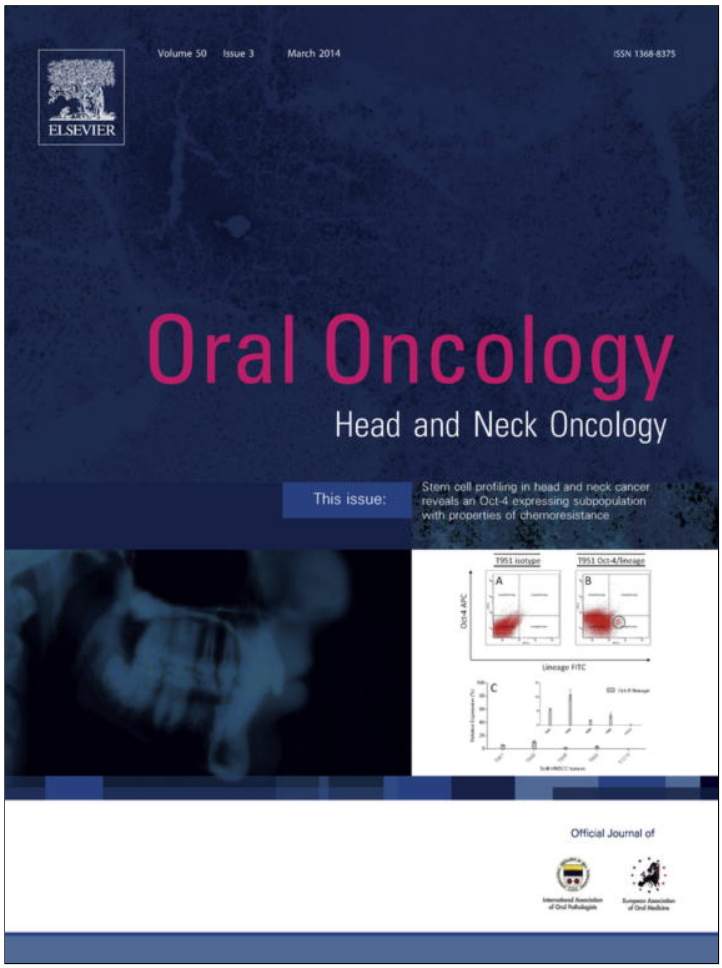

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Review

Clinical significance of head and neck squamous cell cancer biomarkers

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SUMMARY

Head and neck tumors belong among the six leading causes of cancer death worldwide. The predominant type of head and neck tumors consists of squamous cell carcinomas (HNSCC). Early detection of primary tumor and relapse is a key factor for enhancing the survival rate of HNSCC patients, because high rates of cases are recognized at advanced stages. Accordingly, biomarkers suitable for the early detection of HNSCC are sorely needed to improve patient outcomes. HNSCC evolve through a multistep process by the accumulation of genetic and phenotypic changes. Searching for specific biomarkers capable of characterizing each degree is therefore really essential.

In this review, genomic and gene expression alterations of HNSCC are summarized and associated with HPV status, clinicopathological conditions, and patient history from the perspective of potential biomarker utilization. The emphasis is placed on non-invasive markers detectable from saliva and blood and clinically relevant studies are mentioned in particular. These include analyses of tumorous tissues, saliva, and blood from patients with histologically defined tumors; cell culture- and other in vitro-based studies with no clinical correlations are rather excluded.

Introduction

Head and neck cancers include several types of cancer originating from the head or neck region, not including thyroid or skin cancers. The predominant (95%) type consists of squamous cell carcinomas whilst 4–5% are salivary gland (adeno) or other carcinomas [1]. Head and neck squamous cell carcinomas (HNSCC) belong among the six most common cancers worldwide [2]. HNSCC develop from the mucosal linings of the upper respiratory tract. Major risk factors associated with the development of HNSCC are smoking or tobacco chewing, alcohol consumption, use of smokeless tobacco products, and genetic predisposition. Tobacco smoking and alcohol consumption have a synergistic effect [3].

Furthermore, human papillomavirus (HPV) infection was identified as one of the primary causes of HNSCC. About 40–80% of oropharyngeal tumors are inflicted by HPV infection in the USA, whereas HPV cancer incidence in Europe changes from 90% in Sweden to approximately 20% in countries with the highest tobacco consumption [4]. Epstein-Barr virus (EBV) can also be a causative agent of nasopharyngeal carcinoma [5]. Eventually, some inherited disorders, such as Fanconi anemia, predispose to HNSCC [6].

Together with progress in treatment, early detection of primary tumor and relapse is a key factor for improving the survival of patients with HNSCC, because high rates of cases are recognized at advanced stages. Deeper understanding of the molecular biology of HNSCC can provide new insights into its development and progression; it also provides various biomarkers with a potential application for cancer screening and monitoring of the response to therapy. Although there is a number of reviews regarding HNSCC biomarkers [7–11], none of them currently provides a general overview of the topic. There are rather exhaustive reviews dedicated to specific issues, which include e.g. HNSCC and miRNAs, HPV status, molecular characteristics, and others. In this review, genomic and gene expression alterations of HNSCC are summarized and associated with HPV status, clinicopathological conditions, and patient history from the perspective of potential biomarker utilization. The emphasis is placed on non-invasive markers detectable from saliva and blood and clinically relevant studies are mentioned in particular. These include analyses of tumorous tissues, saliva, and blood from patients with histologically defined tumors; cell culture- and other in vitro-based studies with no clinical correlations are rather excluded.

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HPV in head and neck carcinogenesis

HNSCC is a heterogeneous disease containing leastways two divergent groups: (a) tumors caused by HPV infection, and (b) tumors caused by other mechanisms. Approximately 20–25% of HNSCC are HPV-positive, generally arising in the oropharynx [11,12]. A majority of HPV-induced HNSCCs are caused by HPV-16 [13]. HPV-positive HNSCC patients tend to be younger, with no former experience of tobacco and heavy alcohol consumption. Moreover, HPV-positive HNSCC can also be sexually transmitted; a significant association was revealed between HPV-16 positive HNSCC and oral sex [14].

HPV-associated HNSCCs mostly emerge in the lingual and palatine tonsils, because HPV targets preferably the extremely specialized reticulated epithelium of tonsillar crypts [15]. Active HPV infection results in several alterations in key cell signaling pathways that promote tumorigenesis. In particular, the expression of E6 and E7 viral proteins leads to the inactivation of two key tumor suppressors, p53 and Rb (retinoblastoma protein). The E6 protein is a small polypeptide that contains two zinc-binding domains [16] and stimulates p53 degradation [12]; a significant decrease in the expression of p53 and p21 was observed in the HPV 16/18 positive sinonasal-inverted papilloma compared with the HPV 16/18 negative sinonasal-inverted papilloma [17]. Besides, the HPV-16 E6 protein can also activate telomerase [18]. Similarly as the E6 protein, the E7 protein is also a small, nuclear polypeptide. The carboxyl-terminus of E7 contains a zinc-binding domain. By contrast to E6, E7 binds to Rb. The underphosphorylated Rb binds E2F and thus prevents the E2F-mediated S-phase induction (Fig. 1) [4,19]. Under physiological conditions, the intracellular accumulation of p16 protein inhibits the progression of cell cycle through cyclin D1 and CDK4/CDK6-mediated events. By contrast, HPV E7 overrides this important cell cycle control, pushing the cells from G1 into S phase [20], because the disrupted binding of E2F to Rb allows E2F to bind DNA and induce cell growth and proliferation [21]. In sum, both E6 and E7 promote cell cycle progression through its activity at different points of cell cycle regulation. In addition, the E7 protein induces abnormal centrosome duplication, resulting in multipolar, abnormal mitoses, aneuploidy and genomic instability [22].

In the context of HPV positivity or negativity, other molecular changes should be assessed. In Fischer et al. study, p21WAF1/Cip1 was highly expressed in HNSCC samples from larynx and pharynx. Its higher expression was correlated with lymph node metastases, decreased survival rate, and locoregional relapse [23]. HPV status was not given in this study. In a more accurate study, high p21WAF1/Cip1 expression was associated with better outcome in HPV-positive HNSCC [24]. Furthermore, in HPV-negative HNSCC, p53 is often mutated, Rb levels are normal, and p16 protein is decreased. Other known differences include the frequent hypermethylation of *14-3-3σ* and *RASSF1A* promoters and the cyclin D gene amplification in HPV-negative HNSCC [25–27]. The result of the hypermethylation of these genes is similar: it abolishes the cell cycle arrest. RASSF1A is a tumor suppressor, which binds to microtubule-binding proteins and regulates the cell cycle and apoptosis in response to mitogenic or apoptotic impulses. The repression of cyclins A and D1 by RASSF1A results in the cell cycle arrest [28]. Protein 14-3-3σ negatively regulates the cell cycle progression by inhibiting activities of cyclin-dependent kinases and Akt oncogenic signaling [29,30]. Furthermore, it was demonstrated, that inactivation of 14-3-3σ by promoter methylation correlates with metastases in nasopharyngeal carcinoma [31].

In addition, the selective upregulation of TCAM-1 (testicular cell adhesion molecule 1) in HPV-positive HNSCC tissue samples was observed by multiple researchers [32,33].

Major differences between head and neck squamous cell carcinomas (HNSCCs) according to the human papillomavirus (HPV) status are listed in Table 1.

Precursor lesions and genetic progression of HNSCC

Malignant transformation of the mucosal lining is a complex genetic mechanism ensuing from the accumulation of multiple genetic alterations, which influence the probability and rate of progression to invasive carcinoma, see Fig. 2 [7]. Cancerogenesis is a multistep process; numerous studies pointed out the fact that individual steps could be characterized by specific genetic or molecular alterations. Therefore, potential biomarkers with regard to tumor progression are mentioned in this chapter.

Precursor lesions

Genetic analysis of surgical margins indicated that HNSCC frequently develops in the field of genetically altered epithelial

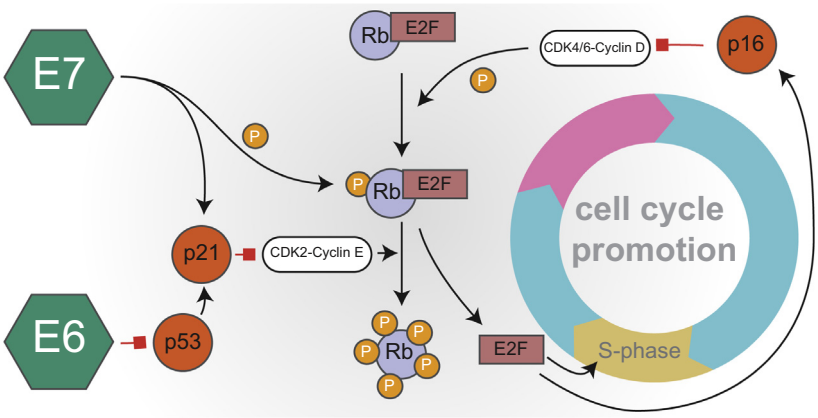

Figure 1. Main mechanisms of HPV-induced oncogenesis – Both E6 and E7 viral proteins can form specific complexes with cellular tumor suppressor gene products. The E7 protein binds and inactivates the retinoblastoma tumor suppressor Rb with a preference for the underphosphorylated, "active" form of Rb. The Rb family of proteins plays an essential role in controlling the cell cycle by governing the checkpoint to S phase. Underphosphorylated Rb binds to the E2F transcription factor forming an Rb-E2F complex, making E2F inaccessible for the transcription of genes associated with DNA synthesis. After the phosphorylation of Rb by cyclin-CDK complexes or Rb inactivation by E7 viral protein, E2F is released from the Rb-E2F repressor complex and can induce the transcription of S phase genes. Inactivation of Rb and inhibition of feedback loop mechanism lead to the overexpression of p16 protein. E7 viral oncoprotein can also interact with other cellular factors that control the cell cycle including the CDK inhibitor p21. Furthermore, HPV E6 proteins can bind to the p53 tumor suppressor protein and promote p53 degradation. Red arrow indicate inhibitory effect. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Major differences between head and neck squamous cell carcinomas according to the human papillomavirus status.

	HPV-positive HNSCC	HPV-negative HNSCC
Risk factors	High-risk sexual practices	Cigarette and alcohol use
Tumor site	Lingual and palatine tonsils	Non-oropharyngeal sites
Histopathology	Basaloid, non-keratinizing, poorly differentiated	Keratinizing, moderately differentiated
p53 pathway disturbances	Degradation of wt p53 by E6	*TP53* mutations, 17p LOH
Protein Rb pathway disturbances	Degradation of wt pRb by E7, p16 overexpression	$p16^{INK4A}$-promoter hypermethylation, 9p LOH
Cyclin D	*Cyclin D* gene amplified less frequently	*Cyclin D* gene is amplified frequently
TCAM-1	Upregulation	Non
14-3-3σ and RASSF1A	Low level of promoter methylation	Promoter hypermethylation
Relative responsiveness to chemoradiation	Better	Worse
Relative prognosis	Improved	Worse

HPV (human papillomavirus); LOH (loss of heterozygosity); pRb (retinoblastoma protein); wt (wild type). Adapted and extended from Pai et al. [11].

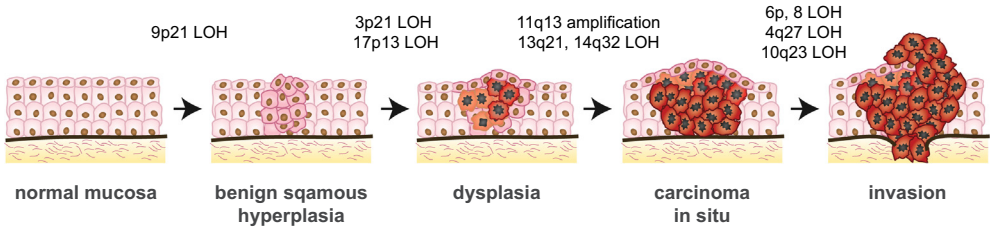

Figure 2. Genetic changes associated with the histological progression of HNSCC based on chromosomal material changes. LOH (loss of heterozygosity); Candidate tumor suppressors include p16 (9p21), p53 (17p13), RASSF1A (3p21), PTEN (10q23), and Rb (13q21). Candidate proto-oncogene includes cyclin D1 (11q13).

cells that are referred to as precursor fields [34,35]. Only a minority of the precursor fields might appear as clinically identifiable lesions, which show as either white or red mucosal areas (leukoplakia and erythroplakia). In such easy-to-diagnose precursor fields a tumor can develop; thus, these tumors are usually soon resected. However, the fields are not always resected entirely and malignant transformation of an unresected precursor field might cause a local relapse that is clonally related with the field and the primary tumor [36,37]. Recently, it became possible to visualize these fields using autofluorescence [38,39]. Furthermore, the development of local relapse was significantly associated with the low expression of keratin 4 and cornulin in the surgical margins of the index tumor [40]. The authors therefore propose using those genes to verify the resection margins. Ploidy studies of dysplastic leukoplakias demonstrated that most aneuploid lesions resulted in tumor occurrence, but only 60% of tetraploid lesions and only circa 3% of diploid lesions did the same [41]. Analogous studies on erythroplakias confirmed the potential of aneuploidy in predicting the SCC progression [42]. Furthermore, poorly differentiated tumors overexpress genes involved in cell adhesion, embryonic development, motility, differentiation, and extracellular matrix, whereas well-differentiated tumors overexpress genes involved in anti-apoptotic pathways, metabolism, and epithelial cell differentiation [43].

Loss of heterozygosity and chromosomal aberations

Loss of heterozygosity (LOH) is an important marker of tumor progression and can cause inactivation of the tumor suppressor gene. Traditional methods of mapping LOH regions include the comparison of both tumor and patient-matched normal DNA samples. LOH studies for HNSCC show that the earliest alterations target genes located on chromosome locations 3p (*RASSF1A*), 9p21 (cyclin-dependent kinase inhibitors), and 17p13 (*TP53*) [44].

A loss of chromosomal region 9p21 is found in 70–80% of dysplastic lesions of the oral mucosa. At 9p21 two functionally and structurally different cell cycle regulators, p16 (INK4a) and p14 (ARF), encoded by the gene *INK4a/ARF* are located. 9p21 LOH and inactivation of the remaining alleles of *INK4a/ARF* by promoter hypermethylation represent early and frequent events in the progression of HNSCC [35,45] together with the overexpression of EGFR, which rises with the increasing severity of dysplasia in premalignant lesions [46,47].

Chromosomal alterations which occur in connection with advanced grades of dysplasia and HNSCC include amplification of 11q13, PTEN (10q23.3) inactivation, and LOH at 13q21, 14q32, 6p, 8p, 4q27 [8,9,35,48]. The amplification of 11q13 was reported in one-third of HNSCC, where amplified cyclin D1 inflicts the cancer phenotype [49,50]. Genetic alterations in PTEN occur in 5–10% of HNSCC lesions and the loss of PTEN expression can be observed in approximately 30% of HNSCCs. The lack of PTEN expression could be an independent prognostic factor of poor clinical prognosis [51]. In sum, losses of heterozygosity were more frequently found in the histologically higher-grade lesions and in the lower-grade lesions when a high proliferation rate was present [48].

Protein markers of tumor progression

Several studies reported increased metallothionein (MT) protein levels in malignant tumors in the head and neck area [52–55]. Metallothioneins seem to support proliferation and anti-apoptotic activity and are considered to be involved in microenvironment remodelling [56]. Sochor et al. determined MT levels in tumor tissues of patients suffering from head and neck tumors using differential pulse voltammetry [54]. The highest MT level was determined in the tissues of oral tumors (170 ± 70 μg/g wet weight tissue, wwt) followed by tumors of hypopharynx (160 ± 70 μg/g wwt) and larynx (160 ± 70 μg/g wwt). The relatively lowest MT level was determined in oropharynx tumors (130 ± 50 μg/g wwt). In the study of Dutsch-Wicherek et al., tissue samples taken from patients with pharyngeal squamous cell carcinomas were analyzed. An increased MT immunoreactivity was observed in tissue samples from tonsillar squamous cell carcinomas in comparison to the reference group with chronic tonsillitis [56].

Furthermore, elevation of cyclooxygenase-2 (COX-2) was described in HNSCC at both mRNA and protein levels [57]. Increased expression of COX-2 resulted in angiogenesis promotion through an elevated level of prostaglandins E2 and VEGF, thus leading to

the promotion of tumor growth [58]. Angiogenesis is a crucial process in tumor growth and subsequent metastasis. Inter alia, oral cancer overexpressed 1 and 2 (ORAOV1 and 2) transmembrane proteins are involved in tumor angiogenesis and regulation of cell growth [59,60]. Overexpression of ORAOV1 as well as ORAOV2 was reported in HNSCC [60–62]. Another mechanism included in the tumorous angiogenesis lies in the intake and utilization of locally stored fibroblast growth factors (FGFs). The role of FGF binding protein (FGF-BP) in this mechanism is considered. Li et al. demonstrated a FGF-BP mRNA expression in primary HNSCC specimens and metastatic tumor specimens but not in adjacent control tissues [63]. Over and above, the supporting stroma of most epithelial cancers contains specialized fibroblasts, known as reactive tumor stromal fibroblasts or cancer-associated fibroblasts (CAFs). A relatively specific molecular marker of CAFs is the expression of fibroblast activation protein (FAP), which is a cell-surface protease [64]. The limited expression of FAP in normal tissues and benign epithelial tumors in contrast to its abundant expression in the stroma of many types of epithelial cancers show FAP as a promising marker of the malignant progression of cancer cells [65].

Growth factors and their receptors play an unarguably important role in HNSCC. EGFR, a member of the cerbB family of tyrosine kinase receptors, has been shown to be expressed in many tumors, including in up to 80% of HNSCC [66], where it was associated with poor patient outcomes [67]. The EGFR expression was namely associated with the higher proliferative index, advanced HNSCC tumor stage, and increased tumor angiogenesis [68]. While all members of the cerbB family were overexpressed in HNSCC, the overexpression of EGFR was usually the highest [68]. Potential biomarkers of HNSCC progression are listed in Table 2.

Tumor progression and miRNAs

Discoveries in the expression profiling of microRNA in head and neck oncology promise a great progress in the diagnosis, prognosis and therapy of HNSCC. Although the microRNAs are important regulatory factors in cancer developement, our understanding of the role of miRNAs in the HNSCC oncogenesis remains unclear. Nevertheless, some alterations consistently identified in head and neck cancer, such as upregulation of miR-21, miR-31, miR-155, and downregulation of miR-26b, miR-107, miR-133b, miR-138, and miR-139 were found [69,70].

Biomarkers of promoting metastases

Regardless of progress in the HNSCC treatment, the survival rate of five years after diagnosing advanced HNSCC remains insufficient, approximately 50%. One reason for high mortality associated with the late stage HNSCC is the inherent capability of tumor cells to go through locoregional invasion due to the presence of a rich lymphatic network and the overall high number of lymph nodes in the neck region [71]. Even in patients without the clinical evidence of lymph node involvement (N0), there is a high incidence of occult lymph node metastases, ranging from 10% to 50% [72]. The presence of lymph node metastases is significantly associated with the poor patient outcome [73]. The diagnosis of neck lymph node metastases is an essential requirement for clinical staging and treatment, and is now widely accepted as the most important factor in HNSCC prognosis [74–76]. HNSCC invasion and nodal metastases constitute a complicated process involving different signaling pathways and proteins; however, some possible markers of the metastatic process were discovered (see Table 3).

HNSCC is known to be associated with extensive chromosomal alterations. Numerous chromosome alterations associated with the propensity to metastasize were found (gains: 4q11–22, and Xq21–28; losses: 8p, 6p, 11q14–24, 4q26–28, 11q, and 17p11–12) [9,35,43]. Several genes associated with cell signaling (*BEX1*, *BEX2*, *ZNF6*, *NGFRAP1L1*, *GPRASP2*) are clustered just on chromosome Xq21–22, whose chromosome gain is associated with HNSCC metastasize [43]. This may be considered an explanation why the female sex is associated with the bad response to organ-sparing therapy and poor outcome [77].

Rickman et al. proposed four-gene model (*PSMD10*, *HSD17B12*, *FLOT2* and *KRT17*) as a predictor of metastases. HPV positive samples were eliminated from the study. The four-gene model was highly associated with the development of metastases (hazard ratio 6.5; 95% confidence interval 2.4–18.1). This four-gene model predicted the occurrence of metastases with 74% sensitivity and 78% specificity [43]. Furthermore, Rickman et al. found downregulation of genes that encode proteins involved in apoptosis (CASP1,

Table 2
Potential biomarkers in head and neck cancers.

Major classes	Member	Function
Cell-cycle regulation	*p16^{INK4A}*	A tumor-suppressor gene regulating senescence and cell-cycle progression [45]
	TP53	A tumor-suppressor gene regulating cell-cycle progression and cell survival [36]
	PTEN	A tumor-suppressor gene regulating signaling pathways controlling cell proliferation and apoptosis [51]
Cell-cycle regulation	*Rb*	A tumor-suppressor gene regulating cell-cycle progression and apoptosis [21,45]
	Cyclin D1	A proto-oncogene regulating cell-cycle progression [49]
	Bmi-1	Controls cell cycle and self-renewal of tissue stem cells [124]
Signal transduction	EGFR	A transmembrane TK that acts as a central transducer of multiple signaling pathways [77]
	VEGF	A transmembrane TK that promotes proliferation, migration, and survival of endothelial cells during tumor growth [125]
	PIK3CA	A gene encoding the p110α subunit of phosphoinositide 3-kinase (PI3K) α [126,127]
Secreted protein	FGF-BP	Fibroblast growth factors binding protein [63]
	FAP	Protein secreted by cancer associated stroma [60]
Transmembrane protein	*TMEM16 (ORAOV2)*	Calcium-activated chloride channel [60]
	ORAOV1	A regulator of cell growth and tumor angiogenesis [59,61,62]
Transcription factors	NFκB	Proinflammatory transcription factor [84]
Prostaglandin metabolism	Cox-2	Catalyzes prostaglandin synthesis from arachidonic acid [57,128]
Metal ion homeostasis, oxidative stress	MT	Low-molecular weight proteins involved in heavy metal detoxification, essential metal ion homeostasis and cell protection against free radicals [53]
Oncoviruses	EBV	A causative agent for most nasopharyngeal carcinomas, plasma EBV DNA load is an independent prognostic factor [129,130]
	HPV	A causative agent for most oropharyngeal cancers [13]

EBV (Epstein-Barr virus); EGFR (Epidermal growth factor receptor); HPV (Human papillomavirus); Rb (Retinoblastoma); TK (Tyrosine kinase); TP53 (Tumor protein 53); FAP (Fibroblast activation protein); VEGF (Vascular endothelial growth factor); MT (Metallothionein); Cox-2 (Cyclooxygenase-2); Bmi-1 (B cell-specific Moloney murine leukemia virus integration site 1); ORAOV (Oral cancer overexpressed gene). Adapted and extended from [11].

Table 3
Biomarkers involved in promoting metastases.

Biomarker	Function	Expression	HPV status	References
CASP1	Apoptosis	↓ mRNA	HPV-negative	[43]
CCR7	Chemokine receptor	↑ mRNA, protein	NA	[131–133]
CD44	Cell–cell and cell-matrix adhesions	↑ mRNA, protein	NA	[134–136]
CEP55	Cell cycle regulation, cytokinesis	↑ mRNA, protein	NA	[137]
c-Met	Proliferation	↑ protein	NA	[138]
COL17A1	Cell interactions	↓ mRNA	HPV-negative	[43]
Cortactin	Cell motility and invasion	↑ mRNA, protein	NA	[139–142]
CXCR4	Chemokine receptor	↑ mRNA, protein	NA	[131,132,143,144]
DAPK3	Apoptosis	↓ mRNA	HPV-negative	[43]
DSG3	Cell-to-cell adhesion	↑ protein	NA	[145]
DST	Regulation of the cell cycle	↓ mRNA	HPV-negative	[43]
Hif-1α	TF, hypoxia	↑ protein	NA	[146,147]
IL18	Apoptosis	↓ mRNA	HPV-negative	[43]
LRP6	Signaling	↑ mRNA	HPV-negative	[43]
MMP-2	ECM degradation	↑ mRNA, protein	NA	[137,148]
MMP-9	ECM degradation	↑ protein	NA	[134,136,149]
MYCN	Signaling	↑ mRNA	HPV-negative	[43]
NBS1	Cell cycle regulation, DNA double-strand break repair	↑ mRNA, protein	NA	[148,150]
NFκB	Proinflammatory TF	↑ mRNA, protein	NA	[84,151,152]
PPP2R1B	Apoptosis	↓ mRNA	HPV-negative	[43]
RSK2	Cell cycle regulation, proliferation, apoptosis	↑ protein	NA	[153]
Snail	TF, regulator of EMT	↑ protein	NA	[146,148]
SPP1 (osteopontin)	Secreted phosphoprotein	↑ mRNA, protein	NA	[60,154]
Survivin	Inhibitor of apoptosis	↑ protein	NA	[155–157]
Twist	TF, regulator of EMT	↑ protein	NA	[132,146]
VEGF/R	Angiogenesis	↑ protein, mRNA	NA	[112,158–160]
14-3-3σ	Cell cycle regulation	↓ mRNA, protein	NA	[31]

MMP (matrix metalloproteinase); ECM (extracellular matrix); TF (transcription factor); EMT (epithelial-mesenchymal transition); VEGF/R (Vascular endothelial growth factor/receptor); NA (not available).

DAPK3, IL18, PPP2R1B), negative regulation of the cell cycle (DST) and cell interactions (COL17A1), and upregulation of genes encoding proteins involved in signaling (MYCN, LRP6) in tumors which have developed metastases. Thereunto, overexpression of secreted phosphoprotein 1 (SPP1) correlated with lymph node metastasis and lymphatic invasion in Kashyap et al. [60].

Prognosis and survival markers

Many factors can influence the prognosis of HNSCC patients. The most important ones include the cancer type and location, stage of disease, and cancer grade. Other factors affecting prognosis include biological and genetic features of cancer cells, age of the patient, and general health condition and response to treatment.

HPV status is one of major factors affecting the prognosis. Specific molecular characteristics of HPV-positive tumors are discussed in the following chapter; in this chapter, the associations of HPV status with the prognosis are only mentioned. There are profound prospective and retrospective evidences that the subgroup of patients with HPV-positive oropharyngeal cancer has a more favorable prognosis as compared to patients with oropharynx cancer negative for HPV [19,78]. A meta-analysis of 23 studies analyzing the survival in 1747 HNSCC patients stratified for HPV status found a hazard ratio of 0.72 (95% CI, 0.5–1.0) for the overall survival in HPV-positive oropharyngeal HNSCC patients, and a hazard ratio of 0.51 (95% CI, 0.4–0.7) for the disease-free survival compared to HPV-negative patients [79]. Two recent studies suggest that patients with HPV-positive HNSCC have a lower risk of second primary cancers when compared to patients with HPV-negative tumors [80,81]. In these studies, the rate of second primary cancers in HPV-positive HNSCC patients was observed to range from 0 to 2.2% compared to 10.2–13% in HPV-negative patients. Moreover, HPV-positive HNSCC seems to be more radiosensitive than HPV-negative disease. It can be consequence of differences in p53 between the tumor types; although HPV-positive tumors have reduced levels of p53 due to E6-mediated degradation, the p53 protein is intact and not mutated, unlike the high percentage of HPV-negative tumors, which have mutated p53. Radiation-induced increases of p53 levels may be sufficient to provoke programmed cell death in the HPV-positive tumors in response to radiation-induced damage [82]. Furthermore, the presence of p53 mutations in surgical margins was found as an independent prognostic indicator for locoregional recurrence (relative risk = 7.1; $p = 0.021$; 95% confidence interval, 0.9–56) [36]. On the contrary, the absence of p53 mutations in surgical margins was significantly associated with no local and locoregional recurrence ($p = 0.027$ and $p = 0.028$, respectively).

For further, low EGFR and high p16 (which correlates with higher HPV titer) expressions were established as markers of good response to organ-sparing therapy, whereas high EGFR expression, combined low p53/high Bcl-xL expression, female sex, and smoking were associated with a poor outcome [10,77]. There was also some evidence of improved overall survival in patients with oropharyngeal squamous cell carcinoma with raised Bcl-2, amplification of 11q3 and loss of 16q genes, and low levels of c-met, Ki67, IMD, PLK, FHIT, nuclear survivin, or nuclear cyclin D1 [10]. On the other hand, the lack of PTEN expression and the loss of heterozygosity could be independent prognostic indicators of poor clinical prognosis [48,51] as well as elevation of mRNA and protein COX-2 levels [57,83].

An important role in HNSCC pathogenesis seems to be played by transcription factors such as NF-κB. NF-κB is a family of transcription factors composed of hetero- or homo-dimers from five different subunits, NF-κB1, NF-κB2, RELA, cREL and RELB. NF-κBs are transiently activated under physiological conditions in response to infection or injury, but these genes are aberrantly overactivated in cancers, promoting pathogenesis and therapeutic resistance. In HNSCC, a major role of NF-κB in the regulation of tumorous transcriptome and proteome was appointed [84]. Human head and neck squamous cell carcinomas were among the first cancers for which a proof was gained for incorrect constitutive activation of NF-κB [85]. Subsequent studies have shown that NF-κB is acti-

vated in squamous dysplasias and carcinomas and correlate with the progression of dysplasia and decreased survival rate in patients with HNSCC [86]. Aberrant NF-κB activation was detected in tobacco-associated as well as in viral-related HNSCC; these include EBV-related nasopharyngeal and HPV associated oropharyngeal carcinomas [85].

A key inhibitor of tumor suppressor p53 (iASPP) was found to be up-regulated in malignant conditions. Immunohistochemical staining indicated iASPP in both cytoplasm and nucleus. Importantly, the overexpression of cytoplasmic and nuclear iASPP was significantly associated with T, clinical stage, lymph node metastasis, and recurrence. Survival analysis demonstrated high iASPP expression in a significantly negative correlation with the disease-free survival and overall survival [87]. Coexpression of MMP7, MMP9, and MMP13 has also been associated with the poor outcome in esophageal squamous cell carcinoma ESCC [88].

Non-invasive biomarkers in HNSCC

Serum, plasma, and saliva contain a number of stable markers, whose differential expression patterns seem to be specific for certain diseases. Noninvasivity of these markers is very important for the acquisition of healthy controls for oncological case-control studies. Obtaining control healthy tissues from patients is not ideal, because of the genesis of unexpected alterations in the expression of selected mRNA, miRNA or proteins in a body burdened with neoplastic processes.

Salivary markers

The uppermost advantage of saliva as a diagnostic tool is the fact that it contains cells detached from the oral cavity and is in a direct contact with oral cancer lesions.

Hu et al. successfully confirmed five candidate biomarkers inclusive of myeloid related protein 14 (MRP14), Mac-2 binding protein (M2BP), profilin 1, CD59, and catalase on oral cancer patients and matched controls [89]. Furthermore, autoantibodies against the aberrantly expressed p53 were found in both saliva and serum of patients with oral cancer. P53 antibodies positivity strongly correlated with the poor treatment outcome in cancer patients [90–92]. Moreover, Sato et al. found higher interleukin-6 (IL-6) concentrations in saliva of patients with oral cancer than in controls [93]. Multivariate analysis revealed that postsurgery salivary IL-6 concentration was an independent risk factor for locoregional recurrence in patients with oral squamous cell carcinoma (OSCC) ($p = 0.03$; relative risk, 0.14) [94,95]. Brailo et al. observed that salivary TNF-α levels and IL-6 levels were significantly higher in patients with oral leukoplakia in comparison with healthy controls [96]. In accordance, Rhodus et al. referred significantly increased salivary concentrations of IL-8, IL-1, IL-6 and TNF-α in the oral cancer group in comparison with the patients with dysplastic oral lesions and controls [97]. IL-8 was also detected at higher concentrations in the saliva ($p < 0.01$) of patients with OSCC compared with healthy controls ($p < 0.01$). These results were confirmed at both the mRNA and the protein levels [98]. Zhong et al. [103] found a 75% positive expression of telomerase in the saliva of OSCC patients. Using quantitative proteomics methods, higher levels of actin and myosin were also observed in the saliva of patients with malignant oral lesions in comparison to those with pre-malignant lesions. Sensitivity/specificity values for distinguishing between pre-malignant lesions and malignant lesions were 100%/75% ($p = 0.002$) for actin, and 67%/83% ($p < 0.00001$) for myosin in soluble saliva [99]. Salivary transferrin was also studied as a biomarker of early stage oral cancer detection. Increased salivary transferrin levels in patients with OSCC strongly correlated with the tumor size and stage [100]. The tumor-specific mRNA in saliva could also be utilized as a biomarker for oral cancer. Elevated salivary mRNA for IL-1B, IL-8, dual specificity phosphatase 1 (DUSP1), ornithine decarboxylase antizyme 1 (OAZ1), H3 histone, family 3A (H3F3A), S100 calcium binding protein P (S100P), and spermidine/spermine N1-acetyltransferase (SAT) were clearly documented as oral cancer biomarkers [101,102].

Melanoma-associated antigen proteins (MAGE) suppress apoptosis and support proliferation therefore have a crucial role in carcinogenesis [103]. MAGE expression was detected in the sputa of HNSCC patients [104]. Since MAGE is not ordinarily expressed in normal tissues, except for the testis, and 5-year survival of pharyngeal cancer patients was lower in cases with MAGE-A expression, it could be considered as a promising marker for the detection of HNSCC [105–108].

With regard to salivary microRNAs, miR-31 was found to be elevated in HNSCC and could serve as a useful predictor for early detection and post-operative follow-up [109]. Furthermore, two miRNAs, miR-125a and miR-200a, were present at significantly lower levels ($p < 0.05$) in the saliva of patients with oral squamous cell carcinoma than in the saliva of control subjects [110].

Blood markers

There have been some studies of cytokines and angiogenesis factors as potential useful serum markers of disease progression, cancer recurrence and survival of patient with HNSCC [111]. For example, the serum levels of VEGF were significantly higher in patients with the advanced T stage (T3 or T4) ($p = 0.001$), lymph node metastases ($p < 0.001$) and advanced stages (stage III or IV; $p < 0.001$) [112,113]. Furthermore, mean serum concentrations of IL-8, hepatocyte growth factor (HGF), and growth regulated oncogene 1 (GRO-1) were increased in patients with HNSCC [114]. Serum concentrations of IL-6 were also significantly higher in patients compared with the levels detected in healthy individuals and subjects with oral premalignant lesions [98,114,115]. The serum IL-6 levels were especially high in patients with the higher pT status ($p < 0.001$), higher pathological stages ($p < 0.001$), positive bone invasion ($p < 0.001$), and higher tumor depths ($p = 0.005$). Patients with higher pre-treatment IL-6 levels (> 1.35 pg/mL, median level) had worse prognoses for 5-year overall survival and disease-specific survival despite the treatment [115].

Changes in the first post-treatment serum cytokine levels were correlated with response, progression, and survival. Post-treatment increases in IL-6 or HGF were observed in patients who had a relapse and inflammatory or infectious complications. Some relationship between the change in the pre-treatment and first post-treatment cytokine measurement with survival was detected for HGF, IL-8, IL-6, and VEGF. The association between longitudinal decreases in IL-6, L-8, VEGF, and HGF throughout the follow-up with survival was detected with a time-dependent Cox model ($p = 0.01$, 0.07, 0.08, and 0.05, respectively) [114].

Furthermore, patients with HNSCCs had significantly higher serum MMP-3, -7, and -9 titers than controls ($p < 0.001$). The elevated MMP-3 and MMP-9, but not MMP-7, correlated with distant metastases and poor survival ($p < 0.05$) [116,117]. Kuropkat et al. showed a significant increase of MMP-8 in the serum of HNSCC patients [117], and Yen et al. reported MMP-10 and MMP-1 to be suitable markers for OSCC disclosure, with gingiva and margin as controls [118].

Chang et al. showed serum levels of C-reactive protein (CRP), matrix metalloproteases MMP-9, MMP-2, transforming growth factor-beta 1 (TGF-beta 1), IL-6, and E-selectin as having power of discrimination between leukoplakia, patients with untreated oral cavity squamous cell carcinoma, and age- and gender-matched healthy control groups with significant elevation trends

of those markers from control to OSCC. All examined markers decreased in relapse-free patients following the treatment. However, in patients with a relapse, IL-6, CRP, and serum amyloid A remained at elevated levels [119].

High levels of MMP-2 or MMP-9 were detected in the plasma of patients suffering from different kinds of cancer, including HNSCC [120].

Krejcova et al. analyzed MT levels in the blood of patients suffering from primary malignant tumor in the head and neck area. Tumor blood samples originated from patients with oropharyngeal cancer, laryngeal cancer, hypopharyngeal cancer, oral cavity cancer and rarely occurring nasal cavity and paranasal sinus cancers. Blood MT levels of healthy controls were lower than blood MT levels of oncological patients [55]. Up-regulation of miR-31, miR-10b, miR-24, miR-181, and miR-184 in the plasma of OSCC patients was also found [121].

Conclusions

Poor prognosis of HNSCC is mainly due to late disease presentation and lack of suitable biomarkers to detect the disease progression. Accordingly, biomarkers suitable for the early detection of HNSCC are sorely needed to improve patient outcomes. HNSCC evolve through a multistep process by the accumulation of genetic and phenotypic changes. Searching of biomarkers specific for high-risk tumors in early stages is therefore really essential. The application of molecular biologic techniques is promising in simplifying earlier detection and may generate protocols for screening of cancer patients. Molecular profiling may also help in the prediction of tumor behavior and responsiveness to therapy. The molecular pathways underlying tumorigenesis are ever better understood today, and therefore the number of possible biomarkers increases. More clinical studies, which will validate the sensitivity and specificity of these biomarkers in clinical settings are needed to translate these findings into potential strategies for early detection leading to improved patient outcomes.

While the detection of biomarkers and the targeted therapy for HNSCC have experienced a great progress, there are still significant facets of HNSCC that are not fully understood.

There is a 4% annual risk of HNSCC patients developing a second primary tumor [122]. These second primary tumors are thought to result from the "field cancerization" [11,123]. The next point of interest could be to identify biomarkers of field cancerization and to develop a target that would prevent disease recurrence or appearance of second primary tumors.

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Conflict of interest statement

None declared.

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