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REVIEW ARTICLE

Molecular diagnostic alterations in squamous cell carcinoma of the head and neck and potential diagnostic applications

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Abstract Head and neck squamous cell carcinoma (HNSCC) is a common malignancy that continues to be difficult to treat and cure. In many organ systems and tumor types, there have been significant advances in the understanding of the molecular basis for tumorigenesis, disease progression and genetic implications for therapeutics. Although tumorigenesis pathways and the molecular etiologies of HNSCC have been extensively studied, there are still very few diagnostic clinical applications used in practice today. This review discusses current clinically applicable molecular markers, including viral detection of Epstein–Barr virus and human papillomavirus, and molecular targets that are used in diagnosis and management of HNSCC. The common oncogenes *EGFR*, *RAS*, *CCND1*, *BRAF*, and *PIK3CA* and tumor suppressor genes *p53*, *CDKN2A* and *NOTCH* are discussed for their associations with HNSCC. Discussion of markers with potential future applications is also included, with a focus on molecular alterations associated with targeted therapy resistance.

Keywords Head and neck squamous cell carcinoma · Carcinogenesis · Molecular biology · Diagnostic test · Targeted therapy · Oncogenes · Tumor suppressor genes · Gene expression

Introduction

Head and neck squamous cell carcinoma (HNSCC) is a relatively common malignancy, and is typically quoted as

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being the sixth most common cancer worldwide. Despite major advances in the diagnosis and management of many other types of cancer, HNSCC remains a malignancy that is difficult to treat and some subsites continue to have a relatively low cure rate. Some of the advances in recent years have come from the application of targeted therapies. Unlike cancers in other organ systems, however, these novel therapies have not been coupled with any reliable companion diagnostic molecular tests to predict response to therapy. Therefore, although the tumorigenesis pathways and the molecular etiologies of HNSCC have been extensively studied, there are very few diagnostic clinical applications in practice today. This review focuses on the better studied molecular alterations in HNSCC, especially those used in current clinical diagnostic testing.

Viral etiology and molecular applications

In few other sites or tumors of the human body are viral associations more important, nor have they been more clearly elucidated than in HNSCC. From the initial work that associated Epstein–Barr virus (EBV) with specific tumors of the nasopharynx, to recent studies demonstrating that human papillomavirus (HPV) drives the growth of some squamous carcinomas, the viral pathway of oncogenesis is well established. These tumors, their viral associations, clinical diagnostic applications, and assays are discussed.

EBV

The tumors of the head and neck that are most strongly associated with EBV are those occurring in the nasopharynx. Other non-mucosally derived tumors have also been associated with EBV, including some lymphomas [1], but these non-squamous tumors are not further discussed here. Interestingly, there is a very strong link to ethnicity and to geography in the incidence of the EBV-associated tumors, with the highest prevalence seen in Southeast Asia [2, 3]. Screening strategies in high-risk areas have included looking for serological evidence of EBV (high titers are associated with the presence of tumor) or analysis for circulating EBV DNA in quantitative assays [4–7]. Most patients who are diagnosed with nasopharyngeal carcinoma typically present with symptoms associated with mass effect or nodal metastatic disease [8].

The terminology for tumors of the nasopharynx, including EBV-associated carcinomas, has changed over the decades. Originally, nasopharyngeal carcinoma was known as the Schmincke–Regaud tumor, after a German pathologist and a French radiologist, respectively [9, 10]. However, subsequent publications of the World Health Organization (WHO) classification system applied a more categorical naming convention designated by histological features (Table 1). Type I referred to conventional squamous cell carcinoma (SCC); type II, differentiated non-keratinizing SCC; and Type III, undifferentiated carcinoma, the latter being the most common morphology for the EBV-associated tumors. In the most recent edition of the WHO classification, terminology has reverted to straight histologic descriptors, thus acknowledging that all variants of SCC have been described in the nasopharynx. These histological types include keratinizing SCC, non-keratinizing differentiated and undifferentiated [which is also referred to as lymphoepithelial carcinoma (LEC)] SCC, and basaloid SCC (BSCC). Importantly, another term that has been used is "lymphoepithelioma"; we believe that this term should be avoided, since it nosologically implies a benign tumor.

Histologically, EBV-associated HNSCC, regardless of the location (but certainly including those in the nasopharynx) often has a characteristic and unique appearance. These tumors are referred to diagnostically as "lymphoepithelial carcinoma", which is an appropriate descriptive diagnosis highlighting the intimate association between the epithelial cells and lymphocytes. The epithelial component

Table 1 Past and current WHO terminology for tumors of the nasopharynx

WHO (1971)	WHO (2005)
Type I carcinoma	Non-keratinizing carcinoma (differentiated and undifferentiated)
Type II	Keratinizing squamous cell carcinoma
Type III	Basaloid squamous cell carcinoma

WHO World Health Organization

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demonstrates sheet-like or syncytial growth of large, pleomorphic cells with vesicular chromatin and prominent nucleoli. In most cases, these cells are relatively conspicuous. However, because of the background inflammatory component, which consists of polyclonal non-neoplastic infiltrating lymphocytes, the epithelial cells can sometimes be inconspicuous and difficult to identify on routine staining. Even though the lymphocytic component is thought to be a benign passenger, it is quite consistently retained in both the primary tumors and in metastatic lesions. The LEC morphology is strongly associated with EBV, especially in endemic regions. In Western countries, carcinomas with a lymphoepithelial morphology can also be seen in the tonsil and tongue base, and these are virtually always associated with HPV (see discussion of HPV) [11–13].

EBV is thought to be etiologically critical, and EBV-associated tumors occur in patients with unique and striking demographics. The patients tend to be younger than those with typical smoking-associated SCC and they often present with bulky metastatic disease in the neck, particularly in level V lymph nodes. There are other associations, such as dietary patterns (salted fish, fermented foods) and there are genetic determinants as well, such as associations with certain HLA subtypes [14]. Although treatment protocols are not standardized, common approaches include combination therapy with radiation and chemotherapy, along with the potential addition of targeted agents [15].

EBV can be detected in a variety of different ways, including polymerase chain reaction (PCR)-based technologies, in situ hybridization (ISH), and immunohistochemistry (IHC). PCR assays from tissue sections are straightforward and practical to perform in a molecular diagnostic setting. These assays are highly sensitive and can detect a single or a few copies of virus. This high sensitivity may be problematic in diagnostics, since a positive result could potentially come from contamination or from incidental viral copies that are not related to tumorigenesis. In other areas of diagnostics, where EBV is detected from blood instead of tissue (as in transplant patients, for example), quantitative PCR is used to provide actual viral copy numbers. There is no role currently for this type of sensitive and quantitative approach in tissue diagnosis. At the other extreme, IHC is not very sensitive and suffers from issues related to consistency in assay technique. Because there are no standardized approaches for antigen retrieval and quantitation, and because of the presence of background non-specific staining, IHC assays have not proven useful in diagnosis.

The most commonly used diagnostic application today is an RNA ISH assay directed against the EBV-encoded small RNAs (EBERs), which are the noncoding RNA molecules expressed in cells with latent EBV infection [16]. RNA ISH for EBER is a highly sensitive and specific test for EBV-related carcinomas and is easy to perform and interpret. There are commercially available probes that allow for simple detection in the routine laboratory setting [17]. ISH relies on the application of synthesized probes that are designed to match the nucleic acid sequence of the viral genome (or RNA, as in the case of EBER). When the probe finds the target, it hybridizes through strong reverse base pairing bonds. A signal is generated through either chromogenic (colorimetric) or fluorescent detection systems. The pathologist can then visualize the presence of the signal and classify as either positive or negative. In genomic probes, one can estimate copy number of the virus, as well. However, currently there are no diagnostic applications for quantification of copy number.

HPV

One of the most interesting and exciting developments in the field of head and neck pathology in the past decade has been the identification of a strong association between certain HNSCCs and HPV [18, 19]. Our understanding of this association has been growing substantially as more and more studies have now supported HPV as tumorigenic and pathogenic in HNSCC, and as the clinical and prognostic ramifications of this association are being unraveled. HPV-associated HNSCC is increasing in frequency, as can be seen from Surveillance, Epidemiology and End Results (SEER) data and from international studies of incidence and prevalence [20, 21]. The occurrence of this tumor is strongly associated with higher prevalence of many sexual partners, including multiple vaginal and oral sexual partners [22]. There is some hope that we will see normalization or decreases in this trend with the use of the HPV vaccine, although there will certainly be a significant lag time [23]. HPV-associated HNSCC has been clearly shown to have a better treatment response and better prognosis than non-HPV carcinomas in large numbers of retrospective and prospective studies and in patients treated with all different clinically accepted treatment regimens [24–27].

The form of the virus is critical to its biological and clinical relevance. The HPV must be transcriptionally active to have any clinical relevance for the behavior of the tumor. The most common site for transcriptionally active HPV-associated SCC is the oropharynx. The anatomical areas of the oropharynx include the palatine tonsils, lingual tonsil (tongue base), lateral and posterior pharyngeal walls, and the soft palate. The two most common subsites for HPV-associated SCC are the lingual tonsil area (tongue base) and palatine tonsils. SCC of the oropharynx can be difficult to detect clinically, because the tumors are frequently small and inconspicuous, and because they arise from the deep crypts rather than from the surface mucosa.

The diagnosis may require a diagnostic tonsillectomy (rather than biopsy) and/or multiple deep biopsies. Patients with HPV-associated SCC can present with large bulky lymph cervical node metastases [26, 28]. These metastases are frequently cystic, which can complicate the cytologic diagnosis through fine needle aspiration (FNA), when only cyst debris is obtained rather than a cellular specimen [29]. Some patients, therefore, are initially diagnosed when a positive lymph node is resected, and the primary tumor is subsequently identified after extensive diagnostic workup [29, 30]. In fact, despite extensive biopsies and workup, there is still a small group of patients in whom a primary is never found (metastases from carcinoma of unknown primary) [31].

Despite the challenges inherent in finding the primary tumors, once found most are histologically and diagnostically straightforward. Most HPV-associated tumors have unique morphologic features, including a propensity to be either non-keratinizing SCC or BSCC [32–36]. These two variants both show minimal keratinization and cells that are more like those found in the basal epithelial zones. In fact, some have suggested that tumors have this morphology because they are actually differentiating into the basal-appearing cells that normally populate the non-neoplastic crypts. These features include high nuclear-to-cytoplasmic ratios, hyperchromasia, lack of prominent nucleoli, ovoid to almost spindle-shaped nuclei, polarization, and high mitotic and apoptotic rates. They typically have comedo-type necrosis and invade as large and rounded nests with little stromal desmoplasia. Both can give rise to cystic metastases in the neck [29, 37]. Recently, it has also been recognized that undifferentiated carcinoma or LEC arising in the oropharynx is almost always associated with transcriptionally active HPV [11–13].

HPV detection has also been reported through a variety of technologies. Many of the initial studies used DNA-based PCR approaches, which are very sensitive [38, 39]. There are several different approaches for a PCR-based application, including using either common PCR primers or type-specific ones that detect the majority of HPV subtypes [40, 41]. Quantitative assays have also been reported, though these have not been proven to have clinical value beyond the qualitative aspects of identifying HPV. HPV DNA is fairly common in SCC and in other types of head and neck tumors as well. Recent studies have indicated that it is not the presence of the virus alone, but whether it is transcriptionally active that confers pathogenicity. This has led to the development of an assay that uses reverse transcription and PCR (quantitative RT-PCR) to detect high-risk HPV mRNA, usually for the E6 and E7 transcripts [42–45]. There are also specific Federal Drug Administration (FDA)-approved assays for HPV used in cervical cytology (Pap) tests for screening for carcinoma of the uterine cervix. Though these have not been FDA approved for use in HNSCC, there are several reports that illustrate the technology is valid and can be used especially for FNA (after appropriate validation as a laboratory-developed test) [46]. HPV testing in lymph node FNA specimens can be particularly useful in cases where there is no known primary tumor, as the presence of HPV is a strong indicator of an oropharyngeal primary [29, 47, 48]. Not all oropharyngeal carcinomas are HPV positive, and therefore the inverse is not true—in other words, a negative HPV test does not preclude an oropharyngeal primary tumor.

The most commonly applied direct diagnostic assay for HPV is probably DNA ISH. Probes for the more common HPV subtypes are commercially available [27]. The most common subtype, accounting for up to 90 % of HPV-associated HNSCC is HPV16 [21]. There is no association between the low-risk subtypes (6 and 11) and oropharyngeal carcinoma, although these are associated with some benign squamoproliferative lesions in the head and neck (squamous papilloma and Schneiderian papilloma). There are also commercially available assays that allow for the detection of E6/E7 RNA directly by ISH [49, 50]. This technique allows for sensitive and specific HPV detection, but also for confirmation of the transcriptional activity [50]. The results have been shown to correlate very well with other types of HPV test and with clinical outcomes.

In recent years, it has also been recognized that $p16^{ink4a}$, which is one of the protein products of the *CDKN2A* gene (chromosome 9p21), is over-expressed as a result of viral expression of the E7 gene [33, 40, 51–54]. This over-expression can be easily identified using IHC in a simple and practical diagnostic assay. Because p16 is also a tumor suppressor protein, expression can be altered secondary to gene mutation or methylation [55]. Most non-HPV-related carcinomas thus lack p16 expression, but some can have expression by other mechanisms. Thus, it is important to identify strong and diffuse staining as a surrogate marker of transcriptionally active HPV. In one recent study, this was validated as either >75 % of the cells having nuclear and cytoplasmic staining, or alternatively >50 % with >25 % of areas with confluent staining [56]. The staining can be done on biopsy samples prior to definitive therapy [57]. The correlation between p16 over-expression and active HPV is very high in oropharyngeal SCC, but not 100 %. Interestingly, when considering results across cohorts of patients, p16 positivity alone conveys a better prognosis, even in the absence of identifiable HPV by other techniques [58]. However, the clinical and biological significance of p16 over-expression in the absence of HPV RNA is unclear.

DNA-based assays

Oncogene alterations

Oncogenes are critical tumor-associated genes that are found across almost all tumor types. Oncogenes have been extensively studied and investigated, and are frequent targets for clinical molecular diagnostic applications. For tumorigenesis pathways, only one of the oncogenes in a cell needs to be altered or mutated for the pathway to be activated. This is in contrast to tumor suppressor genes, which are discussed in the next section.

Epidermal growth factor receptor (EGFR)

There is a vast literature about EGFR in HNSCC. The original papers described abnormal expression patterns of EGFR at the protein level, mostly using IHC as a means for detection. It is known that over 90 % of HNSCC have over-expression of EGFR at the protein level, most with marked over-expression [59]. Increased levels of EGFR have been associated with local or regional recurrence and with an overall decreased survival [60–62]. One caveat is the recognition that quantitation of EGFR protein using IHC has been notoriously difficult, and several studies have demonstrated that different antibodies and different protocols for antigen retrieval will affect the intensity and distribution of staining, and can lead to high variability between studies [62, 63].

The expression pattern of EGFR is actually altered in many different types of tumors, including lung and colon cancer [64, 65]. However, over the years, a mechanism for over-expression in HNSCC has not been clearly elucidated, despite many studies that have examined EGFR at all levels. Despite the lack of understanding of EGFR expression in HNSCC, anti-EGFR therapy has become a mainstay in the treatment of HNSCC, particularly in combination with other therapies [62, 66].

It is clear that primary somatic mutations in *EGFR* are uncommon in HNSCC. This is in contrast to lung adenocarcinomas, which are known to have somatic mutations which are clustered in the tyrosine kinase domain, particularly in exons 18–21 [67, 68]. In multiple studies, HNSCC has been shown to have a negligible rate of *EGFR* mutation, when the alterations typical of lung cancer are studied [60, 69–71], though a few studies have identified these mutations [72, 73]. There are, however, high-level amplifications of the *EGFR* gene in approximately 10 % of HNSCC, and polysomy is present in even more [70, 74–76]. Amplification is best identified using fluorescent in situ hybridization (FISH), which can also identify cases with polysomy. Currently, however, there are only very limited data to suggest that increased copy number conveys any prognostic significance or impacts on therapy [70, 74–76].

Finally, there is a known mutation in *EGFR* called the EGFRvIII, which encodes for a truncated protein that lacks part of the extracellular ligand-binding domain. This altered form of EGFR appears to decrease the sensitivity of tumor cells to EGFR inhibitor therapy. The EGFRvIII mutation can be detected at the DNA level, using PCR-based approaches, or by using a variant-specific IHC antibody [77]. Approximately 20–40 % of HNSCC harbor this mutation [60, 61]. Few studies have examined the impact of the EGFRvIII on prognosis, response to therapy, or overall survival, although there are some early indications that this mutation may have some prognostic significance [61].

Unfortunately, because there has been no consistently identifiable alteration in the *EGFR* gene or protein that correlates to response to therapy with EGFR inhibitors, EGFR analysis has not been incorporated into the pathologic evaluation of HNSCC [78].

CCND1

CCND1 is the gene that encodes for cyclin D1. It is located on chromosome 11q13. Amplification of the *CCND1* gene has been identified in as many as 40 % of HNSCC, and over-expression of cyclin D1 protein has been seen in as many as 75 % of HNSCC [79–84]. A number of studies indicate that there may be prognostic value for protein expression and gene amplification and early results suggest a possible link to therapeutic responses [81, 82]. Other studies suggest that the prognostic significance attributed to *CCND1* amplification could be due to frequent co-amplification with other genes located at the 11q13 locus [85, 86]. These conflicting results, the lack of consistency in methods, and a lack of large, randomized trials have hindered definitive clinical applications for this testing.

RAS

The *RAS* gene family consists of three separate genes: *KRAS*, *HRAS*, and *NRAS*, which function as small GTPase molecules through binding of GDP/GTP (Table 2). The role of the *RAS* genes in cell signaling is critical, as they are part of the RAS–RAF–MEK–MAPK pathway that controls many cellular functions. Mutations in the *RAS* genes have been identified in a wide variety of different tumors and have been known for decades. The mutations are activating ones, as are most oncogene mutations. Across many organ systems and different types of tumors, the most consistent and regular mutations involve codons 12, 13, and 61. There is a high frequency of mutation in colon and pancreatic cancer, and smoking-associated lung

Table 2 The *RAS* gene family members and their chromosomal location

Gene	Full name	Location
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	12p12.1
NRAS	Neuroblastoma RAS viral (v-ras) oncogene homolog	1p13.2
HRAS	v-Ha-ras Harvey rat sarcoma viral oncogene homolog	11p15.5

cancers. The importance of these mutations is that they confer resistance to EGFR inhibitor therapy [87, 88]. Interestingly, *KRAS* mutations are very uncommon in HNSCC [78]. *HRAS* mutations have been described in HNSCC, but again only in a small subset of patients [89–91]. Currently, there are no clinical applications for RAS testing in HNSCC.

BRAF

The *BRAF* gene is located at chromosome 7q34, and is also part of the important RAS–RAF–MEK–MAPK pathway. Mutations in the *BRAF* gene are also seen in the papillary thyroid carcinomas, many gliomas, and in melanomas. However, they are much less prevalent in other carcinomas [87, 92]. Similar to *KRAS* gene mutations, the *BRAF* gene mutation confers resistance to EGFR inhibitor therapy [87]. *BRAF* gene mutations are quite rare in HNSCC [91, 93], and thus there is no current clinical role for testing in HNSCC.

PIK3CA

The PI3K–PTEN–AKT pathway is another critical pathway in oncogenesis and our understanding of pathogenesis of some common tumors. This pathway is one of the most frequently altered in HNSCC, with defects being found in nearly 50 % of tumors in one study [94]. This pathway can be activated through a variety of receptor tyrosine kinases, including several discussed in other areas of this review (e.g., EGFR and MET). The class 1a PI3 kinases are composed of two subunits, one of which is the 110 kDa p110a subunit encoded by the *PIK3CA* gene. *PIK3CA* is located on chromosome 3q26. Mutations and amplifications of this gene have been identified in a variety of different human malignancies, including HNSCC [91]. In HNSCC, the prevalence of this mutation is probably around 5–15 % [89, 90, 95–97]. These mutations are oncogenic and thus are commonly activating point mutations. The most frequent mutations occur in exons 9 and 20, with hotspots at H1047R, E542K, and E545K. There are currently a variety of different inhibitors that are being studied for targeting the PI3K pathway, including specific inhibitors that target *PIK3CA* mutations [98, 99].

Within the same pathway, *PTEN* mutations have also been identified in HNSCC [71, 96, 100]. PTEN is a tumor suppressor gene and thus inactivation requires two hits, often a point mutation coupled with a deletion on the second gene copy [89, 96]. The result of *PTEN* inactivation appears to be constitutive activation of the *PI2K* pathway, similar to that seen with *PIK3CA* mutations [96].

MET

MET is a receptor tyrosine kinase for which the ligand is hepatocyte growth factor (HGF). This gene is located at chromosome 7q31. It has been shown that amplification and mutations of *MET* are present in several different types of carcinoma, including lung and kidney cancers [87, 101–104]. Importantly, these mutations appear to cause activation of signaling pathways that then confer resistance to EGFR inhibitor therapy [105]. In lung cancer, for example, *MET* amplification has been shown to be present in up to 20 % of patients with EGFR inhibitor therapy resistance [106]. In HNSCC, *MET* mutations and amplifications are seen in up to 25 % of cases in some series [107–111]. Over-expression at the protein level is also seen [61, 112, 113]. Studies of therapeutic implications of *MET* gene alterations are very early, but there are ongoing trials of small molecular MET inhibitors [104, 107]. Given the implications from the lung cancer literature, there may be a significant role for MET in defining resistance to EGFR inhibitors in patients with HNSCC [104, 114–116].

Tumor suppressor gene alterations

Tumor suppressor genes are probably the most widely studied tumor-associated genes in all tumor types. They are thought to be responsible for initiation of tumorigenesis for a large number of tumor types. For tumorigenesis pathways, both tumor suppressor genes in a cell need to be altered or mutated for the pathway to be inactivated. The foundation for this concept came from Dr. Alfred Knudson, with the famous Knudson Hypothesis [117, 118]. Of the mechanisms involved in the pathogenesis and progression of keratinizing HNSCC, those that govern the transition of the cell cycle from G1 to S phase have been studied in the most detail. Among them, alterations in the tumor suppressor genes *p53*, *p16*, and *cyclin D1* are relevant, while alterations in the retinoblastoma gene are infrequent [119].

p53

One of the first well-studied tumor related genes in HNSCC was the *p53* gene [120–122]. This gene is a tumor

suppressor gene located on chromosome 17p13. *p53* is frequently altered in a wide variety of human cancers. At the nucleic acid level, the most frequent mutations include point mutations, deletion mutations, and epigenetic alterations, including promoter methylation. Most of these alterations cause inactivation of *p53*, and according to the basic premise of tumor suppressor gene carcinogenesis, both copies of the gene need to be mutated in order for tumorigenesis to occur. New evidence also shows that gain of function *p53* mutations may be important in tumorigenesis [123].

In HNSCC, *p53* alterations have been detected in up to 80 % of tumor cases [89, 90, 124, 125]. In many studies of loss of heterozygosity (LOH), which detects deletion mutations, the frequency of tumors with LOH ranges between 50 and 70 % of cases [126, 127]. Promoter methylation is also seen, as are somatic sequence mutations [128]. The least effective test to detect alterations in the *p53* gene is to identify over-expression at the protein level [129]. While over-expression is frequently associated with neoplasia, there is not a high concordance between genomic alterations and over-expression; in other words, the protein can be over-expressed for many reasons, not just because of tumor suppressor gene mutation.

p53 has not been typically used as a clinical biomarker in HNSCC, although some studies have confirmed an association with prognosis and survival [122, 124, 130–132]. There are no targeted therapies that use *p53* as a companion diagnostic, however, and therefore it is not used in the general workup of HNSCC. There is some evidence, though, that *p53* mutations confer resistance to radiation therapy and may be associated with treatment failures [133]. Other interesting clinical applications of *p53* that have been studied over the years have been mutational assessment of surgical resection margins and assessment of clonality to differentiate second primary tumors and metastatic carcinoma [124, 126, 129, 134–138]. Although neither of these have been applied in routine clinical use, probably because of the intensive nature of this type of assay, *p53* mutation detection at surgical resection margins does appear to be associated with local recurrence and tumor clonality can be used to resolve challenging diagnostic dilemmas.

CDKN2A

CDKN2A is the gene that encodes for the protein products $p16^{INK4A}$ and $p15^{INK4A}$ The gene is located at 9p21 [125]. It is considered to be a tumor suppressor gene that is frequently inactivated in HNSCC through deletion, or through less common point mutations and epigenetic promoter methylation. $p16^{INK4A}$ functions as a cyclin-dependent kinase inhibitor in regulation of the G1/S checkpoint of the cell cycle. Although inactivation of *CDKN2A* is clearly part of the pathogenesis of HNSCC, there are currently no clinical or diagnostic applications of testing for genetic alterations. The most important clinical role for p16 testing is by immunohistochemical over-expression, through its association with HPV (see above). The over-expression of p16 protein that occurs in HPV-associated tumors is not driven by gene alterations, but rather through the viral HPV E7-mediated transcriptional dysregulation. The *CDKN2A* gene is wild type in such tumors.

Table 3 The *NOTCH* genes and their chromosomal location

Gene	Location
NOTCH1	9q34.3
NOTCH2	1p13-11
NOTCH3	19p13.2-13.1
NOTCH4	6p21.3

NOTCH

NOTCH is a family of receptors with 4 members (Table 3) that bind with *NOTCH* ligands. The receptor undergoes heterodimerization. The signaling events for the *NOTCH* pathway are not entirely understood, but are likely important for development and proliferation [139]. *NOTCH* gene mutations have been reported to occur in less than 5 % of malignancies that have been studied, including lung, breast and ovarian carcinomas and glioblastoma [139]. Based on the types of genetic mutations and the function of the gene, it is likely that *NOTCH* functions as a tumor suppressor gene in HNSCC [115, 139].

Recently, two studies have shown that *NOTCH* mutations also occur in HNSCC. These used state-of-the-art technology to study molecular events in HNSCC, including next generation sequencing [89, 90]. These studies corroborated the presence of several known mutations, including those discussed elsewhere in this article (*PIK3CA*, *HRAS*, *p53*, and *CDKN2A*). But, they also identified a few novel targets that had only been noted occasionally in previous studies. One of the potentially important mutations occurred in the *NOTCH* genes (Table 3) [89, 90]. In these two studies, *NOTCH1* mutations were seen in 11–15 % of cases studied. *NOTCH2* and *NOTCH3* mutations were also detected [89].

RNA-based assays

Gene expression profiling

All of the discussion above focuses primarily on the genomic pathways and the proteins that result from over-

expression or inactivation at the DNA level. Another entire field of study in tumors has evolved from the ability to examine the mRNA from tumor cells in comparison to reference standards. While this has always been possible at the single analyte level, new technologies in the past decade have enabled multi-analyte testing through arrays that query thousands of targets simultaneously. One of the most powerful is the expression microarray, which allows for identification of over- and under-expressed mRNA. The most significant results from expression array applications are usually in the discovery phase of investigation. This technology allows us to identify novel targets that are altered in tumors, and explore those in greater depth with other technologies. The clinical applicability of microarray assays has been limited, likely due to several substantial technical limitations [140]. First, mRNA is inherently unstable in samples and requires special handling, and the technology remains relatively expensive and labor intensive in the laboratory. But, even more importantly, major concerns repeatedly arise about whether expression arrays have adequate quality control to be used clinically, since results are frequently not reproducible and overlap in gene profiles from study to study is often low [141, 142].

In HNSCC, expression microarray studies have been used to investigate signatures in different clinical scenarios, including prediction of metastasis, treatment responsiveness, and prognosis. All of these have yielded some interesting, but limited results [59, 143–146]. The major limitations continue to be the small sample sizes and low overlap between studies. For this technology to be useful in the clinical setting, large-scale studies, prospective analyses, and randomized trials will likely be needed to validate results [141, 143, 146]. However, recently, a validation study of a previously reported gene expression profile for nodal metastasis has been published which may bring clinical implementation of gene expression profiling a step closer [147–149]. Studies using RNA, either as targeted or large expression signatures, have also been done in peripheral blood and in lymph nodes to detect rare tumor cells [150–152].

microRNA

Another area of active investigation that has not yet been introduced in clinical practice is the study of microRNA signatures or expression patterns in tumors. MicroRNAs are short RNA molecules that are involved in the regulation of gene expression. There have been some initial studies indicating that there are different patterns of microRNA expression in HNSCC, and that these may have the ability to discriminate tumors for etiology (HPV vs. non-HPV-associated tumors, for example), prognosis, and therapeutic response [153–156]. MicroRNAs are smaller and more stable than messenger RNA, and therefore, variability should be reduced and reproducibility improved in the expression patterns as the field evolves.

Current clinical applications

In HNSCC, there are surprisingly few clinical applications of molecular diagnostic tests that are used in common practice today. The most significant and universally applied are the tests for viral associations in SCC. Most pathologists are currently testing for HPV or the surrogate marker p16 (or both) in oropharyngeal carcinoma and for EBV in nasopharyngeal (or lymphoepithelial) carcinomas. The other assays discussed above are not widely available today in routine clinical diagnostic laboratories. With relatively few clinical implications, there are few drivers for widespread uptake in clinical care.

Growth in clinically appropriate molecular diagnostic testing in HNSCC as companion diagnostics is certainly anticipated, particularly as novel somatic oncogene mutations are identified that can be targeted by drugs. For example, with up to 10 % of HNSCCs harboring *PIK3CA* mutations, and drug production that specifically target *PIK3CA*, there may a real drive for establishing this as a diagnostic test. Similarly, the identification of *NOTCH* mutations will be critical when drugs are clinically available that target tumor cells with this molecular alteration. Another important area of potential clinical applicability will come from testing for mutations that confer drug resistance, especially to the EGFR inhibitor therapy. Even if these mutations are rare, there may be significant value in testing, if their presence implies that the tumor will not respond to therapy.

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