, p<0.001). Significant immunophenotypic differences were observed in patients with and without *MLL* gene rearrangements in both ALL and AML. Number of ALL cases in those tumor cells expressed CD10, CD20, CD45, CD133, CD15, CD65 NG2 significantly varied between *MLL*-positive and *MLL*-negative groups (p<0.001, p<0.001, p=0.002, p<0.001, p=0.004, p=0.019 and p<0.001 respectively). NG2-positivity represented the highest overall correct prediction (OCP) rate for presence of *MLL*-rearrangements (90.6%). Diagnostic accuracy of CD20-negativity and CD45-positivity was lower (81.2% and 81.9% respectively) while OCP for other markers was not sufficient enough. Number of AML cases in those tumor cells expressed CD99, CD133, CD15, CD65, CD4, CD11b, CD61, NG2 varied between *MLL*-positive and *MLL*-negative groups ((p=0.019, p=0.012, p=0.002, p=0.004, p=0.005, p<0.001, p=0.015 and p<0.001 respectively). Thus CD61-negativity, high CD99, CD15, CD133, CD65, CD11b, CD4 and NG2 expression were immunophenotypic signatures of *MLL*-rearranged infant AML, although CD11b and NG2 had the highest diagnostic efficacy (95.0% and 89.7% respectively).
Conclusion: Thus infants' ALL and AML immunophenotype varies greatly due to the presence of *MLL* gene rearrangements. Complex diagnostic immunophenotyping of infants' AL allows predicting presence of *MLL* rearrangements while NG2 and CD11b are the most applicable single markers for ALL and AML respectively.
Conflict of interests: None

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New Aspects of Genetic High Risk Stratification in Pediatric AML: A Report of AML-BFM-STUDY 2004

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Purpose: The aim was to evaluate the prognostic impact of specific chromosomal aberrations in a large cohort of uniformly treated children with AML.
Methods: 765 patients <18 years with de novo AML (WHO criteria, no Down Syndrome) were included in Study AML-BFM 2004. In 702 (92%) patients genetic data were conclusive for either cytogenetic, FISH analyses or RT-PCR (initial bone marrow or blood samples).
Results: Several aberrations were confirmed as unfavorable factors (see table). Furthermore, patients with trisomy 8 exclusively had a poor outcome. Interestingly, monosomies (excluding -7) were detected in 19 patients, out of whom 12 belonged to the genetically high risk group due to other aberrations. However, the remaining 7 patients showed a dismal outcome, too.

	n	5y pEFS%(SE)	p-value*
MLL-rearr. others than t(9;11) or t(11;19)	75	46(6)	0.018
t(10;11)(p12;q23)	27	34(10)	0.0035
der.12p	26	23(9)	0.00030
isolated +8 without favorable genetics	16	25(11)	0.0034
-7	8	13(12)	0.0011
complex karyotype**	58	42(7)	0.04
monosomy without other high risk factors	7	14(13)	0.03
genetically high risk group***	41	29(7)	<0.0001

*Logrank compared to the total group,**≥3 chromosomal aberrations, ≥1 structural,***no favorable cytogenetic and one of these aberrations: t(9;22)(q34;q11), t(7;12)(q36;p13), t(6;11)(q27;q23), t(6;9)(p23,q24), t(8;16)(p11;q13), -7, -12p.
Conclusion: Our study confirms the unfavorable prognosis of several cytogenetic aberrations. Further, we identified any monosomy and a trisomy 8 (without additional cytogenetic aberrations) as potentially poor prognostic factors in pediatric AML.
Conflict of interest: None

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Development of Patient-Specific Assay for MRD Detection in Pediatric Patients with CEBPA-Positive NK AML

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Introduction: Detection of MRD in AML patients with normal karyotype (NK-AML) is challenging and mutational (NPM1, FLT3-ITD) or abnormally expressed (WT1) markers are used for this goal. Up to 20% of patients with NK AML harbor CEBPA mutations in leukemic blasts. CEBPA is a key transcriptional factor, regulating granulocytic differentiation and leukemia with CEBPA mutations is provisional entity in WHO classification (2008). Mutations of CEBPA can be potential markers for

MRD monitoring. At the same time there are limited publications concerning MRD monitoring using CEBPA.
Study aim: Aim of this work was to develop patient-specific approach for MRD detection in pediatric NK AML patients using CEBPA mutations as marker of MRD.
Results: In retrospective study 10 patients harboring mutations in CEBPA gene were found (7,3%). Mutations included 7 deletions, 3 insertions and 1 indel distributed in the gene functional domain in the following way: 2 mutations in TAD1, 4 - in TAD2 and 5 - in bZIP region. For 7 patients sets of mutation-specific primers/probe were developed using Primer3plus to monitor the persistence of MRD. The specificity of each RQ-PCR assay was tested by comparison of amplification of patient's BM DNA versus wild type (wt) control DNA. Range of sensitivity was assayed in duplicates by serial dilution of patients' diagnostic BM DNA in wt DNA. In 3 of four cases there was clear discrimination between patient's and wt DNA, in one case there was nonspecific amplification of wt DNA 10 cycles later. Additionally, for patients with GCG deletion in TAD2 domain we have found useful detection of mutation using HRM.
Conclusion: Detection of CEBPA mutations can be useful for MRD detection of NK AML patients, being at the same time challenging task for RQ-PCR approach because of high GC-content of gene and its structure.
Conflict of interest: None

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Minimal Residual Disease in Peripheral Blood and Bone Marrow of Infants with MLL-Rearranged Acute Lymphoblastic Leukemia: Concordance and Prognostic Significance

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Objective: To estimate prognostic significance of minimal residual disease (MRD) in bone marrow (BM) and peripheral blood (PB) by qualitative detection of different *MLL* fusion gene transcripts (FGt) in infants with ALL treated by MLL-Baby protocol.
Methods: Fifty three infants (20 boys and 33 girls) and with defined *MLL* rearrangements were included in the current study. Median age was 5.3 months (range 0.03-11.80). MRD detection was performed from BM and PB samples by real-time quantitative PCR and nested RT-PCR with sensitivity non-less than 1E-04. MRD-negativity was defined as absence of FGt in the both assays. Median of follow-up period in the observed group was 5.2 years. TPs for MRD assessment were as follows: day 15 of remission induction (time point (TP) 1), at the end of remission induction (TP2), after each course of ATRA administration (TP3-TP9). Informed consent was obtained in all cases.
Results: We estimated 142 paired BM/PB samples. 79 samples were double positive, 41 were double negative Thus concordance between MRD results in BM and PB samples achieved 84.5%. Concordance varied between different TPs of MLL-Baby protocol from 79.0% to 100%. The highest concordance rate was at TP4 and TP7 (92.3% and 100% respectively). All discrepant results (22 samples 15.5%) were BM-positive/PB-negative. MRD-positivity at TP4 in BM led to unfavorable outcome. Event-free survival was significantly lower in MRD-positive group in comparison to MRD-negative one (9.9±6.1 vs 75.9±8.0, p=0.001). MRD-positivity at this TP in BM was the only significant factor in the diagnostic model where initial risk factors (age at diagnosis, initial WBC count, immunophenotype, CNS disease, presence of *MLL-AF4*) were combined to response criteria (number of blast cells at day 8 of dexamethasone prophase) (Table). We could not find any TP when MRD data obtained from PB samples had prognostic values.
Conclusions: Despite high qualitative concordance rate between BM and PB samples we could not show prognostic significance of MRD monitoring by FGt detection in PB. Univariate and multivariate analysis revealed that MRD-positivity at TP4 in BM was significant and independent prognostic factor of unfavorable outcome.
Conflict of interest: None

IX. IMMUNOTHERAPY OF ACUTE LEUKEMIAS

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Targeting Acute Myeloid Leukemias with TCR-Transgenic T CELLS

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Introduction: Treatment of high risk or relapsed myeloid leukemias by allogenic hematopoetic stem cell transplantation (HSCT) followed by the administration of donor lymphocyte infusions (DLI) is a therapeutic concept with curative potential. Leukemia-specificity of DLI with simultaneously reduced risk for Graft versus host disease (GvHD) may be achievable by genetic modification of the DLI with T cell receptors (TCR) recognizing leukemia-derived human leukocyte antigen (HLA) ligands presented on the surface of leukemia cells.
Method and results: We recently identified 19 HLA ligands that are presented on different HLA molecules and are derived from 7 different genes suitable for targeting in leukemia. We are currently establishing a biobank of TCR with specificity for these HLA ligands. So far, four TCR could be isolated with specificity for 3 different myeloperoxidase (MPO) and 1 ITGA2B epitope. In depth analysis of the specificity of one TCR (TCR2.5D6) that recognizes an HLA-*B*07:02*-restricted MPO epitope revealed a safety profile suitable for clinical translation. TCR2.5D6-transgenic T cells recognize HLA-*B*07:02*$^{+}$MPO^{+} acute myeloid leukemia (AML) cells in vitro and adoptive transfer in a xenogenic mouse model of human AML resulted in significant prolonged survival compared to control groups. A clinical trial with TCR2.5D6-transgenic central memory T cells administered to patients with AML after haploidentical or HLA-*B*07:02*-mismatched transplantation is prepared at the moment.
Conclusion: In conclusion, our data indicates that targeting of leukemia-associated HLA ligands with TCR-transgenic T cells is a highly promising strategy to improve treatment options for patients with this highly aggressive disease.
Conflict of interest: None

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PD-1/PD-L1 Blocking Enhances CD33/CD3-Bispecific BiTE® Antibody Construct (AMG 330) Mediated Lysis of Primary AML Cells

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Background: In our previous work, we were able to show effective elimination of primary AML cells by a CD33/CD3-bispecific BiTE® antibody construct (AMG 330) using a MS-5 feeder cell-based culture system supporting the growth of AML cells for up to 36 days (Krupka et al, Blood 2014).
Study aim: The present study examined the influence of the immune checkpoint pathway on AMG 330-mediated lysis of primary AML cells.
Results: No constitutive expression of PD-1 and its ligand PD-L1 was found on T cells (n=23) and AML cells (n=193) at time of primary diagnosis. Upon the addition of AMG 330 to primary AML cultures, we observed a strong upregulation of PD-1 on activated T-cells, which correlated with the extent of T-cell proliferation (10/10). This was most prominent within the subpopulation of effector memory T-cells. Furthermore, in response to AMG 330-mediated T-cell activation, we observed an upregulation of PD-L1 on primary AML cells (16/19). This phenomenon was cytokine-mediated as the sole addition of IFN□ and TNF□ also induced PD-L1 expression. Interestingly, we also observed a PD-L1 upregulation on T-cells upon activation with AMG 330, but to a much lower extent compared to primary AML cells (n=17; mean MFI ratio: T-cells: 4.7; AML cells: 12.1). Blockade of the PD-1/PD-L1 interaction increased T-cell proliferation resulting in enhanced cytotoxicity against primary AML cells (with/without PD-1 blocking: 75 vs 44%) and a significant increase in IFN□ production. This effect was most prominent in co-cultures with a low effector : target cell ratio.
Conclusion: We hypothesize that PD-L1 upregulation on primary AML cells is a relevant immune escape mechanism employed by AML cells to escape cytokine-mediated immune responses.
Conflict of interest: C.K. and M.S. received research fundings from AMGEN Research (Munich) GmbH
K.P.R.K., G.Z. and P.B. are employees of AMGEN Research (Munich) GmbH. K.N. and A.S. are employees of AMGEN Inc.

IX. STEM CELL TRANSPLANTATION

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Autologous Hematopoietic Stem Cell Transplantation for Adults with Acute Myeloid Leukemia: A Single-Centre Experience

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Introduction: Hematopoietic stem cell transplantation (HSCT) remains the most efficacious therapy in patients (pts) with acute leukemias. For older pts with high risk or relapsed disease lacking a related HLA-compatible donors autologous transplantation (AutoHSCT) is a valid alternative therapeutic option.
Material: From 1997 till 2014 in Department of Haematooncology and Bone Marrow Transplantation Medical University of Lublin there were performed 29 AutoHSCT in pts with acute myeloid leukemia (AML: 15 M and 14 F; median age: 52.2 years). There were diagnosed following FAB types of AML: M0 - 3, M1 - 4, M2 - 6, M4 - 10 and M5 – 6. The pts with AML were classified into 3 cytogenetic prognostic groups: high risk – 9, intermediate – 16 and low risk – 4. Twenty five pts with AML were in first complete remission (CR) and 4 in second CR.
Methods: The peripheral HSCs mobilised after chemotherapy (mainly second course of consolidation) and G-CSF has been the source of the stem cells in all cases. The median CD34 infused cells has been 3.58x10^6/kg. The conditioning regimen was BuCy in all pts with AML. The intravenous form of busulfan was applied in 15 last pts.
Results: The median time for ANC recovery > 0.5x10^9/L and for PLT > 20.0x10^9/L was 12 and 16.5 days respectively. Treatment related mortality in the whole group was 3.4% (one pt with sepsis in aplastic period). The median follow-up of survivors is 21.9 months (range: 11.7 – 142.4). The 3-year disease-free survival (DFS) and overall survival (OS) was 60% and 68%, respectively.
Conclusion: Our data confirm that AutoHSCT is a very valuable therapeutic option for pts with AML, especially older and lacking a related HLA-compatible donors.
Conflict of interest: None

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Haploidentical 2ND ALLO-HSCT For Treatment of Acute Leukemia (AL) Relapse after 1ST ALLO-HSCT: Retrospective Registry Analysis of 63 PTS

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Background: Relapse of AL after allo-HSCT is frequently treated with 2nd allo-HSCT. Using a haploidentical donor at 2nd allo-HSCT might represent a feasible option.
Study design: A retrospective analysis including 63 consecutive pts (female n=30; AML n=51, ALL n=12) from 9 German centers was conducted. Median age was 40 years (range, 16-65). Grafts at 1st allo-HSCT were from matched related (32%), matched unrelated (33%), mismatch unrelated (18%), haploidentical donors (6%), and other donors, including cord blood (8%). Median duration of CR after 1st allo-HSCT was 414 days (range, 18-1633). Relapse was initially treated by cytoreductive chemotherapy in all cases; stage at start of conditioning for haploidentical second allo-HSCT was CR in 27%, active disease in 66% and not evaluated in 8%. Conditioning for 2nd HSCT was myeloablative/reduced in 14%/86% To overcome the HLA barrier, 23 patients (36%) received ex vivo T-cell depletion (TCD), following either CD3/CD19 negative or CD34 positive selection. 4 pts received in vivo TCD only, 2 received no TCD, 35 pts (55%) received high-dose cyclophosphamide post-transplant according to the Baltimore protocol.
Results: Neutrophil engraftment was achieved after a median of 12 days (range, 8-26). 50 pts (78%) achieved CR after 2nd haploidentical allo-HSCT, out of which 23 (46%) relapsed again. After a median follow-up of 425 days, 47 pts had died, 22 from leukemia, and 25 from treatment-related causes. Kaplan-Meier estimated overall survival at 1 and 2 years from haploidentical 2nd HSCT was 41+/-6% and 19+/-6%.
Conclusions: Haploidentical 2nd allo-HSCT is a promising approach to the treatment of AL relapse after 1st allogeneic transplant. OS rates at least comparable to alternative treatments were observed. Different strategies to overcome the HLA barrier seem feasible. Registered as NCT01997918.
Conflict of interest: None

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Allogeneic Transplantation in Elderly Patients with AML and MDS Comparing two Reduced-Intensity Regimes

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Study aim and method: Different reduced-intensity conditioning (RIC) regimens are available for allogeneic hematopoietic stem cell transplantation (allo-HSCT) in elderly patients. To evaluate the outcome of sequential therapy we retrospectively compared RIC with fludarabine 30mg/m^2, cytarabine 2g/m^2 and amsacrine 100mg/m^2 for 4 days followed by busulfan 10x0.8mg/kg (FLAMSA-Bu) with RIC utilizing fludarabine

5x30mg/m^2, carmustine (BCNU) 2x150mg/m^2 and melphalan 110mg/m^2 in elderly patients treated at our institution between July 2005 and October 2012. 114 patients (AML n=99; MDS n=15) aged ≥59 years (59 pts ≥66 years) who were treated with either FLAMSA-Bu (n=66; n=24 ≥66 years) or FBM (n=48; n=35 ≥66 years) followed by serotherapy with ATG in all pts. Median patient age was 66 years (68 years FBM; 64 years FLAMSA-Bu).

Results: The hematopoietic cell transplantation comorbidity index (HCT-CI) was higher for FBM patients than for the FLAMSA-BU with 26 (54%) vs 24 patients (36%) scoring ≥2 (p 0.085). Graft source after conditioning with FBM/FLAMSA-Bu was bone marrow (1/2), peripheral blood stem cells (40/62) and double cord-blood (7/1). HLA-matched donors were related in 23 pts (20%) and unrelated in 91 pts (80%). Incidence of severe acute (III-IV) and chronic GvHD was 22.9 %/16.6 % for FBM vs 18.2%/19.7% for FLAMSA-Bu, respectively. Non-relapse mortality after 1 year was 26.8% for FBM versus 25.2% for FLAMSA-Bu group. Incidence of relapse after FBM vs FLAMSA-Bu conditioning was 22.9% vs. 15.2% (1 year) and 31.3% vs 16.7% (2 years). After a median follow up of 31.4 months (range 4.4-97.5) estimated overall survival (OS) and relapse-free survival (RFS) after 2 years was 55.4% and 51.4% for the FBM vs 58% and 56.7% for the FLAMSA-Bu group, respectively. Both conditioning regimens are feasible and provide similar rates of OS, NRM and GvHD.

Conflict of interest: None

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New Strategy in Stem Cell Transplantation for AML: CD96 Antibody TH-111 Removes Leukemic Stem Cells from Autografts

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Background: High relapse rates observed in AML patients after autologous stem cell transplantation (ASCT) due to residual leukemic stem cells (LSC) in the graft limit this approach. The monoclonal antibody TH111, raised in our laboratory, targets the CD96 antigen characteristically expressed on AML-LSC.

Study design: Here, a strategy is developed to remove CD96 positive LSC from autologous grafts by magnetic cell sorting (MACS). In addition, antibody engineering improves antibody dependent cell-mediated cytotoxicity (ADCC) against residual LSC allowing therapeutic targeting in other situations.

The efficiency of antibody mediated LSC purging was determined by spiking a graft with AML cells. Using biotinylated CD96 antibody TH111 and anti-biotin-microbeads, targeted cells could be depleted approx. 1000-fold with MACS technology. Viability of healthy hematopoietic progenitor cells (HPC) as well as their potential to proliferate and differentiate was not affected. Importantly, primary CD96-positive AML-LSC could be efficiently eliminated from bone marrow aspirate of an AML patient. Recombinant DNA technologies were used to generate affinity maturated and ADCC-optimized CD96 antibodies. A chimeric antibody containing affinity maturated variable regions in combination with an ADCC optimized human IgG_1 Fc was generated. In contrast to an Fc knock-out variant, this construct efficiently recruited NK cells and lysed CD96-positive AML-LSC.

Result: Using CD96 as marker typical for AML-LSC, column based purging technology eliminates LSC from mixed cell populations. Moreover, a chimeric affinity maturated and Fc-optimized CD96 antibody was able to recruit NK cells for lysis of AML-LSC.

Conclusion: Therefore, graft-engineering strategies focusing on CD96 may be feasible and avoid contamination with AML-LSC of the autograft. This strategy may help to revitalize ASCT in AML. The design of CD96 antibodies may open additional therapeutic avenues in eliminating residual disease in allogeneic situations as well.

Conflict of interest: None

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Monitoring of Bone Marrow Chimerism after Allogeneic HSCT – Early Relapse Detection by a Quantitative PCR Approach

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Background: Allogeneic hematopoietic stem cell transplantation (HSCT) has become a cornerstone in the treatment of patients at high risk for relapse of their acute leukemia. Initially monitoring of chimerism after HSCT was applied to confirm donor cell engraftment, but, since relapse is host derived, the increase of host chimerism might indicate imminent relapse. However, commonly used STR assays are limited by low sensitivity, whereas XY-FISH, CD34+ cell chimerism and MRD approaches are limited by applicability.

Study aim: In this prospective, non-interventional study we evaluated the accuracy, reliability and feasibility of a quantitative PCR based commercially available assay (Allele SEQR® Chimerism Assay, Abbott) and its potential for early relapse detection. Between 05/11 and 01/13 95 consecutive patients that received allo-HSCT for AML/MDS, were enrolled.

Method and results: According to local standards, bone marrow samples were tested at day 30, 90 and 180 after allo-HSCT and patients were followed up until 03/14. Informative markers were available in all screened patients (n=68). High sensitivity (<0,05% at 100ng DNA) translated in a remarkable relapse prediction/ exclusion rate in 61 repetitively tested patients (relapse rate 86% vs. 15%, p=<0,001, Mantel-Byar) with a median time from positive testing to relapse of 68 days resp. no relapse within >60days after negative testing.

Conclusion: Hence, this approach might facilitate personalized monitoring and chimerism-triggered therapy.

Conflict of interest: None

Late Submissions

AGIHO - EDUCATIONAL SYMPOSIUM

Epidemiology of Lung Infiltrates in Leukemia and Stem Cell Transplant Patients

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Abstract

Lung infiltrates are frequent in patients treated for acute leukemia and in hematopoietic stem cell transplant (HSCT) recipients. The development of pulmonary infiltrates is associated with significant morbidity and mortality in these severely immunocompromised patients. The differential diagnosis of lung infiltrates is wide and should include infectious and non-infectious causes. Pneumonia remains a major cause of mortality among leukemia and HSCT patients, espeially when complicated with bacteremia. Among non-infectious causes of pulmonary infiltrates, diffuse alveolar hemorrhage, and pulmonary edema are particularly common. This short review specifically focuses on the current epidemiology of lung infiltrates in patients with acute leukemia and HSCT recipients.

The development of lung infiltrates in patients treated for acute leukemia and in patients undergoing stem cell transplantation (HSCT) is a frequent

life threatening complication, requiring early diagnosis and treatment. [1-4] In particular, it has been estimated that patients with high-risk neutropenia, develop pulmonary infiltrates with a likelihood of 20-25%.[1,5,6]

Pulmonary infiltrates in leukemia and HSCT patients pose a major challenge for clinicians because of the wide differential diagnosis, including infectious and non-infectious causes.[7,8] In a recent study, non-invasive and bronchoscopic diagnostic techniques were applied to determine the etiology of the pulmonary infiltrates in 200 consecutive patients with different non-HIV conditions, of whom 121 (60%) had hematologic malignancies or were HSCT recipients.[3] The etiology of lung infiltrates was infectious in 23% of cases, non-infectious in 23%, and in 19% remained undetermined. The main infectious etiologies were bacterial (24%), fungal (17%), and viral (10%). The most frequent pathogens were *Aspergillus fumigatus*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. Among the noninfectious etiologies, diffuse alveolar hemorrhage, pulmonary edema, and bronchiolitis obliterans organizing pneumonia were the most common causes.[3] Other non-infectious causes that should be carefully considered in the differential diagnosis of pulmonary infiltrates include lung involvement by the underlying malignancy, immune reconstitution syndrome and lesions caused by chemotherapy or radiation.

Importantly, pneumonia is a common cause of mortality among leukemia and HSCT patients. In a retrospective study of 801 patients with acute leukemia who underwent induction chemotherapy, pneumonia occurred in 233 (29%) of them.[5] One or more pathogens were identified in 79 (34%) cases; gram-positive bacteria were present in 67% of patients, gram-negatives in 30%, fungi in 23%, and viruses in 10%. In a multivariate analysis, age greater than or equal to 60 years, acute myeloid leukemia, low platelet count, low albumin level, neutropenia, and neutrophil count greater than 7,300 were risk factors. The case fatality rate of pneumonia was 17% (40 of 233 patients).[5] In a retrospective study of 458 patients affected by acute leukemia receiving an anthracycline-containing induction regimen, 109 (23.8%) developed pneumonia.[6] Advanced age and total blast count were the only factors independently associated with a higher risk of developing pneumonia. The case fatality rate of pneumonia was 38.5% (42 of 109 patients).[6] Attributable mortality of pneumonia was 55% and overall mortality reached 62.5% in a prospective series of 40 consecutive cases of bacteremic pneumonia in adult neutropenic patients with cancer.[9] In that study, *P. aeruginosa* and *Streptococcus pneumoniae* caused 72.5% of all episodes of bacteremic pneumonia.

According to data from the Hospital Infection Surveillance System for Patients with Hmatologic/Oncologic Malignancies (ONKO-KISS), initiated in Germany in 2000, the pooled mean site-specific incidence density per 1000 neutropenic days was 5.9 for pneumonia (6.1 and 5.6 in patients with allogeneic and autologous transplantation, respectively). After allogeneic transplantation 11 cases of pneumonia per 100 patients occurred, whereas 5.4 cases of pneumonia per 100 patients occurred after autologous transplantation. A nationwide prospective study was conducted in Spain, from September 2003 to November 2005, to determine the epidemiology, etiology and outcome of pneumonia after allogeneic HSCT.[11] A total of 112 episodes in 427 consecutive allogeneic HSCT recipients were included (incidence 52.2 per 100 allogeneic HSCT/year), and 72 of them (64.3%) were microbiologically documented. Bacterial pneumonia (44.4%) was more frequent than fungal (29.2%) and viral pneumonia (19.4%). The most frequent microorganisms in each group were: *Escherichia coli*, *S. pneumoniae*, cytomegalovirus, and *Aspergillus* spp. The development of pneumonia and chronic graft-versus-host disease (GVHD) was associated was associated with increased mortality after allogeneic HSCT, and the probability of survival was significantly lower in patients that had at least one pneumonia episode. Pneumonia development in the first 100 days after transplantation, fungal etiology, GVHD, acute respiratory failure, and septic shock were associated with increased mortality after pneumonia.[11]

The incidence, etiology, outcome, and risk factors for developing pneumonia late after HSCT were investigated in 1359 patients who underwent transplantation in Seattle.[12] A total of 341 patients (25%) developed one or more episodes of pneumonia. Etiological diagnoses was established in 144 patients, including virus (10%), bacteria (9%), *P. jirovecii* (5%), and other fungi (6%). The overall cumulative incidence of first pneumonia at 4 years after discharge home was 31%. Factors independently associated with development of late pneumonia after allografting were increasing patient age (RR 0.5 for <20 years, 1.2 for >40 years, P=0.009), donor HLA-mismatch (RR 1.6 for unrelated/mismatched related, P=0.01), and chronic GVHD (RR 1.5, P=0.007).[12]

Finally, it should be pointed out, that respiratory viruses such as *Influenza*, *Parainfluenza*, Coronavirus, Rhinovirus, *Human Metapneumovirus* or *Respiratory Syncytial Virus* are an important yet underestimated cause of respiratory tract infection and lung infiltrates in immunocompromised patients, particularly among allogeneic HSCT recipients.[13-16] In individual patients, respiratory viruses such as *Influenza A* (H1N1)pdm09 may also facilitate the development of invasive aspergillosis.[17] In immunosuppressed patients affected by these viruses, neutropenia, poor APACHE II score, age over 65 years and severe lymphocytopenia have been reported prognostic factors resulting in a higher risk of fatal outcome.

In summary, the development of lung infiltrates occur frequently in leukemia and HSCT patients and is associated with significant morbidity and mortality. The differential diagnosis of pulmonary infiltrates is wide and includes infectious and non-infectious complications. In particular, pneumonia remains one of the most significant causes of mortality among these severely immunocompromised patients.

Conflict of interest: The author declares that he has no conflict of interest

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MAIN SESSION IV

FLT3 Inhibitors in AML

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Two decades ago when AML blasts were found to harbor FLT3 mutations in approximately 35% of patients[1] there was hope that this gain-of function mutation in a tyrosine kinase inhibitor might be an important therapeutic target in this relatively resistant disease. Moreover, recognition that the most common type of mutation, namely a length or internal tandem duplication in the juxtamembrane region of the FLT3 receptor, was associated with relatively poor prognosis [2] further spurred therapeutic development. A number of FLT3 inhibitors were shown to have important in vitro activity in murine models. Such agents inhibit the growth of leukemic cell lines whose growth factor independence was based on a FLT3 ITD transduced gene[3] or improve outcomes of a fatal myeloproliferative disorder from a FLT3 ITD stem cell transplant.[4] However, the lack of an approved FLT3 inhibitor in 2015 suggests that this potential therapeutic avenue was paved with rocks and stones rather than gold. However, there is still significant hope that one or more FLT3 inhibitors might yet find their way into the therapeutic armamentarium of AML drugs either as a single agent or in combination with chemotherapy. Some of the pitfalls in the therapeutic development of FLT3 inhibitors in AML have been inconsistent potency, protein binding leading to the lack of sufficient drug levels for prolonged enzyme inhibition, off-target effects, non-specificity, high FLT3 ligand levels, and probably most importantly the issue that a FLT3 mutation is a relatively late 'hit' in the pathophysiology of AML. The FLT3 inhibitors which have undergone clinical development in AML to greater or lesser degrees to fall into three categories: 1) "early" agents (tandutinib, lestaurtinib, and midostaurin), "later" agents (quizartinib, PLX3397, and crenolinib) and 3) sorafenib. Tandutinib emerged out of solid pre-clinical testing, had activity in early phase trials[5], but a business decision led to its abandonment as a therapeutic agent. Lestaurtinib displayed single agent activity in a 14 patient trial conducted at Johns Hopkins.[6] However, a randomized trial in which chemotherapy (the choice of which dependent on the disease free interval) plus or minus lestaurtinib was given to patients with relapsed FLT3 ITD mutant AML showed no benefit for the addition of lestaurtinib to chemotherapy.[7] Ancillary studies revealed that the failure of this trial may have been due in part to the fact that insufficient lestaurtinib levels in plasma were present in many patients. Those who achieved adequate lestaurtinib levels for long enough period of time did experience a benefit. Nonetheless, because of the negative results lestaurtinib will likely not be developed further in AML.

The third agent which has been in development for over 10 years, midostaurin, is a multi-targeted kinase inhibitor that inhibits both tyrosine kinases and serine-theonine kinases. Single agent trials with this drug at various doses showed that it had the ability to reduce the blast counts in many patients with FTL3 mutant AML (and in a few patients with FLT3 wild-type AML)[8] but important clinical responses were rare. Nonetheless, the biological activity of this drug was encouraging enough so that it was combined with chemotherapy in a phase IB trial. While higher doses of midostaurin were not well tolerated in combination with standard induction chemotherapy, a tolerable dose of 50 miligrams twice daily in combination with chemotherapy was retrospectively shown to improve the disease free and overall survival of FLT3 ITD patients to a range seen in FLT3 wild type patients.[9] These relatively encouraging results prompted a large phase III trial (CALGB 10603; RATIFTY) in patients aged 18-60 with FLT3 mutant AML in which standard induction chemotherapy and high dose ara-C consolidation was combined with either placebo or midostaurin for 14 days per cycle followed by continuous maintenance placebo or midostaurin for a year. Midostaurin, unlike most of the other FLT3 inhibitors (except for crenolinib to be described below) can inhibit both the FLT3 ITD and FLT3 tyrosine kinase domain mutants, allowing accrual of patients with both FLT3 mutant subtypes (n=714 over 4 years). The primary endpoint of the trial was overall survival (not censored for stem cell transplant) to be analyzed after 504 deaths; however, perhaps because of a high rate of stem cell transplantation (55% overall, 24% in CR1), these events have not yet occurred. Event-free survival data will hopefully soon be available; discussions with regulatory agencies are ongoing to allow release of this data. Thus, it is possible that midostaurin might be approved as a drug to be combined with chemotherapy for mutant FLT3 AML.

One of the newer agents, PLX3397[10], has undergone phase I/II testing in patients with mutant FLT3 AML. This drug is more specific and perhaps better tolerated than the first generation agents. Initial results are encouraging; it is unclear if and when this drug will be developed further in AML. Probably the most promising agent for potential use as a single agent in AML is quizartinib which has the advantage of a high degree of potency and specificity for the FLT3 ITD mutation. In phase I/II trials the drug showed an impressive level of activity in terms of reducing the blast count in blood and marrow,[11,12] although true complete remissions were noted in less than 10% of treated patients. Interestingly for this specific drug, there were also some responses in FLT3 wild-type patients; however, the overall response rate of 60% in FLT3 mutant patients is impressive. One of the potential drawbacks of this agent is that it selects for secondary tyrosine kinase domain mutations, providing mechanism for early resistance.[13] These results have led to registration ongoing trial of this drug vs physicians'-choice chemotherapy for patients with relapsed mutant FLT3 AML. The drug has also been tolerably combined with chemotherapy[14]; the U.S. Intergroup has plans to perform a randomized trial of chemotherapy plus or minus quizartinib during induction, post-remission therapy and specifically after stem cell transplant.

Crenolinib have the potential advantage of inhibiting both FLT3 ITD and FLT3 TKD mutations.[15] Phase I evaluation of this agent has suggested that there is a significant level of activity. Potentially this drug will also be combined with chemotherapy in preparation for a phase III chemo plus or minus crenolinib trial.

Sorafenib and sunitinib, putative VEGF receptor inhibitors approved for use in metastatic renal cell carcinoma are relatively good FLT3 inhibitors. Sorafenib in particular has been shown to have both single agent activity[16] and has been combined with both standard induction chemotherapy [17] and hypomethylating agent[18] therapy. A phase III trial of chemotherapy plus or minus sorafenib in newly diagnosed AML patients (not restricted to mutant FLT3) suggests that sorafenib may lead to a superior event free survival with a trend toward improved overall survival.[19] The frequent and appropriate use of stem cell transplant to consolidate first remissions in AML patients with FLT3 mutations may cloud the ability to note an overall survival advantage with any new agent in AML, and FLT3 agents in particular, when used early in the course of the disease. Moreover, FLT3 inhibitors such as sorafenib have shown impressive responses in patients with FLT3 AML relapsing after a stem cell transplant.[20] Why this setting might yield a high number of responses with FLT3 inhibitors is unclear, but may have to do with changes in the immunologic millieu engendered by these compounds or perhaps by the different disease biology present in the post-transplant relapse setting. In any event, most subsequent trials will likely employ FLT3 inhibitors after transplant alone and/or in addition to early use

In summary, the use of FLT3 inhibitors as single agents or in combination with chemotherapy in AML has not yet led to an approved agent. However, the story is far from over. Both sorafenib and midostaurin as adjuncts to standard chemotherapy in FLT3 mutant AML (and sorafenib in all AML), quizartinib as a single agent in AML, and crenolinib with its relatively unusual ability to inhibit both FLT3 ITD and TKD, may each have a role in AML. While FLT3 inhibitors are unlikely to have major long term disease mediating activity as single agents, drugs which can 'bridge' patients to transplant and/or potentiate the effects of chemotherapy may be useful. Thus, while FLT3 inhibitors have not been the straightforward hoped-for panacea in AML, it is much too early to 'close the book.'

Conflict of interest: The author declares that he has no conflict of interest

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