Head and Neck Cancers: Evidence-Based Treatment presents a practical, state-of-the-art resource for any clinical oncologist treating or managing patients with head and neck cancers, including oropharyngeal cancer, cancer of the oral cavity, laryngeal cancer, nasopharyngeal cancer, hypopharyngeal cancer, cancer of the sinuses and the skull base, salivary gland cancer, and neck lymphadenopathy.

Section I of the book covers the most pertinent details on the epidemiology, biology, diagnosis and staging of the disease including topics such as the genomic landscape of head and neck squamous cell carcinoma and novel imaging modalities. Section II discusses the evidence-based treatment modalities for conventional and novel chemotherapy regimens, the evidence behind emerging radiation therapy techniques and the minimally invasive surgical advances changing the landscape of care. The chapters in Section III are dedicated to site-specific management, including management guidelines, tables with FDA-approved therapies and relevant ongoing clinical trials as well as instructive clinical cases with important discussion on outcomes and follow-up care. Finally, Section IV focuses on recurrent and metastatic disease and Section V provides the essentials on supportive care, including managing the elderly, managing patients suffering from dysphagia and oral complications, and must-know details of quality of life assessment and patient-reported outcomes.

Emphasizing the practice-changing techniques and the latest evidence-based treatment advances including targeted therapies, immunotherapy, transoral robotic surgery, and radiation therapy precision, this comprehensive yet accessible textbook is indispensable for any clinical oncologist of each discipline wanting a balanced and evidence-based reference on managing patients with head and neck malignancies.

Key Features:
- Includes didactic clinical cases for each type of head and neck cancer
- Numerous tables highlight FDA-approved therapies and ongoing clinical trials
- Provides evidence-based recommendations for treating head and neck cancers at each stage of the disease with conventional and novel treatment strategies
- Covers strategies for managing acute and late complications to treatment
- Includes access to the fully-searchable downloadable ebook
HEAD AND NECK CANCERS
HEAD AND NECK CANCERS

Evidence-Based Treatment

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Head and neck cancers are challenging diseases that require complex management and exemplify the value of collaboration among multiple specialties. Very appropriately, we the Editors are representatives of each of the three major treatment modalities. The genesis of this work was driven by recognition that, despite evolution in various approaches and new emerging data, high quality clinical care is optimal when there is mutual respect of each other’s discipline, and when each patient has a treatment recommendation made by multidisciplinary input.

When discussing the need for a new textbook we recognized and agreed that critical developments have been transforming the field of head and neck oncology. Indeed, in the past few years there have been significant advances in our understanding of the molecular biology, immune evasion, and genetic landscape of head and neck cancers, as well as the multidisciplinary management of these malignancies, including immunotherapy, targeted therapies, precision radiation therapy, robotic surgery, and supportive care capabilities. With the rising epidemic of human papillomavirus-associated oropharyngeal cancer, customizing treatment is of utmost importance, in particular taking into consideration long-term treatment effects.

A major breakthrough worth reiterating has been the introduction of immunotherapy as a standard therapy that establishes the significance of host immune response in the initiation and progression of squamous cell carcinoma of the head and neck. Taken together, these developments provide the basis and justify the timely publication of “Head and Neck Cancers: Evidence-Based Treatment”.

This textbook captures key advances and aims to become a quick and handy reference tool for head and neck cancer management issues, and features didactic patient cases in each site-specific management chapter. We anticipate that physicians involved in the care of these patients will find the material relevant to their clinical practice. Evidence-based approaches are emphasized throughout the textbook. The chapters have been authored by internationally recognized experts, with a focus on multi-modality representation. We are thankful and much obliged to their diligent and thoughtful contributions.

Finally, we owe a debt of gratitude to our patients. Their journeys and life stories are teaching us to become better doctors.

Athanassios Argiris, MD, PhD, FACP
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David I. Rosenthal, MD, FACP, FASTRO
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Head and Neck Cancers: Evidence-Based Treatment
Every cell division somatic mutations occur and accumulate (4). From the first cell division in the developing embryo, mutations start to accumulate. DNA replication is not foolproof, causing mutations to occur from the day of conception, and when the wrong genes are hit, cancer may result. However, when certain critical genes are hit, such as TP53 and other genes in cell cycle regulation or DNA maintenance, replication stress may develop and the accumulation of genetic changes will dramatically increase in such cells (5). After a couple of mutations in critical control genes, the carcinogenic process may drive itself (6). To summarize, there is an intrinsic risk for cancer development by accumulating somatic mutations in every cell division, but exogenous factors that trigger or increase genetic alterations boost the cancer risk.

**The Genomic Landscape of HNSCC**

*Steffen Wagner*

*Jens P. Klussmann*

*Ruud H. Brakenhoff*

**CANCER AND SOMATIC GENETIC ALTERATIONS**

Since the 1990s, it was broadly accepted that cancer arises by an accumulation of somatic genetic changes. These genetic alterations were deciphered by studying cancer and their recognizable precursor stages at the molecular level. The number of genetic changes roughly increases in parallel to the morphological severity of the lesions, and although there seems a certain order, the accumulation was considered more important than the order (1,2). Most of these concepts are still valid in 2018, although the complexity has been tremendously enhanced by the rapidly increasing knowledge during the past few years. The relatively simple linear genetic progression models became more complex and more variable (3).

Smoking and excessive alcohol consumption are important risk factors for head and neck squamous cell carcinoma (HNSCC), causing gene mutations, and these tumors still form the bulk of HNSCC. However, a subgroup of tumors is caused by infection with the human papillomavirus (HPV), and the incidence of these tumors has also increased. Hence, exogenous factors play an important role in HNSCC.

Notwithstanding these causative exogenous carcinogens, it is important to realize that by every cell division somatic mutations occur and accumulate (4). From the first cell division in the developing embryo, mutations start to accumulate. DNA replication is not foolproof, causing mutations to occur from the day of conception, and when the wrong genes are hit, cancer may result. However, when certain critical genes are hit, such as TP53 and other genes in cell cycle regulation or DNA maintenance, replication stress may develop and the accumulation of genetic changes will dramatically increase in such cells (5). After a couple of mutations in critical control genes, the carcinogenic process may drive itself (6). To summarize, there is an intrinsic risk for cancer development by accumulating somatic mutations in every cell division, but exogenous factors that trigger or increase genetic alterations boost the cancer risk.

**MUTATIONS, COPY NUMBER ALTERATIONS, AND EPIGENETIC CHANGES**

Somatic genetic changes are broadly defined. The term “mutations” links to genes, and usually relates to genetic alterations at the base pair level, stretching from a single nucleotide to small deletions and insertions (indels).
However, many genetic changes encompass gains and losses of almost entire chromosomes. How critical these huge losses and gains are in the development of cancer remains somewhat elusive, but for sure they add to the inactivation of tumor suppressor genes and activation of oncogenes (3,7). The CDKN2A locus encoding p16^{INK4A} is frequently lost usually by a large chromosomal loss at 9p21 on one allele and a focal loss around the CDKN2A gene, or a mutation or epigenetic event in the gene itself, on the second allele. Hence, we can assume that these chromosomal losses clearly play a role.

The more frequent gene amplifications (up to 10–20 copies) are also relevant. There are frequent amplifications in HNSCC at chromosome locus 11q13 where the CCND1 gene is residing encoding cyclinD1 or at the 7p12 locus of the epidermal growth factor receptor (EGFR) gene (7), both bona fide oncogenes in HNSCC. What is less clear is the precise role of the large chromosomal gains that increase gene copy numbers just twofold.

Besides mutations and chromosomal alterations, epigenetic alterations occur frequently. At the DNA level these encompass methylations at CpG sequences, most particularly in the promoter region of genes, thereby shutting them off. Also, the CDKN2A gene is frequently inactivated by methylation. However, many more epigenetic alterations are described that encompass, for example, histone code modifications. The DNA is wrapped around histone octamers, and these proteins play an active role in the expression of the genes in the vicinity of where they bind to the DNA strand. These histones can be acetylated and methylated at different amino acid residues, with major effects on gene expression (8).

Last but not least are the microRNAs (miRNAs). These small noncoding RNAs of approximately 22 nucleotides can bind to the 3’ untranslated region (3’UTR) in the mRNA of genes targeting them for degradation by the RISC complex. Hence, one miRNA may target a multitude of genes. Many changes in miRNA expression are reported in cancer, including HNSCC. In addition, major changes in general miRNA biogenesis have been described in cancer (9).

next-generation sequencing methods (7), a major problem became apparent. Many somatic mutations were identified, but mostly not in cancer driver genes but in so-called passenger genes. As indicated earlier, during every cell division cells accumulate mutations and this holds true for tumor cells as well. Hence, the number of mutations will increase, but not all are in genes that drive cancer progression. In 2013 this problem of drivers and passengers was comprehensively evaluated, and some solutions were suggested (11). It became apparent that there is a considerable mutational heterogeneity between tumors causing bias in the mutational profiles, and which is not corrected for to weigh whether a mutated gene is indeed a driver gene in a particular cancer. A most important factor of this mutational heterogeneity was a consequence of the tumor type and exposure to mutagens, as some tumors such as melanomas were shown to accumulate many mutations, while others show hardly any mutations. In addition, the type of mutations differs between tumors: in melanomas frequent TT-dimers occur causing a specific mutational profile. Summarized, some mutations are just more likely to occur in a particular tumor type.

Most remarkable was the observation that the number of mutations accumulates in genome regions that replicate late during S-phase encompassing the regions that are not actively transcribed. Hence, genes in these locations are found to become frequently mutated, but usually do not encompass cancer driver genes. The authors suggested that nucleotide shortage may play a role here.

When the authors developed a correction algorithm that takes all the biases into account, and used that on the sequencing data of 178 lung squamous cell carcinomas (SCCs), the number of candidate cancer driver genes reduced from 450 to 11 (11). Hence, mutation data should be interpreted with great caution to identify potential cancer genes. Obviously, the frequency with which mutations occur in genes underpins their relevance as cancer drivers, but in those cases that genes are mutated infrequently, additional functional studies should elucidate the role of these genes.

**DRIVERS AND PASSENGERS**

Since modern cancer genomics was initiated by large-scale Sanger sequencing of cancer genomes (10), and emerged rapidly by

**CLASSIFICATION OF HNSCC**

The very large majority of head and neck cancers (>90%) are SCCs that arise in the mucosal linings of the upper aerodigestive tract, and in a
single cell type, suggesting that it is a relatively homogeneous disease. However, HNSCC is a remarkably heterogeneous disease, and this led to several subclassifications of the disease in relation to anatomy, etiology, and clinical or molecular characteristics. Some of these are overlapping. In this section, we describe these classifications and take one as the main topic in the subsequent chapter.

**ANATOMIC CLASSIFICATION OF HEAD AND NECK CANCERS BY SUBSITE**

In combination with histology (morphology) obtained from microscopic tumor examination, anatomical classification is used routinely in cancer registries to describe the kind (morphology, behavior, and grading) and origin of neoplasms, which can be used as tool for epidemiology, health management, and clinical purposes. Nine subsites of head and neck cancer are distinguished according to anatomical localization and molecular properties ($p16^{INK4A}$ expression and HPV status) of the disease (12). In Table 3.1 the major subsites of head and neck cancers are listed including corresponding *International Classification of Diseases for Oncology*, third edition (*ICD-O-3*) codes and the further molecular subclassification in the case of oropharyngeal and hypopharyngeal cancer.

In consideration of risk factors, each subsite has its particular set of usually more than one environmental factor that contributes to the genetic alterations associated with carcinogenesis. For example, sunlight exposure is more relevant for cancers of the lip than for laryngeal or oropharyngeal cancers, for which lifestyle factors

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<td>1 Lip and oral cavity</td>
<td></td>
<td>C00.0-6, C00.8-9, C02.0-3, C02.8-9, C03.0-1, C03.9, C04.0-1, C05.0, C05.8-9, C06.0-2, C06.8-9</td>
</tr>
<tr>
<td>2 Major salivary glands</td>
<td></td>
<td>C07.9, C08.0-1, C08.8-9</td>
</tr>
<tr>
<td>3 Nasopharynx</td>
<td></td>
<td>C11.0-3, C11.8-9</td>
</tr>
<tr>
<td>4 Oropharynx</td>
<td>$p16^{INK4A}$-positive, HPV-mediated</td>
<td>C01.9, C02.4, C05.1-2, C09.0-1, C09.8-9, C10.0, C10.2-3, C10.8-9, C11.1</td>
</tr>
<tr>
<td>5 Oropharynx and hypopharynx</td>
<td>$p16^{INK4A}$-negative, HPV-unrelated</td>
<td>C01.9, C02.4, C05.1-2, C09.0-1, C09.8-9, C10.0, C10.2-3, C10.8-9, C11.1 and C12.9, C13.0-2, C13.8-9</td>
</tr>
<tr>
<td>6 Nasal cavity and paranasal sinuses</td>
<td></td>
<td>C30.0, C31.0-1</td>
</tr>
<tr>
<td>7 Larynx</td>
<td></td>
<td>C10.1, C32.0-2, C32.8-9</td>
</tr>
<tr>
<td>8 Mucosal melanoma of the head and neck</td>
<td></td>
<td>C00.0-6, C00.8-9, C01.9, C02.0-4, C02.8-9, C03.0-1, C03.9, C04.0-1, C04.8-9, C05.0-2, C05.8-9, C06.0-2, C06.8-9, C09.0-1, C09.8-9, C10.0-3, C10.8-9, C11.0-3, C11.8-9, C12.9, C13.0-2, C13.8-9, C14.0, C14.2, C14.8, C30.0, C31.0-1, C32.0-2, C32.8-9</td>
</tr>
<tr>
<td>9 Cutaneous squamous cell carcinoma of the head and neck</td>
<td></td>
<td>C00.0-2, C44.0, C44.2-4, C44.8</td>
</tr>
</tbody>
</table>

such as tobacco and alcohol exposure or viral infection have the highest impact. HPV-related tumors are typically found in the oropharynx and even more particularly in the tonsils, which is discussed in more detail in the following.

In summary, head and neck cancers are diverse concerning anatomical localization and contributing risk factors. The current knowledge shows that HPV-related cancers in the head and neck region are primarily localized within the oropharynx, mainly within the tonsil (13). Up to a certain extent, HPV-negative cancers of the oropharynx might be very comparable to nonopharyngeal head and neck cancers, but HPV-related cancers of the oropharynx are a true distinct disease entity, and classification on the basis of HPV status is the leading topic in this chapter.

**SUBTYPES IN HNSCC BASED ON GENE EXPRESSION**

Initially microarray hybridizations and later RNA sequencing studies allowed researchers to identify all genes in a tissue that are expressed. These gene expression profiles have been explored in different tumor types including HNSCC by several groups. In 2004, four expression subtypes were defined in HNSCC by cDNA microarray hybridization, resembling those in lung cancer found before (14,15). In a later study, four comparable profiles were identified using Agilent 44K microarrays, and these groups were classified and termed as basal, mesenchymal, atypical, and classical gene expression subtypes (16). While HPV status was not included in the earlier study, an enrichment of HPV-associated gene expression was observed in the atypical subtype with elevated expression of *CDKN2A, LIG1*, and the transcription factor *RPA2* (16,17). The basal gene expression profile showed signatures found in basal cells from airway epithelium (e.g., high *COL17A1* expression associated with the extracellular matrix production, and high *TGFA, EGFR*, and *TP63* expression) (16,18). Genes associated with epithelial-to-mesenchymal transition (EMT), mesenchymal markers, and transcription/growth factors were enriched in the mesenchymal subtype, while genes enriched in the classical subtype were associated with exposure to cigarette smoke, xenobiotic metabolism genes, and transcription factors (16). Also four subtypes were identified in a third study using Illumina Expression BeadChips (19). Although one cluster closely matched the classical gene expression profile, the other clusters correlated less well with the former studies. This could illustrate the heterogeneity of HNSCC regarding gene expression, but may also indicate differences in collecting and processing of samples and the used profiling platforms. However, an important finding of this study was that the samples containing transcriptionally inactive HPV16 DNA shared characteristics with HPV-negative samples regarding gene expression and frequency of *TP53* mutations, indicating that the presence of HPV16 DNA is not indicative for a role of the virus. Further, the so-called disruptive *TP53* mutations and an immune response–related gene expression cluster were associated with lymph node metastasis, independent of HPV status (19).

Five subtypes of HNSCC, including two biologically distinct HPV subtypes, were identified by Keck and coworkers in 2015 in a clinically homogeneous cohort of 134 locoregionally advanced HNSCCs with 44% HPV-positive tumors. All patients were treated with concurrent chemoradiotherapy. Gene expression profiling was done using Agilent 4x44Kv2 expression arrays and combined with previously published and publicly available data, generating a final dataset of more than 900 patients. One of the subtypes showed an immune and mesenchymal phenotype and contained both HPV-positive and HPV-negative tumors, while the other subgroup resembled the classical expression pattern according to prior nomenclature in other cancer types and molecular characteristics (20–22), but also contained both HPV-positive and HPV-negative tumors. A basal expression subgroup encompassed the remaining HPV-negative samples with significant enrichment of hypoxia-responsive genes (e.g. *HIF1A, CA9*, and *VEGF*), neuregulin signaling (including *EGFR, AREG, NRG1*), and overexpression of epithelial marker genes like P-cadherin (*CDH3*) and cytokeratins (*KRT1, KRT9*). In contrast to the basal expression subgroup, both HPV-positive subtypes showed low expression and no copy number events for *EGFR/HER* ligands (23).

Several gene expression signatures have been established from microarray and RNA-Seq studies. However, none of these approaches could be implemented in the standard of care for HNSCC so far. A recent study demonstrates the use of formalin-fixed paraffin embedded (FFPE) oropharyngeal squamous cell carcinoma (OPSCC) tumor tissue for mutation and transcriptional profiling by using Ion Torrent
AmpliSeq cancer panel tNGS and NanoString gene expression assays (24). In this pilot study, 230 cancer-relevant genes were analyzed in four HPV-positive and four HPV-negative samples. The expression of several genes was highly likely to correlate with HPV status (e.g. WNT1, PDGFA, and OGG1). Further, by unsupervised hierarchical clustering six groups of differentially expressed genes were identified in this dataset. Technical replicates of this gene expression study proved a reproducible pattern of gene expression for each tumor with respect to the underlying biology (24) and it may be assumed that the ability to use FFPE samples will advance gene expression studies.

The expression profiling either by protein or RNA expression suggests differential benefit of target specific therapies for different patient groups with HNSCC. Tumors with high and amplified levels of EGFR may qualify for cetuximab treatment. Especially in sight of current antibody-based therapies, this might be of translational importance and further efforts are needed for biomarker development and improvement of personalized care for patients with HNSCC in the future.

In summary, there are several ways to classify head and neck tumors, based on anatomical subsite, based on gene expression profiles, but most prominent at present is the classification on the basis of HPV status, at least in the oropharynx. HPV-positive tumors are different at the molecular level and show a very different clinical behavior, which recently led to an adaptation in the staging (25) and clinical trials to de-intensify therapy. The role of HPV in nonoropharyngeal tumors is less clear, but all in all we separate HNSCC primarily in HPV-negative and HPV-positive tumors, and discuss the genomic landscape likewise.

**HPV-NEGATIVE AND HPV-POSITIVE HNSCC: CARCINOGENESIS**

**HPV-NEGATIVE TUMORS**

Most HNSCC patients present with tumors de novo. However, there are precancerous lesions in the mucosal linings of the upper aerodigestive tract that are either white (leukoplakia) or red (erythroplakia) areas of the mucosa. These lesions may progress and develop into SCCs (26). Leukoplakia is the most common precursor lesion of oral squamous carcinomas and its prevalence varies between 0.1 and 0.5% (27,28). The standard policy is to treat the lesion when possible and analyze the specimen or a biopsy by microscopic examination for dysplasia, graded as mild, moderate, or severe. Although consensus criteria have been defined by the World Health Organization (WHO), it remains problematic to make an objective categorization of dysplasia owing to a high inter- and intraobserver variation. Patients are subsequently monitored by active surveillance and biopsy on indication.

The percentage of oral leukoplakias that develop into cancer depends on various factors such as the study population, the definition of leukoplakia used, and the length of the observation time, but an annual transformation rate of 1% to 2% per year is a reasonable assumption (26). Risk factors for progression are female gender, size, and the presence and grade of dysplasia.

Besides these recognizable lesions in the mucosal linings, it has been well established that mucosal abnormalities may exist that are only visible under the microscope. When tumors are diagnosed and excised, the specimen is transferred to the pathology laboratory to perform definitive staging and investigate the surgical margins for tumor invasion. The pathologist also scores for changes in the mucosal epithelium, and grades these as mild, moderate, or severe dysplasia. Particularly severe dysplasia is considered as a preneoplastic change in the mucosal epithelium. Already in 1953, the term “field cancerization” was proposed to describe these dysplastic changes that might also explain the high propensity that local recurrences develop after HNSCC treatment and the high risk that multiple independent tumors develop in the mucosal linings of the upper aerodigestive tract (29).

In the nineties, these precancerous changes were comprehensively studied with genetic markers, and it was shown that the number of genetic changes runs more or less in parallel to the severity of the lesions. This led to the first genetic progression model of head and neck cancer (2). Loss of heterozygosity at chromosomes 3p, 9p, and 17p appeared to occur in dysplasia, apparently reflecting early carcinogenesis, while other alterations at 11q, 4q, and 8 were typically present in carcinomas, likely corresponding to a relatively late phase in carcinogenesis.

These genetic markers were employed to study the presence of genetically altered mucosal epithelial cells, also coined as “fields” in
relation to the field cancerization concept of Slaughter et al. (29). Indeed many more pre-malignant changes characterized by tumor-associated genetic changes were observed, and shown to be clinically relevant as they formed an important source of local recurrences and second primary tumors at least in surgically treated patients (30–32). Most recent studies have identified these same genetic changes at chromosome arms 3p and 9p together with mutations in TP53 as the best predictors of malignant transformation in leukoplakia (33) and in surgical margins (34).

The tumor suppressor gene on chromosome arm 9p is CDKN2A encoding the p16INK4A protein, which binds and disrupts the cyclinD/CDK4-6 complex that drives cells through the G1-S checkpoint. The p16INK4A cell cycle inhibiting protein is frequently inactivated in HNSCC by mutation or methylation in combination with chromosomal loss, or by homozygous deletion (7,68). On chromosome 17p13 the TP53 gene is located that is also frequently inactivated in HNSCC, mostly by missense mutations combined with allelic loss. Somatic mutations are found in 60% to 80% of the tumors (7,35–37). Hence, there is quite some information on the precancerous fields and the molecular changes in these fields, although more detailed DNA sequencing data are still lacking.

Furthermore, there is only very limited data on what precedes the development of these fields. Van Houten et al. described small p53-positive focal patches in tumor-adjacent mucosal epithelium (35), and showed that these indeed contained mutations, but not related to the index tumor of these patients. These mutated p53-positive patches were considered equivalent to the “clones” or “clonal units” and defined as a family of daughter cells that originate from an adult stem cell that makes up that part of the squamous epithelium, and that has now become detectable by the mutation in p53. These p53 mutated clonal units were considered to represent the first oncogenic changes in the mucosa, and formed together with the genetically defined fields the basis of the hypothetical patch-field-tumor-metastasis progression model for HNSCC development (3). This model was deduced from the existing descriptive studies but recent data in engineered mouse models support such a model. By Axin2 lineage tracing experiments the stem cells and the patches they form have been shown recently, at least in mouse skin (38).

The initial genetic progression model, later adapted and simplified, are elementary linear models (3). However, the plethora of new candidate cancer genes that are mutated in HNSCC will make these simple linear models much more complex and diverse, and the research field has to deal with the fact that there are likely multiple ways that lead to mucosal epithelial cell transformation.

In most recent molecular profiling studies it was shown that another subgroup of tumors exists that is characterized by very few copy number alterations. This subgroup of HPV-negative and TP53 wild type tumors typically displays HRAS and CASP8 mutations and a more favorable prognosis (7). In previous studies, it was shown that this group is diploid, proficient in DNA mismatch repair and seems to occur more frequently in females without a history of smoking and alcohol consumption (39).

**HPV-POSITIVE TUMORS**

HPV infection causes a subgroup of tumors, most particularly those arising in the oropharynx. HPV-positive OPSCCs form a separate disease entity that led to an adapted staging system (25). In addition, several studies to de-escalate therapy have been initiated and the results are awaited. Most assays to test for HPV in tumor specimen are based on the detection of viral DNA. There are many HPV types that may cause cancer, but most prominent is HPV16, particularly in the head and neck region. As mentioned earlier, the virus encodes two oncogenes E6 and E7. The E7 protein binds the pocket proteins pRb, P130, and p107 thereby creating an S-phase environment in the cell. Usually an unscheduled S-phase with high E2F activity causes p53 activation by p14 inhibition of MDM2 and induction of apoptosis, and therefore the virus expresses the oncoprotein E6 that targets p53 for degradation. Hence, the virus hits precisely the same pathways as is noted in HPV-negative tumors: the p53–p21 pathway and the p16–cyclinD1–Rb pathway. Typically in HPV-positive tumors, p16INK4A is active and often overexpressed, while cyclinD1 at 11q13 is not amplified and p53 is wild type (7,40,41). Hence, at the genetic level, most prominent in HPV-positive tumors is the absence of TP53 mutations as well as the absence of the loss of chromosome arms 3p and 9p and the amplification of 11q13, while these changes are very common in HPV-negative tumors.
Both E6 and E7 expression remains critical in HPV-positive HNSCC cell lines (42) and the cancer-associated phenotype caused by inactivation of the p53 and pRb pathways in oropharyngeal keratinocytes is at least cellular immortalization (43). This phenotype would also fit with the timing of the genetic events early in the progression of HPV-negative HNSCC as abrogation of the p53 and pRb pathways by loss of 9p21, the location of CDKN2A as well as TP53 mutations are frequently found in the precursor fields (2,30,44), and are considered as the earliest genetic changes. In HPV-positive HNSCC these same pathways are likely also the first to be inactivated by the viral E6 and E7 oncoproteins, strongly suggesting that HPV infection is indeed the initial carcinogenic event as it is in cervical cancer (45).

The key precursor lesions in the mucosal linings are leukoplakias, and it was therefore logical to study HPV presence in these lesions, but the results are highly discordant. A major problem in the different studies is the false positive results by the applied ultrasensitive HPV DNA assays. Most reliable studies suggest a very low prevalence of less than 1%; this is reviewed by Ha and Califano (46). Given the second observation that 3p and 9p losses of heterozygosity are the key genetic changes predicting malignant transformation of leukoplakias (33), although these alterations are typically absent in HPV-positive tumors (40), we consider progressing leukoplakia lesions as being HPV-negative, and they should not be considered the precursor lesion of HPV-mediated carcinogenesis.

Are there any other indications for HPV-induced precancerous changes in the upper aerodigestive tract? They are very common in the cervix, and in fact the target lesion for screening (45). The highest attributable fraction of HPV in the head and neck is found in the tonsils, and therefore both precancerous changes and sites of infection are expected in the tonsils. Several studies have been carried out with the largest reporting on over 4,000 tonsils of which over 3,300 could be analyzed. No HPV was found (47). Hence, the precursor lesions of HPV-driven carcinogenesis in the upper aerodigestive tract are still an enigma. There have been indications that tonsillar cancers may be surrounded by p16INK4A-positive mucosal fields, but these might have been superficially growing tumors and without proper genetic analysis of these lesions this remains unclear (48).

Finally, it was also studied using HPV assays whether HPV-positive tumors in the oropharynx are surrounded by large fields of infected cells, a surrogate for the fields of genetically altered cells that precede HPV-negative tumors. It was assumed that HPV infection is the first carcinogenic event in HPV-positive tumors, as it is in the cervix, and that HPV presence and E6 transcripts could be used to study the presence of these fields surrounding HPV-positive tumors. Remarkably, in none of the analyzed tumor-free surgical margins E6 transcripts could be detected. Again, strongly suggesting that HPV-induced field cancerization seems not to occur in the upper aerodigestive tract. Alternatively, HPV infection may also not be the first carcinogenic event (49). In contrast to HPV-mediated carcinogenesis in the cervix that can be followed by inspection and biopsies of the acetowhite lesions, there are at present no indications for HPV-related precancerous changes in the upper aerodigestive tract. Hence, the molecular pathogenesis of HPV-induced squamous cancers in the upper aerodigestive tract remains unsolved, and currently relies on the extrapolation of data collected in the invasive carcinomas.

**HPV-NEGATIVE AND HPV-POSITIVE HNSCC: PROTEIN EXPRESSION, GENETICS, AND EPIGENETICS**

A most interesting feature of head and neck cancers in comparison to other tumor entities is the existence of virus and chemical agent driven cancer within one anatomically well-defined sublocalization (the oropharynx) in a somewhat balanced numerical ratio. For example, in lung cancer, smoking is the dominant risk factor while viral infection is negligible. In contrast, almost 100% of cervical carcinomas are associated with HPV infection. During the past year it became evident that HPV-related OPSCCs are a distinct entity with characteristic molecular and clinical features such as superior survival of patients. Consequently, it was to be expected that HPV was introduced for classification of oropharyngeal cancer in the latest version of American Joint Committee on Cancer (AJCC) staging albeit HPV status is determined only by p16INK4A immunostaining as a surrogate and not in combination with HPV testing (12).

The choice to rely on p16INK4A immunostaining was that it is easier to standardize compared to viral oncogene (mainly E6 and E7)
mRNA detection, and that it is a good although not perfect surrogate marker. Overexpression of p16\(^{\text{INK4A}}\) protein is indirectly linked to the oncogenetic activity of HPV encoded protein E7. In contrast, CDKN2A, the gene encoding p16\(^{\text{INK4A}}\), is one of the most frequently inactivated tumor suppressor genes in HPV-negative tumors (50,51).

**DIFFERENTIAL PROTEIN EXPRESSION IN HPV-POSITIVE AND HPV-NEGATIVE HNSCC**

The most prominent protein markers in HNSCC include p53, EGFR, and VEGF. The 53 kDa nuclear phosphoprotein p53 is coded by the tumor suppressor gene TP53. It is one of the most important tumor suppressors involved in DNA synthesis/repair and programmed cell death and, when activated, inhibits the cell cycle by induction of p21. Expression of p53 is usually not detected in healthy cells owing to its short half-life, but it is frequently observed in cancer cells. Accumulation of p53 occurs by enhanced expression upon cellular stress, for example, induction by carcinogenic processes. However, several mutational hot spots within TP53 in cancer cells have been identified, which lead to mutated protein with abrogated tumor suppression function. Therefore, selection processes cause tumor cells to accumulate TP53 mutations. Although not fully understood, the consequence of more TP53 mutations/higher p53 expression level could be a worse prognosis. These findings are superimposed by effects of HPV-induced carcinogenesis, and right now, it is unclear whether TP53 mutations have prognostic impact independently from HPV status. Some studies do report this (52), but the studied cohorts are somewhat small and based on heterogeneous patient populations. HPV-positive tumors rarely contain TP53 mutations compared to HPV-negative tumors (40) and prognosis is usually better. Although pointing in the same direction, underlying mechanisms differ. In HPV-driven cancers, viral E6 oncoprotein expression impedes p53 function via binding and recruitment of ubiquitinating proteins and labeling p53 for proteasomal degradation. Therefore, no selection pressure to acquire TP53 mutations exists in HPV-driven cancers. However, as already mentioned another protein marker (p16\(^{\text{INK4A}}\)) is overexpressed in HPV-related cancers. The p16\(^{\text{INK4A}}\) tumor suppressor protein is generally inactivated by mutation, methylation, and losses in HPV-negative tumors. In HPV-driven tumors, p16\(^{\text{INK4A}}\) expression is usually detectable in the majority (>70%) of tumor cells by immunohistochemistry, with a strong, diffuse expression pattern in both nucleus and cytoplasm (classical pattern of HPV-induced transformation) (48,50,53,54). Inactivation of retinoblastoma (Rb) by the HPV E7 oncoprotein overcomes cell cycle arrest and E7 enhances p16\(^{\text{INK4A}}\) expression by inducing epigenetic changes (55). However, it has to be mentioned that HPV-independent p16\(^{\text{INK4A}}\) overexpression is described for different cancers and about 5% of oropharyngeal cancers (50), probably by alternative alterations in the p16-signaling pathway (e.g., inactivation of retinoblastoma). Previous studies have shown that respective disadvantages of HPV detection by polymerase chain reaction (PCR; low specificity) or by p16\(^{\text{INK4A}}\) immunohistochemistry (HPV-independent overexpression) can be ruled out by the combination of both techniques.

**MUTATIONS AND COPY-NUMBER ABERRATION (CNA) PROFILES**

Complex karyotypes are frequent in HNSCC, including several numeric and structural alterations. A high variability and heterogeneity among cells indicates selection processes and clonal evolution in HNSCC. This led to one of the first genetic progression models for HNSCC, in which chromosomal changes (gains, losses, and translocations) accumulate in an ordered manner with resulting growth advantage of a favored cell population (2). In a 2014 review, cytogenetic aberrations in HNSCC have been summarized (56).

The current knowledge indicates that certain genetic alterations are shared by HNSCC irrespective of HPV status, while others are restricted to either HPV-negative or HPV-positive HNSCC (57). This has been largely confirmed by data from The Cancer Genome Atlas (TCGA) consortium. For example, amplification of the 3q26-28 region, including PIK3CA, TP63, and SOX2 are frequent to both (7). Deletion (14%) or mutation (8%) of TRAF3 (tumor necrosis factor receptor–associated factor 3), which is involved in innate and acquired antiviral immune response, was found in high frequency in HPV-positive HNSCC (7). In this context, higher mutation rates in CYLD (cylindromatosis lysine 63 deubiquitinase) have also been identified in HPV-positive HNSCC and both aberrations correlated with NFkB (nuclear factor kappa-light-chain-enhancer of activated B cells) activation, episomal HPV status of tumors, and improved patient survival (58).
Amplifications of EGFR and FGFR1 appear to be restricted to HPV-negative HNSCC, which is confirmed by other studies (7,59–61).

A comparison of HPV-positive and HPV-negative tumors was done for mutations and CNAs in 617 cancer-associated genes in 120 matched tumor/normal samples (42.5% HPV-positive tumors) (62). In contrast to the general expectation, mutational burden did not differ between HPV positive and HPV negative. The mutational spectrum of HPV-negative tumors resembles that of published lung SCC analyses with high mutation frequency in TP53, CDKN2A, MLL2, CUL3, NSD1, PIK3CA, and NOTCH genes. In HPV-positive tumors a unique profile with high mutation rates in DDX3X, FGFR2/3 and aberrations in PIK3CA, KRAS, MLL2/3, and NOTCH1 was found (62).

A most important progress in recent years was the publication of 279 head and neck cancer genomes characterized by next-generation sequencing and array analysis by TCGA consortium (7). In this dataset, 36 cases were identified to be HPV positive. The analysis confirmed differential genetic patterns described earlier. Further, frequent structural changes in TRAF3, a gene at chromosomal region 14q32 and frequently involved in antiviral immune responses were found in HPV-positive tumors. In contrast, amplifications of EGFR and FGFR1 appear to be restricted to HPV-negative HNSCC (7,59–61). TpC mutations were found more frequent in HPV-positive tumors, although the total number of somatic mutations was not different between HPV-positive and HPV-negative tumors. Missense mutations in PIK3CA coding for the catalytic subunit of PI3-kinase were identified in HPV-positive tumors as well as amplifications of E2F1. PI3-kinase activity activates AKT signaling and PIK3CA mutations in HPV-positive tumors have been reported before [e.g., in 8/33 HPV-positive tumors analyzed (63)]. Data from TCGA and other studies have recently been summarized to describe the genetic landscape of HPV-associated HNSCC, including structural alterations, mutation signatures, viral integration, and their relation to cellular pathways (64).

Although latest sequencing results are a great progress for cancer research, one has to mention that total numbers of analyzed samples are still small. For example, the aforementioned mutation in KRAS was found in 5.8% of HPV-positive tumors (3/51 patients) (62), and this finding could not be validated in another study (63).

Among the best known and highly validated differences between HPV-negative and HPV-positive HNSCC are somatic mutations in the TP53 gene, which are found in 60% to 80% of HPV-negative but are virtually absent in HPV-positive tumors (7,35–37). This reflects the low selection pressure to acquire TP53 mutations in HPV-positive cancers due to p53 inactivation by the HPV E6 oncoprotein.

Although the majority of HNSCCs are either driven by HPV or harbor TP53 mutations, a remaining 20% cases seem to have p53 wild type (39). It might be that other genes in the p53 pathway are altered in selected cases (66) or that p53-independent tracks of malignant progression are followed. For example, this subgroup of HPV-negative and TP53 wild type tumors typically show HRAS and CASP8 mutations, as was recently shown by molecular profiling studies (7).

HPV-driven tumors with E6 transcription were shown to have a different genetic pattern (e.g., absence of chromosome arms 3p, 9p loss, and 11q13 amplification) compared to HPV-negative HNSCC with respect to loss of heterozygosity analysis and microarray comparative genomic hybridization (40,41,67). The genes located at 9p and 11q13 are CDKN2A and CCND1, and both are established cancer genes in HPV-negative HNSCC and often affected by genetic alterations (7,41,68). In HPV-positive HNSCC, chromosomal instability seems to be associated with HPV DNA integration into the host’s genome and, albeit being linked to cancer progression in general, is associated with favorable prognosis in tonsillar cancer (69).

In the classical view of HPV-related carcinogenesis, integration of the viral DNA into the host genome disrupts control of E6 and E7 activity by inactivating E2 protein by linearizing the HPV genome within this E2 coding open reading frame. Although this model appears to be reasonable, in an analysis of 73 HPV-associated OPSCCs, episome-derived PCR products were only detected for more than 60% of samples (70). Also, HNSCC cell line analysis indicated that viral oncogene expression was independent of viral copy number and the presence of viral integration. One of the studied cell lines did not show HPV DNA integration at all, indicating that HPV DNA integration is not a necessary event in carcinogenesis (71). Very recently, RNA-Seq and whole genome sequence data from TCGA HNSCC samples were analyzed for the possible HPV genome states (episomal only, integrated state, and a state in which the viral
EPIGENETICS AND miRNAs

Epigenetic alterations, either DNA methylation or histone modifications of viral and host genomes, are two important processes effecting gene expression. Especially the role of DNA methylation in HPV-positive OPSCC is well established, and affects not only pathogenesis of OPSCC but also clinical behavior of tumors. Gene promoter methylation often causes transcriptional silencing of tumor suppressor genes involved in DNA damage repair, detoxification, cell cycle regulation, and apoptosis, and may be as important as inactivation of genes by deletion or somatic mutation (73,74).

Viral E2 protein expression and binding to E2 binding sites (E2BSs) in the viral genome are important regulators of E6 and E7 expression during the normal HPV life cycle. Loss of E2 function during carcinogenesis is considered a prerequisite for consistent activation of viral oncogene expression and malignant transformation (75). Methylation marks at E2BS in the upstream regulatory region of E6 and E7 regulate transcription in the presence of intact E2 expression, which is particularly important in tumors with episomal (nonintegrated) HPV (76,77). Distinct patterns of DNA methylation in the host genome are described for viral driven cancers including HPV (78–80). Since DNA methylation profiles persist or even increase during disease progression, this is of particular clinical relevance. A trend toward a higher level of gene promoter methylation in HPV-related as compared to HPV-negative OPSCC was shown, which is most likely due to accelerated expression and enzymatic activity of DNA methyltransferases (DNMTs) (80–84). In patients with HPV-associated SCCs distinct subgroups were identified based on viral DNA integration and the E2BS methylation status, causally linked with viral oncogene expression (70,76,77). Recently, a methylation signature for HPV-related cancers was determined based on quantitative evaluation of differentially methylated regions in the proximal promoter of five host genes (ALDH1A2, OSR2, IRX4, GRIA4, and GATA4), predicting the clinical outcome of HNSCC patients, including HPV-related OPSCC (78,85).

MiRNAs are the best studied noncoding RNAs involved in gene regulation since their discovery in 1993 (86). After their processing from 60 to 70 nucleotide, long hairpin-like precursors, miRNAs are incorporated together with Dicer1 and Argonaute (AGO) proteins in the miRNA-induced silencing complex (miRISC). This complex is guided by sequence complementarity of the miRNA to its target mRNAs, and causing gene suppression by targeted mRNA degradation and translational repression. Although altered miRNA expression is known in various cancers and carcinogenic processes, invasion and metastasis, less is known in head and neck cancer. A definite conclusion from the large-scale profiling studies is hampered by the fact that miRNAs do not have “one” target and one function. Because of their small size of about 22 bases, miRNA may bind to more or less conserved target sequences and target genes themselves can have several binding sites for one or different miRNAs. This generates rather complex networks of molecular interactions regulated by miRNAs. Although only partially documented, most cellular pathways involved in carcinogenesis of head and neck cancers are influenced by miRNA regulation.

Several differentially expressed miRNAs have been described until now for HPV-negative and -positive HNSCC and with the technical progress, their number and reliability of data will certainly rise. Hsa-miR-363 is among the best known miRNAs found to be upregulated in HPV-positive compared to HPV-negative HNSCCs (87–89). Among others, its predicted target sequences are within CDKN1A (cyclin-dependent kinase inhibitor 1), CASP3 (Casase-3), and CD274 (programmed cell death 1 ligand 1), indicating regulatory function in apoptosis, cell cycle, transcriptional regulation, and immunity. Downregulation in HPV-positive compared to HPV-negative HNSCC was reported for hsa-miR-125a, -143, -145, -199a, -126, -181b, and -31 in at least two studies each (87–91). The most prominent target genes are: CD34 (hematopoietic progenitor cell antigen CD34), CDKN1A, TP53 (p53), ERBB2 (receptor tyrosine-protein kinase erbB-2), KRAS (GTPase KRas), SOX2 (transcription factor SOX-2), MET (hepatocyte growth factor receptor), MTOR (serine/threonine-protein kinase mTOR), HIF1A (hypoxia-inducible factor 1-alpha), VEGFA (vascular endothelial growth factor A), PIK3R2 (phosphatidylinositol 3-kinase regulatory subunit beta), TERT
(telomerase reverse transcriptase), BCL2 (apoptosis regulator Bcl-2), and FOXP3 (forkhead box protein P3). Other miRNAs reported in at least two studies are hsa-miR-26b, -29a, -155, and -222 (87–91). Predicted target sequences are within genes already mentioned or with similar important functions. However, expression data are conflicting for these miRNAs indicating the need for further investigations in this field of gene regulation. Although miRNA regulation is highly complex, miRNAs may serve as diagnostic and prognostic markers and miRNA-mediated gene regulation could be the target of future therapy concepts.

**HPV-NEGATIVE AND HPV-POSITIVE HNSCC: ALTERED PATHWAYS**

For development and progression of every malignant disease, a critical set of cellular pathways has to be dysregulated. Although involved factors may differ depending on the originating tissue and impact of environmental factors (chemicals, radiation, or carcinogenic viruses), a basic set of pathways seems comparable for most cancers. Generally, proliferation and cellular survival are increased, while cell cycle control is changed, and DNA-repair and linked apoptotic programs are suppressed (Figure 3.1). This is achieved by the action of the major HPV oncoproteins E6 and E7 in HPV-induced carcinogenesis, and is independent from inactivation of anticancer sentinels like p53 on the genetic level. Further steps involve dedifferentiation of tumor cells and activation of immune escape mechanisms. Downregulation of HLA-A and –B alleles has been reported for HPV-driven cancers in 1995 (92) and recent sequencing data point to enrichment of mutations in HLA and beta-2-microglobulin genes in HPV-positive HNSCC (7). This is particularly interesting, since attraction and activation of CD56+ natural killer cells is expected by HLA I loss and higher numbers of CD56+ natural killer cells were reported in HPV-associated OPSCC recently, and correlate with improved survival independently from HPV status (93).

**FIGURE 3.1** Molecular pathways frequently altered in HPV-positive HNSCC.

HNSCC, head and neck squamous cell carcinoma.
Further, an increased disturbance in NOTCH signaling was observed in both HPV-positive and HPV-negative HNSCC (7,59), indicating a certain need for dedifferentiation in carcinogenesis for both entities. For development of invasive properties, downregulation of cell surface adhesion molecules and activation of extracellular matrix degrading enzymes have to take place. Further, enhanced angiogenesis and adaptation of metabolic processes regarding changing environmental conditions during tumor growth or migration of tumor cells, is needed for tumor progression.

The driving forces, details of the molecular participants, and potentially the sequence of events differ between HPV-positive and HPV-negative HNSCC. Therefore, a detailed knowledge of molecular processes is needed for disease classification and development of more specific and effective treatment concepts for distinct tumor types.

**IMPLICATIONS FOR THERAPEUTIC TARGETING**

Molecular and genetic classification of patients’ tumors will help to guide treatment decisions in the future. Further, the increasing knowledge of altered pathways in HNSCC by investigating underlying genetic alterations will lead to the prediction of novel therapeutic targets and reevaluation of already known target/therapy combinations, which in clinical trials with genetically unselected patients, have not been as successful as expected (e.g., EGFR-directed therapies). As a prerequisite, genetic profiling of patients has to become possible by routine, which can be assumed regarding developments in high-throughput sequencing technologies.

Generally, oncogenic alterations are more susceptible to pharmacologic inhibition than changes in tumor suppressors or related pathways. HPV-positive HNSCC might be more eligible for therapeutic targeting than HPV-negative HNSCC, as they appear to be more homogeneous regarding altered pathways and activation of cancer-associated pathways seems to be more frequent. To date, the most obvious candidates are PIK3CA and CDK4/CDK6 pathways, due to high rates of PIK3CA mutations and CCND1 amplifications. Furthermore, suitable therapeutics are already available for interfering with both pathways. STAT3 might be another promising therapeutic target. Hyperactivation of the STAT3 transcription factor is a hallmark of HNSCC, although oncogenic mutations are not described. STAT3 activation is driven by upstream growth factor receptors like Janus kinase (JAK) and EGFR and associated with oncogenic behavior and resistance to standard therapeutics in HNSCC (94).

Options to interfere with dysregulated pathways in cancer are numerous and have been recently summarized for HNSCC (95,96). However, as HPV-negative HNSCC is mainly caused by tumor suppressor gene inactivation, synthetic lethal interactions with other genes need to be explored. Logical candidates might be the remaining genes regulating the head and neck cancer cell cycle. In tumors the G1/S checkpoint is inactivated by the abrogation of pRb and p53 pathways, and molecules such as Wee1 kinase that keep the cyclinB/CDK1 complex inactivated till cell division occurs, are now becoming regulation nodes (97). Increasing data of genetic alterations driving carcinogenesis, functional analysis of the cancer genes, and future development of methods for their therapeutic targeting will provide further options for the treatment of HNSCC. It remains to be seen whether novel targeted treatment options have the power to complement or replace conventional therapy, and the immunotherapies that are discussed in other chapters.

**SUMMARY**

The genomic landscape of head and neck cancer is known in greatest detail. The main classification in our view at present is between HPV-positive and HPV-negative tumors, certainly in the oropharynx as these tumors differ enormously with respect to etiology, molecular changes, patient characteristics, and most importantly, clinical outcome. HPV-positive tumors are staged differently in TNM8 (12), and likely may be treated differently in time. Within the oral cancers, there is also a subgroup of tumors that are devoid of copy-number changes, and this group needs to be characterized in more detail.

The most important driver pathways in head and neck cancer are p53-p21, p16-cyclinD1-Rb, EGFR-PI3K-AKT, TGF-beta, and NOTCH1. Many other genes have been found mutated in much lower frequencies and are likely to play a role, but precisely how and in which pathways remains to be investigated. Disappointingly, the number of oncogenes involved in HNSCC is very limited. EGFR and PIK3CA seem the main oncogenes involved but only in subsets of tumors. Oncogenes are interesting targets for therapy, but HNSCC seems more a disease of
inactivated tumor suppressor genes, which are no direct targets, enforcing the exploration of other strategies. But new opportunities have emerged. Head and neck cancers belong to the group of tumors with a relatively high mutational load, which may make them susceptible to immunotherapies. Thus far, treatment is still mainly based on stage and site, and not on genetic or other molecular classifications. This may change on short notice for the oropharyngeal tumors that are caused by HPV.

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