The biological significance of methylome differences in human papilloma virus associated head and neck cancer

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Abstract. In recent years, studies have suggested that promoter methylation in human papilloma virus (HPV) positive head and neck squamous cell carcinoma (HNSCC) has a mechanistic role and has the potential to improve patient survival. The present study aimed to replicate key molecular findings from previous analyses of the methylomes of HPV positive and HPV negative HNSCC in an independent cohort, to assess the reliability of differentially methylated markers in HPV-associated tumors. HPV was measured using real-time quantitative PCR and the biological significance of methylation differences was assessed by Ingenuity Pathway Analysis (IPA). Using an identical experimental design of a 450K methylation platform, 7 of the 11 genes were detected to be significantly differentially methylated and all 11 genes were either hypo- or hypermethylated, which was in agreement with the results of a previous study. IPA's enriched networks analysis identified one network with msh homeobox 2 (MSX2) as a central node. Locally dense interactions between genes in networks tend to reflect significant biology; therefore MSX2 was selected as an important gene. Sequestration in the top four canonical pathways was noted for 5-hydroxytryptamine receptor 1E (serotonin signaling), collapsin response mediator protein 1 (semaphorin signaling) and paired like homeodomain 2 (bone morphogenic protein and transforming growth factor-β signaling). Placement of 9 of the 11 genes in highly ranked pathways and bionetworks identified key biological processes to further emphasize differences between HNSCC HPV positive and negative pathogenesis.

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Introduction

The results from a number of studies involving next-generation sequencing sequence analysis, gene expression microarrays, whole-exome sequencing and genome-wide methylation studies support the observation that human papilloma virus (HPV) positive squamous head and neck cancer (HNSCC) is a distinct entity and therefore exhibits a particular set of somatic alterations (1-6). Data from epidemiological and clinical studies have also indicated that HPV positive HNSCC is clinically different from HPV negative HNSCC in terms of patient characteristics, sensitivity to treatment and molecular biology (7,8).

Global characterization of the HNSCC methylome demonstrates the different landscapes in HPV positive tumors compared with HPV negative tumors. CpG methylation is higher in HPV positive cells in both repetitive and non-repetitive regions (genic and non-genic) (9,10). Methylome analysis of HPV positive and HPV negative primary formalin-fixed paraffin-embedded tumors using the Illumina HumanMethylation450K Beadchip platform (2) have confirmed previous reports (9,11) that the DNA methylation signature of HPV positive HNSCC is different from that of HPV negative HNSCC and that HPV positive HNSCC has a strong tendency to undergo hypermethylation. A previous study (2) highlighted 11 genes: Chromosome 14 open reading frame 162/coiled-coil domain containing 177 (*C14orf162*/*CCDC177*), cadherin 8 (*CDH8*), collapsin response mediator protein 1 (*CRMP1*), engulfment and cell motility 1 (*ELMO1*), 5-hydroxytryptamine receptor 1E (*HTR1E*), meiotic double-stranded break formation protein 1 (MEI1), msh homeobox 2 (*MSX2*), protocadherin 10 (*PCDH10*), protocadherin β-11 (*PCDHB11*), paired like homeodomain 2 (*PITX2*) and synapsin II (*SYN2*) as undergoing significantly different methylation processes in HPV positive and HPV negative HNSCC (2) (Table I).

It has been established that patients with HPV positive HNSCC have better survival outcomes than those with HNSCC that are HPV negative, possibly due to improved responses to chemoradiation (12). However, it remains unclear why HPV positive patients experience improved prognoses-the underlying mechanisms responsible for these outcomes have not yet been investigated. A mechanistic role for promoter methylation is beginning to be established, with the aim of

improving survival outcomes of patients with HPV positive HNSCC (13). The aim of the current study was to verify that the 11 aforementioned genes undergo significantly different methylation in HPV positive and HPV negative HNSCC tumor samples and to assess their biological significance using Ingenuity Pathway Analysis (IPA) for biomarker potential in HPV-associated HNSCC.

Materials and methods

DNA extraction and amplification. DNA from 4 HPV positive and 4 HPV negative freshly frozen (-80˚C) primary HNSCC tumor samples, resected from patients admitted between December 2010 and January 2012 in the Department of Otolaryngology/Head and Neck Surgery (Henry Ford Hospital, Detroit, USA), were subjected to comprehensive genome-wide methylation profiling using the Infinium HumanMethylation450K BeadChip kit (Illumina, Inc., San Diego, CA, USA). Tumor site and demographic characteristics are presented in Table II. For the present study, patient informed consent for inclusion of surgically resected tissues was given, and the study was approved by the Henry Ford Health System Institutional Review Board committee.

DNA was extracted according to the manufacturer's protocol (Qiagen, Inc., Valencia, CA, USA). Tumor HPV DNA concentrations were measured using real-time quantitative PCR (qPCR) as previously described (14,15). Briefly, primers and probes to a housekeeping gene (β-globin) were run in parallel to standardize the input DNA. By using serial dilutions, standard curves were developed for the HPV viral copy number using CaSki (American Type Culture Collection, Manassas, VA, USA) cell line genomic DNA, which 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) (14-16).

The Infinium HumanMethylation450 BeadChip kit includes 485,764 cytosine positions in the human genome and covers 99% of Reference Sequence genes. The Reference Sequence collection aims to provide an integrated, comprehensive, well-annotated non-redundant set of sequences, including genomic DNA, transcripts and proteins (17). The interrogated CpG sites are distributed among all 23 human chromosome pairs (17).

Processing samples for the Infinium HumanMethylation450. The Infinium HumanMethylation450 assays were performed at the Applied Genomics Technology Center, Wayne State University (Detroit, MI, USA). Following DNA checks of original DNA quality, quantification and bisulfite conversion, 4 μl of bisulfite-converted DNA was used for hybridization according to Illumina Infinium methylation protocols. Data were normalized using the Controls Normalization method (Illumina, Inc.). The methylation score for each CpG was represented as a beta (β) value according to the fluorescence intensity ratio and each β-value in the Infinium HumanMethylated450 BeadChip kit was accompanied by a detection P-value. β-values could take any value between 0 (non-methylated) and 1 (completely methylated) and were determined using the GenomeStudio® 2009.2, Methylation Module ver. 1.5.5., ver. 3.2 (Illumina, Inc.). Probes were discarded if P>0.05. All normalized and raw data were submitted to the GEO (Gene Expression Omnibus, NCBI) according to the instructions provided (GEO accession numbers: GSE67114). The resulting β-values were exported to Microsoft Excel, JMP and SAS (SAS Institute, Inc., Cary, NC, USA) for data analysis.

Pathway analysis. To determine the biological processes occurring within the 11 differentially methylated genes, they were uploaded to the Ingenuity pathway Analysis (IPA; Ingenuity Systems, Inc; Qiagen, Inc.). IPA integrates genes and molecules that are part of the same biological functions or regulatory networks interacting together.

Statistical methods. There was *a priori* evidence that the 11 genes were differentially methylated and each in a particular direction, therefore, P<0.05 was considered to indicate a statistically significant difference.

Results

HumanMethylation450K BeadChip analysis. For all genes, 99.97% of the CpGs and 100% of the CpGs associated with the 11 genes of particular interest, met the detection threshold of P<0.05 and therefore were eligible for analysis.

Of the 11 genes of interest, the methylation status of 7 differed significantly between HPV positive and HPV negative HNSCC (Table I). *CDH8*, *PCDHB11*, *ELMO1*, *MSX2*, and *HTR1E* were significantly hypermethylated and *MEI1* and *C14orf162*/*CCDC177* were significantly hypomethylated (Fig. 1). The methylation status of all 11 genes as either hypo- or hypermethylated was consistent with the results of a previous study by Lechner *et al* (2) even though the methylation of four genes did not differ significantly (P>0.05).

Pathway analysis. *C14orf162*/*CCDC177* was excluded from the list of genes in IPA as it was unaccounted for in the IPA knowledge database. IPA connected 7/10 genes in a 35 gene network characterized by the following functions: Cellular Development, Skeletal and Muscular System Development and Function, and Embryonic Development as the sole enriched top network (Fig. 2). The seven genes exhibiting significantly different methylation were *MSX2*, *CRMP1*, *ELMO1*, *CDH8*, *SYN2*, *PCDH10* and *PITX2* (Fig. 1).

The top five molecular and cellular functions included cell morphology, cellular assembly, cellular movement, cell-to-cell signaling and interaction and cell death and survival (Table III). *ELMO1* was represented in all the top five functions followed by *MSX2* and *PTX2* (4/5); *CRMP1* and *SYN2* (3/5); *PCDHB11* (2/5); and *CDH8*, *HTR1E* and *PCDH10* (1/5).

The highly ranked canonical pathways (P<0.05) indicate sequestration of *HTR1E* in serotonin receptor signaling, *CRMP1* in sematophorin signaling, and *PITX2* in bone morphogenic protein (BMP) and transforming growth factor (TGF) β signaling (Table IV).

Discussion

Squamous cell carcinomas primarily develop in the larynx, pharynx and oral cavity, and make up the vast majority of

Table I. Infinium HumanMethylation450 Beadchip assay data for HPV positive vs. HPV negative HNSCC samples for 11 genes.

Gene name	Chromosome	HPV status	Mean β	SD	Ratio of HPV positive vs. HPV negative	P-value	Methylation
C14orf162/CCDC177	14	Positive	0.161	0.041	0.579	0.019[a]	Hypo
		Negative	0.278	0.034			
CDH8	4	Positive	0.479	0.062	1.640	0.004[a]	Hyper
		Negative	0.292	0.055			
CRMP1	4	Positive	0.208	0.058	1.155	0.749	Hyper
		Negative	0.180	0.058			
ELMO1	7	Positive	0.578	0.067	1.234	0.026[a]	Hyper
		Negative	0.468	0.033			
HTR1E	6	Positive	0.433	0.062	1.357	0.027[a]	Hyper
		Negative	0.319	0.060			
MEI1	22	Positive	0.318	0.072	0.849	0.008[a]	Hypo
		Negative	0.531	0.087			
MSX2	5	Positive	0.394	0.081	2.213	0.009[a]	Hyper
		Negative	0.178	0.065			
PCDH10	4	Positive	0.333	0.103	1.524	0.166	Hyper
		Negative	0.220	0.023			
PCDHB11	5	Positive	0.548	0.074	1.302	0.042[a]	Hyper
		Negative	0.421	0.076			
PITX2	4	Positive	0.416	0.112	1.386	0.138	Hyper
		Negative	0.300	0.088			
SYN2	3	Positive	0.399	0.115	1.013	0.781	Hyper
		Negative	0.394	0.104			

[a]P<0.05, indicates a statistically significant difference. Cohort of 8 samples (4 HPV+HNSCC, 4 HPV-HNSCC). HNSCC, head and neck squamous cell carcinoma; HPV, human papilloma virus; chrom, chromosome; SD, standard deviation; meth, methylation status; hypo, hypomethylated; hyper, hypermethylated; C14orf162/CCDC177; chromosome 14 open reading frame 162/coiled-coil domain containing 177; CDH8, cadherin 8; CRMP1, collapsin response mediator protein 1; ELMO1, engulfment and cell motility 1; HTR1E, 5-hydroxytryptamine receptor 1E; MEI1, meiotic double-stranded break formation protein 1; MSX2, msh homeobox 2; PCDH10, protocadherin 10; PCDHB11, protocadherin β-11; PITX2, paired like homeodomain 2; SYN2, synapsin II.

Table II. Patient cohort.

Tissue ID	Site	Age	Ethnicity	Gender	HPV status
HFHS-HN19	Oropharynx	60	AA	M	Positive
HFHS-HN28	Oropharynx	64	CA	M	Positive
HFHS-HN30	Oropharynx	62	AA	M	Positive
HFHS-HN48	Tonsil	53	CA	M	Positive
HFHS-HN51	Larynx	49	CA	M	Negative
HFHS-HN23	Larynx	67	AA	M	Negative
HFHS-HN26	Tongue	71	CA	M	Negative
HFHS-HN42	Oropharynx	66	AA	F	Negative

AA, African American; CA, caucasian; M, male; F, female; HPV, human papilloma virus.

mucosal head and neck cancer (18). It has been challenging to accurately and reliably stratify HNSCC to predict outcomes, primarily due to the numerous anatomic sites and subsites from which tumors can arise. Globally, 600,000 people are annually diagnosed with HNSCC (19). The number of cases of HNSCC has gradually increased over the past three decades and currently account for 5% of all malignancies (20).

Just two risk factors, tobacco and alcohol (21) are responsible for 72% of HNSCC cases (22). However, previous studies have also implicated HPV status as a possible cause of certain

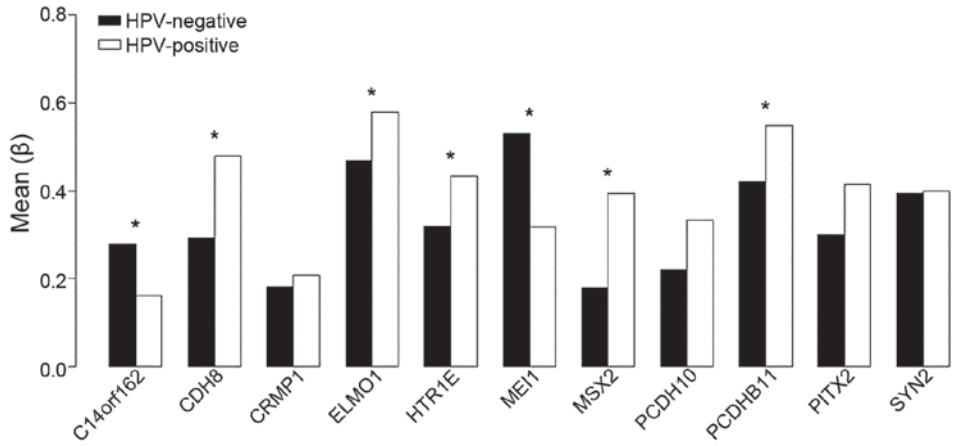

Figure 1. Illumina 450K methylation beadchip beta values associated with HPV positive and HPV negative HNSCC. Of 11 differentially methylated genes, 7 demonstrate statistically significant differential methylation in HPV positive vs. HPV negative HNSCC (*P<0.05). For all 11 genes, hypermethylation and hypomethylation directional changes were concordant with the results from Lechner *et al* (2). Among these genes, *MEI1* and *C14orf162* were hypomethylated and the remaining 9 were hypermethylated. Differences in methylation in HPV negative and HPV positive HNSCC were significant in 7 of the genes analyzed. HPV, human papilloma virus; HNSCC, head and neck squamous cell carcinoma; *C14orf162*/*CCDC177*; chromosome 14 open reading frame 162/coiled-coil domain containing 177; *CDH8*, cadherin 8; *CRMP1*, collapsin response mediator protein 1; *ELMO1*, engulfment and cell motility 1; *HTR1E*, 5-hydroxytryptamine receptor 1E; *MEI1*, meiotic double-stranded break formation protein 1; *MSX2*, msh homeobox 2; *PCDH10*, protocadherin 10; *PCDHB11*, protocadherin β-11; *PITX2*, paired like homeodomain 2; *SYN2*, synapsin II.

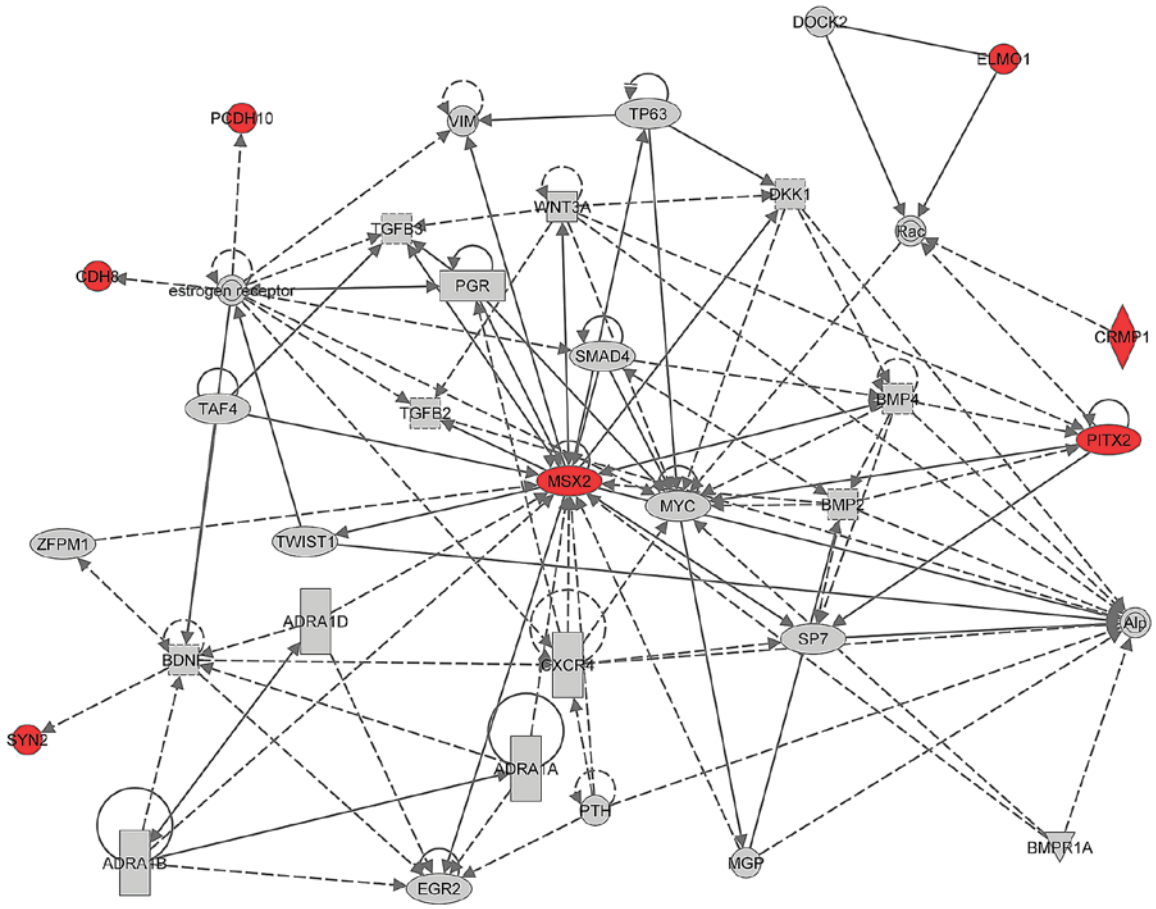

Figure 2. Top network. IPA identified 'Cellular Development, Skeletal and Muscular System Development and Function, Embryonic Development' as the only enriched top network. This network is built with 35 genes from IPA's knowledgebase and includes 7 (*MSX2*, *CRIMP1*, *ELMO1*, *CDH8*, *SYN2*, *PDCH10*, *PITX2*) of the 11 differentially methylated genes (highlighted in red). *MSX2*, msh homeobox 2; *CRIMP1*, collapsing response mediator protein 1; *ELMO1*, engulfement and cell motility 1; *CDH8*, cadherin 8; *SYN2*, synapsin II; *PDCH10*, protocadherin 10; *PITX2*, paired like homeodomain 2.

HNSCC cases (23) and an independent risk factor for oropharyngeal cancer (OPSCC) (24). The biological significance of HPV as an additional independent risk factor is highlighted by the improved prognosis of patients with HPV positive

Table III. Top molecular and cellular functions.

Name	P-value	Differentially methylated genes
Cell-to-cell signalling and interaction	<0.0001-0.028[a]	*PCDHB11, HTR1E, CDH8, PITX2, ELMO1, MSX2, SYN2*
Cellular movement	<0.0001-0.048[a]	*CRMP1, PCDH10, PITX2, ELMO1, MSX2*
Cellular assembly and organization	<0.001-0.027[a]	*PCDHB11, CRMP1, ELMO1, SYN2*
Cell death and survival	<0.001-0.038[a]	*PITX2, ELMO1, MSX2*
Cell morphology	<0.001-0.04[a]	*CRMP1, PITX2, SYN2, ELMO1, MSX2*

[a]P<0.05; indicates a statistically significant result. C14orf162/CCDC177; chromosome 14 open reading frame 162/coiled-coil domain containing 177; CDH8, cadherin 8; CRMP1, collapsin response mediator protein 1; ELMO1, engulfment and cell motility 1; HTR1E, 5-hydroxytryptamine receptor 1E; MEI1, meiotic double-stranded break formation protein 1; MSX2, msh homeobox 2; PCDH10, protocadherin 10; PCDHB11, protocadherin β-11; PITX2, paired like homeodomain 2; SYN2, synapsin II.

Table IV. Top canonical pathways.

Pathway	P-value	(Name) of differentially methylated genes/total pathway genes
Serotonin receptor signaling	0.02[a]	1 (*HTR1E*)/33
Semaphorin signaling in neurons	0.03[a]	1 (*CRMP1*)/52
BMP signaling	0.04[a]	1 (*PITX2*)/80
TGF-β signaling	0.056	1 (*PITX2*)/89

[a]P<0.05; HTR1E, 5-hydroxytryptamine receptor 1E; CRMP1, collapsin response mediator protein 1; PITX2, paired like homeodomain 2; BMP, bone morphogenic protein; TGF-β, transforming growth factor β.

HNSCC compared to HPV negative HNSCC (23,24), which may partly be due to improved therapeutic responses to chemoradiotherapy (12).

It has been demonstrated that HPV positive OPSCC is a distinct type of HNSCC characterized by overexpression of cyclin-dependent kinase inhibitor 2A (CDKN2A), improved patient prognoses, nonkeratinizing histology and a high prevalence of HPV infection (25). At present, HPV status is the most robust and valid molecular diagnostic and prognostic biomarker for HNSCC (26). The expression of E6 and E7 HPV oncoproteins may influence the genetic profile of HPV positive HNSCC (23). HPV positive tumors are characterized by E6 suppression of wild-type p53 function (27), retinoblastoma pathway inactivation by E7 and overexpression of wild-type CDKN2A (28), and infrequent amplification of cyclin D (29), whereas for HPV-HNSCC, the opposite occurs (30). Tumor protein 53 mutations are detected in all HPV negative cases and in the majority of HPV negative tumors, downregulation of CDKN2A and/or cyclin D1 amplification occur (1). The improved prognosis of HPV positive patients compared to HPV negative HNSSC patients has been confirmed (12,16,24), however the mechanistic explanation for this phenomenon remains elusive.

Continuing to adapt the idea of assessing the methylation status of patients with HPV positive HNSCC compared to those with HPV negative HNSCC, Lechner *et al* (2), analyzed 32 cases of HNSCC (18 HPV positive and 14 HPV negative), detected 11 genes that were methylated differently between HPV positive and HPV negative HNSCC and analyzed mRNA expression for two genes: *CDH8* and *PCDH10*. The mRNA expression data for the remaining 9 genes were obtained from a different study by Pyeon *et al* (6). The study also confirmed previous reports (9,11) that HPV positive patients have a distinct DNA methylation signature from their HPV negative HNSCC counterparts.

The present study, in an independent HNSCC sample set using an identical experimental design of the 450K methylation platform, confirmed that 7 of the 11 aforementioned genes were significantly differentially methylated. Additionally, replication of complete concordance of methylation direction (hypo- vs. hypermethylated) was observed for all 11 genes. A recent analysis demonstrated that 9 genes, *CDH8*, *CRMP1*, *ELMO1*, *HTR1E*, *MEI1*, *MSX2*, *PCDHB11*, *PITX2*, *SYN2*, were observed to be significantly differentially methylated in HPV positive compared to HPV negative HNSCC (31).

Differentially methylated genes can be investigated by the pathway analysis framework, which is able to identify distinct signaling pathway networks and therefore may provide a biological basis for further exploration of methylated 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 a specific phenotype for example, the context of disease, to identify gene groups associated with biological systems (32,33). The pairing of biological relationships allows for a strategic knowledge base approach and has been used to improve understanding of the systems biology of disease processes and more intuitively identify potential therapeutic targets (34-36).

A previous study by our group (5) demonstrated that genes involved in signal transduction pathways of HPV positive HNSCC tumor genomes exhibited a predominant hypermethylation profile. In the current study, 9 of the 11 genes that were represented in IPA's top functions, pathways and networks were hypermethylated. Hypomethylated *C14orf162* was not registered in IPA's knowledge database and thus was not included in pathway analyses. Hypomethylated *MEI1* did not place among significantly ranked pathways/functions/networks. *C14orf162*/*CCDC177*, also

known as myelin proteolipid protein-like protein, is encoded by a gene that maps to human chromosome 14q24.1. MEI1 is expressed almost exclusively in the gonads and is required for normal vertebrate meiotic chromosome synapsis (37). Its role in cancer has not yet been investigated and the results of the present study suggest opportunities for additional studies to further investigate the mechanism by which *MEI1* operates in cancer.

The IPA network generation algorithm iteratively constructs networks that strive for optimization of the number of focus genes (10 in the current study) and interconnectivity, under the constraint of a maximum network size of 35 genes. The former identifies how user-specified genes interact with each other or with neighboring genes, thus highlighting the molecular biology implied in a dataset. In the present study, IPA's enriched networks analysis produced only one network: Cellular development, skeletal and muscular system development and function and embryonic development. This network included 7/10 focus genes, *MSX2*, *CRMP1*, *ELMO1*, *CDH8*, *SYN2*, *PCDH10*, *PITX2* that completed the 35 gene network from IPA's knowledgebase. Locally dense interactions between genes tend to reflect significant biology therefore, the role of the MSX2 gene in particular has been highlighted as significant.

MSX2 is a homeobox gene implicated in bone metabolism, organ development, and breast and colorectal tumorigenesis (38-41). Its involvement in HPV-associated HNSCC has been previously documented (2) and was confirmed in the present independent study, which presents additional rationale for further investigations into the role of MSX2 in HNSCC.

ELMO1 was represented in all top five functions, followed by *MSX2* and *PTX2* (4/5), *CRMP*1 and *SYN2* (3/5), *PCDHB11* (2/5), and *CDH8*, *HTR1E*, and *PCDH10* (1/5) (Table III). ELMO1 is an evolutionarily conserved cytoplasmic engulfment protein that promotes actin-dependent phagocytosis and cell migration in a number of invertebrate models and immortalized mammalian cell lines (42). Its role in tumorigenesis and increased invasiveness has been investigated in glioma cells (43), esophageal adenocarcinoma (44) and breast cancer (45). Furthermore, it has been demonstrated that hypermethylated *ELMO1* was part of one of the three methylation epigenotypes for colorectal cancer (46).

The top four canonical pathways indicated the inclusion of *HTR1E*, *CRMP*1 and *PITX2*. *HTR1E* is one of the 33 genes in the highest ranked serotonin receptor signaling canonical pathway (Table IV). Serotonin (5-HT) is a growth factor and a cell cycle mediator that regulates DNA synthesis (47). In the mammalian nervous system, 5-HT receptors are part of the superfamily of G-protein-coupled receptors. Treatment of tumor cells with EGF (Epidermal Growth Factor) and 5-HT may decrease the number of G0/G1 cells that are dormant, resulting in more active, dividing cells that consequently have increased sensitivity to chemotherapeutic treatment (48). Differential methylation of the promoters of *HTR1E* has been detected in patients with schizophrenia (49).

CRMPs are cytosolic phosphoproteins involved in axonal guidance and neuronal differentiation (50). *CRMP1* is one of 52 genes in the second ranked semaphorin signaling canonical pathway implicated in semaphorin-induced growth cone collapse during neural development and axonal guidance (50). Semaphorin signaling affects focal adhesion assembly/disassembly and induces cytoskeletal remodeling, which consequently impacts cell motility, cell shape, cell migration and attachment to the extracellular matrix (51). Oncology studies are thus increasingly focused on axon guidance molecules.

CRMP1 has been characterized as a novel invasion-suppression gene. It has been demonstrated that low expression of CRMP1 mRNA in lung cancer tissue is significantly associated with lymph node metastasis, early post-operative relapse, advanced disease and shorter patient survival times (52). How CRMP1 inhibits cell invasion remains unclear, however, DNA methylation in the context of a hypermethylated *CRMP1* suggests low expression and therefore implicates epigenetic mechanisms in HNSCC tumorigenesis.

The *PITX2* gene was placed in 4/5 of the top molecular and cellular functions (Table III) and two of the four top canonical pathways (Table IV). *PITX2* is 1 of 80 genes in the BMP and 1 of 89 genes in the TGF-β signaling pathways. TGF-β/BMP signaling pathways are involved in bone formation during mammalian development with various regulatory functions (53) and their disruptions have been implicated in multiple bone diseases, including tumor metastasis (54). *PITX2* encodes a transcription factor of the paired-like homeodomain protein family, a crucial component during normal embryonic development (55) that has diverse roles in cell differentiation, proliferation, organogenesis and hematopoiesis (56,57). PITX2 is downstream of TGF-β and fibroblast growth factor (FGF) signaling and may regulate cell proliferation by activating the expression of cyclins D1 and D3 (58). It has been demonstrated that methylation of *PITX2* in human tumorigenesis occurs in several types of human cancer, including colon, breast, ovarian and prostate cancer (59-61). A previous study indicated that PITX2 downregulation with associated promoter hypermethylation predicted good clinical outcomes following radical prostatectomy (61).

Confirmation of hypermethylation of *CDH8* and *PCDHB11* in the independent sample set of the current study is consistent with findings from previous studies demonstrating that cadherin genes are targets for transcriptional silencing in HNSCC and HPV-mediated hypermethylation (2). Cadherins are targets of Polycomb repressive complex 2 and are involved in different types of cancer and cancer specific processes (62) including the epithelial to mesenchymal transition, a process that facilitates cell invasion and metastasis (63). Polycomb group target genes are more likely to contain cancer-specific promoter DNA hypermethylation than other genes (64). This supports the theory that cancer originates from the stem cells, where permanent silencing replaces reversible gene expression thus locking the cell into a perpetual state of self-renewal that predisposes it to malignant transformation (65).

In conclusion, the current study investigated an independent HNSCC sample set. The concordance of methylation direction (hypo- vs. hypermethylated) from previous analyses of the methylomes of HPV positive and HPV negative HNSCC was replicated for all 11 genes. Of the 11 genes, 9 were represented in IPA's top functions, pathways and network, and 7 genes were reported as undergoing significantly different methylation. The present study suggests that cadherins CDH8 and PCDHB11, together with C14orf162/CCDC177, ELMO1, HTR1E MEI1

and MSX2, are biomarkers for HPV-associated HNSCC. Cell signaling events are critical in the execution of key biological functions and insights into how complex cellular signaling cascades and networks are programmed may be important in aiding the development of novel biological agents with multiple targets. Placement of 9 of the 11 genes in key pathways and biological processes focuses attention on the unique biological processes in HPV-associated HNSCC tumors and identify the differences between the pathogenesis of HPV positive and HPV negative HNSCC at the epigenetic level.

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