Summary and Future perspectives
SUMMARY AND FUTURE PERSPECTIVES

Despite major improvements in surgical techniques and radiotherapy in the clinical management of squamous cell carcinoma in the head and neck (HNSCC), the long term survival of HNSCC patients has only moderately improved during the last 20 years [1]. Significant improvements may be reached when the mechanisms underlying HNSCC carcinogenesis would be better characterized and the causative genes identified. The main risk factors for the development of HNSCC are tobacco smoking and excessive alcohol consumption [2]. Recently, also the involvement of the human papillomavirus (HPV) in a subset of HNSCC cases was firmly established [3-8]. The reported frequencies of HNSCC with high-risk HPV involvement vary tremendously, ranging from 0 to 100% [9;10]. Part of the variation in HPV prevalence could be explained by differences in the location of the tumor, i.e. the prevalence is relatively low in the oral cavity and high in the tonsil [5;6;11]. More importantly, variations in the type of tissue material studied and the HPV detection method used, may have a major impact on the discrepancy in the reported prevalence rates [5;9;12;13]. In Chapter 2, an explanation is provided for the discrepancy in the literature about HPV prevalence in HNSCC. We evaluated commonly used HPV detection methods and used that experience to find a combination of techniques to reach the most reliable HPV detection. This HPV detection algorithm had to be applicable for archival formalin-fixed paraffin-embedded (FFPE) material, since the preparation of FFPE specimens is routine in histopathological diagnostics worldwide. We compared quantitative reverse transcriptase polymerase chain reaction (RT-PCR), fluorescence in-situ hybridization (FISH), general primer PCR (GP-PCR), HPV protein detection in sera, and p16-immunostaining. As a golden standard we used the presence of viral transcripts (oncogenes E6/E7) in frozen tissue samples of the same tumors, a method that reflects active virus involvement. None of the tested methods was optimal with respect to sensitivity and specificity, but an algorithm based on a combination of two frequently used diagnostic techniques reached 100% for both parameters. All HNSCC tumors should be tested first for p16 overexpression by immunostaining, followed by a GP5+/6+ DNA PCR on the p16-positive cases.

In this thesis we also focused on a genetic fingerprint of HNSCC in relation to clinical and molecular variables. For this purpose, a micro-array CGH platform with a genomic resolution of 1 mega base (Mb) was established. This platform allows mapping of the genome-wide numerical genetic alterations in a high throughput manner. When genomic data is generated in a high-throughput manner good bioinformatics becomes necessary. In Chapter 3 a 'bioinformatics'-tool is described
which enables calculation of significance for genetic alterations that differ between tumor groups [14]. Specific statistical approaches need to be applied for ordinal data as produced by array CGH. Furthermore, a correction for multiple testing was incorporated by defining a false discovery rate (FDR) according to Benjamini and Yekutieli [15]. The established array CGH platform and developed bioinformatics were applied to genetically characterize HNSCC that are positive or negative for transcriptionally active HPV16 (Chapter 4). The main question was if genetic differences between HPV-positive and HPV-negative HNSCC could be detected, which would be indicative for two different routes of carcinogenesis. Furthermore, this study also defined the relevant genetic regions that differ and are shared between these two groups of tumors. A gain of 18q was shown to be specific for HPV-positive HNSCC. HPV-negative tumors showed more specific genetic alterations such as loss of 9p21, amplification of 11q13.1 and TP53 mutations. These changes all relate to the abrogation of the p53 and pRb-pathways, and are consistent with and provide further evidence for the hypothesis that HNSCCs develop by two different etiologies [16-18]; one driven by exposure to environmental carcinogens (i.e. tobacco and alcohol) without HPV involvement, and one involving infection with transcriptionally active HPV16. On the other hand, it seems that the two carcinogenic routes also partly overlap. The alterations in common may be necessary events in HNSCC irrespective of the etiological factor, and a comprehensive genetic progression model of multi-step head and neck carcinogenesis was proposed.

In Chapter 5, we studied the hypothesis if additional subgroups within HPV-negative oral and oropharyngeal squamous cell carcinoma (OOSCC) could be distinguished on basis of the pattern of chromosomal aberrations, which might be relevant for further stratification. Thirty-nine OOSCCs were classified on basis of their genetic pattern determined by array CGH using a recently developed cluster algorithm. The tumors clustered in three groups, one (n=8) characterized by a low chromosomal instability index (low CIN), another by a relatively high chromosomal instability index (n=26), and one with a very high chromosomal instability index (n=5). This classification was significantly (p=0.003) associated with survival, with the best survival in the low CIN group and the worst survival in the group with very high CIN. Low CIN was also significantly (p<0.05) associated with the presence and type of TP53 mutation, absence of alcohol consumption and a female gender. In addition, we excluded that these tumors showed microsatellite instability, and they were shown to be diploid. We confirmed this classification of OOSCC on basis of low CIN
and high CIN with an independent set of 89 oral carcinomas [19]. To elucidate the biological basis of the low CIN genotype, these tumors were tested for microsatellite instability, but this could not be detected. The discovery of these new classes of OOSCC with unique genetic and clinical characteristics might have important consequences for future biological and clinical studies. The most important conclusion is, taking also HPV-positive HNSCC in consideration, that at least three and possibly four genetically distinct subgroups of HNSCC exist which might each have their own different route of carcinogenesis. It is too early to speculate on the role of this classification for clinical management. There is a consensus in the field that at least HPV-positive HNSCC should be considered separately in clinical trials, but even for this specific group adjustment of clinical management is not yet implemented. An important problem at present is that there is no consensus on the most reliable assay to assess HPV, which has consequences for the claims on the more favorable prognosis of HPV-positive tumors. Our algorithm might help to assess the involvement of HPV in large retrospective series.

Currently, the role of HPV in a subset of HNSCC is under intensive research. In contrast to the well established causal role of transforming hrHPV infections in carcinomas of the uterine cervix [20], less is known about the precise role of hrHPV infections in HNSCC. We hypothesized that the genomic patterns of HPV-positive HNSCC might show similarity to that of cervical cancers, irrespective the difference in anatomical site. In Chapter 6 genomic profiles of HNSCC, positive and negative for transcriptionally active HPV16 (described in Chapters 4 and 5) were compared to those of cervical carcinomas (all HPV-positive). This comparison showed that a number of alterations common to HPV-negative head and neck carcinomas were rarely detected in HPV-positive carcinomas either from the cervix or the head and neck, which included losses at 3p, 5q, and 8p as well as amplifications at 11q13.3 (CCND1 locus). On the other hand gains of chromosome 20 and losses at 13q seemed specific for HPV-induced tumors, irrespective the anatomical site. Frequently shared changes between all tumors encompassed 3q gains and 11q losses, suggesting critical cancer genes at these loci. Finally, a number of organ-specific alterations were found, including a gain at 8q in HNSCCs and losses at 17p in cervical carcinomas. The high frequencies in which these alterations are detected in these tumor types suggest that they are crucial in carcinogenesis.

HNSCC carcinogenesis is driven by a few genetic events that entail the impairment of the p53 and the pRb pathways, which are involved in cell cycle regulation and apoptosis [21]. Based on epidemiological data and in vitro transformation
experiments, it has been estimated that four to six genetic events are required in humans to transform a normal cell into a malignant cell [22;23]. The current high-throughput methods that allow genome wide analysis of genetic changes with high resolution such as array CGH described in this thesis, but also the increasing body of sequencing data, have tremendously accelerated the identification of candidate cancer genes and chromosomal regions harboring candidate cancer genes [24]. Of many of these genes, the role and importance in the pathogenesis is unclear. Tumors are intrinsically genetically unstable and many DNA changes or aberrantly expressed genes should be considered a consequence and not as a cause of carcinogenesis. It is therefore of utmost importance to discriminate the carcinogenic ‘driving’ from the ‘passenger’ events.

At present suitable transgenic mouse models are absent for HNSCC carcinogenesis, and functional characterization of candidate cancer genes in established HNSCC cell lines only allows analysis of phenotypes in the highly deregulated cellular environment of an established invasive carcinoma cell line. We therefore generated an in vitro model of conditionally immortalized primary oral squamous keratinocytes. Establishment of this model system and its application to investigate the phenotypes associated with p53 and pRb pathway abrogation in HNSCC are described in Chapter 7. The abrogation of the p53 pathway, either by knock-down of the p53 gene by overexpression of dominant-negative p53, or by the human papillomavirus oncoprotein E6, led to an extended life span. Despite the seemingly identical phenotypes, dominant-negative mutant p53 showed most severe molecular changes including upregulation of the hypoxia signaling (HIF1) and WNT pathways. Knock-down of p16, overexpression of cyclin D1 or HPV E7 alone did not seem to have effect on the life span. In combination with abrogation of the p53 pathway this led to an immortal phenotype. Our data suggest a critical order of genetic hits in squamous carcinogenesis with abrogation of pRb and p53 pathways as essential and likely primary hits. This in vitro model of conditionally immortalized primary oral squamous keratinocytes further allows the functional analysis of other candidate cancer genes, as well as high-throughput functional genetic screens.

FUTURE PERSPECTIVES

The Human Genome Project has provided an enormous amount of knowledge about the human DNA sequence. This allowed the global profiling of copy number imbalances in tumors and the precise determination of the breakpoints of regions that are gained and/or lost. During the last few years, micro-array and sequencing
technology rapidly became the main techniques for studying chromosomal aberrations. Array CGH has proven valuable for a better understanding of the biology of human cancer [25;26] and has the potential to improve diagnosis, outcome prediction and treatment response. Diagnostic genomic signatures, as are already present for gene expression [27], will soon be at hand [28] and will improve tailored therapy in the near future. Also for HNSCC we have shown by array CGH analysis the existence of subgroups of tumors with distinct genomic profiles. These subgroups with different genomic entities may also have specific underlying mechanisms of progression and patient prognosis. For instance, HPV-positive HNSCC have distinct genomic alterations similar to those found in cervical cancers, in which HPV has a confident causal role, and should therefore be investigated as a separate tumor group. Use of reliable detection methods as RT-PCR, FISH or the algorithm provided in this thesis, are necessary to select the true HPV-positive patients and to estimate the world-wide prevalence for HPV in HNSCC. The question remains if HPV-induced HNSCC can be prevented by vaccination. Currently prophylactic HPV vaccination is carried out for cervical carcinoma prevention and in theory could be implemented for the prevention of HPV-induced HNSCC as well. To date, there is only evidence that the vaccine prevents infection and it will take years before beneficial effects on the cervical carcinoma incidence will become clear. Nevertheless, no serious side effects have been reported so far, supporting the view to start vaccination studies in the near future in both girls and boys.

Within the group of HPV-negative HNSCC additional subgroups can be distinguished on basis of the genetic profile. This classification seems associated with p53 mutation status, the number of genomic alterations, etiological risk factors, and prognosis. Investigation of larger cohorts should substantiate this initial observation. Prognostic separation of patients in different subgroups based on different aetiology, genomic classification and other parameters clearly has major clinical implications. If unequivocally proven, these subgroups of patients need to be analyzed as separate groups in future studies for several reasons. First, the effect of targeted drugs could be very different and this needs to be considered in future clinical trials. Second, the current clinical management could be adapted either for the tumors that show a clear favorable prognosis (treatment less intense) or for the tumors that show with current treatment regimen a more unfavorable prognosis (more intense: shift to primary surgical treatment with adjuvant chemo- or bioradiation). Thirdly, novel therapies could be developed directed to the cell signaling pathways involved in the different subgroups of tumors (e.g. directed to HPV as causative factor).
Our array CGH studies were of value for tumor classification, but did not improve our understanding of the underlying mechanisms driving cancer because of its descriptive nature. The genomic resolution of the array CGH platform we established and used had a limited resolution of 5 Mbs. The identified genomic aberrations were therefore too large to pinpoint candidate driver genes. Nevertheless, the differential analysis showed the genomic regions that we have to focus on in the future, mainly 3q26, 8q24 and 11q. At present, the resolution and accuracy of array CGH platforms are improving enormously and will soon enable us to detect genomic aberrations at the gene level. Furthermore, sophisticated data analysis tools facilitate the integration of array CGH with expression, single nucleotide polymorphism (SNP), methylation and proteomics data. This and other upcoming high resolution applications like massive parallel sequencing will lead to an excess of candidate cancer genes [24;29;30]. Functional characterization of all these genes and their relevance to HNSCC development necessitates in vitro models. The in vitro model we established will be of importance in understanding the underlying gene interactions and pathways. Large high-throughput genetic screens using cDNA, short hairpin RNA or microRNA libraries are at hand to identify novel candidate cancer genes. The elucidation of the driving cancer genes and signaling pathways causing head and neck cancer, taking the HNSCC subgroups into account, will be a major step to the identification of novel drug targets and to the improvement of the clinical outcome of this disease in the future.
REFERENCES


