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Epigenetic Modulation of Signal Transduction Pathways in HPV-Associated HNSCC

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Abstract

Objective—Human papilloma virus (HPV) positive and HPV negative head and neck squamous cell cancer (HNSCC) are biologically distinct with a prognostic advantage for HPV positive patients compared to HPV negative cases. DNA promoter methylation is central to human diseases such as cancer, including HNSCC, with reported genome-wide hypomethylation and promoter hypermethylation in HPV positive HNSCC tumors. The goal of this study was to identify differentially methylated genes in HPV positive versus HPV negative primary HNSCC genomes with clues to signaling networks.

Study Design—Laboratory-based study.

Setting—Primary care academic health care system.

Subjects and Methods—DNA from 4 HPV positive and 4 HPV negative freshly frozen primary HNSCC were subject to comprehensive genome-wide methylation profiling. Differentially methylated gene lists were examined using the Signal Transduction Pathways (canonical) filter in the Genomatix Pathway System (GePS).

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Maria J. Worsham, substantial contributions to conception and design, acquisition of data, analysis, and interpretation of data; drafting the article and revising it critically for important intellectual content; and final approval of the version to be published; **Kang Mei Chen**, substantial contributions to conception and design, acquisition of data, analysis, and interpretation of data; drafting the article and revising it critically for important intellectual content; and final approval of the version to be published; **Tamer Ghanem**, substantial contributions to conception and design, acquisition of data, and interpretation of data; drafting the article and revising it critically for important intellectual content; and final approval of the version to be published; **Josena K. Stephen**, substantial contributions to conception and design, acquisition of data, and interpretation of data; drafting the article and revising it critically for important intellectual content; and final approval of the version to be published; **George Divine**, substantial contributions to conception and design, acquisition of data, and analysis and interpretation of data; drafting the article and revising it critically for important intellectual content; and final approval of the version to be published.

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Supplemental Material

Additional supporting information may be found at <http://oto.sagepub.com/content/by/supplemental-data>

Results—Twofold methylation differences were observed between HPV positive and HPV negative cases for 1168 genes. Pathway analysis applied to investigate the biological role of the 1168 differentially methylated genes revealed 8 signal transduction pathways forming a network of 66 genes, of which 62% are hypermethylated.

Conclusion—Our study reveals a predominant hypermethylation profile for genes in signal transduction pathways of HPV positive HNSCC tumor genomes. Because signaling events in the cell play a critical role in the execution of key biological functions, insights into how complex cellular signaling cascades and networks may be programmed in HNSCC are likely to be critical in the development of new biological agents designed to hit multiple targets.

Keywords

hypermethylation; gene networks; pathways; signal transduction

Background

The overwhelming majority of mucosal head and neck cancers are squamous cell carcinomas (HNSCC)¹ that primarily develop in the oral cavity, pharynx, and larynx. Accurate and reliable stratification of HNSCC for prediction of outcomes has been challenging, mainly because of the numerous anatomic sites and subsites from which tumors can arise.

In the absence of a single risk factor attributable to developing HNSCC, the 2 well-studied important risk factors, tobacco and alcohol,² are responsible for 72% of HNSCC cases.³ More recent epidemiological and laboratory evidence indicate the human papilloma virus (HPV) as a causative agent for some HNSCC⁴ and an independent risk factor for oropharyngeal cancer (OPSCC).⁵ A systematic review of 5046 patients with HNSCC reported an overall prevalence of HPV infection of 25.9% and concurs with a more recent meta-analysis of 5681 HNSCC.⁶ The prevalence of HPV infection was significantly higher among patients with OPSCC (35.6%) than among those with oral (23.5%) or laryngeal (24%) SCC.⁷ Approximately 95% of these HNSCC HPV subgroups contain high-risk HPV type 16 (HPV-16) genomic DNA sequences.⁸ Its contribution to neoplastic progression is predominantly through the action of the viral oncoproteins E6 and E7.⁹ Expression of these proteins is sufficient for the immortalization of primary human epithelial cells and induction of histologic atypia characteristic of pre-invasive HPV-associated squamous intraepithelial lesions.¹⁰ The biologic significance of HPV as another independent risk factor is underscored by the improved prognosis for patients with HPV positive HNSCC relative to HPV negative HNSCC,^{4,5} due in part to a better therapeutic response to chemoradiotherapy.¹¹

Global characterization of the HNSCC methylome is beginning to uncover differential landscapes in HPV positive versus HPV negative tumors. HPV positive cells had higher CpG methylation both in nonrepetitive regions (genic and nongenic) and in repetitive regions.^{12,13} Querying differentially methylated genes into the Pathway analysis framework to identify distinct signaling pathway networks has the potential to provide a molecular basis for further exploration of these genes as differential targets in HPV positive and HPV negative HNSCC. In this type of analysis, a biological system is surveyed in the context of disease (or other interesting phenotypes) to identify gene groups associated with biological systems (bionetwork).^{14,15} Bionetwork coupling affords a strategic knowledge base approach and has been used to better understand the systems biology of disease processes and identify potential therapeutic targets.^{16–18} Signal transduction is the means by which cells respond to extracellular information. The major signaling systems have been conserved to a remarkable extent in all animals.

While it is becoming more firmly established that HPV positive HNSCC have better survival outcomes than HPV negative HNSCC, believed to be because of better response to chemo radiation,¹¹ the underlying mechanism for these improved prognosis outcomes remains underexplored. In this exploratory study, we hypothesized that differentially methylated genes in HPV positive versus HPV negative tumors can point to biological processes encased within signaling networks functioning in HNSCC.

Materials and Methods

DNA from 4 HPV positive and 4 HPV negative freshly frozen primary HNSCC were subject to comprehensive genome-wide methylation profiling using the HumanMethylation27 BeadArray (San Diego, California). Tumor site and demographic characteristics are presented in Table 1. This study was approved by the Henry Ford Health System Institutional Review Board committee.

DNA was extracted according to the manufacturer's protocol (Qiagen Inc, Chatsworth, California). Tumor HPV DNA concentrations were measured using real-time quantitative PCR (qPCR) as previously described.^{19,20} Briefly, primers and probes to a housekeeping gene (β -globin) are run in parallel to standardize the input DNA. By using serial dilutions, standard curves are developed for the HPV viral copy number using CaSki (American Type Culture Collection, Manassas, Virginia) cell line genomic DNA, known to have 600 copies/genome equivalent (6.6 pg of DNA/genome). The cut-off value for HPV16 positive status was 0.03 (3 HPV genome copy/100 cells).^{19–21}

The Infinium 27k assays were performed at the Applied Genomics Technology Center (AGTC), which is part of Wayne State University School of Medicine's Department of Obstetrics and Gynecology (Detroit, Michigan). The 27k platform measures methylation status of over 27,000 CpGs located in more than 14,000 gene promoters. The methylation score for each CpG is represented as a beta (β) value according to the fluorescent intensity ratio. Every β value is accompanied by a detection *P*-value. β values may take any value between 0 (non-methylated) and 1 (completely methylated) and is determined using the Genome Studio (Illumina, San Diego, California). Probes are discarded if this detection *P*-value is more than .05. The only corrections that are made to the data are background subtraction and normalization. The resulting beta values were exported into Microsoft Excel and JMP (SAS Institute, Cary, North Carolina) for data analysis.

The Genomatix Pathway System²² (GePS) uses information extracted from public and proprietary databases to display canonical pathways or to create and extend networks based on literature data. Genomatix Pathway System software was applied to build a network from differentially methylated genes. Biological processes enriched in the differentially methylated gene lists using the Signal Transduction Pathways (canonical) filter in GePS were identified and ranked starting with the lowest *P*-values determined using Genomatix analysis software.

Statistical Methods

To avoid gender-specific methylation bias, sex chromosome loci ($n = 1092$) were excluded, resulting in a final data set that consisted of 26,486 autosomal loci associated with 13,890 genes. Criteria for differential methylation was set as CpG sites with a ratio of the mean β for the HPV positive samples to the mean β for the HPV negative samples of at least 2.0 or less than 0.5 (ie, a twofold difference).

Results

Of the 26,486 autosomal CpG loci, the 1355 (5%) differentially methylated CpGs between HPV positive and HPV negative samples were assigned to 1168 genes (Figure 1, Supplemental Table 1, available at www.otojournal.org). Of the 1168 genes, 686 (59%) were hypermethylated, 467 (40%) were hypomethylated, and 15 (1%) had both hypo- and hypermethylation (ie, these 15 genes each had more than 1 CpG with a twofold change, and at least 1 of those was hypermethylation and 1 was hypomethylation).

Pathway analysis applied to investigate the biological role of the 1168 differentially methylated genes using the Signal Transduction Pathways (canonical) filter in GePS formed a network of 66 genes (Supplemental Figure 1), of which 62% are hypermethylated (41 of the 66) and 25 (39%) hypomethylated (Supplemental Table 2). Of the 66 genes, 62 were accounted for in 8 signal transduction canonical pathways ($P < .05$). These included the Wnt/ β -catenin degradation ($P = .003$) (Figure 2A), basic mechanism of action of peroxisome-proliferator-activated receptor (PPAR) elements ($P = .006$), E-cadherin ($P = .018$) (Figure 2B), RXR (retinoid X receptor) and RAR (retinoic acid receptor) heterodimerization ($P = .036$), ion channels and their functional role in vascular endothelium ($P = .036$), ErbB receptor ($P = .04$) (Figure 2C), signaling events mediated by stem cell factor receptor (c-Kit) ($P = .04$), and Integrin signaling (Filopodium formation) ($P = .041$) signaling events (Supplemental Table 3).

Of the 66 genes, 8 were represented in more than 1 pathway (Supplemental Table 4) and included *CAV1*, *CD44*, *EGFR*, *ERB2*, *GPC1*, *PPARA*, *PPARG*, and *SDC2*.

Discussion

Molecular subtyping has shown that HPV positive HNSCC differ from HPV negative HNSCC in several ways. HPV positive HNSCC have genetic alterations that are indicative of HPV oncoprotein function⁸ and are characterized by wild-type TP53,^{23,24} wild-type CDKN2A (p16),²⁵ and infrequent amplification of cyclin D,^{26–28} whereas the converse is true for HPV negative HNSCC. HPV positive HNSCC also differ from HPV negative HNSCC in their patterns of allelic²⁹ and chromosomal^{30,31} loss and in their global gene expression profiles.^{26,32} Whole-exome (protein coding genes) mutational profiling^{33,34} recently confirmed mutations in *TP53* as a potential genomic stratifier for HPV status.

To test the possible involvement of epigenetic modulation by HPV in HNSCC, we conducted a genome-wide DNA methylation analysis. In this hypothesis-generating study, albeit one with a small sample size, HPV status appears to modulate or influence promoter methylation as evidenced from this global examination of over 27,000 CpGs. The results point to HPV-associated genomic differences involving epigenetic events of differential DNA methylation that warrant consideration in addition to copy number changes and genomic mutation differences. The differentially methylated genes between HPV positive and HPV negative HNSCC in this study indicate more hypermethylated than hypomethylated profiles (59% vs 40%) and support higher gene promoter hypermethylation levels in HPV positive tumor cells.

From a clinical significance standpoint, recent studies are beginning to establish a mechanistic role for promoter methylation with improved survival outcomes in HPV positive HNSCC. Gubanov et al showed that promoter hypermethylation and concordant low *SMG-1* expression correlated not only with HPV positive status and improved patient survival, but also enhanced response to radio therapy in HPV-positive HNSCC cell lines.³⁵

Our study not only highlights and confirms previously reported studies of HPV-associated differentially methylated profiles, but also attempts to relate them to biological contexts of signal transduction pathways. The biological processes enriched within the differentially methylated genes point to likely functional consequences and biological roles as highlighted by the emergence of 8 signal transduction canonical pathways.

Pathway analysis has become the first choice for extracting and explaining the underlying biology for high throughput molecular measurements, as it reduces complexity and has increased explanatory power. Wnt/ β -catenin signaling is a branch of a functional network that developed around a class of proteins called armadillo proteins and dates back to the first anaerobic metazoans.³⁶ In vertebrates, Wnt signaling acts to prevent β -catenin degradation and promote its ability to activate transcription. In the canonical Wnt pathway, β -catenin acts as the central component.^{37,38} Wnt/ β -catenin signaling is involved in a broad range of biological systems, including stem cells, embryonic development, and adult organs. Deregulation of components in this pathway has been implicated in a wide spectrum of diseases including a number of cancers and degenerative diseases.

Activation of the canonical Wnt pathway at multiple levels³⁹ (plasma membrane, cytoplasm, or nucleus) supports transformation of HPV-infected primary human keratinocytes.⁴⁰ Cytoplasmic and nuclear expression of β -catenin, a hallmark of the activated Wnt pathway, was reported in archived human cervical carcinoma samples.^{40–42} A follow-up study provides a potential link between activation of the Wnt signaling pathway and its contribution to HPV-mediated cervical cancer.⁴³ β -catenin is accumulated in the nucleus of HPV16-positive oropharyngeal cancer cells as a likely direct consequence of E6 and E7 HPV oncogene expression.⁴⁴ In the absence of β -catenin mutations, epigenetic changes in Wnt pathway regulators are thought to explain the activation of the canonical Wnt pathway.⁴⁵ Methylation of WNT-antagonists SFRP-1, SFRP-2, SFRP-4, SFRP-5, WIF-1, and DKK-3 in oral cancer implicates the WNT pathway in oral cancer pathogenesis.⁴⁶ The latter supports the finding of our study, indicating hypermethylation of 11 of 17 Wnt/ β -catenin pathways genes to include SFRP-4 and DKK-3 (Supplemental Table 3).

E-cadherin is a member of the cadherin family of transmembrane glycoproteins and mediates formation of adherens junctions in epithelial cells.⁴⁷ Studies using normal epithelial cell cultures suggest that E-cadherin loss of function may provide a growth advantage.^{48,49} E-cadherin can dictate the activity of several mitogenic signaling pathways, as it differentially regulates EGFR/ERK, PI3-K/AKT, and β -catenin/TCF.⁴⁹ In HNSCC, reduction of E-cadherin results in upregulation of epidermal growth factor receptor (EGFR) and its downstream signaling pathways.⁵⁰

The *EGFR* gene is located at 7p12 and makes a 170-kD transmembrane glycoprotein.⁵¹ It is a member of the receptor protein tyrosine kinase family with several extra-cellular growth factor ligands, including epidermal growth factor (EGF) and transforming growth factor (TGF) α . Overexpression of EGFR is observed in 42% to 80% of HNSCC studied,^{52,53} and *EGFR* gene amplification occurs in up to 30% of HNSCC tumors.^{54,55} The majority of evidence suggests that increased EGFR expression and gene copy number are linked to poorer patient outcomes in HNSCC^{56–59} and may be useful in identifying subgroups of patients at high risk of tumor recurrence and in guiding therapy.^{52,60,61}

Downregulation of the E-cadherin pathway with upregulation of EFGR as a consequence⁵⁰ indicates cross-talk between E-cadherin and EGFR pathways. In our study, genes accounted for in both pathways were hypermethylated (7/7 genes in E-cadherin, 4/4 genes in the EGFR pathway), suggesting downregulation or loss of function of these pathways in HPV positive HNSCC.

Integrin signaling critically contributes to the progression, growth, and therapy resistance of malignant tumors, including HNSCC.⁶² Integrins are transmembrane cell surface receptors comprised of 18 α and 8 β subunits in close noncovalent association that form structural and functional bridges between the extracellular membrane (ECM) and cytoskeletal linker proteins within a cell, with roles in cell survival, proliferation, invasion, and cancer therapy resistance.^{63,64} Targeting of $\beta 1$ integrins with inhibitory antibodies enhances the sensitivity to ionizing radiation and delays the growth of HNSCC cell lines in 3D cell culture and in xenografted mice⁶⁵ and suggests that robust and selective pharmacological targeting of $\beta 1$ integrins may provide therapeutic benefit to overcome tumor cell resistance to radiotherapy.

Peroxisome-proliferator-activated receptors (PPARs) are nuclear hormone receptors that mediate the effects of fatty acids and their derivatives at the transcriptional level. PPARs regulate gene expression by binding with RXR (retinoid X receptor) as a heterodimeric partner to specific DNA sequence elements termed PPRE (peroxisome proliferator response element).⁶⁶ This heterodimeric transcription factor complex then binds to cognate sequences in promoter regions of target genes involved in the catabolism of fatty acids.⁶⁷ Three isotypes, PPAR α , PPAR β/δ , and PPAR γ , have been identified. PPARs have been implicated in many normal and disease-related biologic processes relevant to the heart and vasculature, including lipid and energy metabolism, inflammation, embryo implantation, diabetes, and cancer.⁶⁸ Increased expression of PPAR β/δ is reported in HNSCC.⁶⁹

Retinoids are natural and synthetic vitamin A derivatives that regulate development,⁷⁰ cell proliferation,⁷¹ and differentiation.⁷² Retinoids control cell proliferation and differentiation by binding to the nuclear retinoic acid receptors (RARs) or heterodimeric retinoid X receptors (RXRs) that in turn affect target gene expression.⁷³ Retinoids have chemopreventive effects in various tumor types, including the skin, prostate, ovary, leukemia, and breast,⁷⁴ and are useful tools to uncover therapeutic and chemopreventive pathways that can reduce carcinogenesis as illustrated by the *UBE1L* gene as a candidate-pharmacologic target for lung cancer chemoprevention.⁷⁵

It is widely accepted that the PPARs must heterodimerize with RXR to carry out most of their functions, making PPARs integral partners of the RXR-dependent signaling network. Cross-talk between these 2 pathways is an active area of research investigation.⁷⁶

Membrane ion channels are essential for many physiological processes. Recent studies have shown that abnormal expression and/or activity of a number of ion channels (eg, voltage-gated K⁺, Na⁺, Ca²⁺-channels, transient receptor potential [TRP] channels, and epithelial Na⁺/degenerin family of ion channels) are involved in the growth/proliferation, migration, and/or invasion of cancer cells.⁷⁷ Ion channels represent promising targets for developing novel and effective cancer therapies.

HNSCC cells have the ability to exploit diverse signaling pathways for growth advantage, cell survival, and evasion of apoptosis, with advancement along the tumorigenesis progression continuum. Current signal transduction based therapies under investigation in HNSCC include growth factor pathways (eg, epidermal growth factor) and nuclear receptor pathways (eg, RAR, RXR signaling).^{78–80} Cetuximab remains the sole FDA-approved molecular targeted therapy available for HNSCC, and though there are several new biological agents targeting EGFR and other pathways in the regulatory approval pipeline, the complexity of aberrant signaling in HNSCC may explain why interfering with only single steps in these pathways have not shown marked clinical response in HNSCC patients.

When compared to the genome, which is identical in every cell and tissue in the human body, the epigenome is highly variable over the life course, from tissue to tissue and from

environment to environment.⁸¹ Also, unlike genes that are inactivated by nucleotide sequence variation, genes silenced by epigenetic mechanisms are still intact and thus retain the potential to be reactivated by environmental or medical intervention.⁸¹ There are several current human therapeutic intervention trials to reverse deleterious epigenetic changes. Some examples include epigenetic therapeutic trials to treat T-cell lymphoma based on reactivation of tumor suppressor genes⁸² and similar trials to prevent colorectal cancer by inhibiting the enzyme responsible for DNA methylation.⁸³ Such therapies have shown promise in halting tumor growth by reactivation of the tumor suppressor gene(s) or by blocking progression of precancerous epigenetic lesions. Increased genome-wide methylation has been found to be more pronounced in HPV positive HNSCC¹³ and suggests additional treatment options for HPV positive tumors with demethylating drugs. Additionally, demethylating drugs in combination with therapeutic HPV DNA vaccines have been found to control more effectively a variety of HPV-associated malignancies due to the fact that DNA methylation is capable of decreasing expression of the encoded antigen of the DNA vaccines.⁸⁴ In fact, preliminary studies already suggest that there is promise of improving preventative HPV DNA vaccine therapy by the addition of the demethylating drug 5-aza-2' deoxycytidine.⁸⁴ The potential for specific hypermethylated sites in HPV negative HNSCC may allow these patients to also benefit from demethylation treatment strategies.

An interesting feature of this study is the observation of a preponderance of methylated genes in the top ranked signal transduction pathways of HPV positive HNSCC tumor genomes. However, given the small sample size, this observation necessitates confirmation and validation in both studies with larger sample sizes that also include potential confounding factors (smoking, alcohol) and mechanistically designed studies from the standpoint of concordance of methylation status with gene expression and more importantly, the impact of gene methylation on the functional outcomes of the pathways they reside in.

Conclusion

The progressive acquisition of a malignant phenotype in HNSCC is dependent on the aberrant activation of multiple signaling pathways underscoring finely choreographed genomic instability events, likely influenced by etiological factors and risk habits, to achieve biological distinctiveness. The integration of the differentially methylated genes into known biological pathways is a versatile tool to gain insights into the biological complexity of genome-wide promoter methylation. In this hypothesis-generating pilot study, we were able to highlight genes and their association to signal transduction pathways germane to HNSCC. Signaling events in the cell play a critical role in the execution of key biological functions. In-depth understanding of how the complex cellular signaling cascades and networks may be programmed in HNSCC will be instrumental in the development of new biological agents designed to hit multiple targets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Of the 26,486 autosomal CpG loci, the 1355 (5%) differentially methylated CpGs between HPV positive and HPV negative samples were assigned to 1168 genes: 686 (59%) were hypermethylated, 467 (40%) were hypomethylated.

Table 1

Cohort characteristics

Sample ID	Site	Age	Race	Gender	HPV16E6	Smoking
31	Larynx (supraglottis)	51	CA	F	Negative	Yes, past
41	Tongue	41	CA	M	Negative	Yes, past
42	Oral cavity	54	CA	M	Negative	Yes, current
43	Tongue	71	CA	M	Positive	Yes, current
45	Tongue	49	CA	M	Positive	Yes, past
46	Tongue	46	CA	M	Negative	Yes, current
47	Tongue	54	CA	M	Positive	No
48	Oral cavity	52	AA	M	Positive	Yes, current

Abbreviations: CA, Caucasian American; AA, African American; F, female; M, male.