Intratumoral heterogeneity in anaplastic large cell lymphoma of non-common subtype

Dmitriy Abramov · Ilske Oschlies · Dmitriy Konovalov ·
Christine Damm-Welk · Wilhelm Wössmann ·
Wolfram Klapper

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Abstract *ALK*-positive anaplastic large cell lymphoma (ALCL) with identical primary genetic aberration present with variable morphology. The eponymous anaplastic large cell morphology can be detected in the majority cases which are described as being of common type morphology. However, morphological variants displaying small tumor cells that are barely distinguishable from reactive lymphocytes occur often in combination with classical large tumor cells and characterize so-called non-common type ALCL. This subgroup has recently been shown to be associated with a weak immune response to the *ALK1* protein and a poorer outcome in children. Expression of T cell markers like CD3 is often reduced or absent in these lymphomas, probably as a direct effect of the *ALK1* protein. In non-common type ALCL, the expression of *ALK* is restricted to the nucleus, suggesting reduced activity of the enzyme. The goal of the present study was to gain insight into the intratumoral heterogeneity of these lymphomas and to understand how T cell marker expression is

distributed among tumor cells. We analyzed the immunophenotype of *ALK1*-positive ALCL in 41 pediatric patients by fluorescence multi-staining and subsequent digital image analysis. The small tumor cells in non-common type ALCL expressed larger amounts of T cell surface markers such as CD3, CD5, and CD8 but less CD30 compared to the large tumor cells. Expression of *ALK1* and phosphorylated STAT3 as well as cell proliferation measured by positivity for Ki67 was reduced in the small cells of non-common type ALCL. These data demonstrate heterogeneity of the immunophenotype of tumor cells within non-common type ALCL suggestive for differentiation of small tumor cells, thereby providing a possible first explanation for the reduced immunogenicity of non-common type ALCL.

Keywords Anaplastic large cell lymphoma · *ALK* · Digital image analysis

Dmitriy Abramov and Ilske Oschlies contributed equally to this work.

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D. Abramov · I. Oschlies · W. Klapper (✉)
Department of Pathology, Hematopathology Section and Lymph
Node Registry, University of Kiel,
Kiel, Germany
e-mail: wklapper@path.uni-kiel.de

D. Konovalov
Department of Pathology, Russian Federal Research Center,
Moscow, Russia

C. Damm-Welk · W. Wössmann
NHL-BFM Study Center, Department of Pediatric Hematology
and Oncology, Justus-Liebig University,
Giessen, Germany

Introduction

ALK1-positive anaplastic large cell lymphoma (ALCL) is a distinct T cell lymphoma entity characterized by genetic aberrations involving the *ALK1* gene and a typical morphology [17, 22]. ALCL are composed of large cells with a characteristic intrasinusoidal or perivascular growth pattern. The cytological appearance is blastic, and horseshoe-shaped nuclei are a characteristic finding in the so-called common type of the lymphoma (cALCL) [22]. The tumor cells are characterized by strong positivity for CD30, expression of *ALK1* fusion proteins and cytotoxic granules, together with loss of surface T cell markers and T cell receptor-associated molecules [17, 22]. Using immunohistochemical staining for *ALK1* to specifically identify the tumor cells, several morphological variants of ALCL have been recognized.

These morphological variants are composed of smaller tumor cells which are sometimes indistinguishable from the abundant reactive bystander cells. Among the non-common type morphologies (ncALCL), the small cell type and a lymphohistiocytic type are most frequent. They may also occur in combination with the common type within one lymphoma (mixed type) [18, 19, 22, 23].

ALCL occurs primarily in children and young adults. Therefore, risk factors have largely been analyzed in pediatric patients. Adverse prognostic factors are visceral, mediastinal, or skin involvement [14]; minimal disseminated disease [7, 16]; low or absent immune response to the *ALK1* protein [1, 16]; and a ncALCL morphology (small cell, lymphohistiocytic, or mixed ncALCL) [7]. Interestingly, even the presence of a minor component of ncALCL in otherwise cALCL is associated with a poor outcome [7]. We detected a strong correlation between the extent of minimal disseminated disease, low immune response, and ncALCL subtype [7]. We thus asked whether lymphoma cell characteristics of ncALCL might be involved in the inhibition of the host immune response against *ALK1*. Furthermore, the immunophenotypes of ncALCL have not been studied in detail since conventional immunohistochemistry cannot reliably distinguish between lymphoma cells and reactive bystander cells [6, 19]. Fluorescence multi-staining was performed in cALCL in detail [3, 10], but data on ncALCL are missing so far.

A variety of *ALK1* translocations have been described in ALCL, which ultimately lead to expression of chimeric proteins in lymphoma cells [17]. The most frequent translocation partner of *ALK1* is the nucleophosmin gene (*NPM*), resulting in the aberrant expression of the chimeric protein *NPM-ALK1* [8]. *NPM-ALK1* protein exhibits tyrosine kinase activity and has been shown to be a major factor in lymphomagenesis of ALCL [5]. Interestingly, in a cell line model, *ALK1* activity influenced the immunophenotype of the lymphoma cells by downregulation of surface T cell markers including T cell receptor molecules via activation of *STAT3* [2, 4, 27]. Thus, active phosphorylated *STAT3* (*pSTAT3*) can be used to assess *ALK1* activity. *ALK1* expression is restricted to the nucleus in the small tumor cells of ncALCL [23], and nuclear *ALK1* protein has been suggested to display less kinase activity and *STAT3* activation [25]. We thus asked whether the immunophenotype between small and large cells within the ncALCL differ.

We analyzed the immunophenotype of *ALK*-positive ALCL in a cohort of 41 pediatric patients including 17 cases of ncALCL applying a novel approach involving fluorescence multi-staining and digital image analysis. We were able to assess the immunophenotype of small and large cells within each ncALCL separately. The phenotype of the small cell component in ncALCL suggests that *ALK1* activity is reduced in the small lymphoma cells.

Material and methods

Patient specimens

Formalin-fixed paraffin-embedded specimens from 41 patients younger than 18 years of age were analyzed. The cohort was composed of 27 (64%) male and 14 (36%) female patients with 14 of 41 (32%) being younger than 9 years. The cases were selected on the basis of sufficient biopsy size. In 17 cases, a tissue microarray (TMA) was constructed using tumor-rich areas. The remaining 24 cases were analyzed on full slides. In 19 of 41 cases (46%), the analyzed tissue was lymph node. All lymphomas were *ALK1* positive according to conventional immunohistochemistry (nuclear and cytoplasmic staining suggestive of *NPM-ALK* translocation in 32 cases (78%) and only cytoplasmic staining in nine cases (22%)). Histological subtyping was performed according to previous publications [7], the guidelines of the WHO classification [23], and the European Pathology Panel for Pediatric Lymphomas [13]. The histological subtype of the lymphomas was common type in 24 of 41 cases (60%) and non-common type (mixed type composed of common type and small cell or lymphohistiocytic) in 17 of 41 cases (40%). The latter group was composed of the mixed types: common type+small cell type ($n=9$), small cell+lymphohistiocytic type ($n=5$), or single differentiation lymphohistiocytic ($n=1$) and small cell ($n=2$). Representative examples are shown in Fig. 1. The median age was 10.8 years; 18% were in stage II, 65% in stage

Fig. 1 Representative example of a cALCL (a) and a ncALCL (b) of the lymphohistiocytic subtype (both H&E)

III, and 18% in stage IV at diagnosis. All available clinical data are shown in supplementary Table 1. For the 37 cases from the archives of the Lymph Node Registry Kiel, Germany, informed consent was obtained from the patients at registration in a Non-Hodgkin Lymphoma study of the Berlin–Frankfurt–Münster study group. For the four cases from the Russian Federal Research Center Moscow, Russia, the study was performed according to local ethical guidelines.

Fluorescence double staining

The slides were dewaxed in xylene (1 h) and rehydrated in a series of ethanol. Antigen retrieval was performed in citrate buffer (pH 6) for 3 min in a pressure cooker. The primary antibodies were incubated on the slides for 1 h. The slides were then washed with phosphate-buffered (PBS) saline and incubated with the secondary antibodies for 1 h. After this, they were washed with PBS, and a 1:5,000 DAPI solution (Molecular Probes, Eugene, OR, USA) was applied. After a final washing with PBS, a mounting medium (Dako, Glostrup, Denmark) was used and the slides were covered. All incubations were performed in a dark, moist chamber at room temperature.

For the detection of tumor cells, *ALK1* mouse monoclonal antibody (mAb) (Dako, 1:10 dilution) or *ALK/p80* rabbit mAb (Epitomics, 1:50 dilution) was used. The *ALK1* stainings were combined with a second marker using the following antibodies: CD30 mouse mAb (self-produced clone BerH2, 1:5 dilution), CD3 mouse mAb (1:100 dilution), CD2 mouse mAb (1:50), CD4 mouse mAb (1:25), CD5 mouse mAb (1:25, all Novo Castra, Newcastle, UK), CD8 rabbit mAb (1:25, NeoMarkers, Fremont, CA, USA), Zap-70 (1:100, Upstate Biotechnology, Waltham, MA, USA), and anti-pSTAT3 rabbit mAb (1:25, Epitomics, 1:100, Burlingame, CA, USA). The secondary fluorescence-labeled antibodies were Alexa Fluor^R 555 and 488 donkey anti-mouse IgG (1:100) and Alexa Fluor^R donkey anti-rabbit IgG 555 and 488 (1:100) (Molecular Probes, Eugene, OR, USA).

Digital image analysis of tumor cell immunophenotypes

All slides were analyzed with an Axioplan 2 microscope (Zeiss, Jena, Germany). Areas considered to be representative of the tumor (reflecting tumor morphology as determined by conventional staining) and of the staining (e.g., providing sufficiently stained positive controls) were selected and photographed with a digital camera SPOT RTTM slider (Diagnostic Instruments Inc., Burroughs, Sterling Heights, MI, USA) and VisiView 1.7.2 software (Visitron Systems, Puchheim, Germany). For each slide and each staining combination, three-color photos were taken: *ALK1*

to identify tumor cells, DAPI to identify cell nuclei, and the third marker (see above). Pictures were subsequently transformed into black and white and further analyzed with TissueQuest 2.2 software (TissueGnostics, Vienna, Austria). The software uses the pictures of each single staining, combines the pictures to virtual overlays, and quantifies the number of double positive cells expressing *ALK1* and the second marker, the mean intensity of staining and the area (size) of the cells. The cutoff to determine positivity was set manually within the software.

Digital image analysis to distinguish small and large tumor cells

The TissueQuest software recognizes and calculates cell masks in a pixel format. Thus, the microscope was calibrated by VisiView such that the size of each pixel obtained by the camera and the VisiView software in each magnification was known. In our project, all photos were done at a magnification of $\times 200$. The size of 1 pixel was $0.1369 \text{ pixel}/\mu\text{m}^2$ and one side of a square measured $0.37 \mu\text{m}$. The diameter of normal lymphocytes varies from 3.5 to $10 \mu\text{m}$ [21]. We first converted the diameter of a normal lymphocyte to the pixels and defined a cell size $<10 \mu\text{m}$ as “small” and a cell size of $>10 \mu\text{m}$ as “large.” The corresponding cell size was 600 pixels, assuming a round cell in the described setting. Using this cell size cutoff, the *ALK1*-positive tumor cells were subdivided into small and large cells and the expression of the second marker was analyzed in each tumor cell subpopulation separately. Objects smaller than 200 pixels are smaller than the smallest lymphocytes ($5.8 \mu\text{m}$ diameter) and were excluded from the analysis to reduce artifacts.

Statistical analysis

All statistical analyses were performed using GraphPad Prism software. To compare marker expression between common and non-common type *ALK*+ ALCL, *t* tests were performed. Paired *t* tests comparing small and large cells within each tumor sample were used to analyze intratumoral heterogeneity.

Results

Immunophenotype of common and non-common type *ALK*+ ALCL

Forty-one *ALK1*+ ALCL were stained with multiple marker combinations using *ALK1* as a marker to identify the tumor cells and a second marker to quantify coexpression within the *ALK1*+ tumor cell population (Fig. 2). *ALK1*-negative cells were considered to be reactive bystander cells and

Fig. 2 Representative stainings for *ALK1* (red) and CD30 (green) in a non-common type ALCL. The arrows indicate a CD30-negative lymphoma cell (a). Double staining for *ALK1* (red) and CD3 (green) in a non-common type ALCL with positive tumor cells indicated by the arrows (b)

excluded from the analysis. In each lymphoma, a representative area was evaluated. The number of *ALK1*+ tumor cells within this area varied from 33 to 1,791. Interestingly, when *ALK1* staining was used to identify tumor cells, a variable number of these were CD30 negative (Fig. 3). Overall, the expression of surface T cell markers was low in *ALK1*+ ALCL, confirming previously published results [3, 10]. Nevertheless, a subset of these lymphomas expressed CD3

or other markers such as CD5, CD2, CD4, and CD8 to a variable extent (Fig. 3). The percentage of CD30-, CD3-, CD2-, CD5-, and CD4-positive tumor cells was not statistically different between cALCL and ncALCL (Fig. 3). However, significantly more cells expressed CD8 in ncALCL compared to cALCL ($p=0.0418$, unpaired t test, Fig. 3). ZAP70 expression was not detectable (data not shown).

Intratumoral differences in non-common type *ALK*+ ALCL

Morphologically, cases of ncALCL are usually composed of heterogeneous tumor cells ranging in size from lymphocytes to large anaplastic cells (also referred to as mixed-type ALCL). Rare cases of ncALCL contain small cells only. We distinguished small *ALK1*+ tumor cells (size of lymphocytes or smaller) from large ones within each lymphoma by digital image analysis and analyzed the surface marker expression in each tumor cell subpopulation separately.

To evaluate the validity of the technique, we determined the percentage of “small” cells (<10 μm diameter) in cALCL and ncALCL. The percentage of “small” cells was significantly higher in ncALCL than in cALCL ($p<0.0001$, t test, data not shown), suggesting that the digital image analysis technique is capable of detecting the microscopically smaller appearing cells.

The surface marker expression level had a high inter-case variability prohibiting comparison of expression levels between tumors. We, therefore, analyzed the differences in expression between large and small lymphoma cells within each individual ncALCL. This method also eliminated the problem that immunohistochemistry is dependent on tissue fixation and quality, since comparisons were only made within a tissue/lymphoma but not between different tissues/lymphomas. The expression of CD30 was significantly lower in small tumor cells compared to large ones ($p=0.0009$, paired t test, Fig. 4). There was a trend toward higher CD5 expression in the small cells, though it did not reach statistical significance ($p=0.066$, paired t test, data not shown). Similar findings were obtained for CD3. Only five cases within the group of ncALCL expressed CD3.

Fig. 3 Expression of surface markers in % of all tumor cells identified by *ALK1* staining. *ALK1*-negative cells excluded. *c* common type, *nc* non-common type histology. Each dot represents one lymphoma; the vertical bar represents the median

Fig. 4 Paired analysis of the CD30 (a), CD3 (b), Ki67 (c), *ALK1* (d), and *pSTAT3* (e) expression of small and large tumor cells. The analysis was performed in *ALK1*-positive tumor cells only. *ALK1*-negative reactive bystander cells were excluded. The expression of CD30, CD3, *pSTAT3*, and Ki67 is indicated in % positive tumor cells, *ALK1* expression as mean staining intensity. Each bar represents one positive case. The bars connect the values of the small and the large tumor cells within each lymphoma (*p* values by paired *t* test). Lymphomas in which the tumor cells showed no staining were included in the statistical analysis of the paired *t* test but omitted from the figure

Interestingly, in only one of these cases, CD3 expression was higher in the large cells than in the small cells of the same lymphoma. All other cases expressed CD3 in a higher percentage of small than large tumor cells (Fig. 4).

The analytical strategy described above was used to quantify *ALK1* and *pSTAT3* expression in small and large cells of *ALK*+ ALCL. *ALK1* intensity was significantly reduced in the small tumor cell population ($p=0.036$, paired *t* test, Fig. 4). We analyzed possible readouts of *ALK* activity in the lymphoma cells, trying to confirm the lower expression in the small cells. In analogy to *ALK1*, the small tumor cells tended to express

pSTAT3 less frequently ($p=0.0514$, paired *t* test, Fig. 4), suggesting that *ALK1* activity is reduced in this tumor cell subpopulation. Interestingly, in ncALCL, the cell proliferation as assessed by Ki67 staining was lower in the small tumor cells than in the large ones ($p=0.0002$, paired *t* test, Fig. 4).

Discussion

ALK+ ALCL is a well-defined entity with variable morphology [23]. In a previous publication, we showed that the

ncALCL are associated with a poorer outcome after multi-agent chemotherapy, independent of the lymphoma stage and other prognostic factors [7]. Furthermore, patients with ncALCL display a reduced immunological response to the *ALK1* protein, resulting in lower anti-*ALK1* antibody titers in ncALCL compared to those in cALCL [1]. So far there is no explanation for the specific biology of ncALCL. However, the treatment outcome for this subgroup of ALCL patients is still unsatisfying although bone marrow transplantation has been shown to rescue children suffering from a relapse [26].

Research on ncALCL is hampered by its rarity (about 10% of all cases of pediatric ALCL) and its cellular composition as a tumor in which the lymphoma cells are often sparse and intimately admixed with abundant reactive bystander cells [19], making gene expression and genomic profiling of this subtype of ALCL difficult or impossible [12, 20]. ALCL cell lines are available for research, but it is uncertain whether they reflect the biology of ncALCL. Thus, an analysis of the tumor biology of ncALCL is dependent on developing methods that are applicable on small amounts of isolated tumor cells or in situ techniques using biopsy specimens. We used an innovative methodological approach to analyze the immunophenotype of ALCL quantitatively and eliminate any bias due to reactive bystander cells. The method of fluorescence multi-staining differs from conventional immunohistochemistry in several ways. First, immunofluorescence staining includes only a minimum amplification of the signal. Thus, it may be less sensitive than conventional immunohistochemistry, although the sensitivity of the cameras may compensate this effect to some extent. Second, immunofluorescence staining allows multiple markers to be combined in one staining and opens the possibility for complex immunophenotyping [3, 9]. Using *ALK1* staining in combination with a second marker, we were able to quantify the immunophenotype of ALCL precisely. Our data confirm previous results showing that surface markers of T cells such as CD3, CD5, CD2, CD4, and CD8 are absent or are expressed at low levels in ALCL [3, 10]. Similar results were also obtained for Zap70, a T cell receptor-associated molecule (data not shown). T cell surface marker expression did not differ between cALCL and ncALCL if all lymphoma cells were analyzed, although our series was enriched for ncALCL compared to the normal prevalence [7]. Given the variability of marker expression within each group, even larger series might have to be analyzed in future studies. Nevertheless, our study demonstrates that assessment of the immunophenotype is only possible in ncALCL by double staining to avoid confusion with reactive T cells.

Our analytical method allowed us to assess the immunophenotype in small and large tumor cells within the group of ncALCL separately. When we did so, we found that the

small cells expressed less CD30 but more CD3 and other T cell markers. In other words, the immunophenotype of the small tumor cells in ncALCL more closely resemble the phenotype of normal lymphocytes. Interestingly, the proliferation was lower in the small tumor cells than in the large ones. This fact might provide a possible explanation for why the ncALCL are less sensitive to chemotherapy. We have to stress that the cutoff chosen between “small” and “large” tumor cells is simply based on the size of lymphocytes (as small cells). It remains to be determined whether this cutoff is reflecting any biologically significant groups. Additionally, our study is limited by the use of both TMA and full slides and biopsies from various locations, which is primarily due to the rarity and the limitation of biopsy specimen in this disease. However, the use of fluorescence multi-staining might overcome the limitations by the heterogeneous material, since the immunophenotype can be precisely measured in the tumor cells.

What might be the reason for the differences in the immunophenotype between large and small tumor cells? Since *ALK1* has been demonstrated to influence expression of T cell surface markers via activation of Stat3 [2], we analyzed *ALK1* and *pSTAT3* expression in small and large cells of ncALCL. The small tumor cells expressed *ALK* less intensely and showed less frequent expression of *pSTAT3*, suggesting that *ALK1* expression and activity are reduced in small tumor cells compared to large cells. These data provide a possible explanation for the upregulation of T cell surface markers in the small cell tumor subpopulation. The *ALK1* expression often seems to be restricted to the nucleus in small lymphoma cells of ncALCL, even if a translocation between *NPM* and *ALK1* is detectable [17, 22]. Since the nuclear *ALK1* might be inactive, this intracellular distribution might additionally contribute to a reduced *ALK1* activity in small cells of ncALCL [25]. The reason for the downregulation of *ALK1* expression in small cells of ncALCL remains uncertain so far. Future studies will have to address the question whether the downregulation is the cause or a consequence of the differentiation of the tumor cells.

In addition, our data might provide a possible explanation for why patients in ncALCL show a weaker humoral response to the *ALK* protein [1], simply because the level of expression is lower. However, not only the level of *ALK* expression but also the immunophenotype of the tumor cells might influence the interaction with the host immune system. Our data show that the small tumor cells in ncALCL express more T cell surface markers such as CD3, CD5, and CD8. Since all of these surface markers mediate cellular interaction and activation in normal T cells [11], one might speculate that this expression will also influence the interaction of the lymphoma cells with the microenvironment in ncALCL. Promising results were recently obtained using

vinblastine monotherapy for relapsed ALCL [15]. In these protocols, vinblastine probably develops its effect not by a tumor-toxic mechanism, but by an immunomodulatory mechanism [24]. It will thus be of interest to use our method of immunophenotyping in future trials assessing this therapy regimen to understand how differentiation of ALCL as measured by T cell surface marker expression might influence the response to immunomodulatory therapy.

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

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