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Lessons learned from next-generation sequencing in head and neck cancer

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Abstract

Scientific innovation has enabled whole exome capture and massively parallel sequencing of cancer genomes. In head and neck cancer, next-generation sequencing has granted us further understanding of the mutational spectrum of squamous cell carcinoma. As a result of these new technologies, frequently occurring mutations were identified in *NOTCH1*, a gene that had not previously been implicated in head and neck cancer. The current review describes the most common mutations in head and neck cancer: *TP53, NOTCH1, HRAS, PIK3CA*, and *CDKN2A*. Emphasis is placed on the involved cellular pathways, clinical correlations, and potential therapeutic interventions. Additionally, the implications of human papillomavirus on mutation patterns are discussed.

Keywords

next generation sequencing; mutations; oncogene; tumor suppressor gene; head and neck squamous cell carcinoma

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) represents the sixth most common cancer in the world, with more than half a million cases diagnosed each year.[1,2] Because of the critical location in the upper aerodigestive tract, these cancers and their treatment significantly impair patient quality of life by affecting breathing, swallowing, speech, and even appearance. Despite recent advances in imaging techniques, surgical techniques, and intensification of treatment with the increased use of chemoradiation, the survival rates for HNSCC have remained largely unchanged in the past 3 decades with a 50% 5-year survival rate.[2,3] Understanding tumor biology offers the potential of individualizing treatment and developing targeted therapies to increase cure rates and minimize morbidities. Our review offers a current overview of the mutations in HNSCC, emphasizing the recent results of large-scale sequencing efforts using next-generation sequencing technology.

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Next-generation sequencing refers to the newer sequencing technologies that have followed the traditional Sanger method. These technologies allow for massively parallel sequencing, resulting in highly accurate reads that provide quick and relatively inexpensive whole exome and genome sequencing. Being able to screen all known human genes in multiple specimens has ultimately made cancer genome sequencing a reality. In 2011, the first reports of whole exome sequencing of HNSCC were published simultaneously by our group and investigators working at the University of Pittsburgh and the Broad Institute.[4,5] All exons for known human genes were sequenced in a total of 106 tumors and matching normal DNA. Mutations were confirmed in genes that had been previously described as key players in HNSCC such as *TP53, CDKN2A*, and *PIK3CA*. Interestingly, these 2 independent research groups reported for the first time mutations in *NOTCH1*. In fact, *NOTCH1* mutations were the second most common mutation in HNSCC (Table 1). To date, the study of genetic mutations in cancer has led the quest in identifying driver events and critical pathways in oncogenesis, ultimately leading the rising wave of biologically targeted therapies. The following review highlights the most common mutations in HNSCC and the resulting alterations in the involved cellular pathways. Emphasis will be placed on clinical correlations and potential therapeutic interventions of the involved genes and pathways. Additionally, the impact of human papillomavirus (HPV)—a causative agent in a growing proportion of oropharyngeal HNSCC—on the mutational landscape of these tumors will be discussed.

TP53

Approximately half of HNSCC tumors harbor mutations of the *TP53* gene located on chromosome 17p13.1, making this tumor suppressor gene the most commonly mutated gene in this tumor type.[4-6] Genetic alterations in *TP53* function are a common occurrence in many human cancers and *TP53* is often called “the guardian of the genome” whose function is to assist with cell cycle arrest, DNA repair, and apoptosis. When DNA damage is detected, cellular sensors activate *TP53*, which in turn regulates the transcription of genes that can lead to cell cycle arrest, providing the necessary pause for the cell to attempt DNA repair. If the damage cannot be repaired, *TP53*-induced factors will direct the cell to apoptosis or senescence.[7] These critical functions prevent damaged cells from propagating and accumulating further cellular damage. In cells in which loss of function of *TP53* has occurred, restorative processes, apoptosis, and/or senescence are unable to occur, allowing for the survival of damaged cells that can eventually give rise to malignancy.

Tumor protein p53 is made of 11 exons, of which the first is noncoding, and consists of 393 amino acids divided into 4 main regions. The protein contains a central region with a critical DNA-binding domain, a C-terminal domain that contains both a tetramerization region and a regulatory region that can bind to the central portion of the protein to inhibit specific protein–DNA interactions, and finally the N-terminal domain that is a transactivation region.[8] Several stress-induced kinases can activate p53 by phosphorylating specific residues in the C-terminal regulatory domain, allowing for conformational changes in the protein that ultimately promotes DNA binding.[9] Pathways known to induce activation of p53 include the ATM, CHK2, p14, and ATR pathways. ATM activation is stimulated by double-stranded DNA breaks and activates p53 through CHK2 activation.[10] Several oncogenes lead to p53 activation through p14.[11] In addition, the levels of p53 are primarily regulated by MDM2, which binds to the N-terminal of the protein leading to ubiquitination and proteolytic degradation.[8,12] The presence of activated p53 leads to activation of several cell cycle regulation and apoptosis pathways. Cell cycle regulating genes require lower levels of p53 to be activated than proapoptotic genes do.[13] Notably, p53 induces the expression of the cell cycle regulator p21 that can inhibit progression through the cell cycle or induce cell senescence. Other important transcriptional targets of p53 include the proapoptotic genes *BAX* and *PUMA*.[11,14,15] Disruptive *TP53* mutations that lead to loss of

protein function preferentially affect the DNA-binding domain of the protein (L1–L2 region, exons 5 to 8).[16] In our series, more than 63% of *TP53* mutations were missense, with the remainder predicted to be inactivating (16% nonsense, 16% insertion or deletion [indel], 8% splice site mutations).[4] Similarly, Stransky et al[5] reported 50% of *TP53* mutations as missense, the remainder of which were various predicted inactivating mutations. The functional effect of the different mutations adds to the complexity of the system, where some mutations completely disrupt DNA-binding capabilities and some mutations allow p53 to interact only with a subset of genes. Furthermore, p53 can also exert functional effects through direct protein–protein interactions with a number of important cell regulatory proteins; and because missense mutations alter its tertiary structure, mutant p53 can interact differentially and disrupt the function of these proteins. This phenomenon, known as "gain of function," was initially described by Levine and colleagues several decades ago.[17,18] In this manner, gain of function mutant *TP53* can function as a dominant oncogene and promote tumor progression in a variety of ways.[7]

Mutations of *TP53* have been identified as an early event in HNSCC, present even in premalignant disease. Oral premalignant dysplastic lesions have been shown to harbor *TP53* mutations in 15% to 27% of cases.[19,20] The presence of *TP53* mutations has been associated with increased risk of progression to malignancy and indeed the incidence of mutations increases with histologic progression from mild dysplasia to invasive carcinoma.[20,21] Tobacco and alcohol exposures have long been regarded as a risk factor for HNSCC. Exposure to these substances has been associated with increased *TP53* mutation rates in patients with HNSCC, where the rate of mutation is almost double in exposed patients compared with nonsmokers.[22,23]

The presence of *TP53* mutations in HNSCC is associated with poor clinical outcomes and disease progression. Our group recently published a large multicenter trial analyzing 420 patients using a hybridization approach with a *TP53* chip that carries all known *TP53* mutations. This analysis showed a decrease in survival by more than 1.5-fold in patients with HNSCC with disruptive *TP53* mutations.[6] It is important to highlight that the use of *TP53* as a prognostic marker in HNSCC had for a long time remained controversial largely due to differences in testing techniques. Prior to the more widespread use of sequencing techniques, the use of immunohistochemistry (IHC) for mutation assessment was a popular approach. Subsequent studies demonstrated discordance in up to 40% of the cases when comparing IHC to sequencing.[24] Additionally, many earlier studies had not analyzed the entire length of the coding sequence, focusing on only certain regions of the gene, thus underestimating the true incidence of mutations. Studies that perform whole exon sequencing of *TP53* and take into account the different types of mutations (disruptive vs nondisruptive) have continued to confirm the negative impact on survival.[25]

Poor tumor response to chemotherapy and radiotherapy has been another variable associated with the presence of *TP53* mutations. The mutation status of *TP53* has been associated with poor response to cisplatin and fluorouracil in patients with HNSCC.[26] A prospective trial analyzing 106 patients showed *TP53* mutation status was an independent predictor of response to cisplatin and fluorouracil, with a risk of nonresponse 2.7-fold higher than that of patients with wild-type *TP53*.[27] *TP53* mutations are strongly associated with locoregional recurrence following primary radiotherapy[28,29] and in some studies predict locoregional failure in patients receiving radiotherapy as adjuvant therapy following surgical resection.[7,30] A recent study by Skinner et al[7] showed that in postsurgical patients with HNSCC receiving radiotherapy, disruptive *TP53* mutations were associated with increased locoregional recurrence when compared with nondisruptive mutations; in support, in vitro data demonstrated a decrease in radiation-induced senescence in HNSCC cell lines with disruptive *TP53* mutations—a major mechanism facilitating locoregional recurrence.

In light of the significant prognostic implications of *TP53*, the molecule continues to be explored as a potential biomarker, although it has not reached the sensitivity and specificity necessary for the use in a broad population-based screening study. Saliva and serum antibody detection assays continue to be developed for early detection.[31,32] Detection of *TP53* mutations in histologically negative surgical margins of patients with HNSCC may be able to identify patients at high risk of recurrence.[33]

Therapeutic strategies aimed at restoration of wild-type *TP53* using tumor injections of viral vectors continue to show promising results in patients with HNSCC. Phase I clinical trials have established the safety profile of the use of intralesional injections. The most commonly reported side effects included transient low-grade fever, intralesional discomfort, and inflammation.[34] Subsequently multiple phase II trials have shown encouraging responses to therapy.[35-37] Recently, a phase III trial comparing adenovirus *TP53* gene therapy, Advexin (Introgen Therapeutics Inc., Austin, TX), versus methotrexate for recurrent advanced HNSCC showed that wild-type *TP53* patients had better response to Advexin, whereas patients with mutant *TP53* responded better to methotrexate.[38] Therapies targeting the *TP53* pathway will continue to be explored in clinical trials and are expected to soon translate into approved therapeutic options. The drug ONYX-015 (Onyx Pharmaceuticals Inc., San Francisco, CA), a *TP53* adenoviral-based treatment for patients with HNSCC has recently been approved for use in China.[39]

NOTCH1

As mentioned earlier, next-generation sequencing has identified *NOTCH1* as the second most commonly mutated gene in HNSCC. The reported incidence of *NOTCH1* mutations was found to be 15% and 14% in each study.[4,5] Given the size of the *NOTCH1* gene, which is composed of 34 exons, next-generation sequencing was an important tool in detecting these mutations across such a large gene. *NOTCH1* is believed to play important roles in regulating normal cell differentiation, lineage commitment, and embryonic development, all biological processes deranged in cancer. A dual biological role of some genes as either tumor suppressors or oncogenes has been described and, in HNSCC, *NOTCH1* appears to act as a tumor suppressor gene, whereas initial reports of *NOTCH1* mutations in leukemia described a constitutively activated truncating oncogenic mutation.[40] Bialleic loss of function was often seen in our HNSCC specimens. In fact, in our study, 7 of 21 patients with *NOTCH1* mutations had 2 independent mutations and loss of heterozygosity (LOH) for this gene was seen in an additional 2 tumors.[4] Recent evidence from several other tumor types supports the possibility that *NOTCH1* can function as a tumor suppressor gene. Murine models have highlighted the importance of *NOTCH1* in squamous epithelial differentiation, because loss of *NOTCH1* in these models contributes to skin carcinogenesis.[41,42] In cutaneous SCC, recent sequencing efforts also suggest a similar loss of function mutational pattern of *NOTCH1*.[43]

The NOTCH1 protein is a transmembrane ligand receptor/signal transducer that is structurally divided into extracellular and intracellular domains. Cleavage of the NOTCH1 intracellular domain (NICD) and translocation to the nucleus is necessary for transcriptional activation and downstream signaling. Proteasomal degradation and downregulation are mediated through the PEST intracellular domain. The extracellular domain is comprised of multiple epidermal growth factor (EGF)–like repeats and 3 LIN12/Notch repeats (LNR). Five NOTCH1-receptor ligands have been described: Jagged 1 and 2, Delta 1, 3, and 4. After receptor activation through ligand binding, 2 cleavages are necessary for release of NICD. First the extracellular portion of the protein is released by protease TNF-α–converting enzyme (TACE). Cleavage 2 by the γ-secretase complex releases the NICD.[44] In the nucleus NICD activated transcription by binding to CBF1 in the presence of coactivators from the Mastermind-like family (MAML). Downstream target genes of NOTCH1 signaling

are crucial for cell differentiation and normal embryonic development of numerous organ systems including keratinocytes and neural tissues. The *Hrt* and *Hes* family of genes, for example, are major effectors of NOTCH1 signaling.[45]

Activating mutations and loss-of-function mutations preferentially occur at different regions of the *NOTCH1* gene. Deletions and mutations of the PEST regulatory domain may prevent proteasomal degradation and prolong downstream activation. Likewise, mutations of the extracellular heterodimer domain may allow constitutive *NOTCH1* signaling in the absence of ligand binding. These previously reported mutations help to explain the oncogenic role of *NOTCH1* in some cancers. In contrast, the majority of *NOTCH1* mutations in HNSCC affect either the EGF-like ligand-binding domain or the NICD domain, suggesting loss of function.[4] The first functional study of putative inactivating *NOTCH1* mutations in skin and lung SCCs was published confirming loss of function of mutations affecting these regions of the gene.[46] In addition to *NOTCH1* mutations, *FBXW7* mutations were identified in 5% of HNSCC specimens sequenced.[4] *FBXW7* forms part of the ubiquitin ligase complex that can mediate NOTCH1 degradation.[47] Thus *FBXW7* mutations could also be affecting the *NOTCH1* pathway, although *FBXW7* is also known to target other oncogenic pathways such as cyclin E and c-myc.

Therapeutically targeting NOTCH1 presents a dilemma, considering the pathway has both oncogenic and tumor suppressor activity depending on temporal and tissue-dependent contexts. A variety of γ-secretase inhibitors (GSI) are available and these can target the constitutively active *NOTCH1* pathway by preventing NICD cleavage and nuclear translocation.[48] GSIs have shown promise in animal and in vitro studies of melanoma and Kaposi sarcoma.[49] Likewise, human trials of GSIs are being investigated in T-cell acute lymphoblastic leukemia (T-ALL) and in advanced breast cancer.[50,51] However, a recently halted phase III trial of GSI for treatment of Alzheimer's disease raises a cautionary tale. Unfortunately, treated patients had an elevated incidence of skin cancer, possibly through their adverse affect on squamous epithelial differentiation.[52] In the context of cancer treatment, successful abrogation of *NOTCH1* oncogenic activity at one site may be complicated by loss of its tumor suppressor function at another site.

Ras-mediated pathways: Raf/MEK/ERK and PI3K

Mutations of *Ras* genes have been implicated in at least a third of human cancers.[53] In HNSCC, particularly oral cancer, the true incidence of mutations in the *Ras* genes has long been debated. Mutations in one of the *Ras* genes, *HRAS*, had been reported in as high as 35% of oral cancers from India, whereas similar studies from the United States failed to identify any mutations.[54-56] With the advance of deep sequencing, *HRAS* mutations have been confirmed as one of the most common mutations in HNSCC in the United States. Mutations of *HRAS* were the sixth most common mutation, with an incidence of 4% in our whole exome sequencing project.[4] Similarly Stransky et al[5] reported *HRAS* mutations as the eighth most common mutation, with an incidence rate of 5%.

The *Ras* family includes 3 genes: *HRAS* (Harvey), *KRAS* (Kirsten), and *NRAS* (neuroblastoma). Considering all human cancers, *KRAS* mutations are the most common type of *Ras* mutation, with *HRAS* being the least common. In head and neck cancer, *Ras* mutations appear to be exclusively *HRAS* mutations.[57] Mutations of Ras proteins preferentially affect codons 12, 13, and 61.[53,58] Oncogenic *Ras* mutations cause the protein to be perpetually active in the GTP-bound state resulting in increased proliferation and survival signaling.[59] Ras proteins are GTPases of approximately 21 kilodaltons localized to the plasma membrane that function as signaling switches by alternating between the GTP-bound active state and the GDP-bound inactive state. All 3 proteins are similar except for their C-terminal domain, which helps determine the distribution of these proteins within the

plasma membrane.[60] Ras downstream effector pathways include the Raf pathway and the phosphatidylinositol 3-kinase (PI3K) pathway. Activated Raf phosphorylates MEK, in turn activating ERK. The Raf/MEK/ERK pathway is involved in the regulation of cell proliferation, differentiation, morphology, and survival. PI3K converts phosphatidylinositol (4,5) biphosphate (P4,5P2) into phosphatidylinositol (3,4,5) triphosphate (PIP3), in turn activating Akt/PKB kinases. PI3K activation promotes cell growth, cell survival, and cytoskeleton reorganization.[61,62]

Overactivation of the PI3K pathway in HNSCC may occur through *PIK3CA* mutations or *PTEN* loss. The *PIK3CA* gene encodes for the catalytic subunit p110alpha, of the PI3K heterodimer, which also contains a regulatory subunit (p85, alpha/beta/gamma).[63] *PTEN* (phosphatase and tensin homolog deleted on chromosome 10) is a key regulator of *PI3K* function, by disrupting *PI3K* products (*PI3, 4P2*, and *PIP3*), downstream targets such as Akt are no longer activated.[64]

Our exome sequencing analysis revealed *PIK3CA* activating mutations in 6% of tumors,[4] whereas Stransky et al[5] similarly reported mutations in *PIK3CA* in 8% and in *PTEN* in 7% of tumors, respectively. Regions of chromosome 3q26 were also amplified in some tumors, including the *PIK3CA* locus, potentially increasing activity of PI3K signaling. Previous studies reported activating mutations of *PIK3CA* and inactivation of *PTEN* in as many as 20% and 10% of tested tumors, respectively.[63,65] Also consistent with our findings, frequent amplification of *PIK3CA* in tumors as well as premalignant lesions has been reported suggesting an early genetic aberration in carcinogenesis.[66,67] Downstream over-activation of Akt and other effectors has many consequences summarized in the following text.

Many receptor tyrosine kinases (RTKs) activate PI3K. Upstream RTKs EGFR, ErbB3, Met, and vascular endothelial growth factor receptor (VEGFR), as well as G-protein–coupled receptors activate PI3K after binding growth factor ligands.[68] Overexpression of *EGFR* is described in HNSCC; perhaps over 90% of tumors demonstrate increased activity, in the absence of somatic mutations.[69,70] Interestingly, although EGFR lacks the necessary C-terminus consensus sites for binding PI3K, it can heterodimerize with ErbB3 and other proteins that possess the appropriate residues to associate with PI3K.[68]

Once PI3K binds to an activated RTK, PI3, 4P2, and PIP3 are generated and lead to activation of a variety of proteins that share a Pleckstrin homology domain.[68] PIP3 recruits Akt to the plasma membrane and activates PDK1/PDK2, kinases that in turn activate Akt. Broad downstream cascades are driven by Akt activation, which is responsible for cell proliferation, glucose metabolism, prosurvival signaling, and angiogenesis. Akt is a serine/threonine kinase that inhibits or activates targets via phosphorylation. Many downstream effects ultimately result in inhibition of apoptosis.[71] For example Mammalian-Target-of-Rapamycin (mTOR), cyclin D1, and NF-κB are activated by Akt, directly and indirectly, promoting cell growth and survival.[68,72-75] Like Akt, phospholipase Cgamma1 (PLCgamma1) has a PH domain and associates with PI3K. PLCgamma1 hydrolyzes PI4,5P2 to IP3 and diacylglycerol (DAG), activating protein kinase C (PKC) that ultimately promotes cell proliferation, migration, and invasion.[68,76]

Various strategies to inhibit Ras signaling have unfortunately been largely unsuccessful in clinical trials, possibly due to secondary alterations in upstream and downstream Ras pathway effectors.[77] Evidence for overexpression of *EGFR*, amplification of *PIK3CA*, and activation of Akt in various tumors including HNSCC, makes it attractive to target multiple levels of this pathway.[78] The EGFR-inhibitory monoclonal antibody, cetuximab, has been approved for locally and regionally advanced HNSCC in combination with radiation. In select cases, improvement of locoregional control and overall survival with the addition of

cetuximab to radiation therapy has been realized.[79] Resistance to EGFR inhibition may be explained by mutant activation or amplification of *PI3K* and downstream effectors autonomous of EGFR and other RTKs.[73] Therapies targeting inhibition of multiple points along the Ras/PI3K/Akt/mTOR pathway are potential approaches to overcome resistance to EGFR inhibition. A 2-part phase I/II clinical trial is under way to investigate combined Mek and Akt inhibition in various solid tumors including HNSCC.[80] Combined cetuximab and lapatinib (inhibiting EGFR and ErbB2) is in phase I clinical trials for patients with HNSCC, colorectal, and lung cancer. The mTOR inhibitor rapamycin, a Food and Drug Administration (FDA)-approved immunosuppressant, is also in phase I trial for treatment-naïve patients with advanced HNSCC.[81] Specific Akt inhibition has been challenging. This is believed to be attributed to sequence similarity with other protein kinases of its family (AGC) that can lead to significant toxicity when Akt is inhibited. An Akt inhibitor, MK2206, is currently in phase II trials for treatment of recurrent and meta-static HNSCC.[82] There is also rationale for addition of PI3K pathway inhibitors to conventional chemo/radiotherapy as an approach to overcoming chemo/radioresistance. PI3K inhibitor PX-866 (LC Laboratories, Boston, MA) is being studied for treatment of HNSCC in individual combinations with docetaxel or cetuximab in phase I and phase II trials.[83,84] PI3K is upstream of Akt and many other effectors, and requires careful evaluation of toxicity that may arise from disruption of normal homeostatic processes.

CDKN2A (p16)

Cyclin-dependent kinase inhibitor 2A (*CDKN2A*), located at chromosome 9p21, is a known tumor suppressor gene involved in the regulation of cell cycle progression and it is often disrupted in HNSCC. LOH at the *CDKN2A* locus has long been recognized as an early event in the progression of premalignant lesions to HNSCC.[85-87] More recently, in our whole exome next-generation sequencing project, we were able to corroborate the importance of *CDKN2A* mutations that were identified in 9% of all tumors. In addition to mutations, gene copy number analyses revealed common LOH and deletions of *CDKN2A*.[4] Furthermore, genetic alterations are not the only silencing mechanism for *CDKN2A* in HNSCC. In fact, *p16* inactivation is present in more than 80% of HNSCC when genetic alterations and epigenetic silencing through DNA hypermethylation are considered.[86,88]

The p16 protein plays a critical role in cell cycle regulation via its interaction with the Rb tumor suppressor. In the G1 phase, Rb complexes with and inhibits transcription factor E2F to promote senescence. Cyclin-dependent kinases CDK4 and CDK6 phosphorylate Rb, releasing E2F, which facilitates S-phase progression.[89-91] p16 is an inhibitor of CDK4/6, inhibition that allows Rb-E2F complexes to stabilize, and halts progression to S phase. This is especially relevant in HPV-positive tumors, where Rb is commonly inactivated by viral oncogenes, as described later in the HPV section.

Although genetic alterations at the 9p21 locus encompassing *CDKN2A* are common early events in HNSCC, these are likely insufficient to drive tumorigenesis by themselves. This is supported by the fact that *CDKN2A* mutations have been reported in benign epithelial lesions that have low potential to transform into malignancy such as benign squamous hyperplasia.[92] However, studies of oral leukoplakia have demonstrated increased malignant potential in lesions with LOH at 9p and other chromosomal hot spots.[93,94] In the later discussion of HPV-associated oropharyngeal HNSCC, we also discuss *p16* testing as a surrogate biomarker for HPV-positive tumors, a distinct HNSCC population with a more favorable clinical prognosis.[95]

Therapeutic targeting of CDKN2A/p16 presents the challenge of restoring tumor suppressor activity, or inhibiting downstream targets that have been rendered overactive. In vitro experiments with demethylating agent 5-aza have demonstrated recovery of *p16*

expression.[96,97] Experimental *p16* gene adenovirus constructs aim to restore cell cycle control.[98] Preclinical elucidation of mechanisms is translating to human trials and, in this case, has prompted inhibition of downstream targets such as CDK4 in various solid and hematologic cancers. The degree to which toxicity will limit such therapies remains to be determined.[99-102]

Human papillomavirus

In recent years, HPV has emerged as a primary etiologic agent in a large subgroup of oropharyngeal cancers. In the United States, the incidence of HPV-positive HNSCC has dramatically increased by more than 200% from the late 1980s to the early 2000s.[103] HPV-positive–associated cancers exhibit different risk factors, epidemiology, and distinct clinical behavior from the rest of the HNSCCs. Not surprisingly, the mutations in this subgroup are also different. Mutations in HPV-positive tumors are at least half as frequent as in HPV-negative tumors. Our group reported a high confidence nonsynonymous mutation rate of 4.8 ± 3 versus 20.6 ± 16.7 (mean ± SD) in HPV-positive versus HPV-negative tumors, respectively[4]; Stransky et al[5] similarly reported a lower mutation rate of HPV-positive tumors compared with HPV-negative tumors. Additionally, although *TP53* mutations are the most common mutation in HNSCC, they are an exceedingly rare occurrence in HPV-positive cancers, pointing to distinct biological mechanisms of tumor formation.[4,104] As the incidence of HPV-associated HNSCCs continues to increase, understanding the role of HPV in tumorigenesis remains critical.[103]

HPV is a double-stranded DNA virus of approximately 7.9 kilobases that was initially identified as the causative agent of anogenital cancers and cervical cancer. In the past 15 years HPV has emerged as an important causative agent in HNSCC. Distinct subtypes of HPV are associated with different clinical lesions. HPV-16 is a high-risk subtype and the main subtype found in HNSCC,[103] whereas HPV-6 and HPV-11 are low-risk subtypes commonly associated with common warts and laryngeal papillomatosis.[105] In the head and neck region, the reticular epithelium of Waldeyer's ring in the oropharynx is hypothesized to be particularly susceptible to HPV infection because of its inherently porous basal membrane that allows entry and processing of foreign antigens and presentation to the underlying lymphoid tissue.[106] The risk of HPV infection in the head neck region increases with the number of orogenital contacts, and sexual history appears to be the main risk factor for HPV-positive HNSCC.[107,108] HPV-positive cancers present in patients that are, on average, 5 years younger than non-HPV-infected patients with HNSCC. In addition, patients with HPV-associated cancer typically have had lower exposure to tobacco and alcohol.[107] Clearly, emerging behavioral patterns in the population at large with decreased tobacco consumption, increased numbers of sexual partners, and oral sex are leading to a decrease in HNSCC associated with traditional risk factors and an increase in HPV-positive oropharyngeal HNSCC.[103]

Although the specific protein interactions are different, HPV targets critical pathways for cell cycle regulation that have previously been described in HPV-negative cancers. HPV oncoproteins E6 and E7 target and degrade the well-known tumor suppressors p53 and Rb. HPV E6 protein targets p53 for ubiquitination and subsequent proteolysis and, in this manner, inactivates the tumor suppressor gene in the absence of mutations.[109] Rb blocks the progression from the G1 phase into the synthesis phase. HPV E7 binds to the cullin 2 ubiquitin–ligase complex and degrades Rb, thereby disrupting cell cycle control. In HPV-negative cancers, the Rb pathway is inhibited by deletion or lack of transcription of *p16*, which is an upstream regulator of Rb. By inhibiting cyclin-dependent kinases 4 and 6 (CDK4/6), p16 allows for phosphorylation and activation of Rb and prevents the cell from progressing into the synthesis phase.[110] Given that HPV-positive cancers express p16, IHC for p16 is often used as a surrogate maker for the presence of HPV.[111] Viral DNA content in

HPV-positive tumors varies greatly from a single copy per human genome to 15,500 copies.[112] Given that only a single copy may be necessary in cancer, relying on copy number to distinguish innocuous viral infection from an HPV-driven cancer is not reliable and establishing that HPV genes are actively transcribed is necessary. At our institution, in situ hybridization for high-risk HPV followed by p16 IHC is used for diagnosis.[109] Original reports of HPV-positive HNSCCs in sites other than the oropharynx largely depended on polymerase chain reaction (PCR) detection of viral DNA and failed to establish active transcription of HPV genes, although this requires further investigation.[113]

Multiple studies have confirmed that HPV-positive patients have a favorable prognosis with better treatment response and ultimately improved survival when compared with HPV-negative patients.[114-116] In 2008, a prospective clinical trial evaluating 96 patients with advanced HNSCC showed better response to chemoradiation (84% vs. 57%) as well as better overall survival (95% vs 62%).[117] In 2010, a retrospective trial evaluating 266 patients with advanced oropharyngeal cancer again confirmed better overall survival based on HPV status (82% vs 57%).[118] Given the favorable outcome of HPV-positive cancers, deintensification trials are currently under way in an attempt to minimize morbidity associated with the use of concomitant chemoradiotherapy. It is important to highlight that not all HPV-positive patients have the same outcome. Patients with heavy tobacco exposure, even if HPV-positive, have been reported to have worse outcomes and are categorized as “intermediate risk” between HPV-positive tobacco naïve and HPV-negative patients.[118] From a genetic perspective, patients with tobacco exposure would be expected to have accumulated different genetic alterations than tobacco naïve patients, perhaps explaining these differences in outcome. These results highlight the importance of multiple biomarkers coming together to allow for accurate prognostic stratification and highlight the importance of continued efforts in the genomic characterization of HPV as well as non-HPV-associated HNSCCs.

Important public health prevention strategies are becoming available for oropharyngeal carcinoma with the emergence of HPV vaccines. In 2006, Gardasil (Merck & Co Inc., Whitehouse Station, NJ), a quadrivalent HPV vaccine against serotypes 6, 11, 16, and 18, was approved by the FDA for the prevention of cervical neoplasia and cervical cancer in girls and women from age 9 to 26 years.[119] In 2009, Cervarix (GlaxoSmithKline Biologicals, Rixensart, Belgium), a bivalent vaccine against serotypes 16 and 18, was approved for the same demographic group.[120] Assessing the efficacy of HPV vaccines in reducing the incidence of HPV-associated HNSCC will require a long-term review of incidence rates in pre- and postvaccine eras, bearing in mind that such cancers manifest primarily in patients over 40 years of age. Reduction in oropharyngeal HPV infection rates may be a more immediate metric for inferring efficacy.[121] In October 2011, the Advisory Committee on Immunization Practices of the Center for Disease Control and Prevention (CDC) recommended vaccination for boys and men for the prevention of both anogenital and oropharyngeal cancers.[122]

Clinical implications and future directions

Designing targeted therapies in HNSCC remains challenging, given the diversity of genetic alterations and frequent occurrence of tumor suppressor inactivation within this tumor’s mutational spectrum. HNSCC represents a heterogeneous group of tumors in which multiple genetic alterations are observed, as opposed to a single dominant translocation or mutation. The mutation rate in HNSCC is consistent with other solid tumors in which single tumors commonly have mutations in more than 3 genes, in contrast to many leukemias and lymphomas in which the most common abnormality is a single genomic translocation that leads to activation of an oncogene.[123,124] Given the heterogenicity of the tumors, multiple

genes, and pathways will need to be evaluated for diagnostic, prognostic, and therapeutic studies.

In HNSCC, the vast majority of tumors harbor inactivating mutations of tumor suppressor genes, whereas activating mutations generating oncogenes are infrequent. The clinical implications of the mutation types are significant as new generation targeted therapies will have the challenge of restoring function rather than selectively inhibiting oncogene activation. Restoring the lost function of a tumor suppressor gene has proven to be a difficult task and no systemic therapies with this purpose are currently available. Because of the difficulties in developing new targeted therapies, early detection and careful surveillance are currently the best approaches to reduce morbidity and mortality in HNSCC. Emphasis should be placed on public health strategies aimed at reducing risk factors, such as tobacco and alcohol cessation programs and HPV vaccination. In the future, somatic mutation detection may influence early diagnosis and tumor monitoring and surveillance. In particular, detection of mutations in plasma or saliva can aid in early detection and tumor surveillance, whereas detection of mutations in surgical margins and lymph nodes may help identify residual disease.

Although this review emphasized mutations in HNSCC, disruption of critical pathways in cancer can occur through nongenetic mechanisms such as posttranslational modifications or epigenetic silencing. Different mechanisms can target the same critical pathways as seen in the case of *p16*, which is inactivated by mutations in a subset of tumors and by DNA methylation in another.[88] *EGFR* is an example of a gene that does not contain sensitizing mutations in HNSCC but is an effective target of therapy in this tumor type.[126] Mutations of *EGFR* in colon and lung studies led to the identification of the EGFR pathway as critical in the pathogenesis of cancer. However, sensitizing mutations affecting the intracellular domain are not prevalent in HNSCC.[127] In HNSCC, overexpression and amplification render tumors sensitive to EGFR therapies.[128] Comprehensive delineation of the pathways and nongenetic mechanisms altering the pathways remain to be explored. In-depth analysis of critical pathways identified through genetic studies may provide additional direction for targeted therapies. Certainly, the role *NOTCH1* plays as a tumor suppressor or an oncogene in specific cell types remains to be elucidated and might depend not only on the characteristics of the mutations but also on other mechanisms regulating the pathway.

CONCLUSIONS

HNSCCs are comprised of distinct diseases at the molecular level. The different genetic landscapes associated with HPV and tobacco exposure are consistent with clinical and epidemiologic data, suggesting the importance of these environmental factors in prognosis and therapeutic response. Another important observation is that activating mutations in oncogenes are rare. In contrast, the majority of the tumors harbored inactivating mutations in tumor suppressor genes, predominantly *TP53* and *NOTCH1*, or inactivation of p53 and Rb through HPV infection. This distinction is critical because targeted therapies for HNSCC may have limited utility. Therefore, prevention and early detection are the optimal approaches for reducing morbidity and mortality from HNSCC. In addition to the therapeutic implications, the recently published cancer genetic studies have potential implications on prognosis and diagnosis that will be defined by future studies. Collectively the genomic studies, with therapeutic, diagnostic, and prognostic significance, provide a framework to make personalized cancer therapy a reality for patients with HNSCC.

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TABLE 1

Frequently mutated genes in HNSCC.

Gene symbol	Gene name	Chromosomal location	Gene function	Selected downstream targets	Targeted therapeutics*	Mutation rate, %	References
TP53	*Tumor protein p53*	17p13.1	Tumor suppressor: "Guardian of the genome." Assists in cell cycle arrest, DNA damage repair, apoptosis, and senescence	*p21, BAX* and *PUMA*	• Adenoviral-based p53 gene replacement	40–60	4, 5, 7, 11, 14, 15, 36-39
NOTCH1	*Notch1*	9p34.3	Tissue-dependent role as tumor suppressor or oncogene. Important in regulation of cell differentiation, lineage commitment, and embryonic development	*Hes/Hey, p21*	• Gamma-secretase inhibitors studied in T-cell acute lympho-blastic leukemia and breast cancer	14–15	4, 5, 40, 41, 42, 45, 47, 48, 50, 51
HRAS	*Harvey rat sarcoma viral oncogene homolog*	11p15.5	Oncogene; GTP binding protein important in promoting cell proliferation and survival signaling through the Raf and PI3K pathway -	Activates Raf/MEK/ERK pathway and PI3K/Akt pathway	• Inhibition of downstream effectors of Ras, including MEK and Akt • Farnesyltransferase inhibitors prevent appropriate localization of Ras to the intracellular membrane • Antisense oligonucleotides against H-RAS mRNA studied in colon and pancreatic cancer	4–35	4, 5, 57, 77
PIK3CA	*Phosphoinositde-3-kinase catalytic alpha polypeptide*	3q26.32	Oncogene. Catalytic subunit of PI3K. Target of Ras activation. Important in regulation of cell differentiation, lineage commitment, and embryonic development	Activates Akt and PLCgamma1	• Inhibition of downstream effectors of PI3K, including Akt and mTOR • PI3KCA inhibitors studied in breast and non-small cell lung cancer	6–8	4, 5, 59, 61, 62, 68, 72-76, 80, 82-84

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CDKN2A	*Cyclin-dependent kinase inhibitor 2A*	9p21.3	Tumor suppressor. Regulates G1 cell cycle progression to S phase	Inhibits CDK4/6	• CDK inhibitors	9	4, 5, 88, 90

Abbreviation: HNSCC, head and neck squamous cell carcinoma.

*Therapeutic investigations performed in head and neck cancers unless otherwise indicated.