BIOMARKERS, GENOMICS, PROTEOMICS, AND GENE REGULATION

Molecular Heterogeneity of Head and Neck Squamous Cell Carcinoma Defined by Next-Generation Sequencing

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Head and neck squamous cell carcinoma (HNSCC) can be divided into two different clinical entities based on their association with high-risk subtypes of human papilloma virus (HPV16 and HPV18). Dissimilarities in prognosis and molecular profiles have attracted much attention in recent years, in part because of increasing rates of HPV infection in HNSCC; however, the underlying mechanisms and detailed genetic profiles that set these tumors apart are still elusive. To elucidate oncogenic pathways in HNSCC with and without HPV infection, we used targeted next-generation sequencing to interrogate single-nucleotide polymorphisms (SNPs) in 50 cancer-related genes. We detected SNPs in 25 of these genes from HNSCC tissue specimens with and without HPV infection. In 5 of the 25 genes, variant patterns were similar regardless of HPV infection status. A greater number of sequence variants in genes from the tyrosine kinase receptors and their associated pathways were preferentially present in HPV⁺ specimens. SNPs in genes related to tumor-suppressor functions were more prevalent in HPV[−] HNSCC specimens. The observations may help to elucidate mechanisms involved in the molecular pathogenesis of two clinically diverse subclasses of HNSCC. Over-representation of SNPs in either HPV⁺ or HPV[−] HNSCC is another indicator of potentially actionable sequence variants for targeted therapy. (*Am J Pathol* 2014, 184: 1323–1330; <http://dx.doi.org/10.1016/j.ajpath.2014.01.028>)

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide. Despite recent advances in chemoradiation therapy, the 5-year survival rate for HNSCC is approximately 50%.¹ Based on epidemiology and disease outcomes, histologically diagnosed HNSCC has been stratified into two distinct prognostic entities directly linked to infection with high-risk human papilloma virus (HPV). Of the high-risk subtypes, HPV16 has been found in approximately 90% of infected HNSCC and HPV18 in approximately 5%.^{2–5} Other high-risk HPV types (eg, HPV31 and HPV33) have been detected in a few cases of HNSCC.² The incidence of HPV-related oropharyngeal cancer continues to increase globally.^{6–9} Compared with HPV[−] HNSCC patients, HPV⁺ HNSCC patients show poor cell differentiation, better response to chemotherapy

and radiotherapy, and better survival.² The 3-year survival of HPV⁺ HNSCC patients is 83%, as opposed to the 57% observed in HPV[−] HNSCC patients, and increased HPV viral copy number is associated with enhanced response to chemotherapy.^{10,11} In general, HPV infection and p16^{INK4a} expression are recognized as predictive and prognostic biomarkers of HNSCC.^{12–15}

Infection with HPV has divergent effects in different cancers; high-risk HPV16 and HPV18 subtypes account for 70% of cervical cancers with poor outcomes, whereas the virus has beneficial effects in patients with HNSCC. These differential

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effects suggest that host elements that interact with the virus may play a role in determining the pathogenesis of the cancer and consequences of the infection. In the present study, we analyzed host factors that play a role in viral tumorigenesis, including the proinflammatory cytokine tumor necrosis factor alpha (TNF- α) and the tumor suppressors p16^{INK4a} (alias CDKN2A) and p53 (alias TP53). Others have shown that *TP53* is the most commonly mutated gene in HNSCC and that this mutation is more prevalent in HPV⁻ tumors.¹⁶ Genome-wide association studies using exome sequencing revealed that functionally disruptive mutations in genes such as *NOTCH1* are major drivers in the carcinogenesis of HNSCC. In a recent targeted next-generation sequencing (NGS) study, Lechner et al¹⁷ showed that genetic alterations in the PI3K pathway are more prevalent in HPV⁺ HNSCC. The SNP profiles of HNSCC tumors differ between HPV⁺ and HPV⁻ subtypes.¹⁸

Using targeted NGS, we systematically analyzed somatic variants in HNSCC to further understand the differential genomic alterations in HPV-infected versus noninfected tumors. We compared the sequence variants of more than 2800 hotspots in 50 cancer-related genes present in HPV⁺ and HPV⁻ HNSCC patients in an effort to obtain fundamental insights into the pathogenesis of the disease that may identify potential diagnostic biomarkers and therapeutic targets.

Materials and Methods

Patients

Formalin-fixed, paraffin-embedded tissue blocks from 100 patients diagnosed with HNSCC between 2001 and 2013 were included in the study. Primary tumors from the tonsils, the base of the tongue, and the palate were selected for analysis. The sex and ethnicity distribution of the patients is summarized in Table 1. DNA from blood of eight healthy adults was included as an additional control in the sequencing analysis. The study was approved by the Institutional Review Board of Rutgers Biomedical and Health Sciences—New Jersey Medical School (protocol no. 0120100117).

DNA Extraction

The tumor-enriched area on H&E-stained slides was demarcated by a pathologist (N.M.), and the corresponding area was identified on unstained tissue sections (4 to 5 μ m thick) taken from the same tissue block. The selected tissue was macrodissected by scraping it off the slides, and the tumor-enriched tissue was used for analysis. DNA from macrodissected tumor tissue ($n = 100$), matched nontumor tissue from HNSCC specimens ($n = 8$), and blood from healthy donors ($n = 8$) was extracted using a DNeasy blood and tissue kit (Qiagen, Valencia, CA) according to the manufacturer’s recommendations. DNA was quantified using either a NanoDrop spectrophotometer (Thermo Fisher scientific, Waltham, MA) for PCR and for Sanger sequencing or a Qubit fluorometer (Life Technologies, Carlsbad, CA) for NGS.

HPV Detection and Identification

The presence of HPV16 and/or HPV18 was detected with a previously validated laboratory-developed test, using allele-specific quantitative real-time PCR on an ABI 7500 system (Life Technologies).¹⁹ Primers were synthesized at the Rutgers Molecular Resource Facility, and TaqMan probes were obtained from TIB MOLBIOL (Adelphia, NJ). Amplification of the β -globin gene as internal control was performed using a TaqMan Assay (Life Technologies). In brief, 2.5 μ L of extracted DNA, control DNA, or water (no-template control) was added to 22.5 μ L of master mix containing 1 $\times$ TaqMan universal PCR master mix (Life Technologies), 2 mmol/L MgCl₂, 400 nmol/L of each primer, and 200 nmol/L TaqMan probe. Real-time amplification was performed using the following thermal profile: 50°C for 2 minutes and 95°C for 12 minutes, followed by 40 cycles of a three-step amplification at 95°C for 15 seconds, 55°C for 30 seconds, and 60°C for 60 seconds. Amplicons were sequenced to confirm the amplification of HPV16/18.

Detection of p16 by Immunohistochemistry Staining

Formalin-fixed, paraffin-embedded tissue sections were deparaffinized and subjected to antigen retrieval using 10 mmol/L citrate buffer (92°C for 30 minutes). Expression of p16 was evaluated using a mouse monoclonal antibody against p16^{INK4a} (Cell Marque, Rocklin, CA) with 3,3'-diaminobenzidine as the chromogen. Specimens were scored as positive if strong and diffuse nuclear and cytoplasmic staining was present in >70% of the tumor specimen (Supplemental Figure S1A).

Detection of the -308 SNP in the *TNFA* Promoter

DNA extracted from 53 HNSCC patient specimens (23 HPV⁺ and 30 HPV⁻) was analyzed for the -308 *TNFA* promoter single-nucleotide polymorphism (SNP) using Sanger sequencing (Life Technologies). Homozygous wild-type (GG), mutant (AA), and heterozygous (G/A) alleles were identified (Supplemental Figure S1B). Primers for sequencing were as described previously.²⁰

NGS

A total of 69 specimens [53 HNSCC tumor samples (23 HPV⁺ and 30 HPV⁻), 8 matched nontumor specimens, and

Table 1 Sex and Ethnicity Distribution in 100 Patients

Demographics	Number of patients	Average age, years
Sex		
Female	28	62.82
Male	72	58.39
Ethnicity		
Caucasian	42	60.43
Hispanic	24	58.44
African American	20	56.8
Other	14	62.14
Total	100	59.07

8 healthy donor samples] were subjected to NGS on an Ion Torrent PGM platform (Life Technologies). Libraries using 10 ng of DNA were prepared with the Ion AmpliSeq 2.0 kit (Life Technologies) according to the manufacturer's protocol. Genomic DNA was amplified in a multiplex PCR reaction using the Ion AmpliSeq Cancer Hotspot Panel v2, which targets 50 frequently mutated cancer genes and includes more than 2800 hotspots (ie, regions that are frequently mutated) (Supplemental Table S1). Details of the genomic positions for all hotspots are available from the manufacturer. Specimens were run in groups of four and were individually barcoded using an Ion Xpress barcode kit (Life Technologies).

The size distribution and quantity of DNA amplicons was evaluated using an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA). In brief, 1 μ L of prepared library was analyzed on an Agilent high-sensitivity dsDNA chip. A representative report generated by the Agilent bioanalyzer is shown in Supplemental Figure S1C. Peaks ranging from 100 bp to 300 bp were considered to be within the requisite amplicon size range. The concentration of amplicons in the library ranged from 1000 pmol/L to 5000 pmol/L. All libraries were then diluted appropriately to a final concentration of 20 pmol/L. Equimolar amounts of four barcoded libraries that passed quality control were pooled, subjected to emulsion PCR using an Ion OneTouch 200 template kit version 2 DL (Life Technologies), and ligated to Ion Sphere beads. The quality of the amplicons and polyclonality was assessed using a Qubit fluorometer (Life Technologies). Amplicons were subsequently enriched to select viable nanospheres for sequencing on a 316 chip (Life Technologies). Enriched Ion Sphere particles were then loaded onto the 316 chip using an Ion PGM 200 sequencing kit according to the manufacturer's protocol (Life Technologies). Homogeneous loading of the 316 chip used for sequencing can be seen in the Supplemental Figure S1D.

Sequence reads generated were sorted into files and aligned to the human reference genome hg19 using Ion Torrent Variant Caller software version 3.4 (Life Technologies), and the Integrative Genomics Viewer (<http://www.broadinstitute.org/igv>) was used to visualize variants. The visual picture of the sequence alignment with the reference as observed with the Integrative Genomics Viewer was used for elimination of false variants caused by possible sequencing errors or strand bias. Stringent criteria were applied for the determination of true variants. A sequencing run was considered acceptable if it had $\geq 150,000$ reads with a quality score of AQ20 (1 misaligned base per 100 bases). All gene variants reported in our study had to meet at least one of the following criteria: variant coverage $>1000\times$ with $\geq 5\%$ frequency, variant coverage $>500\times$ and $\leq 1000\times$ with $\geq 20\%$ frequency, or variant coverage $>300\times$ and $\leq 500\times$ with $\geq 50\%$ frequency. These conditions were set based on validation experiments performed in our laboratory.

Statistical Analysis

Fisher's exact test and *t*-test were used for statistical analysis.

Results

HPV Infection in HNSCC and Correlation with p16

Stratification of the 100 representative patients in the study (Table 1) showed that the number of men ($n = 72$) diagnosed histologically with HNSCC was roughly three times that of women ($n = 28$) in the study population. The average age was similar for both sexes. Analyzed by ethnicity, 42% of the patients were Caucasians, followed by Hispanics and African Americans. Overall, HPV was detected in 27/100 (27%) tumor specimens analyzed (Table 1). Subtyping revealed that almost all of the 27 infected specimens had HPV16 (25/27), 1 had HPV18, and 1 was coinfecting with both HPV16 and HPV18. Of the Caucasian patients, 16/42 (38%) were HPV⁺, compared with 6/24 (25%) for Hispanic patients and only 1/20 (5%) for African American patients. The presence of p16 was examined by immunohistochemistry in all 100 patient specimens (Table 2), with a finding of 68% positive for p16 (CDKN2A). Representative images of stained slides are presented in Supplemental Figure S1A. There was a notable association between the presence of HPV and p16. Consistent with previous report, 23% of the specimens analyzed in our study were positive for both HPV and p16.^{12–15}

TNFA –308 Promoter Polymorphism and HNSCC

We genotyped 53 patient specimens for the *TNFA* –308 SNP by Sanger sequencing (Supplemental Figure S1B); 28.6% of HPV⁺ specimens were shown to have a proinflammatory –308A genotype, compared with 40% of HPV[–] specimens (Figure 1). We observed no significant association between the –308A allele and HPV infection in HNSCC ($P = 0.34$). Overall, 19 (35.8%) of the 53 HNSCC patients genotyped had the –308A allele, compared with 15.3% in the U.S. population (Centers for Disease Control and Prevention, <http://www.cdc.gov/genomics/population/genvar/frequencies/TNF.htm>, last accessed March 8, 2014).

Figure 1 Percentage of *TNFA* –308A allele in HNSCC patients. DNA extracted from HNSCC tissue specimens was subjected to Sanger sequencing using specific primers flanking the –308 SNP in the promoter region of the *TNFA* gene. The overall group represents combined HPV⁺ and HPV[–] HNSCC specimens.

Figure 2 Types of variants detected in NGS analysis. Variants detected in NGS are classified as population variants, germline SNPs, and somatic SNPs based on the genetic alterations detected in healthy donors, patient–matched nontumor specimens, and HNSCC tumor specimens, respectively. Population variants and germline SNPs represent alterations that are not specific to HNSCC and therefore were excluded from further analysis.

Genomic Variants in HNSCC Specimens Using Targeted NGS

A total of 69 specimens [53 HNSCC specimens (23 HPV⁺ and 30 HPV[−]), 8 matched nontumor specimens (6 HPV⁺ and 2 HPV[−]), and 8 healthy donor specimens] were subjected to targeted NGS to detect somatic variants in candidate cancer genes. Variants found in the specimens analyzed were categorized for clarification of overlapping patterns as population variants, germline SNPs, and somatic SNPs (Figure 2). Population variants were common to healthy donor samples and HNSCC patient samples, and these shared variants were excluded from further comparisons. Germline SNPs were variants detected in both tumor and nontumor components of HNSCC patients and therefore were not considered to be tumor-specific. Somatic variants, generally cancer related, were most often found in tumor tissue; these variants were the main focus of study.

Genomic Variants in HNSCC Tumor, Matched Nontumor, and Healthy Donor Specimens

SNPs in five different genes (*FGFR3*, chromosome position 1807894; *PDGFRA*, position 55141055; *APC*, position 112175770; *RET*, position 43613843; and *EGFR*, position 55249063) were present at high frequencies (>90%) in all three specimen groups ($n = 69$) in the study (ie, 53 HNSCC specimens, 8 nontumor counterparts, and 8 healthy controls) (Figure 2). The presence of common or overlapping SNPs is suggestive of population variants, possibly unrelated to the disease or ethnicity, and therefore we excluded these five SNPs from further analysis.

Germline SNPs in Tumor (HNSCC) versus Matched Nontumor Specimens

Matched nontumor DNA from eight HNSCC specimens (six HPV⁺ and two HPV[−]) was sequenced for identification of variants common to tumor and nontumor tissue. Data obtained from the Ion Torrent Variant Caller software was analyzed at the level of the gene and as individual sequence variants. Common SNPs in *KDR* (chromosome position 55972974) and *APC* (positions 112175769 and 112175589) were present in both tumor and nontumor DNA (Figure 2). Variants in other genes (eg, *PIK3CA* and *MET*) were also detected in both tumor and corresponding nontumor specimens. However, there was a notable difference in location of the individual variants within the *PIK3CA* gene. SNPs at four different hotspots of the *PIK3CA* gene present in tumor specimens were not detected in any of the matched nontumor HNSCC DNA samples. Likewise, SNPs at specific positions in the *MET* gene were seen only in the tumor DNA. One of the eight nontumor DNA specimens shared a SNP in the *KIT* gene with tumor DNA.

Somatic SNPs in HPV⁺ and HPV[−] HNSCC Tumor Specimens

The average number of cancer-related genes that were mutated in HPV[−] specimens was 3.23, compared with 2.16 in HPV⁺ specimens (Figure 3). The difference was not significant ($P = 0.069$).

Somatic variants in five candidate genes were found in both HPV⁺ and HPV[−] HNSCC specimens: *PDGFRA*, *PIK3CA*, *KIT*, *MET*, and *APC* (Figure 4). Although variants from four of the five genes appeared to be more frequent in HPV⁺ than in HPV[−] specimens, the difference was not statistically significant. Variants in *PDGFRA* were present in approximately 30.43% of HPV⁺ HNSCC specimens, compared with 16.67% in HPV[−] HNSCC specimens, indicating a possible association of this gene with HPV in HNSCC ($P = 0.52$). Individual SNPs in the *KIT* gene of HPV[−] specimens were restricted to a single chromosome position (55593464), compared with four different positions detected in HPV⁺ specimens. Likewise, 75% of the SNPs in the *MET* gene of HPV[−] specimens were found at a single

Figure 3 Number of variants found in HNSCC patients. HNSCC specimens ($n = 53$) determined to be HPV⁺ ($n = 23$) or HPV[−] ($n = 30$) were examined for SNPs in 50 cancer-related genes using NGS. Data are expressed as means $\pm$ SEM.

Figure 4 Summary of genomic variants in HNSCC patients. Genes were categorized as common gene variants in HPV⁺ and HPV⁻ HNSCC, HPV⁺ trending genes, and HPV⁻ trending genes.

position (116339672), whereas the SNPs in HPV⁺ specimens were detected at three positions (116339673, 116411973, and 116411978).

Sequence variants in 16 genes were present at higher frequency in HPV⁺ HNSCC patients than in HPV⁻ patients: *FGFR3*, *EGFR*, *KDR*, *NRAS*, *FBXW7*, *CSF1R*, *SMO*, *ATM*, *PTPN11*, *HNFI1A*, *SMAD4*, *STK11*, *JAK3*, *KRAS*, *NOTCH1*, and *RBI*. The position of variants in these genes differed between HPV⁺ and HPV⁻ specimens. *FGFR3* ($P = 0.02$), *NRAS* ($P = 0.03$), and *FBXW7* ($P = 0.03$) variants were detected in 43.5%, 21.74%, and 30.43% of HPV⁺ specimens, respectively, which is significantly greater than the corresponding 13.3%, 3.3%, and 6.7% in HPV⁻ specimens. Furthermore, variants in *CSF1R* ($P = 0.03$), *SMO* ($P = 0.01$), and *KRAS* ($P = 0.004$) were found exclusively in HPV⁺ specimens, suggesting that the SNPs in these genes may be substantially associated with HPV infection in HNSCC. *NOTCH1* variants were also present exclusively in HPV⁺ specimens. The *ATM* variants that were present did not show any statistical difference between the two groups

($P = 0.074$). For *HRAS*, only one variant was detected, in an HPV⁺ specimen. Differences in frequency between HPV⁺ and HPV⁻ HNSCC for SNPs in *EGFR*, *KDR*, and *SMAD4* ($P = 0.07$, $P = 0.08$, and $P = 0.15$) were not significant.

Four genes were altered primarily in HPV⁻ HNSCC specimens: *AKT1*, *TP53*, *ABL1*, and *FLT3* (Figure 4). There was no statistical difference in these genes in HPV⁺ and HPV⁻ specimens.

A summary of the genomic variants is presented in Figure 4. The common genes represent population variants distributed similarly in both HPV⁺ and HPV⁻ HNSCC. By contrast, genomic variants occur preferentially in HPV⁺ versus HPV⁻ HNSCC.

Discussion

In contrast to HPV infections of the genital tract, disease progression of HNSCC has a more favorable outcome if the tumor is infected with the high-risk HPV16 and/or HPV18 subtype. The differential effects of the virus in different cancers implies that a combination of viral and host risk factors play an important role in the oncogenesis of these cancers. In the present study, we identified HPV16 in 92.6% of HPV⁺ tumors, compared with 68% to 87% in the literature.^{1,5} The difference may be attributed to the fact that we studied only the HPV16 and HPV18 high-risk subtypes. The higher incidence of HPV infection in men than in women in our study population is in keeping with demographic data for HNSCC.¹⁸ This apparent difference is explained, at least in part, by associated risk factors such as tobacco and alcohol usage and sexual practices.^{2,19} In agreement with previous report,¹⁹ in the present study 68% of the HNSCC specimens were positive for p16 and 33.8% (23/68) were also positive for HPV.

Overall, the sequencing data showed key differences in the genetic alterations identified in the cancer-related genes

Figure 5 Proposed pathway involved in pathogenesis of HPV⁺ HNSCC. In HPV⁺ HNSCC, several genes for RTK receptors and their downstream pathways are found to be altered, leading to increased cell proliferation and eventually resulting in development of cancer. The majority of HPV⁺ HNSCC patients have wild-type p53, which can induce cell apoptosis in response to chemotherapy. Symbols for molecules do not scale to molecular properties.

Figure 6 Proposed pathway involved in pathogenesis of HPV⁻ HNSCC. In HPV⁻ HNSCC, several RTK receptors are altered, but with fewer downstream variants than for HPV⁺ HNSCC. More importantly, a much higher percentage of *TP53* and *ABL1* variants are associated with HPV⁻ HNSCC, possibly leading to cancer development and poor response to therapy. Symbols for molecules do not scale to molecular properties.

and signaling pathways of HPV⁺ and HPV⁻ HNSCC. Our analysis showed a higher incidence of the *TNFA* proinflammatory -308A allele (37.2%) in HNSCC patients, irrespective of HPV infection, compared with the documented allele frequency in different ethnic groups.^{21–24} This observation corroborates earlier report of an association between the -308 *TNFA* SNP and HNSCC in a European cohort²⁵ and points toward a potential role for TNF- α as a risk factor in the pathogenesis of HNSCC.

Targeted NGS analysis of the genes involved in growth-factor signaling showed that a number of genes from the receptor tyrosine kinase (RTK) family and their associated pathways are altered in HNSCC. Sequence variants in genes encoding RTK receptors (eg, *EGFR*, *FGFR3*, *PDGFR*, *MET*, *KDR*, *CSF1R*, and *KIT*) were preferentially associated with HPV⁺ HNSCC specimens (Figure 5). In particular, HPV⁺ tumors exhibited a significantly higher frequency of *FGFR3* variants ($P = 0.02$) and an exclusive

presence of *CSF1R* variants ($P = 0.03$). The importance of RTK pathway variants in oncology and their relevance to responsiveness with targeted therapy has been highlighted in several cancers, including HNSCC. However, monoclonal antibodies directed toward the EGFR receptor, small-molecule tyrosine kinase inhibitors (TKIs) that bind the catalytic kinase domain of the receptor, and TKIs that target multiple tyrosine kinase receptors have met with limited success in HNSCC.²⁶ Recent *in vitro* data from Singleton et al²⁷ support combinations of TKIs targeting *FGFR*, *ERBB* family members, and *MET* for more effective therapeutic management of HNSCC. Our findings show that variants in these genes are more frequent in the clinically favorable HPV⁺ HNSCC. It is possible that variants in the RTK receptors seen in HPV⁺ HNSCC could potentially interfere with downstream signaling to control proliferation.

Two classic pathways downstream of RTKs that are activated on receptor dimerization are the RAS–MAPK and

Table 2 HPV Infection and Presence of p16 in 100 HNSCC Patients

Demographics	Sample size	Patients [no. (%)]				
		HPV16	HPV18	HPV ⁻	p16 ⁺	p16 ⁺ HPV ⁺
Sex						
Female	28	8 (28.6)	0 (0)	20 (71.4)	20 (71.4)	8 (28.6)
Male	72	18 (25)	2* (2.8)	53 (72.6)	48 (68.1)	15 (19.4)
Ethnicity						
Caucasian	42	15 (35.7)	1 (2.4)	26 (61.9)	30 (71.4)	13 (33.3)
Hispanic	24	6 (25)	1* (4.2)	18 (75)	17 (70.8)	6 (25)
African American	20	1 (5)	0 (0)	19 (95)	11 (55)	1 (5)
Other	14	3 (21.4)	0	11 (78.6)	10 (71.4)	3 (21.4)
Total	100	26 (26)	2* (2)	73 (73)	68 (68)	23 (23)

*One patient was double positive for both HPV16 and HPV18.

PI3K–AKT pathways. The *RAS* isoforms collectively are among the most mutated genes in HNSCC patients, and there is a strong association between the presence of *KRAS* variants and tobacco-related cancers, including HNSCC.²⁸ Our results show an increased number of SNPs in *NRAS* and the presence of *KRAS* sequence variants (including the well-characterized activating mutation G12V) primarily in HPV⁺ specimens. Interestingly, mutations at codon 12 of the *KRAS* genes that lead to constitutional activation of *EGFR* have been detected previously in HNSCC, but were not associated with metastatic potential.^{29,30}

Missense mutations and copy number alterations in the *PIK3CA* gene have been previously implicated in the carcinogenesis of HNSCC.^{30,31} Lechner et al¹⁷ reported a high frequency of PI3K pathway activation in HPV⁺ HNSCC, and data from the Cancer Genome Atlas research network suggest that the high mutation rate of *PIK3CA* in HPV-infected cells makes it a more appropriate target than *EGFR* for the treatment of patients with HNSCC.³² Our studies have documented the presence of *PIK3CA* variants in both HPV⁺ and HPV[−] patients, supporting the hypothesis that PIK3CA may be a viable target for HNSCC. Additionally, the presence of *AKT1* variants in our patient cohort corroborates the involvement of the PI3K–AKT pathway in the pathogenesis and progression of HNSCC. As observed previously, we also demonstrated specific sequence variations in *STK11* in HPV⁺ HNSCC.^{17,33} Loss-of-function variants in the tumor suppressor *STK11* gene are associated with tumorigenesis of sporadic and metastatic HNSCC.³³ Because *PIK3CA* and *STK11* regulate cell growth by modulating the mammalian target of rapamycin complex 1 (mTORC1) pathway, the PI3K–AKT pathway appears to be a target of interest in oropharyngeal cancer.

HNSCC tumor cells responsive to chemotherapeutic agents such as cisplatin undergo apoptosis mediated by p53, RB1, and other facilitator molecules (eg, ABL1). Variants in genes (*TP53* and *ABL1*) encoding these proteins are over-represented in the HPV[−] HNSCC specimens analyzed, suggesting that increased levels of the altered protein may result in the lack of apoptosis in the tumor (Figure 6). These findings are consistent with previous observations that wild-type genes encoding p53 and RB1 predominantly present in HPV⁺ HNSCC are able to produce functional proteins, thus leading to a better prognosis.³⁴ In this regard, the allelic substitution at codon 72 of the *TP53* gene (CGC-arginine or CCC-proline) that plays a major role in inducing apoptosis in *TP53* mutant cells^{35,36} merits further investigation as a target for therapy (Figure 6).

In addition to the genes involved in signaling for apoptosis and survival, variants observed in the *FBXW7* and *SMO* genes of the hedgehog pathway may be also involved in development of HNSCC with HPV infection. A recent report highlights differences in the variant landscape of HNSCC, particularly *TP53*, *FBXW7*, and *NOTCH1*, related to the tumor site and the patient's ethnicity.³⁷ We, however, did not observe any significant variants in the *NOTCH1* or

CDKN2A genes. A limitation of the present study is the use of targeted analysis, which provides information limited to the interrogated area of the gene (in contrast to exome sequencing, which can provide additional data related to the entire genome).

In summary, we have identified specific genetic variants that provide potential insight into the differential pathogenesis of HPV⁺ and HPV[−] HNSCC. Previous studies to differentiate HPV⁺ from HPV[−] HNSCC addressed primarily *TP53* and *EGFR* gene variants. Our present findings confirm the importance of other genes (eg, *PIK3CA*), add to knowledge of SNPs in *STK11* and *FBXW7*, and point out differences in SNP profiles of RTK pathway genes (eg, *FGFR3*, *KRAS*, and *CSF1R*) in HPV⁺ and HPV[−] HNSCC. Several of the genes identified here have been speculated to be either driver or candidate targets for therapy in HNSCC by the Cancer Genome Atlas project.³² The diversity of SNP profiles seen in HNSCC patients highlights the complex nature of the disease and emphasizes the importance of improved patient selection using comprehensive variant analyses for the most effective targeted therapies.

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2014.01.028>.

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