Biomarkers of head and neck cancer, tools or a gordian knot?

Evangeli S Lampri¹, Georgios Chondrogiannis², Elli Ioachim³, Anna Varouktsi⁴, Antigoni Mitselou⁵, Aggeliki Galani⁶, Evangelos Briassoulis⁷, Panagiotis Kanavaros², Vasiliki Galani²

Departments of ¹Pathology, ²Anatomy-Histology-Embryology, ⁵Forensic Pathology, ⁷Hematology, Faculty of Medicine, University of Ioannina, Greece; ³Department of Pathology, General Hospital “G. Hatzikosta”, Ioannina, Greece; ⁴Ippokratio Hospital, Thessaloniki, Greece; ⁵Department of Environmental and Natural Resources Management, School of Engineering, Agrinio, University of Patras, Greece

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Abstract: Head and neck tumors comprise a wide spectrum of heterogeneous neoplasms for which biomarkers are needed to aid in earlier diagnosis, risk assessment and therapy response. Personalized medicine based on predictive markers linked to drug response, it is hoped, will lead to improvements in outcomes and avoidance of unnecessary treatment in carcinoma of the head and neck. Because of the heterogeneity of head and neck tumors, the integration of multiple selected markers in association with the histopathologic features is advocated for risk assessment. Validation of each biomarker in the context of clinical trials will be required before a specific marker can be incorporated into daily practice. Furthermore, we will give evidence that some proteins implicated in cell-cell interaction, such as CD44 may be involved in the multiple mechanism of the development and progression of laryngeal lesions and may help to predict the risk of transformation of the benign or precancerous lesions to cancer.

Keywords: HPV, squamous cell carcinoma, molecular, pharyngeal carcinoma, carcinogenesis, genetic

Introduction

Head and neck cancers are markedly phenotypic and clinically heterogeneous neoplasms of different histogenesis. The most common is epithelial derived head and neck squamous cell carcinoma (HNSCC) with over 500,000 new cases expected worldwide [1]. An estimated 49,260 new cases of HNSCC were diagnosed in the United States in 2010 [2]. Advancement in treatment strategies did not influence significantly the survival rates, which remain poor, as recurrence, distant metastasis, and second primaries are developed in many patients [3].

Pathogenically, the development of these tumors has been associated with the mutagenic role of tobacco carcinogens [4], which can induce specific mutations in different genes. Carcinogens are strongly associated with HNSCC, suggesting a possible implication of pathways related with the cellular response to genotoxic damage; in addition, genes that are implicated in DNA repair processes or metabolism of carcinogens play their crucial role [5]. Nationality seems to influence the incidence of these tumors, as the Black population has higher rates than Hispanic and Asian population [6]. A role for certain types of human papillomaviruses has also been suggested.

The majority of patients present with late stage disease and the overall 5-year survival rate have not changed significantly over the last three decades, the fact that most of the patients will present with advanced disease increases significantly the morbidity and mortality. The WHO estimates oral cancer as having one of the highest mortality ratios of all malignancies [6]. Early detection in head and neck cancer has been shown to dramatically increase survival rates when compared to detection at later disease stages [7], being the most important variable leading to positive outcomes [8]. Moreover, there are not many screening tools and markers to discriminate the patients who are to be benefited by adjuvant therapy. Despite great advances in the surgical and non-surgical man-
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Management of these patients with favorable cure rate, therapies are aggressive and frequently significantly affecting the quality of life with severe short-term toxicity and long term functional impairment, such as speech and swallowing difficulties. Thus, there is a great necessity for better patient selection to avoid unnecessary treatment and minimize long-term toxicity. Fortunately, with the help of clinical trials, there is a great progress in the identification and validation of biomarkers as molecular targeted therapies. To determine which patients will benefit from these treatments requires personalized medicine and the analysis of factors must be specific to the individual and their tumor. The twenty-first century can be characterized as “the age of biomarker discovery” which promises an earlier cancer diagnosis and a more fruitful treatment [1, 9].

Presently, the clinical applications for tumor biomarkers include molecular margin control for surgical resection, tumor aggressiveness prediction for specific modulation of treatment, and identification of targets for gene-and protein-specific targeted therapy. The ultimate goal is the creation of markers for detection and treatment of pre-clinical and pre-malignant disease, avoiding the progression to cancer altogether [3]. A large number of biomarkers have been identified and are currently under study. Underscoring the numerous possibilities is an analysis of over 13,000 genes that found 1,260 with differential expression in HNSCC [3, 10].

The following review try to summarize some of the most relevant biomarkers related to head and neck cancer and discriminate those with the greatest predictive and therapeutic value.

Historical perspective

The search for a biomarker for head and neck cancer started many years ago. One potential biomarker was considered to be carcinoembryonic antigen (CEA) in blood and saliva of 439 HNSCC patients in 1976, but it did not prove to have any prognostic value [11-13]. One year later, Kato and Torigoe [14] described tumor associated antigen (TA-4), a number of proteins with a common antigenic determinant; 148 resulting in the development of SCC-antigen assays [15]. However, except from one study [16], all the others demonstrated that the squamous cell carcinoma antigen (SCC-antigens) role was limited to the possible monitoring of disease [17, 18]. Moreover, many of the biomarkers which proved to be useful in other carcinoma, like carbohydrate antigen (CA-19-9), did not show to have important prognostic significance in head and neck cancer [19].

Only one study occupied with the levels of CEA, CA19-9, SCC-antigen, thymidine kinase (TK), and deoxythymidine 5’-triphosphatase (dTTPase) in 26 patients prior to treatment and over time following treatment [20]. However, none of these markers by itself or in combination proved to have any significant correlation with tumor location, stage, or grade [3].

Micronuclei, chromosomal abnormalities, and ploidy

Micronuclei are chromatid fragments formed during abnormal division of cells with damaged DNA. They correlate with cancer risk and the reduction of micronuclei helps chemoprevention, but inconsistent clinical outcomes have minimized their utility [3, 21-23].

Chromosomal abnormalities have been extensively documented in HNSCC [24-26]. The long list of described chromosomal abnormalities involves many chromosomes, including 3, 4, 7, 8, 9, 11, 13, 17, 18, and 19. The changes include losses and gains of genetic mate specific gene alterations [27, 28]. In addition; there is evidence that the potential for malignant progression may be linked to certain chromosomal alterations. One study examined chromosomal changes at 3p, 4q, 8p, 9p, 11q, 13q, and 17p associated with clinical leukoplakias [29]. Changes at 3p or 9p in combination with any other change were associated with a 33-fold increase in risk for progression to cancer [3].

A chromosomal translocation t (11:19) between CRTC and MAML2 is present in the majority of mucoepidermoid carcinoma (50-80%) [9, 30]. Another recently described translocation t (6; 9) between MYB and NFIB in adenoid cystic carcinoma leads to overexpression of the MYB gene and downstream target genes including c-KIT which shows high-expression in these tumors [31]. Frequent loss of 1p32-p36 has also been identified in adenoid cystic carcinoma and correlated with solid/basaloid morphology and poorer prognosis [32]. These observa-
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sections favor potential tumor suppressor genes in this region which are lost and may allow for directed targeted therapy in the future [1].

DNA ploidy has been extensively studied as a marker of prognosis. Anything greater than the normal two (diploid) copies falls under the general category of polyploidy. Finally, if chromosomes are not evenly distributed to the daughter cells the resulting cells are aneuploid. These chromosomal changes are common in HNSCC and seem to have prognostic significance [3, 33, 34]. Based on these results, aneuploidy may prove to be a powerful marker of malignant potential, and poor prognosis [3].

Genetic instability (microsatellite alterations and loss of heterozygosity)

Microsatellites are common genetic elements of the human genome composed of highly polymorphic tandem repeat sequences of DNA. They have been successfully used as markers for tumor formation in a large number of malignancies, including HNSCC [35]. These sequences can be amplified with PCR technology to reveal loss of heterozygosity (LOH) or microsatellite instability. The process greatly enhances tumor suppressor gene mapping and identification of tumor clonality in serum and saliva [3, 35-37]. An early and common genetic event in oral premalignancy includes LOH of 9p21 in dysplasia/hyperplasia (30%) and carcinoma (70-80%) [38]. The detection of 3p and 9p loss by LOH analysis using comparative normal DNA (commonly peripheral blood lymphocytes) is currently being used in a NCI sponsored trial of Erlotinib in the prevention of oral cancer (EPOC trial) [1]. There is study which showed a trend towards increased instability with increased disease state [39]. Tetranucleotide microsatellite instability was recently tested in surgical margins as a predictor for recurrence [40].

Oncogenes and tumor suppressor genes

pP53 (TP53)

Mutations in p53 are one of the most frequent abnormalities in HNSCC. A number of studies have confirmed p53 mutation in 30% to 75% of HNSCC tumors [41, 42], raising the necessity each mutation to be specified. Mutations of p53 are met first in severe dysplasia. p53 overexpression was also correlated strongly with poor survival in invasive carcinomas (50% incidence) [43-47]. Especially, there was a statistically significant decrease in survival for patients with mutations in the DNA binding regions of p53 compared to those with p53 mutations outside the DNA binding sites [44]. The last finding may explain contrary reports showing a lack of prognostic ability of p53 [45, 46].

NOTCH1

NOTCH1 is the second most commonly mutated gene in HNSCC, with mutation rate of 14 to 15%. NOTCH1 is important in regulating normal cell differentiation, lineage commitment, and embryonic development. It appears to function as a tumor suppressor gene in HNSCC based on the position and characteristics of the mutations and the inactivation of both alleles. Most NOTCH1 mutations observed in HNSCC affect the epidermal growth factor (EGF)-like ligand-binding domain [48-50].

CDKN2A

Alterations of cyclin-dependent kinase inhibitor 2A (CDKN2A/p16INK4A), a tumor suppressor gene located on chromosome 9p21, have long been recognized in HNSCC [51, 52]. In the next generation sequencing studies, CDKN2A mutations were identified in 9 to 12% of all tumors [48-50]. Gene copy number analyses also revealed frequent loss of heterozygosity and deletions of CDKN2A [48]. Moreover, CDKN2A is inactivated by methylation of the 5' CpG region [53].

In addition, p16, which is protein product of CDKN2A, is implicated in cell cycle regulation via its interaction with the retinoblastoma (Rb) tumor suppressor. The p16 protein inhibits cyclin-dependent kinases (CDK) 4 and 6, which causes hypophosphorylation of Rb and therefore G1 arrest of the cell cycle 37-39. Although alterations of CDKN2A are common events in early HNSCC development, there are not enough to drive tumorigenesis. The last suggestion finds evidence by the fact that CDKN2A mutations have been reported in benign epithelial lesions with low potential for malignant transformation [50, 54-57].

Genes implicated in cell cycle

Although many papers published to date show a correlation between high proliferative activity
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and poor prognosis in head and neck tumors, most of them failed to show any significant association with prognosis for many reasons. One of them is anatomic site. In the last edition of the TNM classification of malignant tumors [58], head and neck tumors include tumors of the maxillary sinus, nasal cavity, ethmoid sinus, oral cavity, lip, salivary gland, thyroid gland, pharynx and larynx, dealing with heterogeneous tumors from different sites, histological types and prognosis [59-63].

**Ki67/MIB-1**

A high cell proliferation, as expressed by the MIB-1 labeling index, was a significant indicator for treatment failure in a large matched-pair study design of recurrent and non-recurrent oral and oropharyngeal carcinomas initially treated with primary surgery combined with curative post-operative radiation [64]. Also, in another large matched-pair study on recurrent and non-recurrent laryngeal carcinomas, homogeneous for site (glottis), stage (T1 and T2) and treatment (only transoral laser surgery), high index of proliferation, using MIB-1 and PCNA staining, proved to have prognostic significance.

**Nucleolar organizer region associated proteins (AgNORs)**

In general, a high proliferative activity is associated with poor prognosis in SCC of the oral cavity. Almost 30% of the cases were investigated using AgNOR analysis, although this method is not extensively applied in pathology departments. Piffko et al [65], reported that AgNORs at the invasive front of the tumor is the most significant independent prognostic factor. This study provides a functional background to the clinical relevance of the histopathological tumor front grading [66] and suggests the use of an aggressive surgical approach for those patients with a high AgNOR quantity. However, in a large number of studies, cell proliferative activity in oral SCC does not have any prognostic significance [63].

**Cyclin D1**

Cyclin D1 overexpression has been reported in SCC in a variety of head and neck sub-sites including the larynx, hypopharynx and tongue [46, 67-74]. Its prognostic significance in oropharyngeal SCC has been contradictory [75-80].

Cyclin D1 is a well-known oncogene in a variety of human cancers, regulating cell proliferation through G1 phase. Cyclin D1 forms complexes with CDK4 and CDK6, which phosphorylate the retinoblastoma protein pRb [81]. This causes pRb to release the E2F transcription factor, which then activates the genes which are necessary for cell cycle progression from G1 phase to S phase [82]. Cyclin D1 correlates with lymph node metastases, high grade tumors and poor survival, playing mainly a role in the late phase of tumor progression [71].

HPV-positive cancers are thought to be associated with downregulation of cyclin D1 expression [83, 84]. In hypopharyngeal cancers, cyclin D1 overexpression has been correlated with poor outcome in cases which are not related to HPV [71], but without existing a large amount of evidence suggesting its prognostic significance.

**Vaccinia-related kinase 1 (VRK1) protein**

The (VRK1) protein belongs to a new family of serine/threonine kinases and phosphorylates several transcription factors, including human p53 [85-88], and can also cooperate with the c-Jun NH2-terminal kinase pathway by phosphorylation of c-Jun [89] and ATF2 [90]. All these proteins phosphorylated by VRK1 have been associated with cellular responses to stress [91-93]. VRK1 contributes to p53 stability by two mechanisms, one of them dependent on Thr18 phosphorylation [90, 94], resulting in its stabilization and favoring its interaction with the transcriptional coactivator p300 which also seems to be implicated in the control of normal proliferation in the absence of cellular stress [94, 95]. The loss of VRK1 also affects the endocytic transport with a phenotype similar to that induced by silencing of mitogen-activated protein kinase [87, 95]. The expression of human VRK1 was determined in normal epithelium, particularly near the basal layer where cellular proliferation takes place, but it is lost as the epithelial cells differentiate. VRK1 was also detected in many lymphocytes within the follicles.

VRK1 was also detected immunohistochemically in HNSCCs, where it was correlated posi-
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tively with many proteins related with proliferation, suggesting a possible role in cell cycle regulation in the context of HNSCC [87].

Epidermal growth factor receptor (EGFR)

Epidermal growth factor receptor (EGFR), a member of the tyrosine kinase family of receptors, which, in response to extracellular binding with its natural ligands, such as epidermal growth factor and transforming growth factor (TGF)-α, generates an intracellular signaling cascade, resulting in cell proliferation. Cells that acquire the ability to overproduce these ligands or increase the number of EGFRs on their surface can create an autocrine growth pathway, resulting in uncontrolled growth [96, 97]. EGFR activates STAT 1, and 3, phosphatidylinositol 3-kinase (PI3k), the Ras-MAPk/ERk pathway and phospholipase c-c (PLC-c), contributing to cell survival and proliferation in HNSCC [1]. Virtually all HNSCC cases express EGFR [97-104], usually at high levels (2+, 3+).

This very high frequency of EGFR expression in HNSCC greatly limits the usefulness of classifying tumors as simply positive or negative as a marker for selecting treatment. Notably, levels of EGFR expression were also upregulated in normal mucosa from HNSCC patients, suggesting that increased EGFR expression is an early event in the development of HNSCC, and may help to explain the high incidence of synchronous and metachronous disease observed in this type of cancer. Up regulation of this factor occurs early in the progression of dysplasia to HNSCC in the upper aerodigestive tract.

High expression of EGFR is a negative prognostic factor which is often associated with nodal metastases and poor survival in patients with HNSCC [96, 98, 103], because it is implicated in cell motility, alters cell adhesion and promotes angiogenesis [98, 103-105]. Moreover, doses of cetuximab may need to be adjusted in patients with very high levels of EGFR, in order to be more efficacious [106].

EGFR expression is usually assessed immuno-histochemically in paraffin embedded tumor samples, facing the commonest problem with this technique which is the variation of results because of the differences in technique, the type of antibody or fixative used, the species, and the storage time of the sample [100]. The increased EGFR gene copy number, as fluorescent in situ hybridization shows, did not seem to be prognostic factor in patients with HNSCC [97, 101]. Another technique, the automated quantitative analysis of protein expression measures with greater accuracy the total and phosphorylated EGFR expression in tumor (samples) [102].

Mechanisms for EGFR over-expression in HNSCCs show EGFR gene mutations as in lung adenocarcinomas are uncommon and gene amplification is infrequent (< 15%), however a truncated mutant activated EGFRvIII is prevalent (40%) [107].

EGFR variants

A mutant form of EGFR known as EGFRvIII has been detected in up to 40% of HNSCC cases [107]. This truncated, constitutively active receptor is ligand independent and does not bind with antibodies that target the extracellular domain of wild-type EGFR. There are in vitro studies which showed that cells with EGFRvIII expression are less sensitive to the growth-inhibiting effects of cetuximab. Moreover, EGFRvIII is only seen in cells that overexpress wild-type EGFR, that’s why there is the suggestion that mutations are a late stage event caused by the rapid proliferation induced by wild-type EGFR overexpression [107]. Other alterations in EGFR include activating mutations, which have been detected in 10% to 30% of patients with HNSCC and predict increased sensitivity to small molecules that target the intracellular portion of EGFR [84, 85]. However, these mutations are present in just 7.3% of Asian patients with HNSCC who have been evaluated for the mutation and in only 1% in the rest population [9, 108-110].

K-RAS

The ras family of genes, H-RAS, K-RAS, and N-RAS, encode a protein that is located in the cytoplasmic side of the plasma membrane. The RAS gene has been identified in approximately one-third of all oral cancers in India and South East Asia [111]. The protein transmits mitogenic signals in response to a variety of physiological stimuli [112]. In HNSCC, the prevalence of K-RAS mutations is low. One study reported a prevalence of 8% [113], but most reports are generally < 5%, or as low as 2%. Although some
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evidence suggests that K-RAS mutations may indicate enhanced proliferation and an aggressive disease course in HNSCC [114, 115], their extremely low prevalence will likely preclude a major role in personalized medicine. Until now, there is no clinical benefit to testing for K-RAS mutation status before administering EGFR-targeted therapy to patients with HNSCC. Further evaluation of K-RAS, including the implications of overexpression of wild-type KRAS [116], is needed to better define the potential prognostic and predictive role, if any, of this factor in HNSCC [9].

NF-kB

Biopsy specimens from patients with oral squamous cell carcinoma was found to express higher levels of NF-kB in comparison to normal and dysplastic tissue [117].

Aurora kinase A (AURKA/STK15/BTAK)

Aurora kinase A (AURKA/STK15/BTAK) was analyzed in tumor and adjacent normal mucosa from HNSCC patients by both real-time quantitative reverse transcription-PCR and immunohistochemistry using a tissue microarray. AURKA induces chromosomal instability leading to aneuploidy and transformation. It was found increased expression and the association of AURKA elevation with poor prescience [118].

**ERCC1 (excision repair cross-complementation group 1) protein**

The ERCC1 plays an important role in repairing DNA damage caused by platinum agents, that’s why may help to select the patients who will benefit from platinum-based therapy [119-130]. Patients with low levels of expression had a 4-fold greater chance of achieving an objective response to cisplatin-based chemotherapy and a prolonged survival compared with those with high levels of expression. These findings show that ERCC1 expression levels before treatment are inversely correlated with response and survival after cisplatin-based therapy [9, 129, 130]. In head and neck cancer was found high expression of ERCC1. From a practical perspective, it appears that high ERCC1 expression in HNSCC tumors may suggest a low probability of benefiting from platinum therapy, so these patients may be benefit more by alternative approaches [9, 131-134]. For ERCC1, there are numerous studies indicating that low mRNA or protein expression is associated with a better prognosis in HNSCC. However, it is not established that ERCC1 expression is regulated at the transcriptional level. The main problem is that a nonspecific antibody was used to measure protein level. Studies manage to validate the utility of these biomarkers (ERCC1 mRNA levels or 8F1 immunohistochemical signal) for predicting clinical outcomes, but they do not demonstrate that DNA repair levels are altered in tumors [135].

**Base excision repair pathway (XRCC1)**

XRCC1 reduced expression or activity results in increased genomic instability and sensitivity to DNA damaging agents. High XRCC1 expression was correlated with resistance to radiotherapy in HNSCC [135, 136].

**Angiogenic factors**

Overexpression of vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8) play their vital role in angiogenesis of SCC. EGFRvIII leads to autoactivation and upregulated VEGF and tissue factor (TF) further augmenting this pathway [137]. Hypoxia is also a strong driving force in tumor angiogenesis, through the overexpression of hypoxia inducible factor-1 (HIF-1), at the top of a cascade of inducible proangiogenic proteins contributing to angiogenesis in HNSCC. Another marker of tumor hypoxia is lysyl oxidase, which may predict distant metastasis [1].

**Structural related biomarkers**

**Epithelial to mesenchymal transition (EMT)**

Another mechanism contributing to cell migration and metastases in SCC is epithelial to mesenchymal transition (EMT), a process by which carcinoma cells lose adhesion factors (i.e. E-cadherin). This process is highlighted by the spectrum/progression of SCC from well differentiated tumors to poorly differentiate and ultimately sarcomatoid carcinoma with single cell invasion and morphologic features of mesenchymal cells [138]. Moreover, Src is a strong inducer of EMT abolishing loss of cell-cell adhesion and E-cadherin and associates with SCC progression and aggressive feature [1, 138].
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*b-Tubulin*

Isotypes Taxanes exert their anticancer activity by binding to b-tubulin polymers and inhibiting microtubule depolymerization and mitotic process. As preclinical models showed, high expression levels of the isotype III of b-tubulin have been associated with resistance to paclitaxel in several cell types [9, 139, 140]. In HNSCC, docetaxel has become part of the standard induction regimen in combination with cisplatin and 5-fluorouracil. The expression of b-tubulin II measured by IHC (samples were scored by percentage of stained cells and intensity of staining; positive results were those above the median score and negative those below) may be associated with outcomes. Patients with low expression of b-tubulin II had better prognosis, regardless of the treatment of docetaxel, as well as the benefit from the addition of docetaxel seemed to be greater [141]. As the use of taxanes in HNSCC grows, this marker may assume a greater role in therapeutic decision making [9].

**cd44**

CD44 is an integral membrane glycoprotein that has diverse functions in cell-cell and cell-substrate interactions. It has been suggested that it may be a determinant of metastatic and invasive behavior in carcinomas. CD44 expression may be involved in the multiple mechanism of the development and progression of laryngeal lesions and may help to predict the risk of transformation of the benign or precancerous lesions to cancer [142].

**Viral etiologies**

*Epstein barr virus (EBV)*

In 1970, EBV was identified in SCCs arising in the nasopharynx which maybe the earliest tumor in the head and neck with a known biomarker. EBV can be detected in tumor tissue by in situ hybridization for EBV encoded RNAs (EBER) or by the less sensitive method of immunohistochemistry [1].

*Human papilloma virus (HPV)*

Approximately 30 years after EBV’s association with nasopharyngeal carcinoma, human papilloma virus (HPV), especially type 16, proved to be etiologic and prognostic factor in oropharyngeal carcinomas [143]. HPV-positive HNSCC is associated with a younger age of onset than in smokers, and it was proved that HPV is a reliable prognostic marker for improved local control and overall survival at 5 years of 79% for HPV + versus 20% for HPV negative patients [144]. Evaluation for HPV 16 may be performed on archival sections of biopsy tissue or FNA (i.e., neck nodes) by in situ hybridization or PCR amplification of viral related genes (E6, E7).

HPV-positive tumors in HNSCC are almost always oropharyngeal. However, prevalence of the reported HPV infection varies widely, because of the different method of detection applied. About 35% of overall head and neck tumors have been reported to be HPV positive, as detected by PCR [145], with cancers of the tonsils (43.6%) and the base of the tongue (38.4%) to be the most often detected sites [146]. One of the largest and most recent analyses of patients with oropharynx cancer from RTOG (Radiation Therapy Oncology Group) 0129 demonstrated a 64% incidence of HPV-positive tumors, greater than previously appreciated, using in situ hybridization techniques [147]. The prevalence of HPV-related HNSCC may also have geographical differences. While a world-wide survey of oropharynx cancer found that the prevalence of HPV infection was 18%, another report indicated the prevalence was 38% and notably higher in North America (47%) than in Europe (28%) [148].

The prevalence of types of HNSCC potentially related to HPV infection appears to be increasing in the United States [145]. Especially, in younger-age cohorts, the HPV-related tumors (e.g., base of tongue, tonsil, oropharynx) increased the last years, in contrast to the incidence of unrelated to HPV cancers (e.g., tongue, gum, lip, other oral cancers) which has decreased. The previous findings may be in part the result of changes in both sexual behavior and smoking. Of those head and neck tumors that contain HPV DNA, up to 90% present with the oncogenic variant HPV-16 [9, 149-150]. Compared with tumors of the oral cavity and oropharynx, squamous cell carcinoma of tonsils has been most strongly and consistently associated with HPV-16 infection [150]. In an epidemiological study [156], the prevalence of seropositivity of the oncogenic HPV-16 was nearly twice as high.
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HPV-positive oropharyngeal squamous cell carcinoma has distinct risk factors and molecular profiles, as they seem to be related with certain types of sexual habits and not smoking or alcohol use. In a nested case control study involving 130 consecutive patients with oropharynx cancer, the risk of oropharynx cancer was related to sexual behavior [66]. As far as it concerns the molecular profile, HPV-16 has been suggested to immortalize epithelial cells of both cervical and oral origin in vitro [151, 152]. HPV contributes to tumorigenesis through proteins E6 and E7 which inactivates p53 and the retinoblastoma protein pRb. The loss of the pRb negative feedback loop causes increased p16 protein expression [1, 153, 154]. p16 then inhibits cyclinD1-CDK4/6 (cyclin-dependent kinase 4/6) complexes, which regulate the progress from the G1 phase to the S phase of the cell cycle. In that way, HPV-positive tumors are associated with downregulation of cyclin D1 expression [83, 84]. And express wild-type p53 tumor suppressor genes.

p16 over-expression by immunohistochemical assessment may be used as a substitute marker of HPV [144]. HPV may also be detected in saliva promising a screening or monitoring role in oropharyngeal cancer [155]. Positivity of HPV-16 in serum proved to be an independent predictor of oropharynx cancer, after adjusting for age, sex, and alcohol use.

HPV status is not only a risk factor for developing HNSCC, but also define a distinct subtype of HNSCC tumors, promising a more personalized treatment. There are studies which showed HPV-positive tumors have better response and survival after treatment, maybe because these tumors maintain an apoptotic response to radiation and chemotherapy [143]. Alternatively, these patients tend to be younger and healthier compared with other head and neck cancer patients, with fewer smoking related comorbidities; however, it is important to note that in multivariate analyses of clinical trials, the prognostic effect of HPV is not driven by demographics alone [9].

The impact of HPV status on response and survival was evaluated by the Eastern Cooperative Oncology Group (ECOG) in a prospective multi-center phase 2 study of chemoradiation as organ-preserving therapy in 96 patients with resectable stage III/IV laryngeal or oropharynx cancer, using in situ hybridization. HPV DNA was detected in 40% of tumor samples; 63% of oropharyngeal tumors were HPV positive, but none of the laryngeal tumors had HPV DNA. Most positive cases had the subtype HPV-16 (95%). Patients with HPV-positive tumors had a higher response rate after induction chemotherapy and after chemoradiation. Overall survival (OS) was significantly longer in HPV-positive patients than in HPV-negative patients (alive at 2 years: 95% vs. 62%). The lower death and progression risk for HPV-positive patients was still present after adjusting for age, disease stage, and performance status [9].

Recent retrospective studies of large phase 3 trials have solidified the clinical importance of HPV as a prognostic marker for HNSCC. The phase 3 TROG (Trans Tasman Oncology Group) 02.02 (HeadSTART) trial, which randomized patients with stage III/IV HNSCC to receive cisplatin/radiotherapy-tirapazamine. At the 2-year mark, HPV-positive tumors were associated with better OS versus HPV negative tumors as well as improved failure-free survival. In addition, patients with p16-positive tumors had improved 2-year OS and improved 2-year FFS. Patients with both HPV and p16-positive tumors had increased OS and FFS rates compared with double-negative patients [9, 157].

Similarly, analysis from the RTOG 0129 study, randomized patients to receive standard fractionation 70 grays (Gy) + cisplatin or accelerated fractionation with concomitant impulse 72 Gy + cisplatin, correlated HPV status with patient prognosis. OS, PFS, and locoregional failure were all significantly superior among HPV-positive patients at 2 years and locoregional failure. Interestingly, heavy smoking status (> 20 pack-years) seemed to negate partially the benefit conferred by an HPV-positive tumor status; compared with HPV-positive/< 20 pack the year [156].

A retrospective analysis [142] of 646 evaluable samples showed that HPV-positive patients (identified by the surrogate marker p16) had a 5-year survival rate of 49% compared with 19.6% for those who were HPV negative and showed a strong prognostic correlation with HPV status [9, 158].

Together, these studies have definitively established HPV as a meaningful prognostic marker...
for oropharyngeal HNSCC, and are sufficient to warrant accounting for HPV status in the design and analysis of future clinical trials using combined or sequential chemotherapy and radiation [9].

As the prevalence of HPV infection and HPV related HNSCC increases [145, 149, 159], questions regarding screening are raised. Fortunately, only basic laboratory techniques required to screen serum samples for HPV-16, reliably [160]. However, the value of screening high-risk patients for HPV infection has not been demonstrated and remains unknown the best way to treat HPV-related premalignant lesions detected by screening. Furthermore, it is not clear yet whether identifying patients at high risk of contracting HPV may improve early detection of HNSCC by raising patient and physician awareness and increasing monitoring. Moreover, the rising incidence of HPV infection raises other issues, such as vaccination strategies. There is a great need for continued retrospective analyses and epidemiological studies in order the treatment strategies of HNSCC patients according to HPV status to be clarified [9].

Epigenetic modifications

Epigenetic modifications, consisting of aberrant DNA methylation, histone modifications and miRNAs, intervening in regulation of gene expression, induce HNSCC tumorigenesis and perhaps play a more central role in the evolution and progression of this disease [161].

Analysis of DNA extracted from oral SCC tissues and oral premalignant lesions (OPLs) found they exhibit more frequent and higher levels of DNA methylation compared with healthy or corresponding normal tissue from neoplastic tissues [162]. Smoking, a major risk factor for the development of OSCC, has been linked to nonspecific global hypomethylation [163, 164]. In contrast to smokers, patients who drink heavily have an increased risk for CpG hypermethylation of multiple oral SCC-related genes. Chronic inflammation of the oral mucosa is another risk factor that can potentially modify the methylation status of various genes in SCC tumors [165]. The occurrence of multiple CpG methylation sites in a panel of tumor-related genes in OSCC was highly associated with cancer stage and may correlate with lymph node metastasis [166]. To date, most oral cancer-related publications focused on CpG methylation of APC, Survivin, E-cadherin, MGMT, MLH1, p14ARF, p15INK4B, p16INK4A, RARβ and RASSF genes [161, 162].

Post-translational modifications of histones are frequently observed in oral cancer. These epigenetic alterations occur primarily at the N-terminal tails within each of the four histone complexes (H3, H4, H2A and H2B). Various modifications include methylation, acetylation, phosphorylation, ubiquitination, ADP-ribosylation and sumoylation of specific residues within these histone tails, modifying the tertiary DNA structure. There is a study, where the patterns of histone and DNA methylation were positively correlated in normal, OPL and OSCC tissues [162], and in another study, the pattern of H3K4 histone methylation was associated with OSCC malignancy. Moreover, H3K4me2 histones, transcriptionally inactive, are more frequent in oral SCC compared with normal tissues [167]. Interestingly, a similar pattern of histone methylation was detected in OPLs, such as leukoplakia, resembling more like OSCC tissues than with normal tissues. That’s why in many instances, leukoplakia needs to aware great surveillance as a premalignant condition for the development of OSCC and treated accordingly.

Similar to histone methylation during oral carcinogenesis, histone deacetylation, plays its own important role. It has been demonstrated that histone deacetylation, catalyzed by various histone deacetylase 2 (HDAC2) improves the stability of the HIF-1α protein, which may enhance invasion and migration in OSCC [168]. Likewise, expression of HDAC6 was upregulated in oral SCC and was found to be stage-specific; the higher the stage, the greater the activity [169]. In addition to deacetylating histones, HDAC6 was revealed to be capable of deacetylating α-tubulin, thus promoting microtubule-dependent cell motility [170].

Interestingly, Arif et al. discovered that histone H3, primarily H3K14, is hyperacetylated in oral SCC [171]. These investigators discovered that in the KB oral cancer cell line, increased H3 acetylation was nitric oxide-dependent [171].

The poly (ADP-ribose) polymerase (PARP) family of enzymes is responsible for the post-transla-
tional covalent transfer of ADP-ribose to proteins, as well as formation of polymers of poly (ADP-ribose). Actively proliferating OSCC has greater activity of PARP-1, DNA synthesis rates and ADP ribosylation [172], a finding that is ascribed to histones rather than nonhistone chromosomal proteins. The addition of poly (ADP-ribose) to histones loosens the chromatin structure, facilitating DNA repair through large protein complexes such as chromatin assembly factor (CAF)-1, which integrate H3K56-acetylated histones into the chromatin. Worst prognosis in terms of survival and metastasizing behavior may be predicted in PARP-1high and CAF-1/p60high OSCC tissues [173]. Taken together, DNA methylation, acetylation and/or poly (ADP)-ribosylation may all be important factors in the development of OSCC [161].

miRNAs are the most recent entrants into the category of epigenetic gene expression regulators. miRNAs represent small RNA molecules with important regulatory functions, while each miRNA can target multiple mRNAs or gene targets. MiRNAs inhibit protein formation by degradation or repression of translation of the mRNA transcript. MiRNAs are introduced recently in research area, but increased rapidly [161].

Conclusion

There are a growing number of molecular markers that may potentially be used as either prognostic or predictive tools in the treatment of head and neck cancer. The understanding of these biological markers is not as well defined as in other tumor types, such as breast or lung cancer, but recent advances have brought us closer to providing personalized medicine for these patients. Most patients diagnosed with head and neck cancer present with locally advanced disease, for which a combined modality treatment approach with curative intent is usually prescribed. By tailoring treatment to the specific genetic or molecular profile of a tumor in an individual patient, response and survival outcomes could be improved through refined treatment decisions, treatment-related costs, complications reduced, and unnecessary interventions avoided. Such refinements may also facilitate the development of newer, more effective therapies.

To accelerate the validation of available biomarkers and the discovery of new ones, and to address questions emerging from clinical studies, it will be important to associate relevant preclinical models to clinical studies. The development of biomarkers of angiogenesis represents a unique opportunity for clinical oncologists and laboratory scientists to join forces to address relevant questions and design meaningful studies. Despite the great increasing number of biomarkers, further studies will cut the gordian knot and show which biomarkers are the best tools for head and neck cancer.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Evangeli S Lampri, Department of Pathology, Faculty of Medicine, University of Ioannina, Greece. E-mail: evangeli.lampri@gmail.com

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