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ABSTRACTS

TH 01
CULTURED ORAL EPITHELIUM: MODELING OF TUMOR PROGRESSION AND STUDIES OF CANCER RISK FACTORS

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Our laboratory is engaged in the development of culture models that mimic human oral epithelium, including for exploration of the process underlying malignant transformation and for toxicological assessment of risk factors. Normal (NOK), SV40 T antigen-immortalized (SVpgC2a) and malignant (SqCC/Y1) oral keratinocytes were therefore cultured and a serum-free condition established that supported growth in both monolayer and organotypic states. Immunochemical profiling demonstrated that NOK expressed the typical keratins of normal tissue whereas the expression pattern in SVpgC2a and SqCC/Y1 mimicked severe epithelial dysplasia and well-differentiated squamous cell carcinoma, respectively. Transcript profiling by Affymetrix microarray indicated expression of 4-5 x 10^3 genes in the cell lines. The changes from in vitro-immortalization (SVpgC2a versus NOK) outnumbered those associated to malignant transformation (SqCC/Y1 versus NOK). Decreased expression/loss of genes was more common than increased expression/gain of genes. The noted alterations related to cyto-skeleton, cell adhesion, differentiation and oncogenesis. Xenobiotic-metabolizing cytochrome P450 enzymes, conjugation enzymes and enzymes involved in detoxification of aldehydes and reactive oxygen were variably detected. The development of retinoid insensitivity during malignant transformation was also implied from the expression analysis. Microarray results could mostly, but not entirely, be confirmed by other methods, including for mRNA (Northern, in situ hybridization and standardized quantitative RT-PCR) and protein (immunochemistry, 2D gel electrophoresis and enzyme activity). Finally, cancer risk factors, e.g., tobacco and betel nut extracts as well as N-nitrosamines and aldehydes, induced a spectrum of molecular cytotoxic and genotoxic effects in NOK that clearly associated to cancer development. Oral keratinocyte cultures seem to be useful tools for investigating the cytopathology of cancer risk factors, and moreover, may allow for the in vitro modeling of normal tissue as well as of incipient and overt malignancy.

TH 02
MICROARRAY ANALYSIS OF CARBONYL-METABOLIZING ENZYMES IN HUMAN NORMAL AND TRANSFORMED ORAL KERATINOCYTES

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Carbonyl-metabolizing enzymes (CMEs) are involved in a variety of metabolic pathways and detoxification steps, including for agents coupled to the etiology of oral cancer, e.g., N-nitrosamines, polycyclic aromatic hydrocarbons, alcohols and aldehydes. Proliferative, basal-like and terminally differentiated, suprabasal-like oral keratinocytes can be selectively grown by modification of serum levels to mimic the complete phenotype of oral mucosa in vitro. Transformed oral keratinocyte lines are generally differentiation-deficient relative to normal cells. On this basis, normal (NOK), SV40 T antigen-immortalized (SVpgC2a) and malignant (SqCC/Y1) human oral keratinocyte lines were cultured under conditions with or without serum to study expression of CMEs in differentiated epithelium, as well as to analyze for possible differences in transformed states. Transcript profiling by the Affymetrix microarray and application of the recent FOCUS-chip identified members of the ADH, ALDH, SDR and AKR families in all cell lines. Relative to NOK, SVpgC2a and SqCC/Y1 showed similar transcript levels of 47 enzymes, whereas the levels differed more than 2-fold for 9 enzymes. Some of the changed transcripts were altered in both lines, although with the majority in SVpgC2a. In NOK, induction of a suprabasal phenotype caused increased/decreased levels of 4 and 5 transcripts, respectively, whereas SqCC/Y1 and SVpgC2a failed to regulate some of these genes. The overall results implicate that CME families are expressed in oral epithelium of importance in the detoxification of carcinogens, and that suprabasal differentiation may alter such activities. The differences noted in immortalized and malignant cells implicate that deregulated CME expression might be a feature of cell transformation. These findings emphasize the need of further studies of CMEs in normal and tumor tissue, and that these genes have potential as possible future markers of early and late malignancy.

TH 03
DEVELOPMENT OF A CONDITIONAL MOUSE MODEL FOR HEAD AND NECK SQUAMOUS CELL CARCINOMA

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Objective: Despite advances in the management of head and neck squamous cell carcinoma (HNSCC), mortality is still rising. Development of a mouse model for HNSCC, that mimics the human situation, would be beneficial for the identification of new key genes in HNSCC tumorigenesis, and would furthermore serve as an indispensable tool for designing new treatment-modalities.
Methods: Conditional mice equipped with the Cre/LoxP system are especially suited for the development of models of sporadic human carcinomas. The system can control gene (in)activation in a time and/or tissue-specific manner. Mice that express Cre either in Keratin 14 expressing tissues (K14-Cre, epithelial) or after application of ligand (tamoxifen) are crossed with mice, which harbor conditional alleles for p53, LacZ and non-conditional $P16^{ink4a}$. These mice were studied for SCC tumor formation and survival.
Results: We have studied the efficiency of ligand-induced gene switching in R26R reporter mice, that permits the visual-

ization of Cre-mediated recombination via β-galoctosidase staining of tissue sections. After tamoxifen application, specific gene switching could be monitored in affected tissues, like skin and oral cavity. Subsequently, tamoxifen treatment of crosses with conditional p53 and non-conditional $P16^{ink4a}$ mice resulted in the induction of SCC in oral cavity and skin, although with a long latency and low incidence. The use of K14-Cre mice crossed in the same genetic background gave rise to a more efficient tumor induction in skin and oral cavity. Unfortunately, these mice have also a high tendency for mammary tumor development.

Conclusions: We are able to induce SCC in skin and oral cavity with a long latency and low incidence. To increase the reliability of SCC formation in skin and oral cavity, we are currently trying to combine the two above-mentioned systems, to obtain a more tightly regulated Cre-expression. Furthermore, the contribution of other conditional alleles on SCC tumorigenesis is studied.

TH 04
Genotoxicity of Nicotine in human mucosa and lymphocytes

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Cigarette smoke contains a variety of substances being identified as genotoxic mutagens. The aim of this investigation was to discover whether the plant alcaloid nicotine itself has genotoxic effects. DNA migration due to single strand breaks, alkalilabile sites and incomplete excision repair was quantified with the aid of the single-cell microgel electrophoresis (Comet) assay in human mucosal cells of the nose and the tonsils as well as in lymphocytes after incubation with nicotine in concentrations of 1, 2, 4, 8 and 16 mM. N-methyl-N′-nitro-N-nitrosoguanidin (MNNG) and phosphate buffered saline were applied as positive and negative control, respectively. Cytotoxicity, both before and after incubation, was analysed with the trypanblue exclusion test. Increased dose-dependent DNA migration could be demonstrated for nicotine in lymphocytes and in mucosal cells of the nose and the tonsils. The results of this pilot project are of further interest considering the high incidence of tumors in the upper aerodigestive tract. Celltype specific DNA damage after incubation with nicotine demands further investigation.

TH 05
FORMALDEHYDE-RELATED TOXICITY AND GENE EXPRESSION IN CULTURED HUMAN ORAL KERATINOCYTES

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Mouth inhalation of formaldehyde, a known respiratory carcinogen, even below recommended acceptable levels induces genotoxic damage in human buccal epithelium. On this basis, normal (NOK), SV40 T antigen-immortalized (SVpgC2a) and malignant (SqCC/Y1) buccal keratinocytes were cultured and exposed to formaldehyde. Formaldehyde caused dose-dependent cytotoxicity in the cell lines, NOK and SVpgC2a being more sensitive than SqCC/Y1. Formaldehyde induced primarily terminal differentiation in NOK, but apoptosis and necrotic death was also noted. Differently, SVpgC2a and SqCC/Y1 showed apoptosis and necrotic death without detectable terminal differentiation. Glutathione-dependent formaldehyde dehydrogenase (alcohol dehydrogenase 3, ADH3), the primary cellular enzymatic defense against formaldehyde, was expressed at relatively similar levels among the cell types. Transcript levels of ADH3 were higher in proliferative cells, and thus, decreased in growth-inhibited cells, notably without apparent association to the expression of keratinocyte differentiation markers. The levels of the cofactor glutathione were higher in SqCC/Y1 as compared to NOK and SVpgC2a, an effect that associated to the expression levels of glutathione synthetase. Two NOK cultures failed to undergo transformation from repeated formaldehyde exposure. In contrast, repeated formaldehyde exposure of SVpgC2a resulted in the formation of a novel line (termed SVpgC3a) that exhibited multi-focal growth differently to its parental line. Relative to NOK, the immortalized state of SVpgC2a associated to decreased expression of structural proteins (keratins) and cell adhesion proteins (integrins, laminins, CD44), differentiation markers (spr1) among other genes assessed by microarray. Among several changes, the development of the SVpgC3a phenotype was associated with a further decrease in the levels of CD44. We conclude that altered expression of several gene families may couple to formaldehyde-induced keratinocyte transformation, and that susceptibility to formaldehyde toxicity may vary with the stage of transformation. Cell death induction by different pathways, and activity of ADH3 and thiols may cooperate in the protection against formaldehyde.

TH 06
Exposure to cement dust, related occupational groups and laryngeal cancer risk: Results of a population based case-control study

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A population-based case-control study, on laryngeal cancer was performed in the Rhein-Neckar region in Germany to study occupational risk factors of laryngeal cancer (so called "Rhein-Neckar-Larynx Study"). The study included 257 Cases (236 males, 21 females) aged 37-80, histologically confirmed and diagnosed between 1.5.1998 and 31.12.2000 and 769 population controls (702 males, 67 females). 1:3 frequency matched by age and sex. Occupational exposures as well as other risk factors (tobacco, alcohol) were obtained with face-to-face interviews using a detailed standardized questionnaire. The complete individual work history was assessed. A detailed assessment of work conditions was obtained by job-specific questionnaires (JSQs) for selected jobs known to be associated with exposure to potential laryngeal carcinogens. Estimates for total exposure hours by substance were calculated based on JSQs. Published occupational hygiene data were used to infer semi-quantitative scores of exposure intensity for specific job tasks. After adjustment for tobacco and alcohol intake significant elevated Odds Ratios (OR`s) could be demonstrated for persons ever worked as building and construction workers, in particular having been ever exposed to cement in their life. An OR of 2.42 was calculated for workers according to the high

exposed group (95% confidence interval: 1.14-5.15; p<0.001). Smoking is the main confounding factor considering the unadjusted cement OR of 3.20 dropping down to 2.42 after adjustment. It has to be concluded that there is a high statistical probability for cement dust exposure acting as a tobacco, alcohol and asbestos independent risk factor for laryngeal carcinoma. The study gives a base for further investigations on this topic.

TH 07
MLPA: A NOVEL SENSITIVE TECHNOLOGY TO DETECT GENETIC ALTERATIONS IN THE ORAL CAVITY AND OROPHARYNX

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Oral and oropharyngeal cancer (OSCC) arises in genetically altered preneoplastic mucosal lesions. These precursor lesions may be clinically (leukoplakia or erythroplakia) or histologically (dysplasia) identifiable. Histopathologic grading is currently used as a predictor of malignant transformation, but is not sufficiently reliable. Our aim is to improve the early diagnosis of cancer in the oral cavity and oropharynx using a non-invasive test; it will be investigated whether a panel of genetic markers is able to identify the patients at risk. For this purpose we use MLPA, Multiplex Ligation-dependent Probe Amplification. This assay allows the measurements of numerical chromosomal alterations (gains and losses) at multiple target sequences. In practice, forty markers in a single PCR run are analyzed requiring only 20 ng of DNA (approx. 3,000 cells), enabling analysis of small samples. We have obtained reproducible data using three probe sets (120 markers all over the genome) on small samples of exfoliated cells brushed from normal mucosa. We are validating the MLPA method, by analyzing DNA from (pre)neoplastic tissues, on which loss of heterozygosity was previously assessed. These updated results will be presented. The MLPA technique has so far proved to be sufficiently sensitive, reproducible and easy to perform and thus can be considered a promising method to improve the early diagnosis of OSCC.

TH 08
Genetic characterization of multistep head and neck carcinogenesis

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Recent studies have made clear that three functional stages can be discriminated in the multistep process of head and neck carcinogenesis (see Braakhuis et al., Cancer Research 63, 1727, 2003). In the initial phase a stem cell acquires genetic alterations and forms a 'patch', a clonal unit of altered daughter cells. Additional alterations leads to the conversion into a 'field', an expanding preneoplastic lesion. Ultimately, clonal divergence leads to the development of one or more tumors in a field. The aim of the present study is to understand the multistep carcinogenesis, focussing on the genetic characterization of the conversion from patch to field and from field to invasive cancer. First, we showed with TP53 mutation detection and loss of heterozygosity (LOH) analysis that there is a clonal relationship between tumor and most adjacent fields, and that fields can consist of genetically related subclones. Immunostaining with Ki-67 showed that genetically altered fields have a high proliferative activity. Thus, fields are able to replace the normal epithelium by enhanced proliferation without signs of invasive growth. So far, no common LOH marker responsible for the progression from field to tumor was found using 22 microsatellite markers on six different chromosomes. Therefore 'Multiplex Ligation-dependent Probe Amplification' was implemented, a novel genomics technique using minimal amounts of template DNA. This provides information not only about losses, but also about gains with 120 markers dispersed over all chromosomes. Patches have been identified by TP53 immunostaining, and 'Degenerated Oligo-Primed' PCR is used to enlarge the amount of template DNA to allow genetic profiling. Detection and monitoring of these precursor lesions may have profound implications for cancer prevention.

TH 09
MICROSATELLITE INSTABILITY AS AN INDICATOR OF MALIGNANT PROGRESSION IN LARYNGEAL PRECANCEROSIS

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Objective: Being microsatellite instability (MSI) a potential marker of genetic instability in pre-invasive and invasive neoplastic disease, we investigated its usefulness in assessing the risk of malignant progression in laryngeal pre-cancerosis.
Methods: We analyzed MSI in serial biopsies from 10 patients with pre-invasive laryngeal lesions and corresponding metachronous laryngeal cancers in comparison with biopsies of similar lesions without malignant transformation from 20 subjects in a match-paired analysis. MSI was determined by assessing in DNA biopsies the status of 14 microsatellite markers (chromosome loci: 2p16 , 3q21-24, 4q12, 9p21, 13q14, 16q22.1, 17p12 and 21q21).
Results: MSI+ (aberration at 2 or more loci) was detected in 9 out of 10 patients with pre-malignant lesions progressed to carcinoma, whereas only 2 of the 20 biopsies from control subjects showed an unstable phenotype (p<0.001). Interestingly, pre-invasive laryngeal lesions with MSI at hMSH2 loci frequently had instability at two or more additional loci and were considered as MSI+ (overall in 7 out of 11 cases: 6 out of 9 premalignant lesions progressed to cancer and one of the only two cases without progression of the original laryngeal lesion) (p=). The immunohistochemical analysis of the hMSH2 protein expression, however, did not confirm the involvement of this gene in laryngeal carcinogenesis.
Conclusions: Our study suggests that microsatellite status assessment may be useful in determining the risk of progression

in patients with pre-invasive laryngeal lesions for whom chemopreventive and endoscopic protocols can be attempted.

TH 10
HYPOFOLATEMIA AS A RISK FACTOR FOR HEAD AND NECK CANCER. CHEMOPREVENTIVE PERSPECTIVES

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Objective: To evaluate serum folate, homocysteine and vitamin B12 levels in head and neck squamous cell carcinoma, also in order to find new potential metabolic targets for chemoprevention.Methods. We evaluated methionine cycle metabolites concentrations in 150 HNSCC patients, in 45 patients with laryngeal leucoplakia, in 90 healthy smokers, in 120 healthy non smokers. We evaluated statistical differences among groups by variance analysis and Student Neumann Keuls test. We tested also differences among patients with early, locally advanced, and regional metastatic tumors.
Results: Statistical differences for serum folate concentration and, with a lower significance, for serum homocysteine concentration, were evidenced between healthy and cancer subjects and between healthy subjects and patients with leucoplakia.
Conclusions: Our results strongly suggest a role for hypofolatemia as risk factors also for head and neck cancer development as for colon adenocarcinoma and other malignancies. Hypofolatemia has been hypothesized to promote cancer by impairing DNA synthesis and repair, by decreasing DNA stability because of an increased uracil misincorporation, and by altering DNA methylation processes involved in transcriptional regulation. Hypofolatemia could be easily corrected by folic acid supplementation, which might thus be an effective chemopreventive measure. Objectives of chemoprevention in the clinical context of head and neck carcinogenesis are the reversal of premalignant lesions and the prevention of second primary tumors. That's particularly relevant under a clinical viewpoint as SPTs are the leading cause for cancer-related mortality in head and neck cancer patients. Head and neck cancer is a perfect model for chemoprevention trials as precursor lesions (i.e., leucoplakia, erythroplakia), which can be identified early with clinical examination, often precede the development of malignancy, supplying a definite target for secondary prevention and an immediate experimental verification during clinical trials. A chemoprevention trial based on folic acid supplementation in patients with laryngeal leukoplakia is already in progress at our Institution with preliminary encouraging results.

TH 11
IMPAIRED DNA REPAIR IN LARYNGEAL CANCER SUBJECTS. RESULTS OF PHENOTYPIC AND GENOTYPIC STUDIES

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Interindividual differences in susceptibility to pathogens have been recently extensively studied because of an association with (i) an estimation of an individual risk to develop cancer and (ii) a variable progression of cancer. Concerning laryngeal cancer a susceptibility to DNA damaging agents and an ability to remove damage was studied. First, DNA repair damage induced by bleomycin or S9-activated benzo(a)pyrene was determined in peripheral blood leukocytes using alkaline comet-assay. Laryngeal cancer subjects (n=52) were shown to have higher levels of spontaneous and mutagen-induced DNA damage as compared to healthy controls (n=56). A level of spontaneous DNA damage tended to increase with tumour aggressiveness. A percentage of individuals with the arbitrary chosen efficient DNA repair was higher in controls than in cancer subjects for the both used mutagens. Then, a genotyping in the group of laryngeal cancer subjects (males, n=293), subjects with second primary tumours (males, n=118) and in the matched controls (n=322) was performed for the genes coding one activating enzyme, 3 detoxifying enzymes and 3 DNA repair enzymes was performed. The latter group included the genes *XPD*, *XRCC1* and *XRCC3* coding enzymes participating in, respectively, NER, BER and non-homologous ends rejoining. Five polymorphisms were studied in DNA repair genes. There were found only two *XPD* alleles significantly over-represented in laryngeal cancer that could be interpreted as an increased risk. There were not significant differences in distribution of increased-risk and low risk genotypes between primary and second primary tumours. Besides, an accumulation of gene defects in laryngeal cancer substantially contributing to genetic risk was established. Altogether, the established phenotypic deficit of DNA repair in laryngeal cancer subjects was not confirmed by overrepresentation of "risk" genotypes of the studied DNA repair genes.

TH 12
INDUCTED AND SPONTANEOUS MUTAGEN SENSITIVITY IN PATIENTS WITH MULTIPLE PRIMARY TUMORS OF THE HEAD AND NECK REGION.

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Objective: The occurrence of second primary tumors after curative treatment or simultaneous multiple malignancies are current problem in head and neck cancer. The mutagen sensitivity is well known marker to predict which patient are prone to develop the second tumor. The frequency and localization of spontaneous and mutagen induced chromatid breaks in peripheral blood lymphocytes (PBLs) in patients with multiple primary tumors (MPT) may help in defining regions involved in cancerogensis process.
Material and method: The case control study using the bleomycin sensitivity assay (where the chromatid breaks per cell (b/c) were scored) was performed in 36 patients with MPT and two control groups: 52 patients with one malignancy and 47 healthy individuals. The differences between examined patients and control groups were estimated using U Mann-Whitney test. Among the patients with MPT spontaneous and induced b/c level were compared.
Results: The b/c level in PBLs of patients with MPT ranged from 0,26 to 4,12 (mean 1,53) and was significantly higher ($p<0,000006$) both compared with patients with one malignan-

cy (b/c ranged from 0,02 to 3,08; mean 0,74) and healthy controls (b/c ranged from 0,04 to 1,14; mean 0,41). The increase was observed in almost all chromosomal arms. The majority of chromosomal locations with the increased proportion of breaks in the group of patients with multiple tumors were identified as regions where loci involved in DNA repair, cell cycle regulation supressor genes and oncogenes were found.

Conclusion: High incidence of chromatid breaks in MPT patients confirms genetic background and individual susceptibility for cancerogens; confirming this results on bigger population allow for establishing the indications for the chemopreventive treatment. Distribution of chromatid breaks may be helpful for defining the regions responsible for development of second primary tumors.

TH 13
RISK FACTORS FOR MULTIPLE PRIMARY MALIGNANCIES IN HEAD AND NECK CANCER (HNC) PATIENTS

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Objective: In assessing the risk of multiple malignancies in HNC patients, we examined several epidemiological and biological factors potentially associated with second primaries, i.e. family history, tobacco and/or alcohol exposure, inherited chromosome fragility as well as original tumor stage and site, p53 gene status and microsatellite instability.

Methods: Biological parameters were determined in paraffin-embedded tumor samples (p53 and microsatellite instability by SSCP and DNA sequencing) and in peripheral blood lymphocytes (susceptibility to bleomycin-induced chromatid breaks) obtained at the time of initial diagnosis from 76 consecutive HNC patients treated with radiotherapy between January 1985 and October 1989, followed-up for 77 months mean (range 2 to 180 months).

Results: A second primary malignancy was observed in 22 patients (28.9%). A family history positive for HNC was documented in 27 out of 68 (39.7%) patients; a p53 gene mutation at exon 4-11 was detected in 36 (48.7%) out of 74 first primary HNC. Moreover, a mutator phenotype (RER+) (LOH or band shifts at $\geq$ 2 microsatellite loci) was documented in the first primary HNC from 17 out of 63 (30%). An increased susceptibility to bleomycin-induced chromatid breaks (number of breaks per cell $\geq$0.67) was found in 26 out of 54 (48.1%) HNC patients. Furthermore, 9 patients carried on smoking after treatment and 4 (44.5%) developed a second primary. Multivariate logistic regression analysis suggests that both RER+ and chromosome fragility were significantly correlated with an increased risk of developing multiple malignancies (p=0.050 and p=0.008, respectively) as did smoking after treatment (p=0.02).

Conclusions: A genetic susceptibility to DNA damage induced by environmental carcinogens may play a central role in the pathogenesis of multiple malignancies in HNC patients and testing for RER phenotype and chromosome fragility at the time of the original cancer diagnosis may be useful in identifying high risk patients.

TH 14
SECOND FIELD TUMORS: A NEW ENTITY IN HEAD AND NECK ONCOLOGY

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Second primary tumors (SPT) and local recurrences (LR) are a significant problem in head and neck squamous cell carcinomas (HNSCC). Thus far, the definitions of SPT and LR are based on clinical parameters: location and time interval. Our recent molecular studies have provided new insight in HNSCC carcinogenesis leading to a better understanding of how of SPT and LR develop. A crucial step is the outgrowth of a preneoplastic field of genetically altered cells that precedes HNSCC development. This field has a monoclonal origin and clonal divergence takes place during progression and eventually one subclone develops into carcinoma. The population of genetically altered cells have a high proliferative capacity (as measured by Ki-67 positivity), indicating that fields expand and thereby replace the normal mucosal epithelium. Moreover, fields are shown to be very large (over 7 cm in diameter) and are usually not detected by routine diagnostic techniques. An important clinical consequence is that fields often remain after surgery and may give rise to (genetically related) new tumors. We have provided convincing evidence for the development of these new tumors and have designated these: second field tumors (SFT). Considering the etiological differences, we believe it is important to define in molecular terms and discriminate between an SFT, a 'true' SPT, and a 'true' LR. In conclusion, the development of an expanding preneoplastic field is a critical step in HNSCC development with important clinical consequences. Diagnosis and treatment of HNSCC should not be focused on the tumor only, but also on the field it developed from.

TH 15
INTRATUMORAL GENOMIC HETEROGENEITY IN ADVANCED HEAD AND NECK CANCER DETECTED BY COMPARATIVE GENOMIC HYBRIDIZATION

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Little is known about the extent of intratumoral genetic heterogeneity in head and neck squamous cell carcinoma (HNSCC). Therefore, we examined 79 stage III and IV primary HNSCCs and matched lymph node metastases for over- and underrepresentation of specific chromosome regions by comparative genomic hybridization (CGH). The overall ratio of gains and losses was higher in metastases than in primary tumors (4/1 vs 2.5/1). Gains of 3q (78.1% P vs 87.5% M) and 11q (78.1% P vs 62.5% M), and deletions of 3p (43.8% P vs 34.4% M) and 9p (31.3% P vs 15.6% M) were most frequently detected. The highest rate of intratumoral discordance was observed for primary tumors and corresponding metastases (32.8%) compared to matched pairs of two metastases (26.5%), and of two anatomically distinct sides of one primary tumor (24.3%). Further-

more, the discordance rate was dependent on the primary tumor site (oral cavity 49.2%, oropharynx 31%, hypopharynx 30.3%, and larynx 27.3%). In some tumors, the extent of genomic discordance argues against a monoclonal origin. In conclusion, we demonstrate a high individual variation of intratumoral genomic heterogeneity depending on the localization and selection of matched pairs. These findings are of specific importance in view of establishing prognostic markers.

TH 16
CLINICAL AND EPIDEMIOLOGICAL DATA OF PATIENTS WITH MALIGNOMAS OF THE HEAD AND NECK

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Introduction: The present study describes clinical and epidemiological data of 7633 patients with malignomas of the head and neck (SCCHN) documented in the Munich Cancer Register, Germany, who were treated at four departments of head and neck surgery and one of oral-maxillo-facial surgery in the area of Munich from 1978 up to now.
Results: Incidence and mortality as a function of age, sex, and tumor localization are described. Moreover, TNM stages, survival, recurrence, and metastasis rates are presented. Our data document a poor 15-year overall survival rate being only 10% in patients with carcinomas of the hypopharynx and 18% in patients with carcinomas of the oropharynx. The most significant finding is the outcome in case of progression, i.e. recurrence: In, for example, oropharyngeal cancer patients after primary treatment the 15-year survival rate was about 5%, independently of locoregional recurrence, cervical lymph node metastases, or distant metastases.
Conclusion: Based on the documentation of the Munich Cancer Register, our study is the first to present a detailed description of clinical and epidemiological data of SCCHN patients incl. 15-y-survival. Moreover, our data imply a sufficient tumor eradication at the time of first diagnosis thus avoiding cancer recurrence, which can not be cured in an adequate way, and emphasize the need for new preventive, diagnostic, and therapeutic strategies aiming to improve the poor survival rate of cancer patients.

TH 17
P53 CODON 72 POLYMORPHISM AND HPV 16/18 E6 TRANSCRIPT IN SQUAMOUS CELL CARCINOMAS OF THE HEAD AND NECK (SCCHN)

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Within the tumorsuppressor p53, a common sequence polymorphism at codon 72 arises from single-base-pair substitution encoding either prolin (Pro) or arginine (Arg). Several reports have described differences for functional properties of codon 72 polymorphic wild type p53, including susceptibility to malignant transformation, and ubiquitin-mediated degradation by human papilloma virus (HPV) E6 protein. We determined the p53 codon 72 status in 122 unselected SCCHN and lymphocytes of 193 healthy controls. Exon 4 was amplified and restricted with BstU1, which cuts the Arg-coded allele whereas the Pro-coded allele remains intact. To detect a loss of transcript in tumors of heterozygous individuals, a partial transcript analysis was performed, and the proportion of the pertaining guanine and cytidine peaks was calculated. In addition, we determined the HPV 16/18 status with RT-PCR, Southern blot and nested RT-PCR. Within the 193 controls, the genotypes Arg/Arg, Arg/Pro and Pro/Pro were found in 114 (59%), 66 (34%), and 13 (7%) individuals. The distribution of p53 polymorphism genotypes among SCCHN showed a similar pattern: 67 Arg/Arg (54%), 55 Arg/Pro (45%), and 1 Pro (1%). HPV 16/18 E6 was expressed in 17 of the 67 Arg-homozygote (25%) and 20 of the 55 heterozygous tumors (36%). SCCHN showed a minor predominance for the heterozygote genotype, mainly including carcinomas of the oro- and hypopharynx. No association of Arg-homozygote tumors and HPV 16/18 infection was evident in this study. Loss of transcript was observed for 16 (62%) of the Pro-coded and 19 (42%) of the Arg-coded alleles. The frequency for loss of transcription was similar in HPV-positive and -negative tumors (49% versus 45%). In addition, loss of the Pro-coded allele was nearly identical in HPV-negative SCCHN (65%) and in HPV-positive cases (63%), indicating that HPV E6 expression is not involved in selection pressure for loss of transcript.

TH 18
HEAD AND NECK SQUAMOUS CELL CARCINOMAS WITH TRANSCRIPTIONALLY ACTIVE HPV DISPLAY A DISTINCT GENETIC FINGERPRINT

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Transcriptionally active high risk human papilloma viruses (HPV) are found in a subset of head and neck squamous cell carcinomas (HNSCC). We investigated whether these tumors display different genetic profiles compared to those without transcriptionally active HPV. Methods: We selected cases positive for viral DNA and positive for E6 oncogene expression (N=12) and compared these with cases positive for viral DNA and negative for E6 oncogene expression (N=8) and cases negative for both HPV DNA and oncogene expression (N=12). In the tumors, we analysed 1) TP53 mutations and 2) loss of heterozygosity (LOH) with 23 microsatellite markers at 3p, 9p, 17p, 13q, 18q and 8p to obtain a detailed genetic fingerprint. Results: The presence of E6 mRNA was highly and significantly correlated with a low level of LOH, i.e. on average 15% of the tested markers at 3p, 9p, 17p and 18q, versus LOH in approximately 70% of these markers in the HPV DNA+ / E6 mRNA- and HPV DNA- groups. The frequency of LOH at 13q was not different. TP53 mutations were absent in the HPV DNA+ / E6 mRNA+ group, but present in the majority of the other tumors. Conclusion: the presence of transcriptionally active HPV in HNSCC was found to be associated with a distinct genetic fingerprint.

TH 19
P53 MUTATIONS IN SQUAMOUS CELL CARCINOMAS OF THE HEAD AND NECK AND THEIR INFLUENCE ON THE PROTEINS NUCLEO-CYTOPLASMIC DISTRIBUTION

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Introduction: Squamous cell carcinomas of the head and neck region (HNSCC) are among the most common cancers of the upper aero-digestive tract. Mutations found in the tumor-suppressor protein p53 are a frequent event and correlate with tumor progress. To act as a tumor-suppressor p53 has to translocate from the cytosol to the nucleus. Regions of the protein that influence the nucleo-cytoplasmic distribution of p53 are the nuclear localization signal (NLS), the nuclear export signal (NES) and the tetramerization domain (TM) of the protein. We therefore investigated 9 HNSCC cell lines regarding p53 mutations and their impact on nucleo-cytoplasmic distribution.
Methods: Total-RNA out of 9 HNSCC cell lines was isolated to generate cDNA utilizing oligo-dT or p53 specific primers. Keratinocytes served as a control. Utilizing PCR, the whole ORF of p53 was amplified. Sequencing of the products was performed by SeqLab (Goettingen, Germany). Sequence analysis was performed with the MultAlin Software. Protein expression was evaluated by Western blotting utilizing a p53 specific antibody (BP-53-12, Sigma), wich is recognizing an amino-terminal epitope (aa 37-45) of p53. Nukleo-cytoplasmic distribution of p53 was evaluated by laser-scanning microscopy (Olympus).
Results: Keratinocytes that express wild-type p53 exhibited a primarily nuclear localisation, whereas some HNSCC cell lines exhibited a predominantly cytosolic signal, some of them with a total lack of nuclear p53 signal. Mutations leading to a lack of nuclear p53 signal were found to be deletions, involving the NLS. Interestingly, one cell line exhibited an enhanced nuclear signal. Here we found a deletion mutant with defective NES but intact NLS.
Conclusion: p53 mutations in HNSCC frequently involve the nucleo-cytoplasmic distribution of p53 and therefore presumably also its function as a tumor suppressor.

TH 20
p63 isoforms in normal, tobacco exposed and neoplastic tissues

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The human *p63* gene encodes a series of protein isoforms that differ in their N- and/or C-terminal sequences and have widely differing properties in promoting or repressing p53-related functions such as growth arrest and apoptosis. In addition, p63 appears to play important roles in the maintenance and differentiation of epithelial cell populations. Many studies have shown that p63, particularly Np63, is expressed in normal epithelium and also highly expressed in squamous cell carcinomas of surface epithelium.
Objective and Methods: Here, we have refined the expression patterns of p63 isoforms by a quantitative RT-PCR technique applied to micro-dissected normal oral samples. We have also studied whether p63 expression is altered in squamous cell carcinoma in the head and neck compared to normal oral mucosa highly from the same patient. Normal buccal mucosa was also compared to buccal mucosa exposed to tobacco, a known carcinogen for oral epithelium. Immunohistochemical analysis for detection of p53 and Ki-67 proteins was further performed.
Results: Results indicate that all known p63 isoforms are expressed in normal epithelium, with the highest levels consistently found in basal and parabasal layers, although there is a wide variation in expression between different individuals. Extensive use of tobacco had no effect on p63. In tumours, there are high levels of N- and p63 isoforms, but some tumours also express the highly efficient transactivator TA- and p63 isoforms. Comparison with results from immunohistochemical detection of p63 isoforms suggests that Np63 is regulated predominantly at the level of transcription, whereas TAp63 protein levels are subject to post-translational regulation. We could find no correlations between p63-isoform expression patterns and proliferation, p53 status or expression of telomerase.
Conclusions: Data suggest that expression of p63 plays a role in maintaining the differentiation status of tumour cells, rather than playing direct roles in tumour proliferation or immortalisation.

TH 21
EXPRESSION OF P53, P63, AND P73 IN RESPONSE TO CISPLATIN IN CELL LINES OF SQUAMOUS CELL CARCINOMAS OF THE HEAD AND NECK (SCCHN)

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Treatment with DNA-damaging agents causes activation of p53 and, subsequently, cell cycle arrest or apoptosis. In SCCHN, however, the tumorsuppressor p53 has been shown to be frequently inactivated. The p53 family members p63 and p73 share significant homology with p53, and their products can function as sequence-specific transcriptional activators for p53 target promoters. Unlike the p53 gene, p63 and p73 encode for multiple isoforms which vary in their NH2- and COOH-termini. Furthermore, a cryptic promoter generates transcriptionally inert ΔN-isoforms which lack the transactivation domain; they are supposed to exert oncogenic functions. The objective of the present study was to analyze alterations in the expression pattern of the p53 family members in SCCHN upon genotoxic stress caused by exposure to the clinically active antineoplastic drug cisplatin. Using a RT-PCR approach combined with Southern blotting, we determined the p53 expression and p63/p73 isoform pattern in ten established cell lines with well characterized p53 status (3x $p53^{wt}$, 4x $p53^{mt}$, 2x $p53^{wt/mt}$, 1x $p53^{-/-}$). Cells remained untreated (controls) or were subjected to the respective 50% inhibitory cisplatin concentration (IC_{50}), and the expression intensity was normalized for be-

ta-actin. Transcripts of p53 and various p63/p73-isoforms were present in the majority of SCCHN, displaying cell line specific patterns for the composition and intensity of transcript expression. Predominantly, we detected p53-inhibitory variants of p63 and p73, suggesting an oncogenic rather than a tumorsuppressive role in the pathogenesis of SCCHN. Exposure to cisplatin led to the induction of p53 in 4/10 cell lines, including 2/3 $p53^{wt}$ cell lines. Alterations in the p63 and p73 profiles showed considerable variations for both level and pattern of isoform expression. These results suggest a complex regulation of p63 and p73 transcription upon cisplatin treatment which may influence sensitivity to chemotherapy.

TH 22
EXPRESSION OF P16 PROTEIN IS ASSOCIATED WITH HUMAN PAPILLOMAVIRUS STATUS IN TONSILLAR CARCINOMAS AND HAS IMPLICATIONS ON SURVIVAL

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University of Cologne, Germany Our recent analysis of papillomavirus (HPV)-DNA in different malignant head and neck tumors revealed that HPV infections occurred most frequently in the tonsillar carcinomas (58%) and that 84% of positive cases contained the highly oncogenic HPV-type 16. We could also present data in favor of the hypothesis that in view of their clinical behaviour and the involved risk factors HPV-positive and HPV-negative tonsillar carcinomas may represent two separate tumor entities. Looking for a surrogate marker, which in further epidemiological studies could replace the laborious and expensive HPV-detection/typing we analyzed p16 protein expression in 34 tonsillar carcinoma for correlation to HPV-status and load of viral DNA. p16 is an inhibitor of cyclin-dependent kinases 4 and 6 which activate the negative cell cyclus-regulator protein pRB which on its turn down regulates p16 expression. It could be shown that in neoplastic cells of cervix uteri E7 proteins of the high-risk HPVs can interfere with this regulatory circuit by their virtue to inactivate pRB and thus lead to the overexpession of p16. We found 53% of the tested tonsillar carcinoma to be HPV positive. 56% of all tumors tested were immunohistochemically positive for the p16 protein. In 16 of 18 of the HPV positive carcinomas diffuse p16 expression was observed. In contrast, only one of the HPV negative carcinomas showed focal p16 staining ($P<0.001$). As determined by laser-assisted microdissection and quantitative real-time PCR, p16 expression correlated with the presence of HPV-DNA in the individual tumor specimens. Clinical outcome analysis revealed significant correlation of p16 expression with increased disease-free survival ($P=0.02$). These data indicate that p16 is a technically simple immunohistological marker, applicable for routine pathologic histology, and its prognostic value for survival is fully equivalent to HPV-DNA detection.

TH 23
Integration of genomic HPV 16 DNA is associated with $p16^{INK4A}$ overexpression in tonsillar carcinomas

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In a previous fluorescence in situ hybridization (FISH) study, we observed human papillomavirus (HPV) type 16 DNA integration in the tumor cell genome in 21% of head and neck squamous cell carcinomas, particularly tonsillar carcinomas. Because also no p53 mutations in exons 5-8 were detected, this subset of tumors may comprise a distinct pathological entity. In order to further substantiate this hypothesis, we analyzed $p16^{INK4A}$ expression in 81 tonsillar carcinomas, because $p16^{INK4A}$ has been reported to be a specific marker for oncogenic HPV-containing (pre)neo-plastic lesions of the uterine cervix. $p16^{INK4A}$ expression, clinical data as well as tobacco and alcohol consumption of patients were correlated with HPV status. Tissue sections of paraffin-embedded tonsillar carcinomas were subjected to FISH using a HPV 16-specific DNA probe to assess the frequency of tumors exhibiting HPV integration. Subsequent sections were used for immunohistochemical assessment of $p16^{INK4A}$ overexpression. Clinical data and alcohol and tobacco exposures of patients were obtained from medical records. FISH detected HPV 16 integration in 33 of 81 (41%) carcinomas, 32 of which also harbored diffuse $p16^{INK4A}$ immunostaining. In contrast, only 5 of 48 HPV-negative carcinomas did stain for $p16^{INK4A}$. Thus, a very strong correlation was found between $p16^{INK4A}$ overexpression and HPV-containing tonsillar carcinomas ($P<0.0001$). Furthermore, a significant, inverse relation was found between the presence of HPV in the tumor and tobacco and/or alcohol consumption ($P\leq0.0104$). HPV-positive carcinomas also showed to be often smaller ($\leq$4 cm) and less well differentiated than HPV-negative tumors ($P=0.0236$ and 0.009, respectively). Our results indicate a remarkable correlation between HPV 16 integration and $p16^{INK4A}$ overexpression in tonsillar carcinomas, resembling the situation for (pre)invasive lesions of the uterine cervix. This suggests that $p16^{INK4A}$ may be considered as an alternative biomarker for HPV detection. Together with the strongly reduced or absent exposure to tobacco and alcohol in these patients, our study provides further evidence for HPV-positive tonsillar carcinomas representing a different tumor entity.

TH 24
CHARACTERISATION OF HOMOZYGOUS DELETION OF THE $p16^{INK4A}$ GENE AND ANALYSIS OF THE BREAKPOINTS IN HEAD AND NECK CARCINOMAS

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Background: The $p16^{INK4A}$ (also known as CDKN2A/MTS1) tumor suppressor gene at chromosome 9p21 encodes a cyclin dependent kinase inhibitor which plays an important role in the regulation of the G1/S phase cell cycle checkpoint. In different

primary tumors a high frequency of various $p16^{INK4A}$ gene alterations were observed. $p16^{INK4A}$ can be inactivated by several different mechanisms such as point mutation, methylation of the promoter region or homozygous deletion (HD). Our purpose was to determine the frequency and extent of $p16^{INK4A}$ HD in head-and-neck cancer cell lines.
Methods: PCR analysis was performed for $p16^{INK4A}$, $p14^{ARF}$ and $p15^{INK4B}$ exons and flanking microsatellite markers in DNA from 19 cell lines including 16 squamous cell carcinomas (SCC) and 3 adeno carcinomas (ACC) to map HD and to locate the breakpoints.
Results: HD of $p16^{INK4A}$ was found in 12/19 (63%) of all cell lines. 10 samples (53%) showed HD of $p16^{INK4A}$ in all three exons. One line each showed a deletion in exon 1 and 2, and one only in exon 3. In 7 cell lines (37%) the deletion included the adjacent $p15^{INK4B}$ gene. While deletions in $p16^{INK4A}$ occurred alone in 5 cell lines (26%), no sample was found where only $p15^{INK4B}$ was deleted. The shortest deletion extended over 85 kbp, while the longest deletion appeared to encompass nearly 3 Mbp. Further analysis of cell lines without HD revealed that one additional cell line showed promoter hypermethylation with loss of $p16^{INK4A}$ expression.
Conclusions: The data indicate that HD of $p16^{INK4A}$ is an important mechanism in development of head-and-neck cancer, whereas $p15^{INK4B}$ gene deletions are less important. Compared to other carcinomas, e.g. from bladder and kidney, the extent of the deletions found in head-and-neck cancers appear smaller on average. The mechanism by which such a variety of different deletions develop, is the subject of further investigations.

TH 25
P53 MUTATIONS IN MALIGNANT MELANOMA

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Malignant melanoma (MM) is believed to be induced by interacting exogenous factors, in particular ultraviolet radiation, and endogenous factors. A high incidence of mutations in various genes, such as ras and PTEN, have been described in both MM and non-melanoma skin cancers (NMSC). Surprisingly, however, mutations of the tumor suppressor p53 were found to be rare in MM but frequent in NMSC and other solid tumors. On the other hand, immunohistochemical detection of (accumulated) p53 protein, which is considered to indicate aberrant protein, has been reported in up to 80% of MM. In order to further investigate this discrepancy, we analysed the entire coding region of p53 transcript (exons 2-11) in 44 MM and 12 established cell lines of MM: 59% of the tumors and 42% of the cell lines showed p53 mutations, including 22 nonsense, 6 missense, 3 silent, and 4 frameshift mutations in tumors, and 5 missense and 1 frameshift mutation in cell lines, respectively. In contrast to previous reports, these results suggest a substantial frequency of p53 mutations in this disease. Hence, inactivation of p53 appears to be an important event in the carcinogenesis of MM.

TH 26
FREQUENT GENETIC ALTERATIONS OF RAS SIGNALING PATHWAY GENES IN MALIGNANT MELANOMAS

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The Ras signaling pathway is important for the intracellular transduction of mitogenic stimuli from activated growth factor receptors. We have investigated 37 sporadic malignant melanomas (15 primary cutaneous melanomas and 22 melanoma metastases) and 6 melanoma cell lines for mutation, amplification, and mRNA expression of the genes NRAS, KRAS, and HRAS. All tumors and cell lines were additionally analyzed for mutation and expression of BRAF, which encodes a Ras-regulated serine/threonine kinase, as well as for expression of RASSF1A encoding a negative regulator of the Ras pathway. Mutational analysis identified somatic NRAS mutations in 2 primary melanomas, 4 melanoma metastases, and 2 cell lines. One melanoma metastasis showed a somatic KRAS mutation, while HRAS mutations were not detected. Eight primary melanomas, 6 melanoma metastases and 4 cell lines carried somatic BRAF mutations affecting the known hot-spot codon 599. None of the tumors or cell lines with BRAF mutation demonstrated NRAS or KRAS mutations. Real-time reverse transcription-PCR showed reduced RASSF1A transcript levels of ? 50% relative to benign melanoctic nevi and cultured normal melanocytes in 8 melanomas and 4 cell lines. The RASSF1A gene promoter was found to be hypermethylated in 6 of the 8 melanomas and all 4 cell lines. Treatment of the cell lines with 5'-deoxyazacytidine and trichostatine A resulted in an increase of RASSF1A mRNA expression up to the level determined for normal melanocytes. Taken together, 57% of the melanomas and 100% of the investigated cell lines carried mutations in either NRAS, KRAS or BRAF. In addition, 22% of the melanomas and 67% of the cell lines showed reduced RASSF1A transcripts levels. Thus, alterations of Ras pathway genes are of paramount importance in the pathogenesis of sporadic melanomas.

FR 01
GALANIN RECEPTOR 1 AND 2 SIGNALING IN ORAL KERATINOCYTES: ROLE IN PROLIFERATION

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Objective: G-protein coupled receptors, such as galanin receptors, behave as agonist-dependent oncogenes. Galanin Receptors 1 and 2 (GALR1 and GALR2), have been shown to promote clonal growth in small cell lung cancer. Although the receptors and corresponding ligand, galanin (GAL), have been well-characterized in the central nervous system their role in epithelial cells is relatively unexplored. To explore the role of GAL in proliferation of human keratinocytes and elucidate the signaling pathways triggered by GALR1 and GALR2.
Methods: GAL secretion in 24 h conditioned media from squamous cell carcinoma (SCC) cell lines was quantified with a competitive ELISA. Proliferation was investigated in SCC cell lines in the presence of increasing concentrations of anti-

galanin antibody or in immortalized keratinocytes in the presence of anti-GALR1 antibody. GALR1 and GALR2-induced MAPK phosphorylation, and rap1 and rho activation were assayed in immortalized keratinocytes by immunoblot analysis, and pull-down assays respectively.
Results: In secretion studies, UM-SCC-(11A, 11B, 14A, 14B, 17B, 74A, 81B), secreted 175 ng/ml or more of galanin per million cells. UM-SCC(-22A and -17B), which secrete low and high amounts of galanin respectively, were used for proliferation studies. For both cell lines, anti-galanin antibody exhibited dose-dependent inhibition of proliferation, suggesting a cumulative pro-proliferative action when both receptors are stimulated. Treatment of immortalized keratinocytes with GALR1 antibody exhibited an increase in proliferation, consistent with an anti-proliferative action for GALR1. In support of these findings, a GALR2-specific agonist activated the MAPK pathway whereas GALR1 inhibited this pathway. Furthermore, GAL and GALR2-specific stimulation activated Rho, whereas GALR1 inhibited rap1 but had no effects on rho activation.
Conclusions: Secreted GAL has an autocrine pro-proliferative effect in oral keratinocytes. GALR2 and GALR1 stimulate pro-proliferative and anti-proliferative cascades, respectively.

FR 02
Stat 3 plays a major role in intracellular signaling of squamous cell carcinomas

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Objective: Proliferation of squamous cell carcinomas is known to depend on EGFR-induced cell signal transduction. Therefore recent clinical studies on oncological treatment of squamous cell carcinomas have been designed using new substances to block the EGFR cascade. However, it remains still unclear whether blocking or activating the EGFR leads to a homogenous downstream signal. Thus we investigated two different signal pathways of the EGFR cascade (stat 3 / map kinase) after blocking and / or activating the EGFR.
Methods: Squamous cell carcinomas (cell lines, nude mice transplants, patients tumors) were investigated. All tumors were treated with a monoclonal antibody against the EGFR in escalating doses. For *in vitro* studies also treatment with EGF was performed. Protein from tumor cells was isolated and separated using SDS gel electrophoresis. Using the western blot technique different signal transducers of the EGFR cascade were investigated even in their activated (phosphorylated) form.
Results: The two main signal transducers in EGFR activation, Stat 3 and MAP Kinase showed different effects during EGFR activation/ blockade. While Stat 3 was strongly inhibited by EGFR blockade in each tumor, MAP Kinase 1 as well as RAF 1 were differentially regulated depending on the special tumor type.
Conclusion: Our observations show that activation and blockade of the EGFR in squamous cell carcinomas is translated downstream over different pathhways.Therby the STAT pathway seems to play a major role.Implications for EGFR treatment in head and neck carcinomas will be discussed.

FR 03
Semiquantitative evaluation of the EGFR and HER2 expression for Targeted therapy of head and neck squamous cell carcinoma

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Two protooncogene products EGFR (Her-1, c-erbB-1) and HER2 (Her-2/neu, c-erbB-2) have been reported to be frequently overexpressed in head and neck squamous cell carcinoma (HNSCC). In order to identify those patients who may benefit from targeted therapy with inhibitors of EGFR and HER2 signaling pathways, we determined the expression status of EGFR and HER2 in tissue specimens from patients with the oral cavity and pharynx tumours by semiquantitative immunohistochemistry. Two pharmacodiagnostic kits EGFR (pharmDx™ and Hercep-Test™), were used in accordance with manufacturers' instructions to identify HNSCC that overexpress EGFR or HER2. EGFR was overexpressed in 41,3% of 135 specimens, whereas the HER2 overexpression was observed in 2.9% of samples only. Given the necessity for new therapeutic modalities in HNSCC, targeted treatment of patients that overexpress EGFR with its specific inhibitors appears to be reasonable.

FR 04
SENSITIVITY OF HEAD AND NECK SQUAMOUS CELL CARCINOMA TO EGFR-TKI GEFITINIB (IRESSAR) AND EXPRESSION OF EGFR FAMILY MEMBERS.

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FR 05
ANTI-EGFR SUPPLEMENTED TPF CHEMOTHERAPY. PRECLINICAL INVESTIGATIONS TO A NOVEL APPROACH FOR HEAD AND NECK CANCER INDUCTION TREATMENT

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Objective: Recent studies on polychemotherapy of head and neck cancer showed an improved remission rate adding taxanes to the standard cytotoxic drugs cisplatin and 5-Fluorouracil (5-FU). Moreover for enhancing the response rate of chemotherapy today a series of biological response modifiers are of interest. Under these are signal modulators of the epidermal growth factor receptor (EGFR). Therefore we have investigated if the addition of monoclonal antibodies against the EGFR could enhance the response rate of Cisplatin, 5-FU and docetaxel.
Methods: Squamous cell cancer lines were transplanted on nude mice. After tumors began to grow they were treated either with cisplatin, 5-FU and docetaxel alone or in combina-

tion with escalating doses of a humanized monoclonal anti-EGFR antibody.
Results: Comparing with controls docetaxel alone as well as the combination of docetaxel, cisplatin and 5-FU resulted in a significant tumor growth delay ($p< 0,05$). The antibody alone also slowed down the tumor growth significantly at each concentration ($p=0,04$). Nevertheless whether chemotherapy agents nor antibody alone yielded complete tumor remissions over an observation period up to 6 weeks. Only the combination of cisplatin , 5-FU, docetaxel (TPF) and the antibody resulted in high significant complete tumor remissions($p<0,03$).
Conclusions: We can show for the first time that the effect of TPF, which is now used as novel Phase II protocol for induction chemotherapy in head and neck cancer, could be high significantly enhanced through the addition of anti-EGFR antibodies. Because we didn`t observe an increased toxicity in the animal experiments TPF/anti-EGFR therapy may define a new strategy in the induction treatment of head and neck carcinomas.

FR 06
EFFECTS OF ESTRADIOL, TAMOXIFEN AND FASLODEX ON THE EXPRESSION OF INTEGRINS IN ORAL SQUAMOUS CELL CARCINOMAS

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Biological activity of steroid hormones and their antagonists on cancer cells is extensively studied for the reproductive tissues, it has become clear that not only cells of the reproductive tissues but also melanoma cells and neuronal cells are affected by these compounds. It is assumed that the altered integrin expression observed in oral squamous cell carcinomas (OSCC) contribute to the invasive behavior. Little is known about the mechanism of action of estradiol, Tamoxifen and Faslodex on non-reproductive organs and neoplasms as well as their influence on the expression of certain integrins. We have studied the effects of estradiol, tamoxifen and faslodex in *in vitro* experiments on the integrin expression in established OSCC cell lines (UM-SCC 14A, 14B and 14C) on the mRNA level measured by Northern Blot and quantitative real time RT-PCR as well as on protein level determined by flow cytometry and immunofluorescence. Their adhesive and invasive behavior to ECM-Proteins was quantified using adhesion and invasion assays We found significant changes of the transcription of the ß1-integrin subunit, suggesting a regulation at the transcriptional level. Whereas alterations only in cell surface expression but not at the transcriptional level of a certain integrin subunit could be demonstrated for α3. The adhesion and invaion properties to ECM-Proteins are congruent with the change of the level of expression of specific integrins. Further investigations may elucidate the mechanism of action of estradiol and antiestrogens and might lead to new avenues for the use of antiestrogens as adjuvant therapy in patients with OSCC.

FR 07
Localization-dependent EXPRESSION OF ANGIOGENIC GROWTH FACTORS IN NATIVE TISSUES OF HNSCC

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Introduction: For therapeutic inhibition of angiogenesis basic knowledge about secretion of specific angiogenesis-inducing cytokines is required. So far knowledge about distinct expression patterns of angiogenic growth factors in HNSCC is rather incomplete and there is nothing known about whether these expression profiles differ in different regions of the head and neck.
Materials and methods: Native tissues of four representative groups of HNSCC (larynx "L" n=10, hypopharynx "H" n=12, oropharynx "O" n=10 and carcinoma of unknown primary "C" n=10) were snap-frozen immediately after resection. After homogenization and standardization to total protein, levels of the following growth factors were quantified in these tissue lysates by ELISA: HGF, bFGF, VEGF-A, VEGF-D, PDGF-AB, PDGF-BB, GM-CSF und G-CSF.
Results: Irrespective of their localization all tumors examined secreted VEGF-A, HGF, and bFGF. However, in almost all cases highest amounts with up to 7ng/mg total protein were observed for HGF. In contrast, the expression of G-CSF was predominant in larynx carcinomas (70% in comparison to 58% "H" respectively 36% "O" and "C"), while GM-CSF was found more often in carcinomas of unknown primary (73% compared to 58% "H", 50% "L" and 45% "O"). PDGF-AB was produced quite uniformly in 2/3 of all of the tumors while PDGF-BB was found markedly less in hypopharynx carcinomas (33% in comparison to 82% "C", 80% "L" and 72% "O"). VEGF-D was expressed only rarely, in CUP comparatively more often (18%) than in carcinoma of the oropharynx (9%) and larynx or hypopharynx (0% respectively).
Conclusions: We found localization-dependent differences in the secretion of VEGF-D, PDGF-BB,G-CSF, and GM-CSF, while VEGF-A, HGF, bFGF and PDGF-AB seem to be uniformly expressed in all regions of the head and neck. In further studies it has to be proven, whether this reflects the biological behavior of the tumor.

FR 08
ANTI-ANGIOGENIC THERAPY OF HEAD AND NECK SQUAMOUS CELL CARCINOMA BY VEGF ANTISENSE THERAPY

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Angiogenesis is increased in various human cancers, including head and neck squamous cell carcinoma (HNSCC), and correlates with tumor progression and metastasis. Vascular endothelial growth factor (VEGF) has been shown to be a key regulator of angiogenesis. We determined whether VEGF antisense oligonucleotide treatment can decrease angiogenic activity of HNSCC cell lines *in vitro* and of HNSCC xenografts *in vivo*. Established human HNSCC cell lines were screened for VEGF expression at both mRNA and protein levels. By using a 21-mer antisense phosphorothioate oligonucleotide targeting the

translation start site of human VEGF mRNA, we examined modulation of VEGF expression in cell line supernatants by capture ELISA, and in cell lysates by western blotting. Human endothelial cells (HUVEC) were grown in conditioned medium produced from the treated tumor cells. Endothelial cell (EC) proliferation was determined by cell count and EC migration was measured using a modified Boyden chamber. Mice with HNSCC xenografts were treated with PBS, VEGF antisense or sense oligonucleotides (10 mkg; i.p. injection), respectively and tumor volumes were measured for 5 weeks. VEGF antisense oligonucleotide treatment resulted in a significant reduction of VEGF protein expression compared to sense control. Although the growth rate of the tumor cell lines was not affected, addition of conditioned medium from VEGF antisense-treated tumor cells resulted in decrease of endothelial cell proliferation and migration. VEGF antisense oligonucleotide treatment of HNSCC xenografts resulted in a significant tumor growth suppression. These results suggest that down-modulation of VEGF using antisense oligonucleotides may be a potential therapy for the inhibition of angiogenesis in HNSCC.

FR 09
REGULATION OF MMP-2 AND MMP-9 IN SQUAMOUS CELL CARCINOMA CELL LINES

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Matrix metalloproteinases (MMP) play an important role in cancer angiogenesis and metastasis. There is some evidence that wild type p53 can suppress endogenous production of certain MMPs in cancer cells. In addition, the inflammatory intratumoral milieu might stimulate cancer cells to enhanced MMP secretion.
Objective: Evaluate endogenous production of MMP-2 and MMP-9 in two squamous cell carcinoma cell lines (UT-SCC-20A and UT-SCC-24A) before and after cytokine stimulation. The cell lines are established from cancers in the oral cavity and have a different p53 status, UT-SCC-20A being mutated and UT-SCC-24A being wild type.
Methods: Cell culture medium was analysed by zymography and ELISA.
Results: Both cell lines showed up-regulation of MMP-9 secretion after stimulation with TNF-alpha for 24 h in serum-free medium and 96 hours in FCS medium. There was a tendency to a higher MMP-9 secretion in UT-SCC-24A than UT-SCC-20A when stimulated for 96 hours. Up-regulation of MMP-9 was also seen by HGF-stimulation (hepatocellular growth factor) in UT-SCC-24A (wtp53). These results are based on ELISA determinations of cell culture supernatants and were verified by zymography.
Conclusions: Cancer cells can be stimulated to enhanced production of MMP-9 by cytokines which play an important signalling role in immune system.

FR 10
EXPRESSION PROFILE OF MATRIX METALLOPROTEINASES AND THEIR TISSUE INHIBITORS IN THE VX2 CARCINOMA OF THE NEW ZEALAND WHITE RABBIT AS A MODEL FOR SQUAMOUS CELL CARCINOMAS OF THE HEAD AND NECK REGION

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Background: Squamous cell carcinomas of the head and neck region (HNSCC) are among the most common malignancies in this area. The VX2 carcinoma of the new zealand white rabbit metastasizes lymphatically like it is the case in HNSCC and therefore potentially could be used as a model for HNSCC. Since the family of matrix-metalloproteinases (MMPs) is involved in the process of HNSCC invasion, the aim of this study was to investigate the expression level of MMPs and their specific inhibitors (TIMPs) in the VX2 carcinoma to evaluate, if they also play a role in VX2 tumor invasion like observed in human HNSCC.
Materials and Methods: The VX2 carcinoma was generated by tumor implantation in the rabbits ear as previously described. Western blots and immunohistochemical staining were performed under standard conditions, utilizing antibodies against MMP-3, MMP-13, TIMP-2 and TIMP-3.
Results: A positive immunohistochemical signal could be detected for MMP-3, TIMP-2 and TIMP-3 with no significant signal for MMP-13. In the Western blots immunoreactive bands could be observed for MMP-3, MMP-13, TIMP-2 and TIMP-3.
Conclusion: MMP-3, MMP-13, TIMP-2 and TIMP-3 were found to be expressed in VX2 carcinomas of the new zealand white rabbits. The VX2 carcinoma therefore resembles HNSCC tumors not only in its metastatic behavior, but also regarding the expression of MMPs and TIMPs, which are the likely keyplayers during the event of invasion. These observations further underline the significance of the VX2 carcinoma as a model tumor of human HNSCC.

FR 11
CHEMOKINES: PROMOTORS OF METASTASIS IN HEAD AND NECK CANCER?

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Squamous cell carcinoma (SCC) and adenoid cystic carcinoma (ACC) of the head and neck are characterized by a distinct, disease-specific metastatic pattern. While SCC frequently metastasizes to regional lymph nodes, ACC exhibit a high rate (appr. 50%) of distant metastases particularly involving lung and liver, which is associated with dismal prognosis. Tumor cell migration and metastasis share many similarities with leukocyte trafficking, which is critically regulated by chemokines and their receptors. Here we report that the chemokine receptor CXCR4 is highly expressed in human ACC but not in SCC cell lines as determined by flow cytometry while other chemokine

receptors including CCR3 and CCR6 were detected only at low levels in some of the cell lines analyzed. Furthermore, cell surface expression of CXCR4 was downregulated by exposure to its ligand CXCL12/*SDF-1* in internalization assays. Supportingly, only ACC cells showed significant directional migration in response to CXCL12 gradients while SCC cells failed to respond to this chemokine. The described distinct staining pattern for CXCR-4 was confirmed by immunohistochemistry in a broad panel of primary tumors of ACC and SCC. While ACC exhibited strong CXCR4 expresssion, SCC showed no or low expression of this receptor *in vivo*. A correlation of CXCR4 protein expression to clinical parameters (TNM, course ofdisease etc.) is ongoing. Since CXCR4 has been recently demonstrated to play a crucial role in determining the metastatic destination of tumor cells in various malignant diseases, this chemokine receptor may also present a key regulator of metastatis in head and neck cancer explaining the observed bias in the metastatic behaviour of ACC compared to SCC. Ultimately, modulation or neutralization of CXCR4 may lead to clinically relevant inhibition or prevention of distant metastases in patients with ACC.

FR 12
COORDINATE EXPRESSION OF CHEMOKINE RECEPTOR 6 (CCR6) AND CCR7 IN SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK (SCCHN) – IDENTIFICATION OF A METASTATIC PHENOTYPE

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Objectives: Whether tumor metastasis is an active or passive process is controversial. Tumor cells have been shown to express various receptors that enable their access to the lymphatic system and facilitate metastatic spread to lymph nodes (LN) or other organs. SCCHN usually spreads locoregionally to cervical LN with low frequency of systemic metastasis. We hypothesized that SCCHN cell migration and metastasis may share similarities with leukocyte trafficking, which is regulated by chemokines and their receptors.
Methods: Expression of chemokine receptors (CCRs) in SCCHN cell lines and snap-frozen tumor tissues by quantitative RT-PCR was performed, using paired cell lines from primary and metastatic tumors and fresh, paired primary and metastatic specimens. Flow cytometry for CCRs on cell lines and immunohistochemistry of patients' primary and metastatic tumors confirmed the presence of surface protein in these cells, as opposed to surrounding lymphoid tissue. Chemotaxis assays and CCR specific antibody blocking experiments confirmed the functional nature and CCR specific effects observed.
Results: Comparing CCR expression between the primary tumor and its metastatic subpopulations using qRT-PCR and functional migration assays, a consistent pattern arose of CCR6 downregulation and upregulation of CCR7 in metastatic cell lines and tissues. Chemotaxis assays and CCR specific antibody blocking confirmed the qRT-PCR results, showing that functional surface receptors are indeed present. CCR6 was nearly universally downregulated, consistent with the emigration of metastatic cells from peripheral mucosal sites, while CCR7, important for homing of immune cells to secondary lymphoid organs, was consistently upregulated in these metastases.
Conclusions: These results indicate a novel metastatic phenotype in SCCHN, exploiting a mechanism used by human dendritic cells (DC), during the normal maturation and trafficking to LN. Our findings indicate CCR6, CCR7 and their ligands have a critical role in determining SCCHN metastasis, and may help explain the consistent metastatic propensity for cervical nodal metastasis observed in this disease.

FR 13
THE ROLE OF CATHEPSIN D IN FIBROBLASTS AND IN SQUAMOUS CELL CARCINOMA CELLS DURING APOPTOSIS

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High expression of the lysosomal protease cathepsin D in oral squamous cell carcinoma has been shown to closely correlate with carcinoma invasion and progression. We have previously shown that cathepsin D is an early mediator of oxidative stress and staurosporine induced apoptosis in human foreskin fibroblasts. During apoptosis, cathepsin D was relocated from the lysosomes to the cytosol, prior to cytochrome c release and caspase-3 activation, events that were inhibited when cathepsin D activity was blocked. In the present study, the importance of cathepsin D in apoptosis induced by the redox-cycling drug naphthazarin and anti-Fas (clone CH11) was investigated using human foreskin fibroblasts (AG-1518) and two squamous cell carcinoma cell lines (UT-SCC 20A and UT-SCC 24A). In carcinoma cells, the cathepsin D activity was twice as high as compared to the activity in fibroblasts. Immunofluorescence analysis of the intracellular distribution of cathepsin D revealed a distinct granular pattern in all cell types, however in the carcinoma cells also a weak cytosolic staining was detected. In naphthazarin exposed fibroblasts and carcinoma cells cathepsin D was found to relocate from the lysosomes to the cytosol before caspase-3 activity was detected. Pre-treatment of the cultures with the cathepsin D inhibitor pepstatin A (100 µm, 24 h) inhibited the onset of apoptosis in fibroblasts. In carcinoma cells, however, apoptosis induced by naphthazarin or anti-Fas could not be prevented by inhibition of cathepsin D. These results indicate that cathepsin D, which is an important mediator of apoptosis in non-malignant fibroblasts, does not play a role in oxidative stress or Fas induced apoptosis in these two squamous cell carcinoma cell lines.

FR 14
Blockade of FAS/Fas-Ligand (FAS-L)-Interactions does not influence chemoreactivity in squamous cell carcinomas of the head and neck (SCCHN)

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Objective: It is well established that antineoplastic agents exert their cytotoxic action via the activation of apoptotic pathways. Since the Fas system has been identified as a key mediator of drug induced programmed cell death, we sought to investigate it´s functional impact on resistance of five SCCHN cell lines to cisplatin and bleomycin.
Methods: We demonstrated the expression of Fas and Fas-L in tumor samples and the corresponding cell lines on the mRNA- and protein level by RT-PCR, Western-blotting and immunohistochemistry, respectively. To investigate the role of the Fas system in chemotherapy, we determined 1. the behaviour of Fas- and Fas-L-expression (by flow cytometry), 2. the cytotoxic effect (MTT-assay), 3. the fraction of apoptotic cells (AnnexinV-assay) and 4. the effects of metabolic apoptosis-inhibitors and blocking anti-Fas-L-antibodies on treatment with the anticancer agents cisplatin and bleomycin.
Results: We found homogenous basal exppression of Fas and to a lesser extent of the corresponding ligand, both of which are strongly upregulated after exposure to cisplatin and bleomycin. The addition of Caspase-inhibitors – but not antagonistic anti-Fas-L-antibodies – interfered with the induction of apoptosis, thus indicating that the cell lines possess functionally active components of the Fas-system, but fail to enter the apoptotic machinery after treatment with anticancer drugs.
Conclusion: These results suggest that disabling the Fas system may be a critical event in the development of chemoresistance in SCCHN.

FR 15
GENETIC AND EXPRESSION PROFILES OF SQUAMOUS CANCER CELL LINES OF THE HEAD AND NECK CORRELATE WITH CISPLATIN SENSITIVITY AND RESISTANCE

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Treatment strategies for squamous cell carcinoma of the head and neck (SCCHN) are still based on TNM-classification. However, it is clear that biological features of the tumors have an independent impact on the clinical behaviour of SCCHN. Biomarkers may serve as predictive markers for clinical response to specific therapies. The aim of the present study was to examine genetic changes and gene expression profiles that might correlate with sensitivity to cisplatin (MTT assay) in 10 UM-SCC head and neck cancer cell lines. Five cisplatin-sensitive and five cisplatin-resistant cell lines were studied by comparative genomic hybridization (CGH), Spectral karyotyping (SKY), and cDNA microarray analysis. The five cisplatin-resistant cell lines demonstrated significantly more genetic imbalances (regions of loss and amplification) and chromosomal abnormalities than the five cisplatin-sensitive cell lines by CGH and SKY, respectively. Supervised clustering identified approximately 60 genes that clearly distinguish between the two groups of cell lines. Some of these genes are known to be involved in tumor progression, metastasis and drug resistance. By RT-PCR, we further confirmed the differential expressions of tissue inhibitor of metalloproteinase 2 (TEMP and TROP-3. Low expression of the oncogene c-met correlated with chemosensitivity (cDNA microarray). In a clinical material of 30 patients with SCCFIN that received induction chemotherapy, low expression of c-met (immunohistochemistry), one of the gene from the distinguishing set was seen in flow cytometrically diploid tumors ($p<O.01$) and tended to correlate with better disease-specific survival. We conclude that cisplatin sensitivity and resistance are related to distinctive differences in genetic and expression profiles in individual SCCHN tumor cell lines. The genes we have identified may serve as potential targets for novel treatment strategies.

FR 16
BETULINIC ACID: A NEW TUMOR-SPECIFIC COMPOUND ACTS AS A CHEMO- AND RADIOSENSITIZER IN HNSCC CELL LINES

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Objective: To evaluate the cytotoxic potency of betulinic acid, a pentacyclic triterpene derived from white birch trees, on head and neck cancer cell lines.
Methods: Two head and neck squamous carcinoma cell lines (SCC9 and SCC25) were treated with increasing doses of betulinic acid and/or Cisplatin. Surviving cell numbers were counted in an automated cell counter and analyzer system. For measurement of activation of intracellular caspases and determination of apoptosis we used the monoclonal Antibody M30-Apoptosense™ ELISA. Irradiation was performed with a conventional radiation source. The effects of betulinic acid and irradiation on survival of cancer cells were determined in colony-forming assays.
Results: There was a synergistic cytotoxic effect of betulinic acid and cisplatin in SCC9 but not in SCC25 cells. Notably, apoptosis was induced by activation of intracellular caspases in our cell lines. Furthermore, betulinic acid had a radiosensitizing effect over the whole concentration range in the cell lines used when given before irradiation.
Conclusion: Betulinic acid, a new experimental cytotoxic compound, may be of value in head and neck cancer therapy, particularly when given in combination with standard chemotherapeutics or irradiation.

FR 17
COX-2 INHIBITOR NIMESULIDE SYNERGISTICALLY INHIBITS GROWTH IN COMBINATION WITH CHEMOTHERAPY OR IRRADIATION IN HNSCC CELL LINES

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Objective: To evaluate the cytotoxic and apoptosis-inducing effect of the selective COX-2 inhibitor Nimesulide on HNSCC cell lines alone and in combination with Cisplatin or irradiation.
Methods: Two HNSCC cell lines of the tongue (SCC9 and SCC25) were treated with Nimesulide alone or in combination with Cisplatin. Survival was determined 24, 48 and 72 hours after the respective treatment with an automated cell counter and analyzer system. For detection of apoptotic cell death, staining with Hoechst dye and the monoclonal antibody M30 CytoDEATH were carried out. To determine the radiosensitzing effect, clonogenic assays were carried out after irradiating cells subsequent to treatment with Nimesulide.
Results: Our in vitro study demonstrates that Nimesulide has (i) a synergistic cytotoxic effect in HNSCC cell lines in combination with standard chemotherapeutics and (ii) also acts as a radiosensitzer in HNSCC cell lines
Conclusion: Our data provide a rationale for the use of selective COX-2 inhibitors as radio- or chemosensitizers in the treatment of head and neck cancer patients.

FR 18
PHOTOCHEMICAL DEGRADATION OF MITOMYCIN C DISTORTS CYTO-TOXICITY TESTING *IN VITRO*

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Objective: A crucial limitation of cytotoxicity testing *in vitro* is its poor reproducibility. Recently, flavin-mediated photochemical reactions causing oxidative destruction of cytostatic drugs have been identified to cause substantial alterations in cytotoxicity test procedures. To date, such reactions have been described for *Vinca alkaloids*, taxanes, and antibiotics. In this study, we proofed whether cytotoxicity tests using mitomycin C (MMC) are influenced by flavin-mediated photoreactions.
Methods: Wildtype KB cells were cultured in RPMI 1640 (w/o riboflavin, 10% FBS). For chemosensitivity testing, KB-cells were exposed 72 hours to graded solutions of MMC and then the cell number was determined. The MMC concentration causing 50 percent growth inhibition (IC50) was determined graphically. Repetitive chemosensitivity tests (n=5) were done using varying periods (0-120 min.) of exposure to fluorescent light, with, or without the addition of riboflavin to the medium.
Results: A decreased cytotoxicity of MMC in the presence of riboflavin could be found for illumination periods of about 10 minutes. Longer illumination periods increased the functional degradation of MMC. In contrast, illuminated cytotoxicity tests (120 min.) in medium without riboflavin, consistently reproduced the IC50 for KB-cells determined previously in cytotoxicity tests shielded from fluorescent light.
Conclusions: The results indicate that flavin-mediated photochemical destruction of MMC can severely distort the results of cytotoxicity tests *in vitro*. These effects could already be found in tests exposed to fluorescent light for 10 minutes. The omission of riboflavin from the chemical defined part of the culture medium prevented the degradation of MMC effectively. Therefore, the use of flavinfree culture medium provides an methodical option to improve the reproducibility of cytotoxicity tests *in vitro*.

FR 19
IMMUNE RESPONSES TO P53 IN PATIENTS WITH HEAD AND NECK CANCERS: ELEVATED FREQUENCIES AND THE FUNCTIONAL STATUS OF ANTI-P53 PRECURSOR CTL AT TUMOR SITES COMPARED TO THE PERIPHERAL CIRCULATION

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Objective: The presence and distribution in tissues and blood of T cells specific for tumor-associated epitopes are important for generation of anti-tumor responses. *P53* is known to be genetically altered in more than 50% of head and neck cancers (HNC), and T cells specific for wild type sequence (wt) p53 peptides are detectable in peripheral blood of patients with HNC (Hoffmann et al., Cancer Res. 62:1281-1288, 2002). We investigated whether CTL specific for HLA-A2.1-resticted wt $p53_{264-272}$ and $_{149-157}$ epitopes localized to tumor-involved tissues in the HLA-A2+ HNC patients. In addition, we sought to determine whether CD4+CD25+ T-cells, considered to have a downregulatory role in anti-tumor immune responses, also localized to tumor sites.
Methods: Samples of tumor infiltrating lymphocytes (TIL), tumor-involved or non-involved lymph node lymphocytes (LNL) and peripheral blood mononuclear cells (PBMC) from 8 patients were studied by four color flow cytometry. HLA-A2.1/wt p53 peptide tetramer complexes (tetramers) were used to identify and quantitate CTL. In addition, the frequencies of CD4+CD25+ cells in these samples were determined.
Results: The frequencies of CD3+CD8+ anti-wt $p53_{264-272}$ and anti-$p53_{149-157}$ CTL were elevated in TIL relative to PBMC. In one patient, tumor uninvolved LNL and PBMC had equivalent frequencies of these anti-p53 CTL, while tetramer+ CTL accumulated at the tumor site. We also determined that the percentages of CD4+CD25+ T cells in TILs were significantly increased ($p<0.05$) as compared to PBMC.
Conclusions: Our results indicated that anti-p53 CTL as well as downregulatory CD4+ CD25+ T cells preferentially localize to tumor sites in patients with HNC. These findings raise concerns about the functionality of these T cells at the tumor site, and are consistent with our previous results showing dysfunction of T-cells isolated from the tumor in HNC patients. Clearly, for immunotherapy to be successful it will need to circumvent these dysfunctions.

FR 20
IDENTIFICATION OF A NATURALLY PRESENTED HLA-A*0201-RESTRICTED CTL-DEFINED MUTANT P53 PEPTIDE IN SQUAMOUS CELL CARCINOMAS OF THE HEAD AND NECK

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Objective: Determine whether p53 missense mutations that occur within the HLA-A2.1-restricted epitopes, $p53_{149-157}$, $p53_{217-225}$ and $p53_{264-272}$, in HLA-A2.1+ squamous cell carcinomas of the head and neck (SCCHN) are capable of being targeted with tumor specific p53 vaccines.
Methods: Sequence analysis of p53 expressed in 53 HLA-A2+ SCCHN tumors identified 5 distinct mutations, V157F, S149C, T150R, Y220C and E271K, occurring in three HLA-A2.1-restricted p53 epitopes in 10/53 tumors. The Y220C mutation was detected in 6/53 tumors. Synthetic mutant p53 peptides were tested for HLA-A2.1-binding in MHC stabilization assays and induction of anti-mutant peptide CTL from normal donor PBMC. The anti-mutant peptide reactivity of PBMC following *in vitro* stimulation (IVS) with peptide-pulsed autologous dendritic cells was determined in cytotoxic and ELISPOT IFN8 assays using T2 target cells and HLA-A2+ SCCHN cell lines.
Results: All mutant p53 peptides tested, with the exception of Y220C peptide, displayed little to no binding to HLA-A2.1 molecules and failed to induce anti-mutant peptide CTL in IVS cultures. In contrast, the Y220C mutant peptide yielded bulk and cloned populations of CD8+ T cells specific for the mutant peptide and reactive against an HLA-A2+ SCCHN cell line expressing the Y220C mutation, UD-SCC 6 but not PCI-13, an HLA-A2+ SCCHN cell line expressing a p53 mutation at codon 286.
Conclusion: Most of the mutant p53 peptides tested involved non-conserved amino acid exchanges at anchor positions of the epitope that abrogated HLA-A2.1 binding. In contrast, Y220C represents a mutation that can be naturally presented and targeted to induce a potentially robust immune response. We are currently evaluating the immunogenicity of this mutant peptide in the autologous UD-SCC6 system. The notable frequency of the "hot spot" p53 Y220C mutation in HLA-A2+ SCCHN tumors makes development of vaccines targeting it a more practical approach than previously considered for p53 missense mutations.

FR 21
RAPID TURNOVER OF P53 IN HPV+ SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK (SCCHN) LEADS TO ENHANCED CTL RECOGNITION

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Objectives: Human papillomavirus (HPV) infection is associated with a subgroup of SCCHN and may contribute to carcinogenesis due to HPV E6-mediated inactivation by degradation of p53 and Rb. Our objectives were to define the impact of HPV E6 expression on the degradation of p53 in SCCHN cells expressing wt or mutant p53 and determine whether E6 enhanced presentation of CTL-defined p53 epitopes. We also determined whether possible enhanced presentation of CTL-defined wt p53 epitopes by HPV+ tumors correlated with frequencies of anti-wt p53 CTL in peripheral circulation of SCCHN patients with such tumors.
Methods: SCCHN cells expressing mutant or wt p53 were transfected to express HPV-16 E6. Transfectants were analyzed for p53 degradation by immunoblot and recognition by anti-wt $p53_{264-272}$ CTL in ELISPOT IFN-8 assays. Anti-wt $p53_{264-272}$ CTL frequencies in the peripheral circulation of 15 SCCHN (5 HPV+/10 HPV-) patients after tumor removal were determined by flow cytometry using $p53_{264-272}$ peptide-dimeric HLA-A2.1-Ig molecules.
Results: HPV E6 transfected SCCHN cells lines expressing either wt or mutant p53 molecules were sensitive to E6-mediated degradation and ELISPOT assays confirmed that rapid p53 turnover in these cells enhanced $p53_{264-272}$-specific CTL recognition. Finally, frequencies of anti-$p53_{264-272}$-specific CTL were higher and declined after surgery only in patients with HPV-16 E6 DNA in their tumors.
Conclusions: Mutant and wt p53 can be induced to rapidly turnover, an early step in the antigen processing pathway, in HPV E6+ tumor cells. Consistent with higher wt p53 presentation in these tumors, anti-$p53_{264-272}$ specific CD8+ T cells were detectable in patients and appear to decline after removal of their HPV+ tumors. Our findings suggest that HPV-associated SCCHN might represent a particularly attractive population for wt p53 based immunotherapy and application of enhanced antigen processing may expand vaccine-induced p53-specific T cell responses.

FR 22
A WILD TYPE P53 EPITOPE ENCOMPASSES A NATURAL POLYMORPHISM AT CODON 72: IMPLICATIONS FOR VACCINE DESIGN AND HPV-ASSOCIATED SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK (SCCHN)

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Objectives: The tumor suppressor gene, p53, encodes an HLA A2.1-restricted CTL epitope, $p53_{65-73}$, that encompasses a natural polymorphism (R versus P) at codon 72. Such a polymorphic epitope could represent a novel immunotherapeutic target by vaccinating homozygous (R/R or P/P) individuals with the other allelic peptide, which would be recognized as "nonself" and thus would be more immunogenic. This approach would rely on anti -$p53_{65-73}$ CTL cross-reactivity to both allelic peptides. In a preclinical vaccine study, we sought to understand factors influencing the immunogenicity of $p53_{65-73}$ by studying the determinants of peptide presentation and CTL recognition.
Methods: T2 stabilization assays of synthetic $p53_{65-73}$ -derived peptides were used to compare allelic peptide binding to A2.1. CD8+ T cells were generated by *in vitro* stimulation (IVS) using autologous, peptide pulsed dendritic cells. Anti-$p53_{65-73}$ specific CTL activity and allelic peptide cross-reactivity were tested using interferon-γ ELISPOT assays.

Results: HLA A2.1 binding of $p53_{65-73}P$ was found to be higher than $p53_{65-73}R$. Immunogenicity of $p53_{65-73}P$ appeared greater by IVS than $p53_{65-73}R$, and anti-$p53_{65-73}P$ CTL cross reacted with $p53_{65-73}R$-presenting tumor targets. Since HPV-16 E6 has been shown to degrade p53R alleles more efficiently, we examined peptide presentation and T cell recognition of a naturally HPV-16 transformed, HLA A2.1+ cell line derived from a codon72R/P heterozygous SCCHN patient, to determine the allelic peptide generated preferentially.
Conclusions: Vaccine strategies using polymorphic $p53_{65-73}$ peptides would take advantage of the fact that $p53_{65-73}P$ peptide could be recognized as "nonself," and thus more immunogenic, by codon72R/R homozygotes (≅50% prevalence). The published higher sensitivity to HPV E6-mediated degradation of p53 codon72R molecules, increased relative risk of HPV-associated cancers in codon 72R individuals, and low immunogenicity of $p53_{65-73}R$, suggests that this epitope may have particular significance for immunotherapy of HPV-associated cancers.

FR 23
IMBALANCE IN ABSOLUTE COUNTS OF T LYMPHOCYTE SUBSETS IN PATIENTS WITH HNC AND ITS RELATION TO DISEASE

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Objective: Significant levels of spontaneous apoptosis of circulating CD8+ T cells observed in patients with head and neck cancer (HNC) (Clin Cancer Res 8: 2553, 2002), suggest that a lymphocyte imbalance is a common feature of this disease. To evaluate this possibility, absolute numbers and percentages of lymphocyte subsets were examined in the peripheral blood of HNC patients and controls.
Methods: Venous blood was obtained from 150 patients with HNC at various stages of their disease and from 74 normal age-matched volunteers. Absolute numbers of CD3+, CD4+ and CD8+ T lymphocytes were determined using counting fluorobeads in a single- platform flow cytometry-based technique. Percentages of T lymphocyte subsets were also evaluated by flow cytometry.
Results: Patients with HNC had significantly lower numbers of CD3+ and CD4+ T cells than normal controls (NC). Unexpectedly, the mean absolute number of CD8+ T cells was not decreased in HNC patients. In fact, individual CD4+ counts tended to be lower, and CD8+ counts higher in the patients than NCs, accounting for the lower mean CD4/CD8 ratio in patients vs. NC ($p<0.0305$). However, the absolute CD8 cell numbers were highly variable, and three distinct categories of HNC patients were identified: those with a highly elevated, normal or significantly decreased number of circulating CD8+ T cells. Relationships between the number of CD8+ T cells and disease activity, stage, nodal involvement and previous therapy were evaluated. Patients with active disease, particularly those with T3/T4, had the lowest CD8 as well as CD4 T-cell counts. Nodal involvement was not related to T- cell numbers. Patients with NED either normalized their CD8 T- cell count or had a T-cell imbalance (high CD8+, low CD4+ T cells), which often persisted for many years after curative surgery. Previous radiotherapy was associated with low CD4+ ($p=0.0011$) but not CD8+ T-cell counts.
Conclusions: While patients with T3 /T4 disease are lymphopenic due to a persistent decline in both CD4+ and CD8+ lymphocytes in the circulation, numbers of CD8+ T cells tend to recover after curative surgery or radiotherapy. Nevertheless, a significantly decreased CD4+ T-cell count with normal or elevated CD8+ T-cell numbers in the peripheral circulation of the patients with NED after therapy indicates the presence of persistent T-cell imbalance. Possibly, a rapid turnover of CD8+ T cells maintains their homeostasis despite spontaneous apoptosis. We believe that changes in the homeostasis of circulating CD8+ T-cells during disease might provide key information about its progression.

FR 24
GAMMA-DELTA T-CELLS IN PATIENTS WITH SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK (SCCHN)

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Objective: We have previously described a high onset of spontaneous apoptosis in circulating $\alpha/\beta D3^+$ T lymphocytes in patients with SCCHN (Clin Cancer Research 8: 2553-2562, 2002). We now focused on a subset of CD3 lymphocytes described as γ/β T-cells, a cell type with potential relevance in non-MHC restricted anti-tumor immune responses.
Methods: Peripheral blood of 34 SCCHN patients and 34 age-matched controls (CON) was evaluated for frequency of γ/β Tells among $CD3^+$ T-cells and apoptosis (Annexin V binding) by flow multicolor cytometry. Results were correlated with clinical parameters.
Results: Patients with SCCHN (mean age 58,6) had a significantly higher proportion of γ/δT-cells compared to healthy controls (4.3 ±0.4% for SCCHN vs. 3.1 ±0.3% for CON, $p=0.03$). However, this increase was not paralleled with a difference in the onset of apoptosis if compared to CON. There was no correlation between γ/δ T-cells and TNM stage in those patients. However, a significantly higher proportion of γ/δ T-cells was found in 7 patients with metachronous second primary SCCHN (6.8 ±1.1%) compared to the other SCCHN (3.7 ±0.4%, $p=0.03$). In a follow up 6-14 weeks after tumor ablative therapy 17 SCCHN patients showed an increase in the frequency of γ/δ T-cells compared with pre-therapy values (5.7 ±0.0% vs. 3.7 ± 0.6%, $p=0.09$).
Conclusion: Patients with SCCHN have a higher proportion of $CD3^+$ γ/δ^+ T-cells which was not associated with changes in the onset of apoptosis, but may be due to increased de novo generation. For individuals with second primary SCCHN this phenomenon was even more pronounced. Increased frequencies of γ/δ T-cells may be the consequence of tumor-host interactions but apparently fail to build up an effective anti-tumor immune response.

FR 25
LOW THYMIC OUTPUT AND/OR RAPID LYMPHOCYTE TURNOVER IN THE CIRCULATION OF PATIENTS WITH HNC: RECENT THYMIC EMIGRANTS AND SUBSETS OF NAÏVE AND MEMORY T CELLS.

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Objective: Apoptosis of circulating and tumor-associated CD8+ T lymphocytes contributes to immune cell dysregulation in patients with cancer. The output of T cells by the thymus or antigen-driven expansion of T cells in the periphery could compensate for apoptosis. To discriminate between these mechanisms, we studied the frequency of recent thymic emigrants identified by T-cell receptor excision circles (TRECs) formed during T cell receptor rearrangement as well as naïve and memory T cell subsets in patients with HNC and age-matched healthy individuals.
Methods: We obtained peripheral blood lymphocytes from 30 patients with HNC and 30 age-matched normal controls (NC). Multicolor flow cytometry was used to quantify naïve CD8+ (CD45RO-CD27+) and naïve CD4+ (CD45RO-CD27+). DNA was isolated from lymphocytes, and real-time quantitative PCR was used to measure the frequency of TRECs.
Results: We determined that in HNC patients, age-associated decreases in TRECs and naïve $CD8^+$ and $CD4^+$ T cells were significantly greater relative to healthy subjects. Correlations were established between the percentages of naïve CD8+ T cells and the log frequency of TRECs in lymphocytes obtained from patients with HNC ($p=0.0065$) and NC ($p=0.0083$). The memory compartment was expanded in patients with increased proportions of $CD4^+CD45RO^+$ but not $CD8^+CD45RO^+$ T cells.
Conclusions: Our data suggest that lower thymic output combined with rapid turnover of naïve $CD8^+$ T cells set the stage for altered lymphocyte homeostasis and ineffective antitumor responses in patients with cancer.

FR 26
IMMUNOSUPPRESSIVE EFFECTS OF SOLUBLE FACTORS SECRETED BY HEAD AND NECK SQUAMOUS CELL CARCINOMA ON DENDRITIC CELLS AND T LYMPHOCYTES

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Recent observations suggest that the inability of the immune system to mount an effective immune response against head and neck squamous cell carcinoma (HNSCC) could be a result of the immunosuppression mediated through soluble factors that are secreted by tumor cells. Therefore, we investigated the effects of conditioned medium obtained from cultures of three HNSCC cell lines (HNSCC-CM) on the function of dendritic cells (DC) and T cell immune response. In our study, we could not observe any inhibitory effect of HNSCC-CM on the maturation and the cytokine secretion pattern of DC. On the contrary, HNSCC-CM from two of three cell lines consistently decreased the quantity of IFN-γ- and IL-4-secreting T cells upon restimulation in vitro. In conclusion, our data suggest that soluble factors secreted by HNSCC cells directly inhibit the function of effector T cells, rather than impeding the process of antigen presentation. With regard to ongoing efforts in development of therapeutical approaches for treatment of HNSCC, the measurement of intracellular cytokines may represent an essential improvement in the evaluation of cellular immune response in these patients.

FR 27
AUTOLOGOUS HNSCC TUMORS STIMULATE MONOCYTE MCP-1-GENERATED SECRETION VIA LECTIN-LIKE RECEPTORS AND CD14 EPITOPE

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Objective: We have previously shown that freshly isolated autologous monocytes from head and neck squamous cell carcinoma (HNSCC) patients co-cultured with F-spheroids *in vitro* augment secretion of monocyte chemotactic protein-1 (MCP-1) upon 24 hours in co-culture. Presently, the aim was to study the mechanisms of this monocyte secretion.
Method: Biopsies from carcinoma tissue and benign control mucosa from HNSCC patients were used to establish fragment (F)- spheroids *in vitro*. In one experiment, co-cultures of monocytes and F-spheroids were separated by a semi-permeable membrane. In one experiment, Actinomycin D (1 g/ml for 24 hours) pre-treatment of the F-spheroids was studied. In experiments, glucose (100 mM), galactose (100 mM), fructose (100 mM), mannose (100 mM) or -CD14 was added to the co-cultures.
Results: Co-culture of monocytes and F-spheroids separated by a semi-permeable membrane as well as pre-treatment of the F-spheroids Actinomycin D pre-treatment of the F-spheroids decreased the monocyte MCP-1 co-culture response Addition of glucose, galactose and -CD14 antibody diminished the MCP-1 co-culture response.
Conclusions: The monocyte MCP-1 co-culture response is dependent on metabolic active spheroids, secreted stimuli, and is augmented by direct contact with F-spheroids, possibly via lectin-like receptors, and by the CD14 receptor of the monocyte.

FR 28
LYMPHOCYTES OPSONIZED WITH TRIFUNCTIONAL BISPECIFIC ANTIBODIES INDUCE TUMOR CELL LYSIS IN AN AUTOLOGOUS HUMAN *EX VIVO* SETTING

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Background: Trifunctional bispecific antibodies (tbAB) induce the formation of a tri-cell complex of CD3 positive T-cells, EpCAM positive tumor cells and Fc receptor positive cells resulting in tumor cell killing.

Objectives: The anti tumor activity of autologous lymphocytes opsonized with tbAB (tbAB-Mc) was assessed in an human *ex vivo* system.
Methods: Tumor cells and autologous lymphocytes were obtained from patients with head and neck squamous cell carcinomas (HNSCC). PBMC were coincubated with tbAB for two hours. Cytokines released during antibody binding were removed and the tbAB-Mc were incubated with HNSCC. Tumor cells exposed to Cisplatin or cell culture medium served as controls. Tumor cell lysis was assessed by acridine orange staining or by FACS of propidium iodide marked cells.
Results: Coincubation of HNSCC cells tbAB-Mc resulted in tumor cell lysis in 37±9% following 24 hours and in 45±7% following 48 hours ($p<0.05$) when compared with the control. Incubation of the tumor cells with Cisplatin resulted in tumor cell death in 48±7% after 24 h and 49±8% after 48 h.
Conclusion: Autologous lymphocytes opsonized with tbAB induce tumor cell lysis in an autologous human *ex vivo* setting. On the basis of these results a new approach of anti tumor vaccination by cellular therapy could be developed because cytokine release, possible inducing capillary leakage syndrome can thus be avoided.

FR 29
ANTI-TUMOR-VACCINATION IN PATIENTS WITH HNSCC

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Immunotherapy aims at targeting and destructing a tumor by activating the patients immune system. Between January 1996 and December 1997, 20 patients with squamous cell carcinomas of the head and neck (HNSCC) were vaccinated with their own tumor cells in a non-randomized study. Mainly patients with advanced tumor stages were included (stage IV: n=14, stage III: n=4, stage II: n=2). The vaccine was prepared from $1x10^7$ Newcastle-Disease-Virus-modified, irradiated, autologous, cultivated tumor cells. It was applied up to 5 times according to a standardized treatment scheme. Anti-tumor immune reactivity was determined by DTH skin reaction and IFN8 ELISPOT assay. From 63 tumors, which were removed during this time interval, we were able to cultivate 78% successfully. MHC I expression (a prerequisite for antigen presentation) could be shown by immunohistochemistry in all primary cultures. In vaccinated patients we observed no side effects apart from flu-like symptoms. Patients presented with an increased DTH-reaction (up to 3.1-fold). The 5-year survival rate was 100% in stage II and III tumors and 50% in stage IV tumors. In 5 of 8 long-term surviving patients ELISPOT analysis revealed tumor-reactive memory T-cells thus indicating the achievement of an anti-tumor memory. Postoperative vaccination with virus-modified autologous cultivated tumor cells appears to be a feasible and safe new modality of immunotherapy. Both, the observed anti-immune reactivities and the patients overall survival indicate vaccine-induced anti-tumor effects.

SA 01
CHIMERIC MONOCLONAL ANTIBODY U36 LABELED WITH ZIRCONIUM-89 FOR PET IMAGING OF HEAD AND NECK CANCER

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In previously described clinical radioimmunoscintigraphy (RIS) and radioimmunotherapy (RIT) studies at our institute, the potential of the CD44v6-specific monoclonal antibody (MAb) U36 has been demonstrated. For the detection of head and neck squamous cell carcinoma (HNSCC), RIS with technetium-99m-labeled U36 IgG was found to be as valuable as the conventional imaging techniques CT and MRI, but the detection of tumor deposits smaller than 1 cm appeared to be a problem. We hypothesized that introduction of positron emission tomography (PET) might further improve tumor detection because of its high resolution. In addition, PET has potential for quantitative imaging. These features should enable PET to provide proof of principle of MAb targeting and dosimetric determinations prior to RIT. As it takes 2-4 days for intact MAbs to achieve optimal tumor-to-nontumor ratios, commonly used positron emitters like carbon-11 and fluor-18 (half-lives of 20 and 100 min, respectively) are not suitable for labeling of MAbs. Therefore, we now started the exploration of the long-lived positron emitter zirconium-89 ($t_{1/2}$ = 3.27 days). For the first time, ^{89}Zr was produced in large batches and isolated with high purity and yield. Subsequently, ^{89}Zr was stably coupled via the chelate desferal to the chimeric MAb (cMAb) U36. Biodistribution studies were performed in human HNSCC-bearing nude mice showing selective tumor targeting. On PET images, millimeter-sized tumors in the range of 19 to 154 mg were readily visualized. Because of these encouraging results, ^{89}Zr-labeled MAb U36 is currently also evaluated for its capacity to detect primary tumors and metastases in operable HNSCC patients. Initial results from this clinical PET trial will be presented at the meeting.

SA 02
SENTINEL NODE INSTEAD OF ELECTIVE NECK DISSECTION IN HEAD AND NECK CANCER? INVESTIGATION OF TWO DIFFERENT TUMOUR ENTITIES.

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Objective: Detection of sentinel node (SN) aims to avoid unnecessary surgery in absence of lymphatic metastases without running the risk to overlook the presence of micrometastases. Recent investigations have shown a high predictive value of sentinel node biopsy concerning the presence of metastatic disease in tumours of the head and neck. Elective neck dissection is common practice in N0 neck of squamous cell carcinoma (SCC) while in melanoma a negative SN is used to avoid neck dissection.

Methods: 31 N0 patients with carcinoma of the oral cavity and oropharynx and 7 patients with melanoma disease draining to cervical lymph nodes underwent labelling of the SN by peritumoral injection of 99m-Tc-Nanocolloid. Resection of the primary and excision of the SN was performed and neck dissection was completed in all cases of SCC but two as one-step-procedure. Melanoma patients were supposed to have neck dissection in a second step only in the presence of metastases in the SN. The patients had a follow-up of 2-42 months.
Results: Identification of a SN was unsuccessful in 2 cases of patients with SCC. In one case there was false negative result, when a metastases adhering to the primary was resected en-bloc unwittingly whilst the identified labelled SN was tumour-free. Follow-up revealed cervical recurrent tumour in 4 cases of SCC so far. All had had neck dissection completed. Although neck dissection was completed there were 2 cases of cervical recurrences either in N+ and in N0 necks. Among the melanoma patients none had a positive sentinel node and therefore no neck dissection was performed.
Conclusions: We could confirm the high predictive value and accuracy of the sentinel node biopsy in our patients. The patterns of success and failure patients were similar to those reported in the literature. As the next step neck dissection must be set out in case of tumour-free SN biopsy (respecting the possible limitations) and long-term results must be compared. The ENT surgeon should take care of SN biopsy in melanoma draining to the neck and frequently to intraparotideal lymph nodes.

SA 03
COX-1 EXPRESSION IS UPREGULATED IN SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK

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Objective: To evaluate wether Cox-1 has housekeeping gene functions and subsequently to examine a possible linkage between angiogensis and thus flk-1 and Cox-1 expression in squamous cell carcinomas of the head and neck immunohistochemistry and Western blotting were employed.
Methods: Immunohistochemistry was performed in 43 paraffin-embedded tumor specimens of the head and neck and 6 specimens of normal oral mucosa. 10 snap-frozen tumor biopsies and also 6 specimens of normal oral mucosa were analyzed by Western blotting.
Results: Cox-1 was upregulated in all tumor speciemens compared to normal mucosa. The expression pattern of Cox-1 and Cox-2 compared to flk-1 was nearly identical in 31 (72%) and 33 (77%) of 43 tissue biopsies, respectively. Cox-1 and Cox-2 demonstrated the same enhancement in 26 (60%) of 43 tumor samples. In 25 of 43 (58%) biopsies equal protein expression of all three proteins was observed. Western blotting confirmed the immunohistochemistry results showing that Cox-1 is upregulated in tumor specimens compared to normal oral mucosa.
Conclusions: Our findings supports the hypothesis that Cox-1 as well as Cox-2 is an inducable enzym in head and neck squamous cell carcinoma. Thus both enzymes may be of importance for the linkage between chronic inflammation and carcinogenesis as well as angiogenesis and subsequently tumor outgrowth.

SA 04
RADIOSENSITIZING EFFECTS OF COX-2 INHIBITOR ON A HEAD AND NECK SQUAMOUS CELL CARCINOMA CELL LINE

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Objectives: Cyclooxygenase, a key enzyme in prostaglandin synthesis is expressed in human cancer cells. We determined the radiosensitizing effects of a selective COX-2 inhibitor NS398, on the survival of HEp3, a head and neck squamous cell cancer (HNSCC) cell line.
Methods: A dose dependent study was performed with NS398, to determine the IC50. Doses bracketing the IC50 were then used for the radiosensitizing experiments. After incubation for 5 days with and without the drug, cells were irradiated with 0, 2, 4, 6,8 and 10 Gy and radiation survival curves using the clonogenic survival assay were generated. COX-2 protein expression was also determined after irradiation both in the presence and absence of the drug.
Results: NS398 had significant radiosensitizing effects for all radiation doses tested. The SF decreased from 0.79 to 0.41 ($p<0.0001$) at 2 Gy. The significant increase in the radiation induced expression of COX-2 protein in HEp3 cells was inhibited by treatment with NS398. This indicates that NS398 has effects upstream of COX-2 in addition to its effects on COX-2 enzyme activity.
Discussion: Our data shows that NS398 a COX-2 inhibitor has radiosensitizing effects on a HNSCC cell line HEp3. COX-2 inhibitors may act as a radiosensitizing agents with minimal side effects including safety and tolerability. They may have clinical implications for combined therapy of COX-2 inhibitors with radiation therapy.

SA 05
PREOPERATIVE CONCURRENT CHEMORADIATION WITH PACLITAXEL / CARBOPLATIN IN ADVANCED, RESECTABLE CARCINOMA OF THE ORAL CAVITY AND OROPHARYNX (STAGE III/IV): 5-YEAR-FOLLOW-UP OF A PROSPECTIVE MULTICENTER NON-RANDOMIZED STUDY

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The purpose of concurrent chemoradiotherapy is to increase local-regional control and to decrease the incidence of distant metastases. In recent years drug combination using taxane/platinum have been used increasingly in advanced stage head and neck cancer. In the present outpatient phase II trial we investigated the combination of Paclitaxel/Carboplatinum

with 40 Gy radiotherapy in a neoadjuvant setting of operable stage II/IV cancer of the oral cavity and oropharynx. Fifty-three patients were enrolled in this trial during the period from May 1998 to October 2000 and received five cycles of weekly Paclitaxel (40mg/m^2) and Carboplatinum (AUC 1,5). Radiation was delivered in a conventional fractionation schedule with 2,0 Gy/daily. Surgery of the primary tumor and neck followed within 3 to 4 weeks. Fifty-two patients were evaluable for toxicity and response. Complete response was observed in 31 of 52 patients (CR 60%), and partial response was seen in 21 of 52 patients (PR 40%). In 30 of 52 patients complete response was also documented in the resection specimen (pCR 58%). The 5-year overall survival rate was calculated as 72% and the 5-year disease free survival was 75%. Mucositis grade II and III was the most frequently reported side effect. Our present data support again the highly effective combination of Paclitaxel/Carboplatin in in this chemoradiation protocol.

SA 06
ORGAN PRESERVATION IN ADVANCED LARYNGEAL AND HYPOPHARYNGEAL CARCINOMAS

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Up to now treatment for advanced tumors of the larynx and hypopharynx includes total laryngectomy plus, if necessary, paritial pharyngectomy. Surgical advances, the improvement of radiotherapy and active chemotherapeutic regimes have shifted many paradigms. In a prospective study 97 patients were enrolled and treated with induction chemotherapy (200 mg/m^2 paclitaxel and 100 mg/m^2 cisplatin; day 1 and 22). In patients with complete or partial tumor response radiotherapy with 69.9 Gy at the gross tumor and 50.4 Gy in the lymphatic drainage was applied. Non-responders had surgery and radiotherapy with total doses adapted to the radicality of tumor resection. The response rate to induction chemotherapy was 86%(10% complete, 76% partial response). At a median follow up of 25 month the larynxpreservation rate was 80%. The local- regional control rate was 91% and the 3-year estimate to survive with functional larynx is 75%.
The high rates of larynx preservation and the acceptable treatment toxicity of our treatment approach encouraged us to continue our treatment protocol in a multicenter trial.

SA 07
THE ADDITIONAL VALUE OF CHEMOTHERAPY TO RADIOTHERAPY IN LOCALLY ADVANCED NASOPHARYNGEAL CARCINOMA: A META-ANALYSIS OF THE PUBLISHED LITERATURE

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Purpose: The purpose of this meta-analysis was to determine the additional value of neoadjuvant (neoCHT), concurrent (conCHT) and/or adjuvant chemotherapy (adjCHT) to radiation in the treatment of locally advanced nasopharyngeal carcinoma.
Methods: To be eligible, full published studies had to deal with stages III and IV nasopharyngeal carcinoma and to have randomly assigned patients to receive conventional radiotherapy (66-70 Gy in 7 weeks) or radiotherapy combined with chemotherapy.
Results: Ten randomized clinical studies were identified, including 2450 patients. The pooled HR of death for all studies was 0.82 (95%-ci: 0.71-0.95; p=0.01) corresponding to an absolute survival benefit of 4% after 5 years. A significant interaction term (p=0.02) was found between neoCHT, conCHT and adjCHT. The largest effect was found for conCHT with a pooled HR of 0.48 (95%-ci: 0.32-0.72) which corresponds to a survival benefit of 20% after 5 years. With neoCHT, a 6% benefit was found after 5 years, which was however not statistically significant (p=0.12). The pooled RR for LRR for all studies was 0.69 (95%-ci: 0.59-0.81). A significant effect was observed after neoCHT and conCHT, but not after adjCHT.
Conclusion: The addition of chemotherapy to radiotherapy in locally advanced nasopharyngeal carcinoma offers a small but significant effect on the overall survival. The largest efect was noted with the use of conCHT. NeoCHT led to a significant reduction of the incidence of local-regional failures, but no significant effect was observed with regard to the overall survival. No significant beneficial effect was noted with the use of adjuvant chemotherapy for any of the endpoints. Based on these results, the question arises whether the conCHT and adjCHT (based on the results of the Intergroup study) which is currently common practice, could be improved by replacing the adjuvant part by more effective neoCHT combined with conCHT.

SA 08
LOCAL INTRA-ARTERIAL CHEMOTHERAPY WITH SYSTEMIC ANTAGONISATION IN HEAD AND NECK CANCER

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Objective: To prove feasibility and effectivity of selective local intraarterial cisplatin therapy under systemic protection with sodiumthiosulfate (STS). Technical success and complications are parameters for feasibility. Side-effects and survival are parameters for effectivity of therapy and protection. Presentation of the experience in the university of Frankfurt.
Methods: Between December 1996 and January 2003 336 consecutive patients with head and neck cancer stage I to stage IV received 477 intra-arterial treatments. 45 patients were treated with former standard cisplatin (i.a.) and 5-fluouracil (i.v.) and all other got intra-arterial cisplatin with intravenous STS neutralisation. Selectivity was decided according to tumour-size and localisation. A subgroup of 73 patients with oral cancer was analyzed in the frame of a multimodal treatment study for quality of life and survival.
Results: In all intended cases a positioning of the catheter in the external carotid artery was possible. If superselective position of the catheter tip within one of the external carotid branches or even in smaller peripheral branches was intended in most cases the superselective sondation of the intended ves-

sel was posssible. In cases a superselctive catheterposition could not be achieved due to technical reasons a less selective position in the external carotid artery was accepted. Systemic side-effects could be reduced impressively by i.v. administration of STS compared to unprotected therapy. Cumulative survival of the mulimodality treated subgroup of oral cancer was calculated 74% for four years.
Conclusions: Selective high dose IA cisplatin therapy in combination with intravenous administration of the "cisplatin-neutralizer" STS has been shown to be effective and safe for treatment of patients with oral cancer. Systemic STS protection decreases the secondary effects like nausea, renal and bone marrow toxicity resulting in an increased quality of life.

SA 09
CISPLATIN-DNA ADDUCT MEASUREMENTS IN HEAD AND NECK CANCER PATIENTS TREATED BY CHEMORADIOTHERAPY

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Objective: Optimal dose and route of administration for chemoradiotherapy in patients with stage IV head and neck cancer of the pharynx or oral cavity is currently subject of investigation in a randomized phase III trial, comparing intra-arterial (i.a.) supradose cisplatin (150 mg/m^2) (with systemic rescue by sodium-thiosulfate) and intravenous (i.v.) cisplatin (100 mg/m^2). Both arms are combined with standard radiotherapy (70 Gy/7 weeks). In a subgroup of patients we studied levels of cisplatin-DNA adducts in primary tumor and normal tissue.
Methods: We obtained buccal cells, white blood cells (WBC) and/or tumor biopsy before and 23 hours after end of the first course of chemoradiotherapy. Adduct levels were determined by immunocytochemistry using polyclonal antibody NKI A-59 for buccal cells. ^{32}-P postlabeling technique was used to quantify the major adducts (GG and AG) in WBC and tumor.
Results: So far, in 16 patients, adduct levels have been measured; 10 after i.v. and 6 after i.a. cisplatin infusion. Tumor sites were oropharynx (n=10), oral cavity (n=3) and hypopharynx (n=3). Normal tissue samples were obtained from all 16 patients, primary tumor from 10 patients with tumors, accessible for biopsy. See table for results of adduct-levels in tumor and WBC.

Chemotherapy- regime, concurrently with RT:		Cisplatin-DNA adducts (in fmol/μg DNA) in:			
		WBC		Tumor	
		GG	AG	GG	AG
100 mg/m2 i.v.	mean	0,929	0,117	3,946	0,388
	SD	0,249	0,036	1,183	0,067
150 mg/m2 i.a.	mean	0,826	0,093	4,070	0,373
	SD	0,211	0,028	0,523	0,145

The difference between adduct levels in WBC and primary tumor was statistically significant ($p<0.02$) for both i.a. and i.v. treated patients. There were no differences in adduct levels in either WBC or tumor between the i.a. and i.v. group. Analysis of adducts in buccal cells is ongoing.
Conclusions: Cisplatin-DNA adduct levels in primary tumor of H&N cancer are 4-fold increased compared to WBC, both after supradose i.a. and conventional i.v. cisplatin-based chemoradiation. Despite the selective supradose i.a. administration of cisplatin, adduct levels in primary tumor are comparable to levels obtained after conventional i.v. cisplatin. Whether cisplatin-DNA adduct levels correlate with treatment outcome, is subject of current research.

SA 10
INDUCIBLE PROMOTERS FOR GENE THERAPY OF HEAD AND NECK CANCER – AN IN VITRO STUDY

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Objective: The aim of gene therapy includes the tight spatial and temporal control of transgenic expression. There are several approaches concerning externally inducible gene promoters used for the control of suicide genes. Two of the promoters, that might play a role for head and neck cancer gene therapy are the hyperthermia-inducible human heat shock protein-70 (hsp70) promotor, as well as the radiation inducible promoter of the early growth response-1 gene (egr-1).
Methods: We tested the hsp-70 promoter as well as a promoter construct, containing synthetic radioresponsive elements of the egr-1 enhancer for the effect on reporter gene expression in two stably transfected head and neck carcinoma cell lines in vitro and measured the success of gene activation by FACS analysis, westernblot and fluorescence microscopy.
Results: With the hsp70 promoter we reached a 5.83fold increase of reporter gene expression. The radiation inducible construct revealed only weak gene induction and was marked by high background expression. Both systems worked cell type dependent.
Conclusions: The possible use of externally inducible transgene expression in head and neck carcinoma gene therapy is critically discussed.

SA 11
IDENTIFICATION OF NOVEL PROGNOSTICATORS OF OUTCOME IN SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK

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Objective: HNSCC are characterized by an array of DNA copy number changes, the significance of which remains ill de-

fined. We studied the global genomic constitution of 82 previously untreated HNSCC in relationship to clinical outcome using comparative genomic hybridization (CGH).
Methods: CGH data was sub-grouped into 321 individual cytogenetic bands for each patient. Redundancies were eliminated by selecting a single representative of each group of closely linked bands whose abnormalities co-occurred in exact coincidence across patients in the data set. To reduce noise, only genomic deletions or overrepresentations (gains, high-level amplifications) occurring in at least 5% of cases were included in the statistical analyses. This analysis yielded 95 individual gains and 104 losses. Each aberration was submitted to univariate statistical analysis to assess correlations with outcome or with other possible prognostic factors using the log-rank test for survival, Fisher's exact test for dichotomous variables and Spearman correlation for ordinal variables. To calculate the nominal significance level, we estimated the distributions of the ordered chi-square statistics from univariate survival analyses by Monte Carlo simulations. Then we used Hochberg-Benjamini's "false discovery rate" procedure to adjust for multiple statistical testing among the large number of potentially correlated abnormalities. The abnormalities most significantly related to survival were submitted to Cox proportional- hazards regression stratified by TNM stage.
Results: Abnormalities that correlated with cause-specific survival analyses at a nominal, univariate significance level of $p<0.0001$ included gains at 12q24 and 14q11 and losses at 5q11-15, 6q14-21, and 21q11-21. Several more gains and losses were significant at nominal levels of $0.0001<p<0.01$ including amplifications of 8p11 and 11q13. The strongest predictors remained significant when compared with order statistics from the simulations. Five of these (11q13, 12q24, 5q11-15, 6q14-21 and 21q11-21) remained jointly significant when adjusted for TNM stage in multivariate analysis.
Conclusions: Our data confirm association between 11q13 overrepresentation and outcome of HNSCC. Moreover, prognostic significance of 12q24 gain and deletions of 5q11-15, 6q14-21 and 21q11-21 has not been previously described and may aid the treatment planning of HNSCC. Pending confirmation, these alterations merit further consideration to identify the genes driving their selection.

SA 12
DIFFERENTIAL EXPRESSION AND CYTOGENETIC ABERRATION PROFILES OF TUMOR SUPPRESSOR- AND ONCOGENE-PATHWAYS IN HEAD AND NECK CANCERS REVEALED BY TISSUE MICROARRAY ANALYSIS

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Background: Head and neck squamous cell carcinomas (HNSCC) are very heterogeneous in their biological and clinical behavior. Aims: A tumor classification into groups with homogeneous biological and clinical characteristics.
Methods: Tissue microarrays containing a total of 600 HNSCC were used to analyse the expression of proteins with major roles in control of the cell cycle, apoptosis, cytoskeletal architecture and cell adhesion by immunohistochemistry, including tyramide signal amplification. Gene copy number gains and losses were studied by FISH. Results: The relationship between reduced pRb and increased $p16^{INK4a}$ expression which was previously established in both HNSCC and cercical cancer, could be fully confirmed ($p<0.0001$), illustrating the feasability and usefulness of the tissue microarrays. In addition, the pRb-defective, HPV oncogene expressing HNSCC showed an increased prevalence of reduced cytokeratin14 expression. This indicates disruption of cytoskeletal architecture in these tumors, in agreement with their poorer histology. We found significant diferences between the different anatomical tumor sites both in expression profiles as well as in cytogenetic alterations.
Conclusions: Our data demonstrate that different pathogenetic pathways drive the carcinogenetic process in the different sites and contribute to the differential clinical behavior. Molecular classification employing tissue microarrays will eventually lead to a better tumor classification into groups with more homogeneous biological and clinical behavior.

SA 13
MOLECULAR ANALYSIS OF RESISTANCE TO CHEMOTHERAPY AND METASTATIC PROPENSITY IN HEAD AND NECK SQUAMOUS CELL CARCINOMA USING AFFYMETRIX MICROARRAYS

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Resistance to therapy is a central issue of cancer treatment failure. To select the most effective therapy for the patient, it will be necessary to identify parameters that predict responsiveness to specific agents, and to adapt and individualize the treatment modalities. Furthermore, the mechanisms of resistance must be elucidated at the molecular level, leading to novel rational intervention strategies to overcome resistance. Likewise, the genes causally involved in cancer dissemination have the potential to predict the likelihood of metastasis and consequently patient outcome, while also being candidate targets for therapeutic intervention. The identification of associations between gene expression alterations and biological processes is a major theme in biomedical research. Consequently, we are analyzing the gene expression profiles of tumor samples from patients with head and neck squamous cell carcinoma (HNSCC). Comparison of the gene expression profiles of primary HNSCC and normal tissue revealed several differentially expressed genes, to our knowledge not described to be involved in HNSCC tumor formation. Their biological function and contribution to oncogenic transformation is currently investigated by various experimental systems. Although the preliminary hierarchical clustering of the Affymetrix microarray data allowed classifications into groups and subgroups, more samples have to be analyzed to identify statistically significant signatures predictive for resistance to chemotherapy and metastatic propensities.

SA 14
MOLECULAR PROFILING OF AGGRESSIVE THYROID CANCER: PROGNOSTIC SIGNIFICANCE OF MUC1 ALTERATIONS

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Objective: The molecular profile underlying aggressive papillary thyroid carcinoma (PTC) may harbor strong prognostic markers and novel treatment targets. However, the long natural history of PTC and the rudimentary knowledge of its genetic composition have limited the delineation of this profile. In this study we took advantage of differences in clinical behavior between two distinct variants of PTC, the aggressive tall cell variant (TCV) and typically indolent conventional PTC (cPTC), to identify molecular prognosticators of outcome using complementary genome-wide analyses.
Methods: The study population included pathologically confirmed cases of TCV (n=50) and cPTC (n=50). Comparative genomic hybridization (CGH) and complementary DNA (cDNA) microarray (17,840 genes) analyses were used to detect changes in DNA copy number and gene expression. CGH and cDNA microarray data were correlated and validated using real-time PCR and immunohistochemical analyses. Molecular factors identified by this approach were validated in an independent cohort of cPTC, including aggressive (recurrent) and indolent (15-years disease free) cases, matched for established clinicopathological variables.
Results: CGH identified significant differences in the presence (76% vs. 27%; p=0.001) and type of DNA copy number aberrations in TCV compared to cPTC. Recurrent gains of 1p34-36, 1q21, 6p21-22, 9q34, 11q13, 17q25, 19 and 22 and losses of 2q21-31, 4, 5p14-q21, 6q11-22, 8q11-22, 9q11-32 and 13q21-31 were unique to TCV. Gene expression profiling showed significant overlap between TCV and cPTC, but identified 82 dysregulated genes that maximally differentiated the two subtypes. Of these, MUC1 was of particular interest as amplification of 1q by CGH correlated with MUC1 amplification by real-time PCR analysis and protein over-expression by immunohistochemistry in TCV (p=0.005). Moreover, multivariate analysis revealed a significant association between MUC1 over-expression and treatment outcome, independent of histopathological classification (p=0.03). Analysis of the validation cohort confirmed the association between MUC1 over-expression and survival (RR-2.3; 95% CI- 1.1 – 5.5; p = 0.03).
Conclusions: Our data suggest that MUC1 dysregulation is associated with aggressive behavior of PTC and may serve as a prognostic marker and potential therapeutic target in this disease.

SA 15
STRUCTURAL EVALUATION OF PERIPHERAL PARTS OF SALIVARY GLAND ADENOID CYSTIC CARCINOMA (ACC)

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ACC occurs in three major subtypes: tubular, cribriform and solid. Various combinations of mixed structures, including tubular-solid, cribriform-solid, and sometimes all three forms, are found more ofen than the above mentioned uniform histological subtypes. Electron-microscopic studies in our department allowed for the identification of several types of tumor forming cells: cells resembling intercalating ducts, secretory cells, and cells being similar to pluripotent and mioepithelial cells. Secretory cells are mainly found in the tubular type. Tissues resembling intercalating ducts are met in all subtypes and plurpotent tissues are numerous in the solid type. The typical position of mioepithelial cells is rarely seen in the solid type. The aim of the study was to estabish features of the cancer perimeter in various histological subtypes. The analized material consisted of cross-section from 46 ACC specimens. The studied tumor tissue was fixed according to Karnovsky procedure and was subjected to both routine histological assessment as well as transmission electron microscopy. The material for immunohistochemical and routine morphological analysis was fixed in 4% buffered formalin. Fibronectin and laminin immunostainigs were also conducted. The following tumor structures were observed: 16 cribriform , 17 tubular , 13 solid cases. Our grouping into individual histological subtypes, especially the tubular and cribriform ones, was based on quantifying the most prominent structures, because mixed architecture were found in every examined material. Cells were loosely scattered in the stroma and created nests or a range of cells. The characteristic tendency was cellular grouping around the fibrilar structures of stroma, which constituted proteoglicans, glikosaminoglicans and types I V colagen. The stroma of tumors vicinity had a different nature. It was poor in collagen fibers, however, it follows from the immunohistochemical research that fibronectin appears in the stroma as well as in the whole tumor structure. Laminin was mainly present in vessel walls in the main structure both in cribriform parts and in the stroma of solid tumor. In the peripheral parts of tumors laminin positivity was present in the form of a very delicate fibrilar picture. Infiltrating peripheral cells resembled most frequently the pluripotential cells which did not completely lose the characteristics of epithelium but they formed primitive connections only during the contact with the neighboring cells. The characteristics of epithelial differentiation was well expressed (cytokeratin bundles, desmosome like junctions) and found only in one case.

SA 16
PROGNOSTIC VALUE OF PITUITARY TUMOR TRANSFORMING GENE (HPTTG) IN CARCINOMA OF THE HEAD AND NECK

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Objective: In the effort to evaluate the biological behaviour of squamous cell carcinoma many markers were investigated up to now. A quite recently discovered one is the pituitary tumor transforming gene.
Methods: We investigated the mRNA-expression of the gene in 89 samples of squamous cell carcinoma of the upper aerodigestive tract and tried to correlate our findings with clinical parameters.
Results: PTTG-expression showed high correlation with the nodal stage as one of the most important prognostic factors. Furthermore we could prove significant difference of the mRNA-expression between pN0 and pN1-3 stage (p=0,016). Patients acquiring locoregional recurrences revealed higher PTTG-levels than patients without recurrences (p=0,009). Within the pN0-group of patients it was additionally possible to correlate the PTTG-expression with the risk for recurrence (p=0,006).
Conclusion: The investigation reveals that PTTG is an biological marker of clinical relevance. This fact is confirmed by the prognostic subclassification within the pN0-group of patients.

SA 17
SERUM MATRIX METALLOPROTEINASES (MMP) AND THE MACROPHAGE COLONY-STIMULATING FACTOR (M-CSF) IN HEAD AND NECK CANCER

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Objective: Matrix Metalloproteinases (MMPs) and the Macrophage Colony-stimulating factor (M-CSF) have been shown to have tumor marker potential in various malignancies. The tumor marker potential of MMPs and M-CSF was investigated in patients with squamous cell carcinomas of the head and neck (SCCHN).
Methods: The serum concentration of MMP-3, -8, -9, and M-CSF was evaluated in 59 patients with SCCHN and 59 healthy controls by means of a quantitative sandwich enzyme immunoassay.
Results: The serum concentrations of MMP-3, -8, -9, and M-CSF were significantly elevated compared to the healthy control group ($p<0.001$, $p=0.04$, $p<0.001$, $p=0.002$). A significant correlation was observed between the MMP-3 and the M-CSF serum concentration ($p<0.0001$), as well as between the MMP-8 and the M-CSF serum concentration ($p=0.05$).
Conclusions: MMP and M-CSF serum concentrations are of potential interest as tumor marker in SCCHN.

SA 18
THE HEAD AND NECK TUMOR SITES DIFFER IN PREVALENCE AND SPECTRUM OF *P53* ALTERATIONS BUT THESE HAVE LIMITED PROGNOSTIC VALUE

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The tumor site is a strong clinical factor in head and neck squamous cell carcinoma (HNSCC). To clarify the biological and clinical role of p53 alterations in HNSCC, we have examined the prevalence and the nature of p53 alterations in a large cohort of tumors (n=514) from the different sites. For immunohistochemical analysis of p53 protein expression, we introduced tyramide signal amplification immunohistochemistry (TSA-IHC) on a tissue microarray. This allowed the discrimination between normal low level expression and reduced or lost expression. 253 tumors were subjected to mutational analysis by genomic DNA sequencing, employing also the p53 *GeneChip* from Affymetrix. The prevalence of all p53 alterations, i.e. mutations, overexpression and loss of expression, was significantly higher in hypopharyngeal tumors than in the other sites ($p=0.001$). Laryngeal tumors showed the lowest rate of p53 alterations, but revealed a distinct mutation spectrum: most mutations affected exon 5 ($p=0.013$) and the S2' domain ($p=0.002$), and most hotspot 248 mutations occurred in the larynx ($p<0.001$). Although sequencing by *p53 GeneChip* technology proved to be more sensitive than dideoxy sequencing, a large fraction of tumors analysed was p53 wildtype. In agreement with *p53* mutations occurring prior to invasiveness, their prevalence did not increase with tumor stage, and all mutation classes lacked prognostic significance. Interestingly, both p53 overexpression as well as loss of expression increased with tumor stage and with lymph nodal metastasis, but both also lacked prognostic significance after stratification for tumor stage in multivariate analysis, in all tumor sites including the hypopharynx. The large patient cohort of this study showed that *p53* is differentially affected in the different tumor sites of the head and neck, but its mode of inactivation does not play a major role in tumor progression.

SA 19
MCL-1, BUT NOT CD9, MIGHT PREDICT CLINICAL OUTCOME OF PATIENTS WITH ADVANCED HEAD AND NECK CANCER AFTER CONCOMITANT RADIOCHEMOTHERAPY

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Objective: To examine the prognostic potential of Mcl-1 and CD9 in patients with advanced squamous cell carcinomas of the head and neck after concomitant radiochemotherapy immunohistochemistry was employed in 43 untreated tumor biopsies.

Method: Specimens in which more or even 10% of the neoplastic cells showed cytosolic and membraneous immunoreactivity were considered to be immunopositive. Results were correlated with clinico-pathologic characteristics of the subjects.
Results: Both proteins, Mcl-1 and CD9 tested were positive in a proportion of the tumors. The number of cases expressing intense Mcl-1 was higher in the patients without evidence of disease after treatment than in the patients alive with disease or who died of disease (p=0.018). However, no correlation could be found between CD9 expression and the patients with responsiveness to concomitant radiochemotherapy. No correlations could be observed between Mcl-1 and CD9 expression pattern and patients clinico-pathologic data.
Conclusion: We could show that in our study group Mcl-1 might act as a biomarker which predicts clinical outcome of patients with advanced squamous cell carcinomas of the head and neck cancer after concomitant radiochemotherapy.

SA 20
THE SIGNIFICANCE OF THE HEMOGLOBIN LEVEL BEFORE AND DURING SURGERY AND POSTOPERATIVE RADIOTHERAPY IN SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK

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Objective: The objective of this retrospective study was to investigate the prognostic significance of the hemoglobin levels before and during surgery- and postoperative radiotherapy in locally advanced squamous cell carcinoma of the head and neck.
Methods: 122 patients treated with curative intention were included. The hemoglobin levels were assessed before surgery (HbPS), between surgery and radiotherapy (HBAAC), before postoperative radiotherapy (HbRTpre) and at the end of radiotherapy (HbRTend). Patients were classified as anemic when the Hb-levels were <7.5 mmol/l (females) or <8.7 mmol/l (males), respectively. To take into account the duration of anemia during the interval between surgery and radiotherapy, the area above the curve was calculated, defined as the surface between the Hb-level and the normal Hb-value corrected for sex. Higher HbAAC values correspond with lower Hb-levels.
Results: In the univariate analysis, the 3-years local-regional control (LRC) among patients with an HbAAC ≥ median (high HbAAC) was 50% compared to 75% in case of an HbAAC < median (low HbAAC) (p=0.003). In addition, anemia before radiotherapy was associated with higher rates of loco-regional recurrences (p=0.03). No such association was found for the other Hb-parameters. The multivariate analysis for LRC showed that the resection margin, extranodal spread and HbAAC were independent prognostic factors. The overall survival (OS) after 3 years was 76% in case of low HbAAC and 49% in case of high HbAAC (p<0.001). In the univariate analysis, significant associations were also found for HbPS and HbRTend. The multivariate analysis for OS showed that the nodal status, HbPS and HbAAC were independent prognostic factors.
Conclusions: The Hb between surgery and radiotherapy is an important prognostic factor for both LRC and OS. Pre-surgery Hb was also associated with the OS. A prospective study in which the Hb level between surgery and radiotherapy is corrected has been initiated.

SA 21
CLINICAL PREDICTORS OF OUTCOME AFTER TARGETED CHEMORADIATION IN ADVANCED HEAD AND NECK NEOPLASMS

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Objective: To investigate which clinical and radiological parameters are predictive for local control and survival after chemoradiation in advanced head and neck cancer patients.
Methods: Ninety-three inoperable patients with stage III-IV squamous cell carcinoma of the oral cavity, oropharnyx, hypopharynx and supraglottic larynx were treated with superselective-targeted chemoradiation (RADPLAT intra-arterial); all had a minimum follow-up of 2 years. Following parameters were analysed in a multivariate analysis: T-stage, N-stage, lowest involved neck level, gender, age, site, comorbidity, pretreatment haemoglobin, weight loss before treatment and uni-/bilateral infusion. The obtained significant variables in the univariate analysis and common factors like age and gender were analysed using the Cox proportional hazards model.
Results: At 5 years: local control and overall survival for the whole group were 69% and 40%, respectively. Unilateral infusion (P=0.01), oropharyngeal site (P=0.02) and low N-stage (P=0.03) were predictive for local control. Gender (P=0.02), comorbidity (P=0.02), lowest involved neck level (P=0.03), age (P=0.04) and weight loss before treatment (P=0.04) were significant predictors for overall survival.
Conclusion: Aside from tumour and patient characteristics unilateral infusion was an independent factor for local control. Further study incorporating tumour volume and nodal tumour volume is in its final stage.
Conflict of interest. None