Genomewide Gene Expression Profiles of HPV-Positive and HPV-Negative Oropharyngeal Cancer

Potential Implications for Treatment Choices

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Objective: To study the difference in gene expression between human papillomavirus (HPV)–positive and HPV-negative oral cavity and oropharyngeal squamous cell carcinoma (OSCC).

Design: We used Affymetrix U133 plus 2.0 arrays to examine gene expression profiles of OSCC and normal oral tissue. The HPV DNA was detected using polymerase chain reaction followed by the Roche LINEAR ARRAY HPV Genotyping Test, and the differentially expressed genes were analyzed to examine their potential biological roles using the Ingenuity Pathway Analysis Software, version 5.0.

Setting: Three medical centers affiliated with the University of Washington.

Patients: A total of 119 patients with primary OSCC and 35 patients without cancer, all of whom were treated at the setting institutions, provided tissues samples for the study.

Results: Human papillomavirus DNA was found in 41 of 119 tumors (34.5%) and 2 of 35 normal tissue samples (5.7%); 39 of the 43 HPV specimens were HPV-16. A higher prevalence of HPV DNA was found in oropharyngeal cancer (23 of 31) than in oral cavity cancer (18 of 88). We found no significant difference in gene expression between HPV-positive and HPV-negative oral cavity cancer but found 446 probe sets (347 known genes) differentially expressed in HPV-positive oropharyngeal cancer than in HPV-negative oropharyngeal cancer. The most prominent functions of these genes are DNA replication, DNA repair, and cell cycling. Some genes differentially expressed between HPV-positive and HPV-negative oropharyngeal cancer (eg, TYMS, STMN1, CCND1, and RBBP4) are involved in chemotherapy or radiation sensitivity.

Conclusion: These results suggest that differences in the biology of HPV-positive and HPV-negative oropharyngeal cancer may have implications for the management of patients with these different tumors.


ORAL CAVITY AND OROPHARYNGEAL squamous cell carcinomas (OSCCs) constitute a major public health burden worldwide. Approximately 400 000 new cases of OSCC were diagnosed in 2002, and approximately 200 000 patients died of these cancers. The major risk factors for OSCC are cigarette smoking, alcohol consumption, and betel quid chewing. The evidence for human papillomavirus (HPV) as a potential etiologic agent in OSCC was first reported in 1983, when the presence of HPV antigen was shown in oral cancer specimens. Subsequent publications showed an association between infection with high-risk types of HPV and OSCC risk. Molecular and epidemiologic studies strongly suggest that HPV-positive OSCC is a distinctive disease entity that differs from HPV-negative OSCC in molecular, histopathologic, and prognostic characteristics. HPV-positive OSCC (1) being less frequently associated with p53 mutations, (2) occurring primarily in the oropharynx, (3) tending to be poorly differentiated and basaloid subtypes, and (4) having a more favorable disease outcome. To understand the molecular mechanisms underlying these 2 entities of OSCC, we examined genomewide gene expression profiles of HPV-positive and HPV-negative OSCC.
This research was conducted with written informed consent. Thus, 124 patients with cancer were included in this study. Eligible controls were patients who had oral surgery for treatment of diseases other than cancer, such as obstructive sleep apnea, at the same institutions and during the same time period in which the patients with OSCC were treated. In that time period, 45 eligible controls were approached for participation by study staff, and 37 were recruited.

Each patient was interviewed using a structured questionnaire regarding demographic, medical, and lifestyle history, including tobacco and alcohol use. Data on tumor characteristics (site and stage) were obtained from medical records. Two patients who enrolled in the study but did not consent to having their medical records reviewed were excluded from analyses. Thus, 124 patients with cancer were included in this study. This research was conducted with written informed consent and institutional review board approval.

TISSUE COLLECTION

Tumor tissue was obtained at the time of resection from patients with primary OSCC prior to chemotherapy and/or radiation therapy. Normal oral or oropharyngeal tissue was obtained from controls. One control provided 2 normal tissue samples, and 1 cancer case was a large tumor that was divided into 5 pieces. Immediately after surgical removal, each tumor or normal tissue sample was soaked in RNALater (Applied Biosystems, Foster City, California) for a minimum of 12 hours at 4°C and transferred to long-term storage at −80°C prior to use.

DNA MICROARRAY

The DNA and RNA from each specimen were simultaneously extracted using the TRizol method (Invitrogen, Carlsbad, California). To increase DNA purity, we modified the DNA extraction protocol to include the use of a “back extraction buffer” (4M guanidine thiocyanate, 50mM sodium citrate, and 1M Tris; pH 8.0). The RNA was further purified with the use of an RNeasy mini kit (Qiagen, Valencia, California) as per Affymetrix recommendations (Santa Clara, California). For expression array analysis, 1.0 to 2.5 µg of total RNA was converted to double-stranded complementary DNA (cDNA) using a GeneChip Expression 3’-Amplification One-cycle DNA Synthesis Kit (Affymetrix). The cDNA was purified and used in an in vitro transcription reaction to produce cRNA using the GeneChip Expression 3’-Amplification Reagents Kit (Affymetrix). The newly synthesized and biotin labeled cRNA was hybridized to a U133 2.0 Plus GeneChip (Affymetrix) and scanned using an Affymetrix GeneChip Scanner 3000 7G in the Fred Hutchinson Cancer Research Center's Genomics Shared Resources, per Affymetrix protocols.

QUALITY CONTROL OF MICROARRAY RESULTS

We used the quality control (QC) criteria specified by Affymetrix (http://www.affymetrix.com/support/downloads/manuals/data_analysis_fundamentals_manual.pdf) followed by the “affyQCReport” and “affyPLM” packages in Bioconductor (http://www.bioconductor.org) to search for poor-quality GeneChips. These procedures identified 7 GeneChips that did not pass QC tests (5 from cancer samples and 2 from controls), and these were eliminated from further analyses. Thus 123 GeneChips from 119 cancer cases and 36 GeneChips from 35 controls were included in this analysis.

HPV GENOTYPING

We screened all samples for the presence of HPV DNA using a nested polymerase chain reaction (PCR) protocol. All samples that showed a positive PCR result and about 40% of the samples showing a negative result were tested for HPV DNA presence using the LINEAR ARRAY HPV Genotyping Test (Roche, Indianapolis, Indiana) to detect human papillomavirus (HPV) DNA (subject samples in lanes 1, 2, and 4-6). Lane 3 was a negative control (no DNA).
of the samples using HPV-16–specific primers, sequenced the amplified products, and compared them against a known HPV-16 sequence (GenBank 333031).

**DATA ANALYSIS**

Tumors were classified according to site as follows: oral cavity (including tongue, buccal mucosa, gingiva, hard palate, retromolar trigone, and floor of mouth) vs oropharynx (including tonsils, soft palate, uvula, oropharynx, and base of the tongue).

Gene expression values for the approximately 54,000 probe sets were first extracted from probe intensity values of cell intensity (CEL) files using the guanine-cytosine robust multiarray average (gcRMA) algorithm. We then eliminated the probe sets that either showed no variation across the samples (interquartile range/H110210.1 on log2 scale) or that were expressed at very low magnitude (the maximum of the expression value/H110213 on log2 scale). These exclusions helped to limit the number of statistical tests applied when detecting differences between HPV-positive and HPV-negative tumors. After these 2 filtering processes, approximately 21,000 probe sets remained for further analysis.

Statistical tests were carried out to compare HPV-positive and HPV-negative OSCC using a regression framework implemented in GenePlus software (version 1.2; http://www.enodar.com/). To control for the type I error rate, we chose to declare a particular group of genes either “upregulated/overexpressed” or “downregulated/underexpressed” based on a prespecified number of false discoveries (NFD). The choice of NFD, with an appropriate account for the number of genes under investigation (J), dictates the threshold for individual gene-specific P values as NFD/J.

To determine whether the identified probe sets were upregulated or downregulated compared with normal oral tissue, for each probe set we compared the mean expression values of each cancer group with those of controls using linear regression, calculating a robust estimator of variance, and accounting for the fact that multiple samples were tested for some subjects. The probe sets were then placed in order by ascending P value, and a cutoff of .05 was chosen to indicate significant differences in expression.

The functional roles of the genes differentially expressed between HPV-positive and HPV-negative OSCC were assessed through the use of Ingenuity Pathways Analysis (IPA) 5.0 (Ingenuity Systems, www.ingenuity.com). The function analysis identified the biological functions using Fischer exact tests to test the null hypothesis that the set of differentially expressed genes were not representative of each biological function.

**RESULTS**

**PATIENT CHARACTERISTICS**

The characteristics of the study population overall and by HPV status are summarized in Table 1. The patients with cancer were more likely to be older and to be current smokers compared with controls. More case patients presented with oral cavity tumors than with oropharyngeal tumors.

**HPV DETECTION IN OSCC AND CONTROL TISSUE SAMPLES**

Human papillomavirus DNA was found in 41 of 119 tumor tissue samples (34.5%) and in 2 of 35 normal oral tissue samples from controls (6%). Twenty-three of 31 oropharyngeal tumor samples were HPV positive (74%).
whereas only 18 of 88 oral cavity tumor samples contained HPV DNA (21%). Most of the HPV-positive samples (39 of 43) contained only HPV-16. The remaining HPV-positive samples contained HPV-32, HPV-35, HPV-45, or HPV-53. Human papillomavirus type 32 and HPV-53 (low-risk types) were found in oral cavity cancer specimens, whereas HPV-35 and HPV-45 (high-risk types) were found in oropharyngeal cancer specimens. The tumor sample that contained HPV-32 was determined to be positive for HPV by the nested PCR test but negative by the Roche kit (which does not test for HPV-32). Sequencing demonstrated homology to HPV-32.

**GENOMEWIDE COMPARISON BETWEEN HPV-POSITIVE AND HPV-NEGATIVE OSCC**

We used NFD=1 as a selection criteria. This means that we controlled the number of false-positive genes in the discovered gene list to be fewer than 1. We did not find a significant difference in gene expression between HPV-positive and HPV-negative OSCC. When we analyzed oral cavity cancer specimens and oropharyngeal cancer specimens separately, we found no significant difference in gene expression between HPV-positive oral cavity cancer specimens and HPV-negative oral cavity cancer specimens, but we found 446 probe sets (eTable 1, available at http://www.archotolaryngol.com) differentially expressed in HPV-positive oropharyngeal cancer specimens compared with HPV-negative oropharyngeal cancer specimens. This means that the results of 1 probe set among 446 probe sets could be a false-positive finding, corresponding to a false discovery rate of 0.2%. The molecular and cellular functions of these genes that had the lowest P values from the Ingenuity Pathway Analysis 5.0 analysis were DNA replication, DNA recombination, DNA repair, and cell cycle (Table 2).

### Table 2. Top Molecular and Cellular Functions of Genes Differentially Expressed Between HPV-Positive and HPV-Negative Oropharyngeal Cancer Specimens

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Abbreviation: HPV, human papillomavirus.

a Analysis using Ingenuity Pathways Analysis, version 5.0 (Ingenuity Systems, www.ingenuity.com).

b P < .001 for all listed functions.

c Boldface type denotes genes that were upregulated in HPV-positive compared with HPV-negative oropharyngeal cancer.

### COMPARISONS WITH CONTROLS

To determine which, if any, of the 446 probe sets were upregulated or downregulated when compared with normal oral tissue findings, we compared HPV-positive oropharyngeal cancer specimens and HPV-negative oropharyngeal cancer specimens with HPV-negative normal oropharyngeal tissues from controls. Among 446 probe sets, 299 were significantly different between HPV-positive oropharyngeal cancer specimens and HPV-negative oropharyngeal controls (P < .05), with 222 probe sets upregulated and 77 probe sets downregulated. Many of the genes involved in DNA replication, cell cycle, and cell proliferation, such as RPA2, LIG1, POLD1, POLH, MCM2, MCM3, MCM7, NASP, CDC7, CCNE2, CDKN2A, CDK2, RBBP4, PCNA, and Ki67, were upregulated in HPV-positive oropharyngeal cancer specimens. We also found upregulation of genes involved in DNA repair, such as XRCC1, DDB2, FANCG, and TOPBP1. Cell cycle genes that were downregulated were CCND1, APC, and HIPK2. The top 50 probe sets for upregulated and downregulated genes are listed in Table 3. A complete list of the 299 differentially expressed probe sets is provided in eTable 2. When comparing HPV-negative oropharyngeal cancer specimens to HPV-negative oropharyngeal controls using these 446 probe sets, we found 79 upregulated probe sets and 122 downregulated probe sets. Table 4 lists the top 30 probe sets from this analysis. The list of 201 probe sets is provided in eTable 3.

There were 21 probe sets that were upregulated in HPV-positive oropharyngeal cancer specimens but downregulated in HPV-negative oropharyngeal cancer specimens, and 4 were downregulated in HPV-positive oropharyngeal cancer specimens but upregulated in HPV-negative oropharyngeal cancer specimens (Table 5).
Human papillomaviruses are small DNA viruses that are known to be associated with a subset of OSCC. We found significant differences in gene expression on the genomewide level between HPV-positive and HPV-negative oropharyngeal cancer specimens, but not in oral cavity cancer specimens. That our results depend more strongly associated with, oropharyngeal cancers than cancers in other head and neck sites.13 Our results confirm the difference between HPV-positive and HPV-negative oropharyngeal cancers on the molecular level.

The top functions of genes that were differentially expressed between HPV-positive and HPV-negative oro-
Downregulated Genes

- Host cell's cell cycle control and replication machinery do not encode DNA or RNA polymerase but depend on the DNA repair, and cell cycle. Human papillomaviruses do not encode E6 and E7 oncoproteins in HPV-positive oropharyngeal cancers. We found up-regulation of many cell cycle genes in HPV-positive oropharyngeal cancer specimens, such as CDN2E, E2F, CDC7 and CDKN2A. We also found up-regulation of PCNA and Ki67, markers of cell proliferation, in HPV-positive oropharyngeal cancer specimens. This finding is consistent with observations that HPV-16 enhances proliferation of an OSCC cell line.15

Some of the genes that we found differentially expressed between HPV-positive and HPV-negative tu-

Table 4. Top 50 Genes Upregulated and Downregulated in HPV-Negative Oropharyngeal Cancers Compared With HPV-Negative Oropharyngeal Controls

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<td>226257_x_at</td>
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<td>&lt;.001</td>
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<td>STIL</td>
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<td>208854_s_at</td>
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<td>227326_at</td>
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<td>204023_at</td>
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<td>226316_at</td>
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<tr>
<td>206528_s_at</td>
<td>DGUOK</td>
<td>&lt;.01</td>
<td>224801_at</td>
<td>NDFIP2</td>
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</tr>
</tbody>
</table>

Abbreviations: HPV, human papillomavirus; ID, identification; NA, not applicable.
mors have been reported to be associated with chemo
sensitivity to cisplatin, 5-fluorouracil, and paclitaxel,
common chemotherapeutic agents used for treatment of
head and neck squamous cell carcinoma (HNSCC). A study in breast cancer cell lines demonstrated that the
cell line that was most sensitive to cisplatin expressed
low levels of cyclin D1, and that cell lines transfected with
cyclin D1 small interfering RNAs exhibited enhanced sen-
sitivity to cisplatin. Furthermore, cisplatin can sup-
press E6 messenger RNA, restore p53 function and en-
hance radiosensitivity in HPV-16 E6-containing cells of
the SiHA cell line. Since we found lower expression of
RBBP4 than radiation-resistant cell lines, radiation-sensitive cell lines had higher gene and pro-
upregulation of RBBP4 into cell lines induced radio-
sensitization of these cell lines. We found upregulation
of RBBP4 in HPV-positive oropharyngeal cancers. It would
be interesting for future study to examine whether HPV-
positive oropharyngeal cancers are more sensitive to paclitaxel compared
with HPV-negative oropharyngeal cancers. Further
study is needed to confirm this.

Irradiation is a common treatment choice for orophar-
yngeal cancer because of the morbidity associated with
surgical resection. Torres-Roca et al\textsuperscript{22} demonstrated that radiation-sensitive cell lines had higher gene and protein
expression of RBBP4 than radiation-resistant cell lines,
and transfection of RBBP4 into cell lines induced radio-
sensitivity of these cell lines. We found upregulation
of RBBP4 in HPV-positive oropharyngeal cancers. It would
be interesting for future study to examine whether HPV-
positive oropharyngeal cancers are more sensitive to radia-
tion than HPV-negative oropharyngeal cancers.

Although the association between HPV infection and
oropharyngeal cancer is well established, the clinical ben-
efit of testing for HPV in patients with oropharyngeal cancer
has not been established. Our results suggest the pos-
sibility of using HPV status for selecting personalized
therapy for these patients. To translate these findings to
patient management, clinical trials are warranted to evalu-
ate the efficacy of cisplatin, 5-fluorouracil, paclitaxel,
and irradiation in the treatment of oropharyngeal cancer based on
patient HPV status.

\begin{table}
\centering
\begin{tabular}{|c|c|c|}
\hline
Probe Set ID & Gene Title & Gene Symbol \\
\hline
1555878_at & Ribosomal protein S24 & RPS24 \\
1559006_at & cDNA clone IMAGE:4304686 & \\
201677_s_at & Chromosome 3 open reading frame 37 & C3orf37 \\
201687_s_at & Apoptosis inhibitor 5 & AIP5 \\
203017_s_at & Synovial sarcoma, X breakpoint 2 interacting protein & SSX2IP \\
203409_at & Damage-specific DNA binding protein 2, 48 kDa & DDB2 \\
207231_at & Zinc finger DAZ interacting protein 3 & DZIP3 \\
212533_s_at & WEE1 homologue (Saccharomyces pombe) & WEE1 \\
213140_s_at & Synovial sarcoma translocation gene on chromosome 18–like 1 & SS18L1 \\
213573_at & Full-length cDNA clone CSODH006YD11 of T cells (Jurkat cell line) of Homo sapiens & \\
215792_s_at & DNAJ (Hsp40) homologue, subfamily C, member 11 & DNAJC11 \\
219649_at & Asparagine-linked glycosylation 6 homologue & ALG6 \\
224754_at & Sp1 transcription factor & SP1 \\
225340_s_at & GPI-anchored membrane protein 1 & GPAPI \\
225396_at & Zinc finger and BTB domain containing 8 opposite strand & ZBTB8OS \\
225725_at & cDNA clone IMAGE:5261213 & \\
226265_at & Glutamine and serine rich 1 & QSER1 \\
227451_s_at & Coiled-coil domain containing 90A & CCDC90A \\
227545_at & Transcribed locus & \\
228380_at & Transcribed locus & \\
242655_at & Transcribed locus & \\
202305_s_at & Fasciculation and elongation protein zeta 2 (zygin II) & FEZ2 \\
210788_s_at & Dehydrogenase/reductase (SDR family) member 7 & DHR57 \\
211698_at & EP300 interacting inhibitor of differentiation 1 & EID1 \\
217047_s_at & Family with sequence similarity 13, member A1 & FAM13A1 \\
\hline
\end{tabular}
\caption{Genes That Have Expression in Opposite Directions in HPV-Positive and HPV-Negative Oropharyngeal Cancers Compared With Controls}
\end{table}

Abbreviations: cDNA, complementary DNA; DAZ, deleted in azoospermia; EP300, E1A binding protein p300; GPI, glucose phosphate isomerase; HPV, human papillomavirus; Hsp40, heat shock protein 40; ID, identification; SDR, serum deprivation response; Sp1, specificity protein 1.
To our knowledge, 2 previous studies have compared genomewide gene expression between HPV-negative and HPV-positive HNSCC directly, using the same Affymetrix chip used in the present study.23,25 Martinez et al25 were the only ones to specifically examine gene expression in oropharyngeal tissue using 3 HPV-positive and 4 HPV-negative oropharyngeal cancer specimens and 4 normal oral mucosal tissue samples. The researchers identified 124 upregulated and 42 downregulated genes in HPV-positive oropharyngeal cancer specimens compared with HPV-negative oropharyngeal cancers. Only 3 genes (TYMS, TUBGCP3, and SEC24D) from their list overlapped with ours. A greater overlap was seen between the respective lists of genes that were differentially expressed between HPV-positive oropharyngeal cancer samples and normal tissue from controls. These genes included CDKN2A, PCNA, RFC4, MCM2, MCM3, CDC7, TYMS, CCNE2, USP1, and ACTL6A.

Slebos et al23 studied 36 HNSCC specimens from patients, including 15 from the oral cavity, 9 oropharyngeal samples, 9 laryngeal samples, and 3 hypopharyngeal specimens.23 They identified HPV DNA in 7 of the 9 oropharyngeal samples but in none of the oral cavity samples. They found 91 genes differentially expressed between HPV-positive and HPV-negative HNSCC. We found 20 genes overlapping between our results and theirs. These included ACTL6A, ALG6, ASS1, CCDC52, CDC7, CDKN2A, CENPK, DFR2, EIF2B3, EZH2, LIG1, MCM2, MCM6, OPA1, RFC4, RPA2, STMN1, TOPBP1, USP1, and WEE1.

The difference between the results of these studies could be owing to study design, different tumor sites, and different approaches used for statistical analysis. The gene list from our study was based solely on the analysis of oropharyngeal cancer specimens. We did not observe an appreciable difference in gene expression between HPV-positive and HPV-negative oral cavity cancer specimens. In contrast, the gene list generated by Slebos et al23 was based on samples from different head and neck tumor sites, and only a quarter of the tumors were from the oropharynx. To the extent that gene expression associations with HPV status differed in subsets of cases defined according to site, different results would be expected between these 2 studies. Although both our study and that of Martinez et al25 focused on oropharyngeal tumors, our much larger sample size would be expected to detect larger numbers of differentially expressed genes.

Our study was limited by the small number of study subjects, particularly for comparisons among patients with oropharyngeal cancer. Having only 8 HPV-negative oropharyngeal cancer samples, we could not include cigarette smoking, sex, and age as covariates when we compared genomewide gene expression between the HPV-positive and HPV-negative oropharyngeal tumors.

In conclusion, we found differences in gene expression in HPV-positive and HPV-negative oropharyngeal cancer specimens. These differences suggest that (1) HPV-positive oropharyngeal cancer may be more resistant to 5-fluorouracil and paclitaxel than HPV-negative oropharyngeal cancer; (2) cisplatin may be a better choice for treatment of HPV-positive oropharyngeal cancer; and (3) HPV-positive oropharyngeal cancer may also be more sensitive to irradiation. Further study in cell lines and clinical trials is needed to investigate the possibility of using HPV as a guide for the management of oropharyngeal cancer.

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REFERENCES


