original reports

Detection of High-Risk Human Papillomavirus in Oral Cavity Squamous Cell Carcinoma Using Multiple Analytes and Their Role in Patient Survival

abstract

Purpose Accurate detection of human papillomavirus (HPV) in oral cavity squamous cell carcinoma (OSCC) is essential to understanding the role of HPV in disease prognosis and management of patients. We used different analytes and methods to understand the true prevalence of HPV in a cohort of patients with OSCC with different molecular backgrounds, and we correlated HPV data with patient survival.

Methods We integrated data from multiple analytes (HPV DNA, HPV RNA, and p16), assays (immunohistochemistry, polymerase chain reaction [PCR], quantitative PCR [qPCR], and digital PCR), and molecular changes (somatic mutations and DNA methylation) from 153 patients with OSCC to correlate p16 expression, HPV DNA, and HPV RNA with HPV incidence and patient survival.

Results High prevalence (33% to 58%) of HPV16/18 DNA did not correlate with the presence of transcriptionally active viral genomes (15%) in tumors. Eighteen percent of the tumors were p16 positive and only 6% were both HPV DNA and HPV RNA positive. Most tumors with relatively high copy number HPV DNA and/or HPV RNA, but not with HPV DNA alone (irrespective of copy number), were wild-type for *TP53* and *CASP8* genes. In our study, p16 protein, HPV DNA, and HPV RNA, either alone or in combination, did not correlate with patient survival. Nine HPV-associated genes stratified the virus-positive from the virus-negative tumor group with high confidence ($P < .008$) when HPV DNA copy number and/or HPV RNA were considered to define HPV positivity, and not HPV DNA alone, irrespective of copy number ($P < .2$).

Conclusion In OSCC, the presence of both HPV RNA and p16 is rare. HPV DNA alone is not an accurate measure of HPV positivity and therefore may not be informative. HPV DNA, HPV RNA, and p16 do not correlate with patients' outcome.

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INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide with an incidence of 550,000 cases annually.[1,2] Oral cavity squamous cell carcinoma (OSCC) constitutes a majority of HNSCCs, including tumors of the oral anterior tongue and buccal mucosa.[3] The major known risk factors for OSCC are use of tobacco and alcohol and infection with human papillomavirus (HPV).[4,5] Unlike oropharyngeal tumors, in which HPV incidence is reported to be high (up to 90%),[6,7] the prevalence of HPV in OSCC (although it varies greatly among geographies and choice of analyte and assay[8]) is generally accepted to be low.[9,10] In addition, unlike with oropharyngeal tumors,[11-15] the role of HPV in disease prognosis and response to therapy in patients with OSCC is equivocal. Despite the fact that HPV RNA is shown to function as a better screening and patient management tool,[16,17] the presence of HPV DNA is routinely used as a measure of HPV infection in tumors. HPV DNA results do not always match those for HPV RNA, especially in OSCC.

HPV16 and HPV18 subtypes have been epidemiologically linked with head and neck carcinoma.[18] High-risk HPV16 and HPV18 are the most predominant subtypes in oral cavity tumors from Indian patients, whereas the other subtypes (HPV33, HPV6, and HPV11) are rare.[19,20] HPV E6 interacts with p53 to promote its degradation via the ubiquitin pathway, whereas

Table 1. Characteristics of Patients and Tumor Tissues Used in the Study

Characteristic	No. of Patients
Primary site	
Buccal mucosa	41
Oral tongue	112
Sex	
Male	114
Female	39
Age, years	
	112
≤ 40	40
NA	1
Risk habits	
Alcohol	4
Chewing tobacco	43
Smoking	6
Alcohol + chewing tobacco	14
Smoking + alcohol	16
Smoking + chewing	9
Smoking + alcohol + chewing tobacco	10
No habits	44
NA	7
Tumor stage	
I-II	43
III-IV	109
NA	1
Differentiation	
Well	48
Moderate	73
Poor	20
NA	12

Abbreviation: NA, not available.

HPV E7 forms a complex with retinoblastoma (Rb) protein leading to its functional inactivation and dysregulation of the cell cycle.[21,22] In some HPV-related tumors, E6- and E7-mediated inactivation of p53 and Rb result in the accumulation of p16 protein,[23] whereas in others, p16 expression does not directly correlate with HPV positivity.[24] A majority of HPV-negative tumors harbor mutations in *TP53* and *CASP8*, and a significant proportion of HPV-positive tumors harbor mutations in *PIK3CA*.[25-27] In addition, past studies have identified specific mutations in potential drug targets such as *FGFR2/3*, lack of *EGFR* aberrations in HPV-positive patients,[28] and a potential role of *CASP8* in HPV-negative cell lines and patients.[26,29] Despite a wealth of information, questions regarding the accuracy of different HPV tests and whether HPV is an important factor in the stratification and treatment of oral cavity tumors remain to be answered.

In this study, we addressed the following five questions related to HPV in oral cavity tumors. (1) Does sensitivity of the test matter in the detection of HPV DNA? (2) Does the presence of p16 protein and HPV DNA correlate with HPV E6/E7 RNA? (3) Does the presence of high copy number HPV DNA accurately reflect HPV positivity? (4) Are p16 protein, HPV DNA, and HPV E6/E7 RNA individually or together linked with patient survival? (5) Do somatic mutations and DNA methylation at 5-cytosine residues distinguish the HPV-positive from the HPV-negative tumors?

METHODS

Patients, Cell Culture, and Nucleic Acid–Based Assays

Tumor samples (n = 153) from patients with OSCC (buccal mucosa, bone marrow [including from upper and lower gingivobuccal sulcus and retromolar trigone], and oral tongue) were accumulated consecutively and selected for the assay (Table 1; Data Supplement). For nucleic acid–based assays, we tested five sets of primers published in the literature and two that were newly designed in the amplification reactions (Fig 1; Appendix Table A1). Details of the patients and methodology are provided in the Data Supplement.

Immunohistochemistry

For p16 immunohistochemistry (IHC), staining was carried out by using formalin-fixed paraffin-embedded tissue blocks and primary antibody from BioGenex (Fremont, CA; catalog No. AM540-5M; Antip16[NK4], Clone G175-405 in the NordiQC list) and using the PolyHRP detection system (catalog No. QD400-60KE, BioGenex) according to the manufacturer's instructions and a scoring method (Data Supplement). Sections of cervical cancer were used as a positive control.

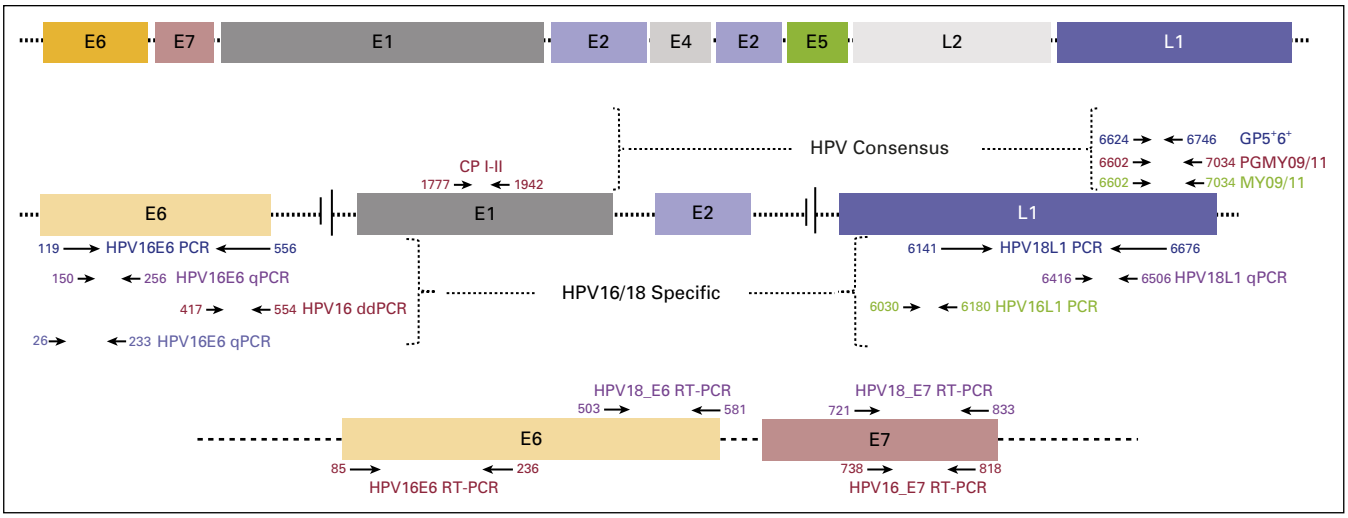

Fig 1. Human papillomavirus (HPV) genome organization and locations of different primers (either consensus or type specific) or probes used in the study to detect HPV DNA and RNA. The numbers before and after the arrows represent the corresponding nucleotide number in the HPV genome. ddPCR, droplet digital polymerase chain reaction; qPCR, quantitative PCR; RT-PCR, real-time PCR.

HPV DNA Copy Number

We deduced the HPV absolute copy number from the quantitative polymerase chain reaction (qPCR) standard curves using cloned HPV16/18. We considered a tumor or cell line to have a relatively high copy number of HPV DNA when the copy number for HPV16 was more than 3.3×10^2 per μg of tumor DNA and that for HPV18 DNA was more than 3.3×10^3 per μg of tumor DNA. To minimize the effect of tumor cellularity, ploidy, and heterogeneity, we expressed the HPV copy number as copies per μg of tumor DNA used in the reaction.

Mutation and Survival Analysis

The mutation data on tumors for *TP53*, *CASP8*, and *RASA1* were retrieved from previously published data.[29] The χ^2 test was used to determine the significance of different clinical parameters of patients. The relationship between tumor HPV status and survival in patients was examined by Kaplan-Meier analysis (Data Supplement). Overall survival (OS) and disease-free survival (DFS) were analyzed, and a log-rank test was used to determine significance ($P < .05$).

Whole-Genome Methylation and Statistical Data Analyses

Whole-genome methylation data were gathered by using the Illumina Infinium Methylation450 BeadChip kit, chip scanning, and data preprocessing; the process was described previously.[30] Statistical methods used to analyze methylation data are provided in the Data Supplement.

RESULTS

p16 Expression and HPV DNA

In our study, 18% of the tumors were p16 positive (Fig 2A; Table 2). We detected HPV DNA at 0.03 ng or with a larger amount of genomic DNA (Appendix Fig A1) from the cell line UMSCC-47/Hep2 when the following primers were used: GP5+6+, MY09/11, CPI-II, PGMY09/11, or HPV16L1 (Fig 2B). However, the newly designed type-specific primers (HPV16E6 and HPV18L1) could detect HPV16 and HPV18 with as little as 0.0003 ng and 0.003 ng of genomic DNA, respectively (Fig 2B). We also tested the effect of cloned HPV DNA amount on amplification efficiency (Appendix Fig A2). Figure 2C shows the efficiency of the consensus and type-specific primers in a set of representative oral cavity tumors (Appendix Fig A3). Widely used primers from the literature (MY09/11, PGMY09/11, GP5+6+, and HPV16L1) yielded either the least sensitivity or moderate (CPI-II) sensitivity of detection, whereas the newly designed HPV16E6 and HPV18L1 primers showed the optimum sensitivity of detection (Fig 2B). We observed inhibition of the amplification reactions at a high concentration of tumor genomic DNA with a positive cell line spike-in experiment (Appendix Fig A4) and therefore higher concentrations of tumor DNA were avoided in the reactions. In addition, an increase in amplification cycles did not aid in the detection of HPV DNA in PCRs as shown in Appendix Figure A5. Results from qPCRs indicated that 33% of tumors (35 of 106) were positive for HPV DNA (Table 2). Although we found a higher incidence of HPV16 (30% [32

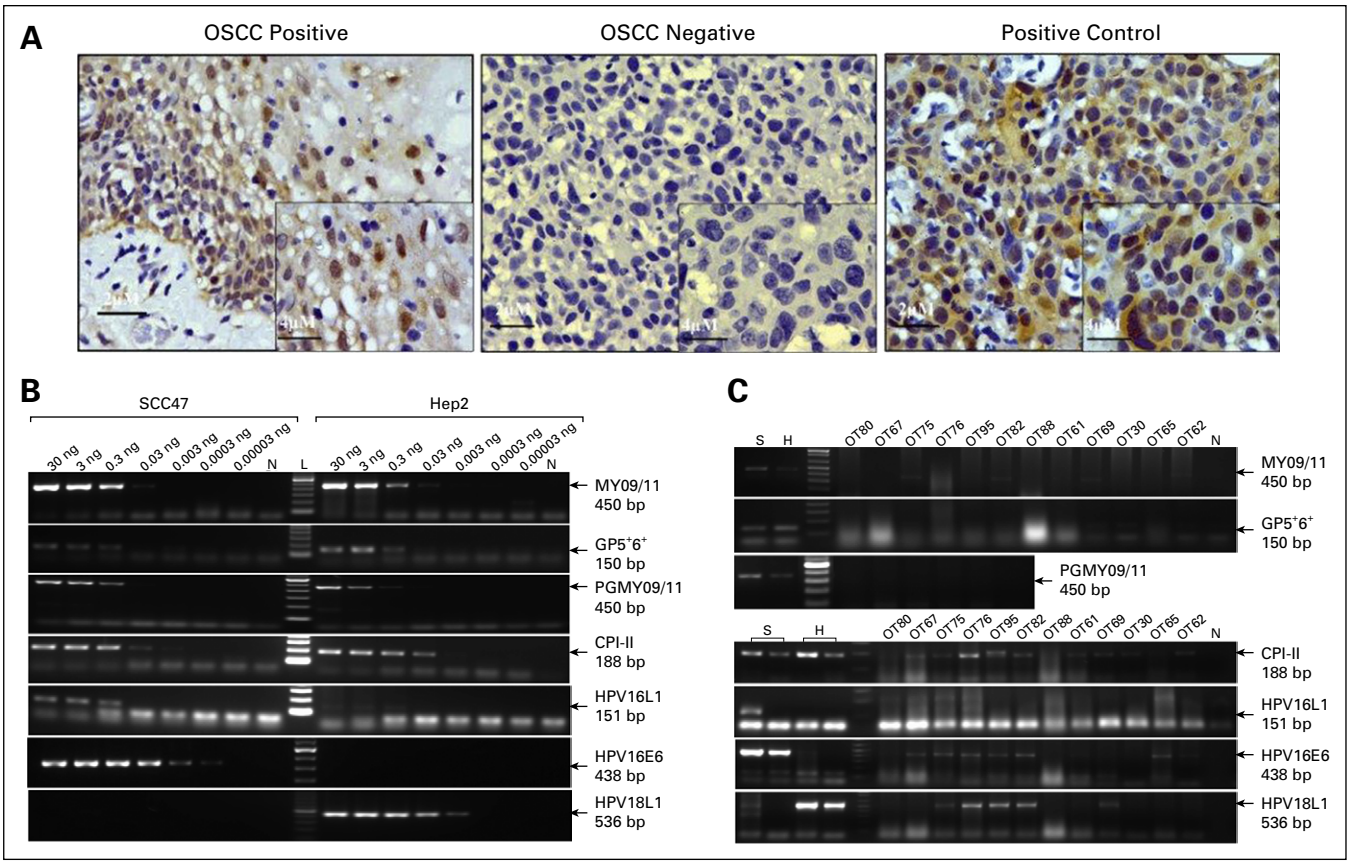

Fig 2. p16 and human papillomavirus (HPV) DNA in oral cavity squamous cell carcinoma (OSCC). (A) Representative images of immunohistochemical staining of p16 in positive and negative OSCC tissue sections; cervical tissue was used as a positive control. (B) Relative polymerase chain reaction (PCR) amplification efficiency and sensitivity of consensus and type-specific primers for detection of HPV using HPV16 or HPV18 individual positive control cell lines (UMSCC-47 and Hep2). (C) Representative HPV DNA PCR in oral cavity tumors with both consensus and type-specific primers. OT, oral tongue.

of 106]) than HPV18 (18% [19 of 106]) type, the HPV18-positive tumors had high copy numbers of viral DNA as reflected in their cycle threshold (Ct) values (Fig 3A iii,vi). Quantitative PCR (qPCR) was performed on oral cavity tumors (n = 106), and the tumors were counted as HPV DNA positive if they had Ct values three times the standard deviation for the mean of negative controls (Fig 3A ii,v). Digital PCR has recently been shown to successfully detect HPV DNA in oropharyngeal tumors in a highly specific manner.[31] Digital PCR results indicated that 43% of oral cavity tumors (59 of 136) were positive for HPV16 DNA (Fig 3B iii; Table 2; Appendix Fig A6).

HPV RNA

Compared with the cell lines, tumors showed low levels of expression of E6 or E7 messenger RNA (mRNA; Fig 3C). Only 15% of the tumors showed expression of E6 RNA and/or E7 RNA (unlike HPV DNA), and 6% of the tumors had both HPV DNA (in all three assays) and transcriptionally active HPV genomes (Table 2). In our cohort, younger patients (age 40 years or younger) had significantly more HPV RNA positivity than older patients when χ^2 analysis was used ($P = .029$).

When the results from all of the assays (p16 IHC, HPV DNA, and HPV RNA) were combined, we found that 6% to 48% of the tumors were positive in various assays combined with PCR (Table 2; Appendix Table A2). We found that 22% of the tumors (23 of 106) had relatively high copy numbers of HPV DNA and/or HPV E6 or E7 mRNA.

Linking Tumor Attributes, Somatic Mutations, and HPV With Survival

We performed Kaplan-Meier survival analyses with various tumor attributes that revealed significant association between tumor differentiation ($P = .03$) and clinical stage ($P < .001$) with OS (Fig 4A). None of the other tumor attributes showed significant association with survival (Appendix Fig A7A-F). In patients with oral cavity tumors, p16, HPV DNA, and HPV RNA did not correlate with the either OS or DFS (Fig 4A; Appendix Fig A8). HPV DNA status alone measured by any of the DNA-based assays alone or in combination

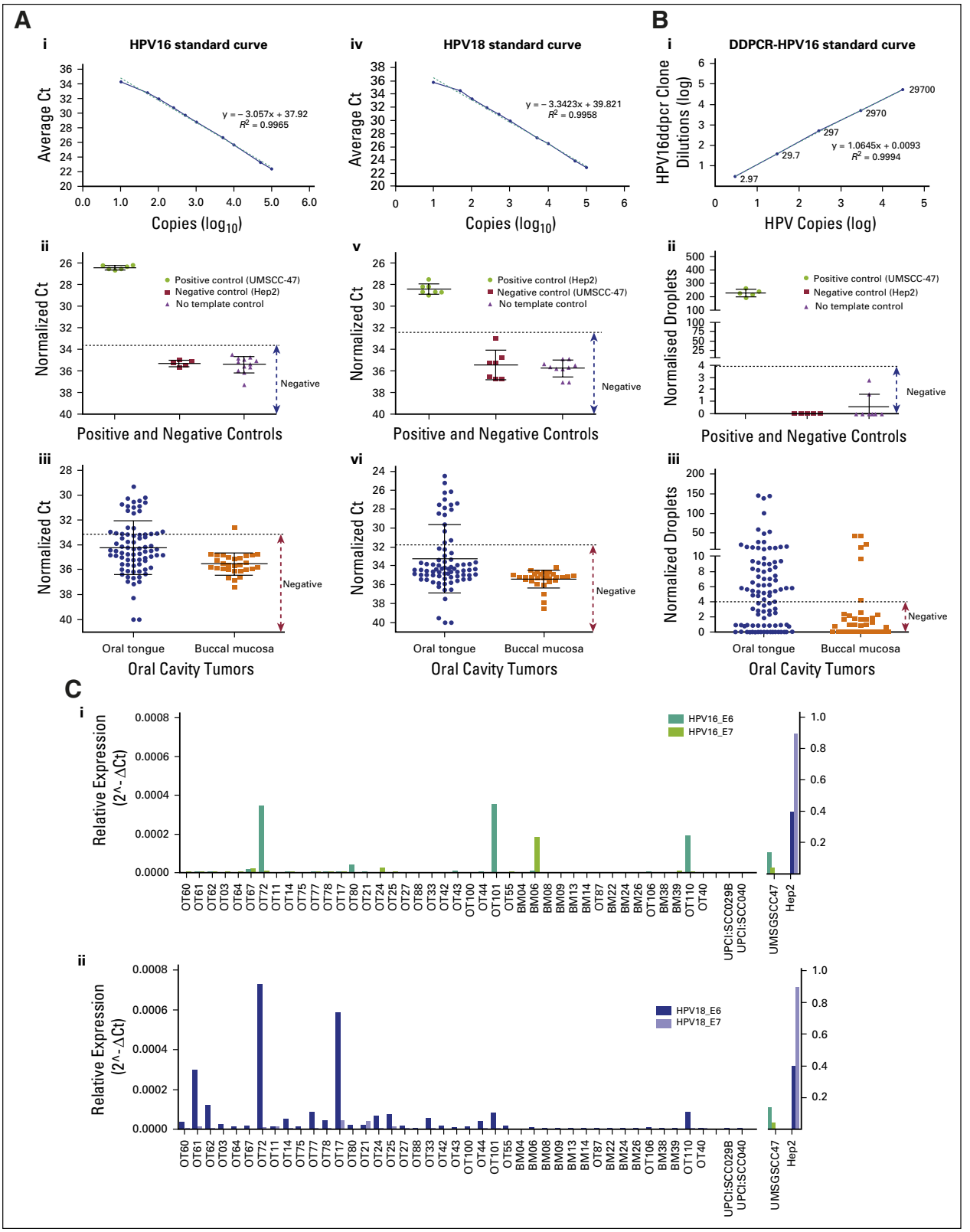

Fig 3. Detection of HPV DNA and RNA. (A i-vi) HPV16/18 assays using quantitative polymerase chain reaction (qPCR) and (Bi-iii) droplet digital PCR (DDPCR) in OSCC. (A i,iv, B i) Standard curves were obtained by using cloned HPV16/18 plasmids. (A ii,v, B ii) Data were subsequently obtained by using the positive (UMSCC-47 and Hep2) and negative (UPCI:SCC29B and UPCI:SCC40) cell line DNA to count HPV DNA in (A iii,vi,Biii) oral cavity tumors. (C) HPV16 (top panel) and HPV18 (bottom panel) E6 and E7 mRNA expression in tumors using qPCR. Horizontal dotted lines: threshold lines for negative samples. BM, buccal mucosa; Ct, cycle threshold; OT, oral tongue.

Table 2. Summary of HPV Assays for Oral Cavity Tumors

Detection Method	% Positivity					
	Oral Tongue		Buccal Mucosa		Combined (oral cavity)	
	Patients Analyzed/ Total No. of Patients	%	Patients Analyzed/ Total No. of Patients	%	Patients Analyzed/ Total No. of Patients	%
p16 IHC						
p16	10/55	18	NA		10/55	18
DNA based						
PCR	39/66	59	2/4	50	41/70	58
qPCR	34/78	44	1/28	3.6	35/106	33
ddPCR	52/95	55	7/41	17	59/136	43
RNA based						
qPCR	5/30	17	1/11	9	6/41	15
Combination						
PCR + qPCR	23/60	38				
PCR + ddPCR	29/60	48.3				
qPCR + ddPCR	27/99	27.2				
PCR + qPCR + ddPCR	20/53	37.7				
p16 + 3/3 methods	2/36	5.5				
RNA + DNA	1/17	6				

NOTE. p16 was measured by the presence of immunopositive cells with both nuclear and cytoplasmic staining using immunohistochemistry (IHC). Polymerase chain reaction (PCR) results indicate the presence of any HPV subtype with consensus primers or HPV16/18 type-specific primers. Quantitative PCR (qPCR) and droplet digital PCR (ddPCR) results are from TaqMan assays with primers and probes for HPV16/HPV18 and HPV16, respectively. HPV RNA results indicate the presence of E6 and/or E7 mRNA for HPV16/HPV18. 3/3 methods, tested with all the 3 DNA-based methods.

did not correlate with survival (Fig 4A; Appendix Fig A8), except when measured with droplet digital PCR (ddPCR) for OS ($P = .03$; Appendix Fig A8E). We tested whether tumors with relatively high HPV DNA copy numbers and/or HPV E6 or E7 mRNA were linked with survival. As shown in Appendix Figure A9A-B, we did not find any significant association with this group of tumors for either OS ($P = .45$) or DFS ($P = .68$).

We also investigated whether somatic mutations in significantly mutated genes in OSCC play a role in survival in patients with HPV DNA–positive tumors. We analyzed three genes (*TP53*, *CASP8*, and *RASA1*) shown to be significantly mutated in oral cavity tumors.[26,29,32] Ninety-five percent of the HPV-positive tumors in the group were wild-type for *TP53* and *CASP1* genes, and 85% of the HPV-positive tumors were wild-type for *RASA1* gene (Appendix Fig A10). We tested whether the mutations in any of the genes, alone or in combination in the HPV-negative tumor group, were linked with survival. We did not find any significant association for this group of tumors with survival (Appendix Fig A9C-D).

Linking Methylation With HPV

Supervised clustering of the first group of patients (a group defined as having high copy number HPV DNA and/or E6 or E7 RNA) resulted in a list of 60 genes of which nine (*FERMT3*, *GIT2*, *HK3*, *PRKCZ*, *ZCCHC8*, *IRF5*, *IFFO1*, *ARID3A*, *HOXA2*) were mapped to the HPV pathway (Fig 4B). Methylation of those genes is involved in the downstream control of the expression of different target genes. For example, *ZCCHC8* methylation is linked with the expression of *RB1*, *PRKCZ* methylation controls state change of *DLG1*, methylation in *ARID3A*, *IRF5*, *IFFO1*, and *HOXA2* are connected with the expression of *TP53*, and *FERMT3*, *HK3*, and *GIT2* genes control the expression of *AP1* (*JUN*) (Fig 4B). All of the genes except *HOXA2* were significantly

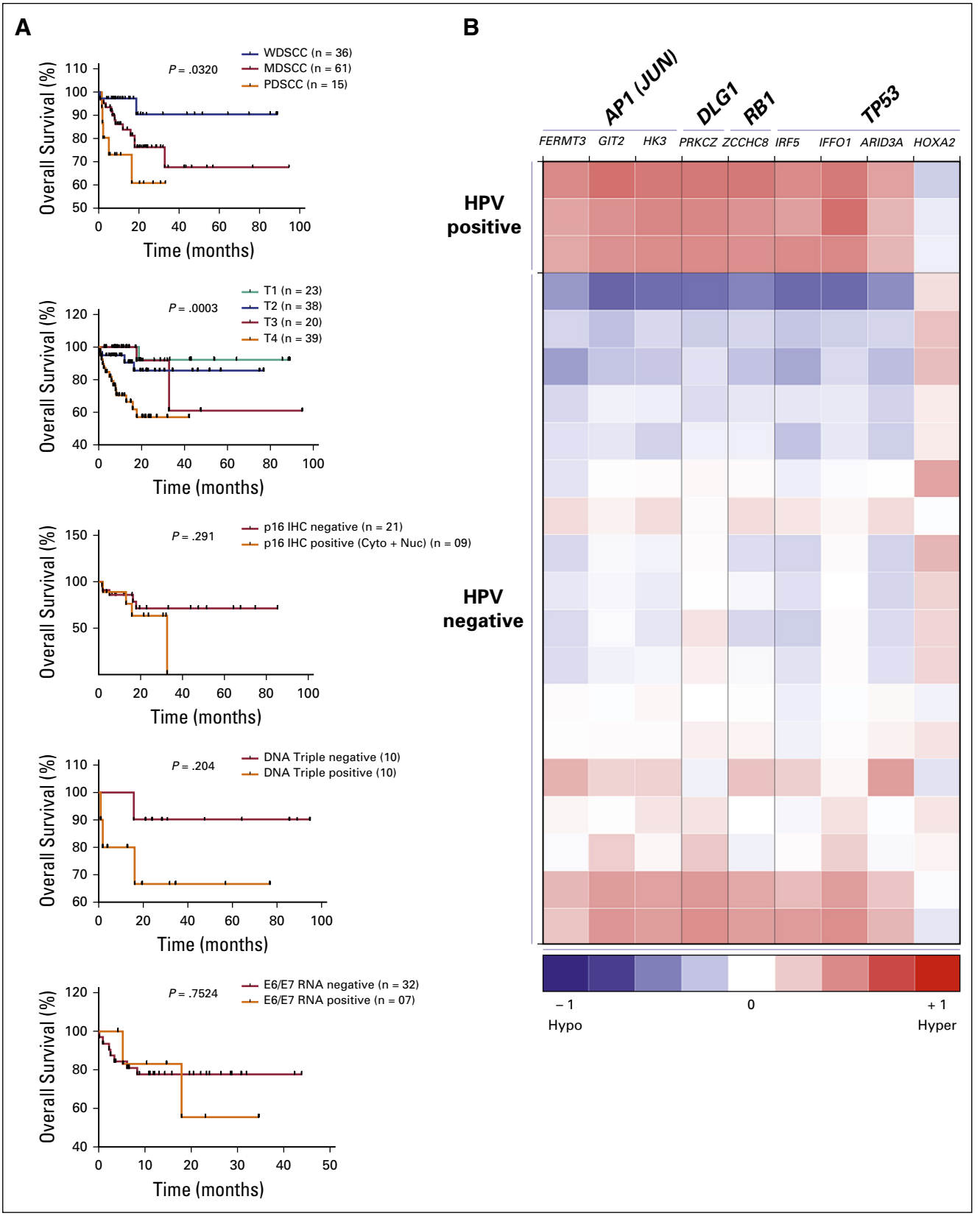

Fig 4. (A) Kaplan-Meier survival plots linking tumors with various attributes such as grade, stage, p16 immunohistochemistry (IHC), HPV DNA, and HPV RNA. (B) Clustering of nine methylated genes stratifying the HPV-positive from the HPV-negative group of tumors along with the HPV-associated pathways in HPV-positive tumors. Cyto, cytoplasmic (staining); MDSCC; moderately-differentiated squamous cell carcinoma; Nuc, nuclear staining; PDSCC, poorly-differentiated squamous cell carcinoma; WDSCC, well-differentiated squamous cell carcinoma.

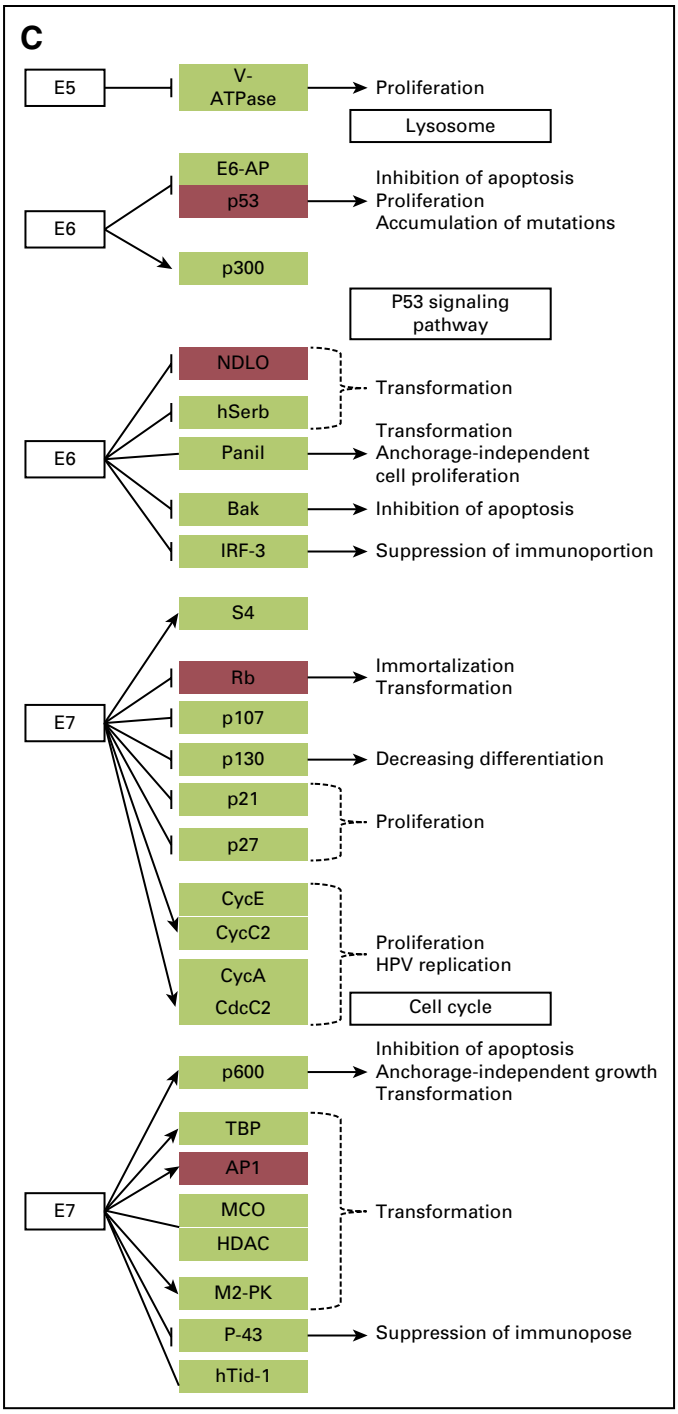

Fig 4. (Continued).

hypermethylated in the HPV-positive group of tumors compared with the HPV-negative group (Fig 4B). The four linked genes obtained from the nine significantly methylated genes were mapped to the pathways involving HPV E6 and E7 proteins (Fig 4B). To test significance, we performed unpaired *t* tests between the two groups: group 1 had relatively high copy numbers of HPV DNA and/or HPV RNA, and group 2 was negative for both HPV DNA and HPV RNA. All of the eight hypermethylated genes and one hypomethylated gene (*HOXA2*) showed high significance ($P < .001$ and $P < .007$, respectively; Appendix Table A3). However, when the patients were grouped on the basis of HPV DNA positivity alone (irrespective of copy number), most of these nine HPV-linked genes did not show a significant association.

DISCUSSION

HPV plays a vital role in the prognosis of patients with oropharyngeal tumors.[33,34] Unlike with disease in the oropharynx, the incidence of HPV and its role in disease prognosis in oral cavity tumors are not well established. Past results regarding HPV DNA incidence in oral cavity tumors varied widely (from low to high; Appendix Table A4) depending on the assay sensitivity, analyte, and patient cohort were used.[34,35] Questions regarding the accuracy of the HPV tests and HPV positivity need to be answered to make confident treatment decisions for treating patients with head and neck tumors.[36] There are only a few studies that used multiple analytes (protein, DNA, and RNA) and various molecular tests (IHC, PCR, qPCR, and digital PCR) to establish HPV positivity in oral cavity tumors and that correlated HPV with tumor attributes (including somatic mutations and methylation) and survival. In this study, we attempted to assess correlations between HPV DNA, RNA, and p16 protein and survival in 153 patients with oral cavity tumors.

Although p16 expression (as measured by IHC) is a commonly used proxy for HPV in HNSCC, its expression is not specific in HPV-associated tumors.[34] Several past studies have correlated p16 expression with HPV,[37-39] but p16 IHC has shortcomings, especially when relating the expression of p16 to patient survival. Limitations, such as variations in staining intensities,[14,40] nonspecific binding of antibodies, and the lack of scoring and interpretive criteria for p16 staining make the method less reliable.[41] Associating p16 status with survival of patients with OSCC has been inconclusive, and some previous reports[42] have suggested additional study to derive any conclusive evidence in this regard. In our study, although we found an unusually high percentage (51%) of tumor cells that showed immunopositive staining, only a small percentage (18%) had both cytoplasmic and nuclear staining, an

accurate reflection of HPV positivity as described earlier.[43-45] Unlike the authors of some of the previous studies,[38] we could not find any correlation (either positive or negative) between p16 expression and survival (Fig 4). A weak correlation between p16 IHC and HPV in situ hybridization was reported earlier.[37] As in previous reports,[46] we found that p16 expression was not a useful surrogate marker for HPV in oral cavity tumors.

Unlike antibody-based methods, nucleic acid–based methods detect HPV with high sensitivity and are therefore widely used.[47] Meta-analysis of 5,478 oral cavity tumors suggested that overall prevalence of HPV DNA was 24.2% with 11% of the tumors being positive for both HPV DNA and E6 or E7 RNA.[48] India has one of the highest incidence rates of oral cavity cancers, and there is a significant difference in the incidence trend between oropharyngeal and oral cavity cancer.[49] Previously, PCR coupled with mass array was shown to provide highly sensitive detection with a small amount of genomic DNA input.[50] Our results showed that 38% of tumors were positive and 13% were negative in all three DNA-based assays (PCR, qPCR, and ddPCR). Overall, the prevalence of HPV DNA (33% to 58%) was dependent on the type of test used; PCR yielded the highest incidence over the more sensitive methods such as qPCR and ddPCR assays (Table 2). This was possibly due to the result of consensus primers used in PCR (but not in qPCR and ddPCR) in addition to the type-specific primers that resulted in the detection of non-HPV16/18 subtypes. As expected, digital PCR, which was the most sensitive of the three DNA-based assays, showed more tumors being HPV16 DNA positive, which resulted in the detection of low copy number viral genomes in tumor samples. On the basis of several levels of evidence, we conclude that the presence of low copy numbers of HPV DNA alone may not be a reflection of functionally active HPV. First, we found that only a fraction of the tumors (15%) had HPV E6 or E7 RNA. Second, only 6% of the tumors were positive for the presence of both the HPV genome and E6 or E7 RNA. Third, almost all of the tumors with relatively high copy numbers of the HPV genome and/or HPV RNA had wild-type *TP53* and *CASP8* genes, which was not the case for tumors with low copy numbers of HPV DNA. Both *TP53* and *CASP8* are known to be wild-type primarily in HPV-positive tumors. In our study, we found that this corresponds to tumors with high copy numbers of the HPV genome and/or a transcriptionally active genome only (Table 2). High prevalence of HPV DNA, as demonstrated in some assays, might suggest the presence of passenger HPV genomes coming from adjacent normal cells (as reported earlier[51-53]), or it could be a reflection of inactive or passenger viruses in oral cavity tumors. Although the numbers are low (n = 3), we cannot explain why some tumors in our study with HPV E6 or E7 RNA did not show the presence of HPV DNA. It is possible that the genomic DNA for those tumors was degraded and therefore could not serve as an ideal template for DNA-based assays. An additional factor that might have added to this is the presence of inhibitors for DNA-based assays in those tumors.

The fact that there were only two tumors that were p16 positive and HPV RNA negative means that a definitive conclusion on the lack of correlation between p16 and HPV RNA cannot be made from our study. Similarly, there were two tumors that were positive for HPV RNA and negative for p16. In HNSCC, p16 is often mutated or silenced, which results in its loss of expression. This could have led to the lack of p16 expression in those two tumors. We did not find any significant correlation between p16, HPV-DNA, and/or HPV RNA and disease outcome (Fig 4A; Appendix Fig A8). Even the tumors with relatively high copy number of HPV genomes and/or E6 or E7 RNA did not support the role of HPV in patient survival (Appendix Fig A9A-B). Our study has highlighted that understanding HPV prevalence in OSCC is complicated. In fact, in oropharyngeal tumors in which p16 has been a definite prognostic marker, a recent study[54] recommended additional HPV DNA testing to accurately predict prognosis.[55] These aspects need further study and analysis.

Although more research is needed to determine how HPV gets to the mouth cavity, it is believed that oral sex and/or bad oral hygiene are two responsible factors. However, a causal role between bad oral hygiene and HPV infection is unclear.[56] In addition, recent data show the role of the oral microbiome in HPV-positive and HPV-negative oral tumors.[57] Future studies linking oral sex and bad oral hygiene with HPV in the mouth cavity among patients belonging to different sociogeographic strata might shed additional light on this problem. Although our study is comprehensive, it has several limitations. Not all

the tumors were assayed with all of the analytes, which makes the sample number different for different methods. We could not perform additional survival analyses for the tumors that were HPV RNA positive, given the small sample size. In our study, we did not perform in situ hybridization, which could have provided additional information on p16 positivity and HPV prevalence. It is possible that the presence of high copy numbers of the HPV genome in the tumors studied does not correlate with the presence of the biologically active virus. Further studies may help answer this question.

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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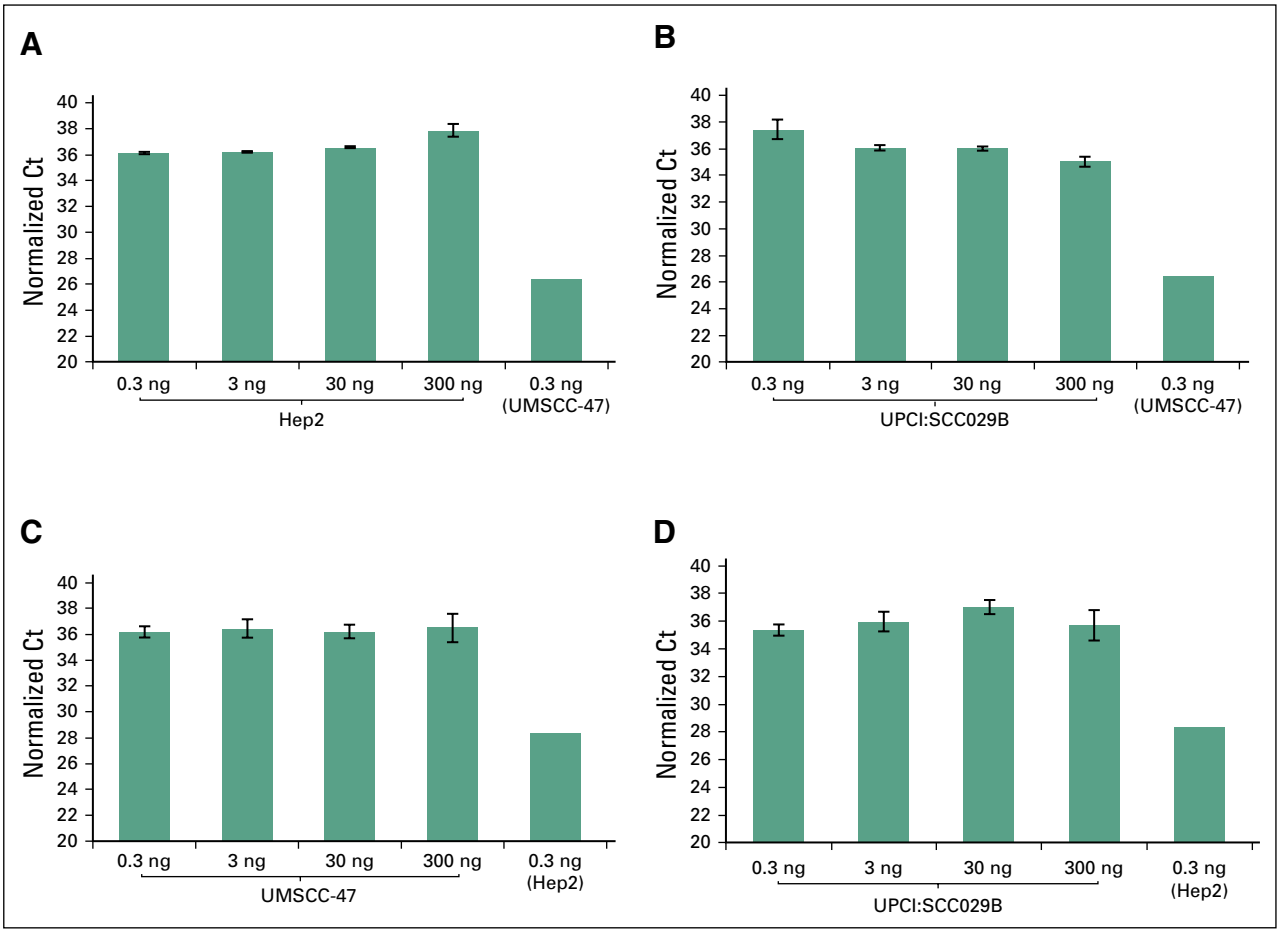


Fig A1. Increasing amount of genomic DNA from cell lines used as positive or negative controls for human papillomavirus16 (HPV16) and HPV18 quantitative polymerase chain reaction (qPCR). HPV16 qPCR using negative control (A) Hep2 and (B) UPCI:SCC029B. HPV18 qPCR using negative control (C) UMSCC-47 and (D) UPCI:SCC029B. Error bars are drawn using data from three independent experiments. Ct, cycle threshold.

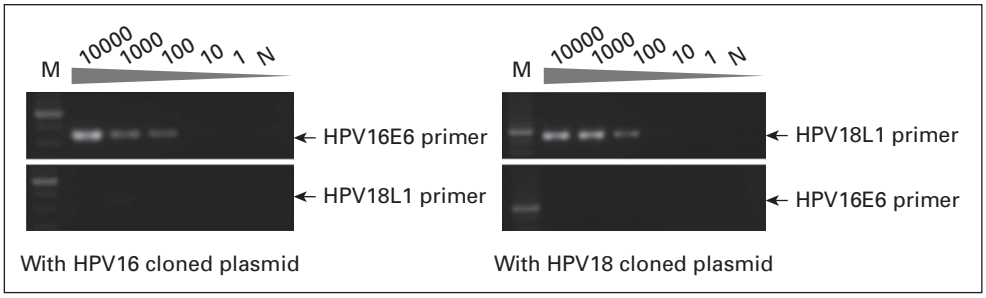


Fig A2. Amplification efficiency of human papillomavirus16 E6 (HPV16E6) and HPV18L1 primers measured by polymerase chain reaction amplification of serially diluted HPV16/18 cloned plasmid copies. M, marker; N, negative control.

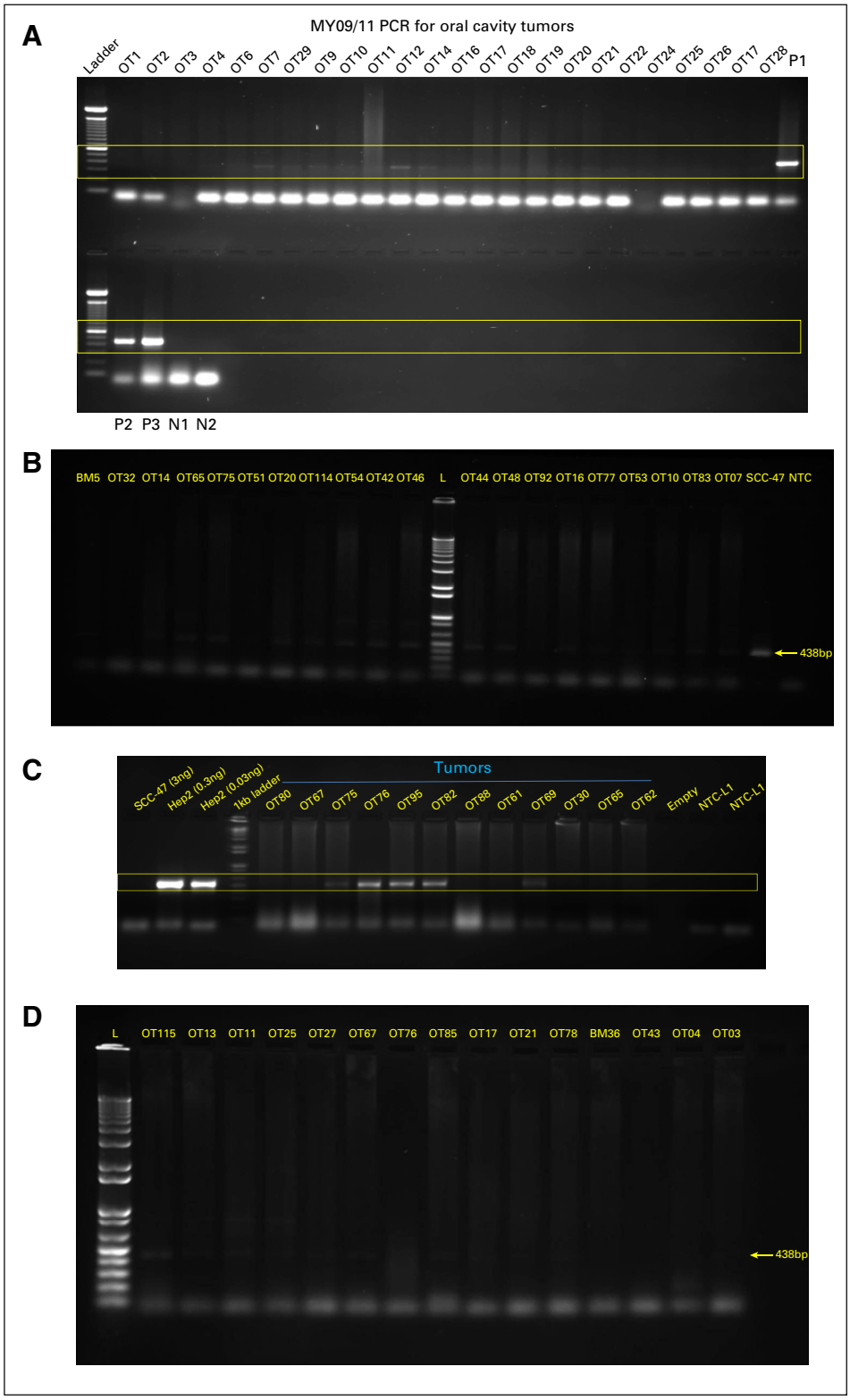


Fig A3. Human papillomavirus (HPV) polymerase chain reaction (PCR) performed by using different sets of consensus or type-specific primers with oral cavity squamous cell carcinoma tumor DNA. PCR for oral cavity tumors with (A) MY09/11, (B) HPV16E6, (C) HPV18L1 using cell lines as positive and negative controls, (D) HPV16E6 for batch 2 (15 tumors), (E) HPV CPI-II using cell lines as positive controls, and (F) HPV16L1 using cell lines for positive controls. BM, buccal mucosa; L, DNA ladder; N1, UPCI:SCC029B DNA (300 ng); N2, no template control (NTC); OT, oral tongue; P1, positive control cervical DNA sample 1; P2, positive control cervical DNA sample 2; P3, UMSCC-47 DNA (HPV16-positive cell line).

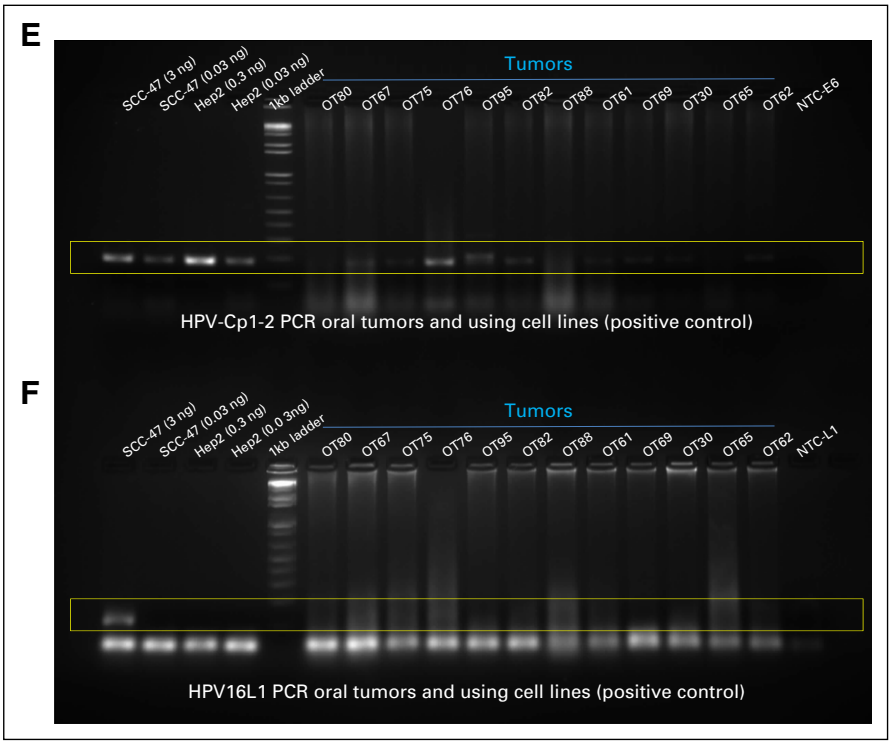
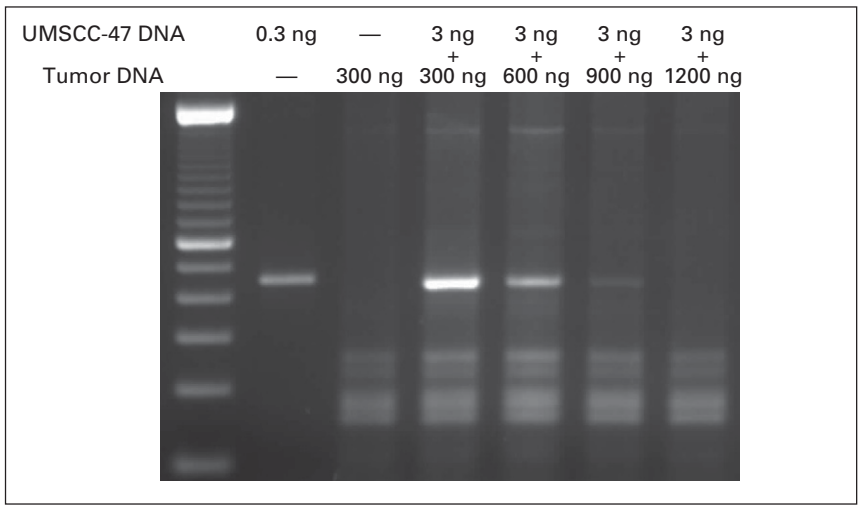


Fig A3. (Continued).

Fig A4. Inhibition of amplification reactions for detecting human papillomavirus (HPV) in polymerase chain reactions at high concentrations of tumor genomic DNA (used with PGMY09-11 primer) spiked with HPV-positive UM-SCC-47 DNA.



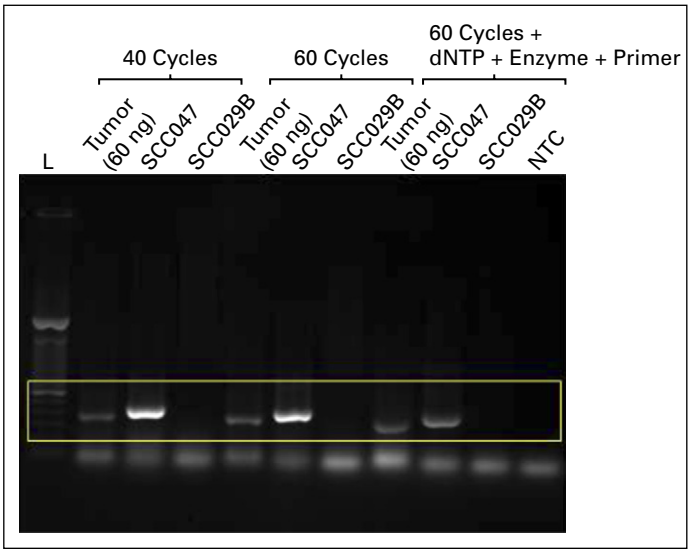


Fig A5. The effect of amplification cycles on polymerase chain reactions. The genomic DNAs used for positive control (UMSCC-47) and negative control cell lines were 63.0 ng and 300 ng, respectively. dNTP, deoxynucleotide triphosphates; NTC, no template control.

Fig A6. Positive and negative cell line DNA used for threshold in droplet digital polymerase chain reaction experiment. OTSCC, oral tongue squamous cell carcinoma.

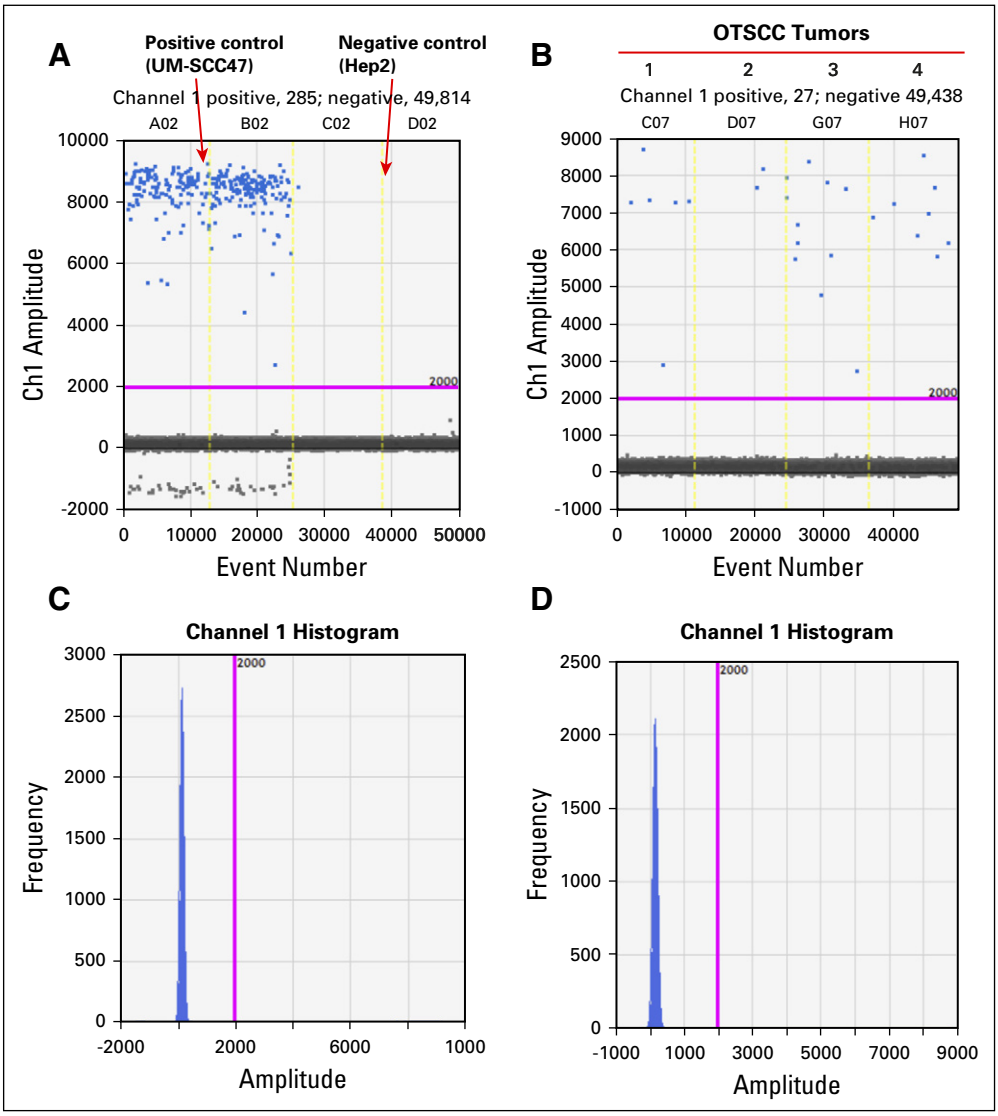
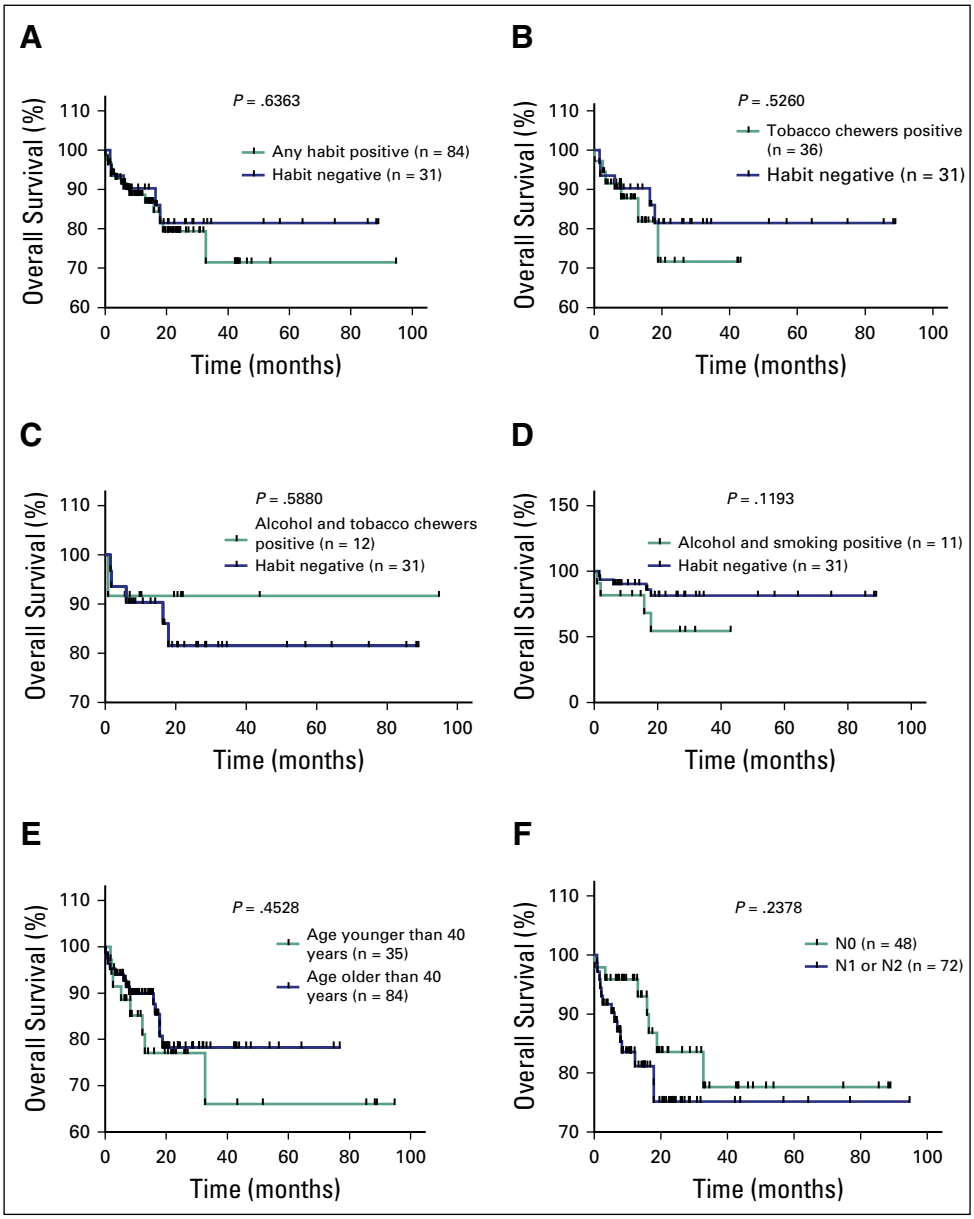


Fig A7. Kaplan-Meier survival analysis with tumors from patients according to habits, age, and nodal status. Overall survival (OS) percentages for patients who were (A) positive *v* negative for any habit, (B) positive for tobacco chewing *v* no habit, (C) positive for alcohol consumption and tobacco chewing *v* no habit, (D) positive for alcohol consumption and smoking *v* no habit, (E) age older than 40 *v* age younger than 40 years, and (F) their nodal status.



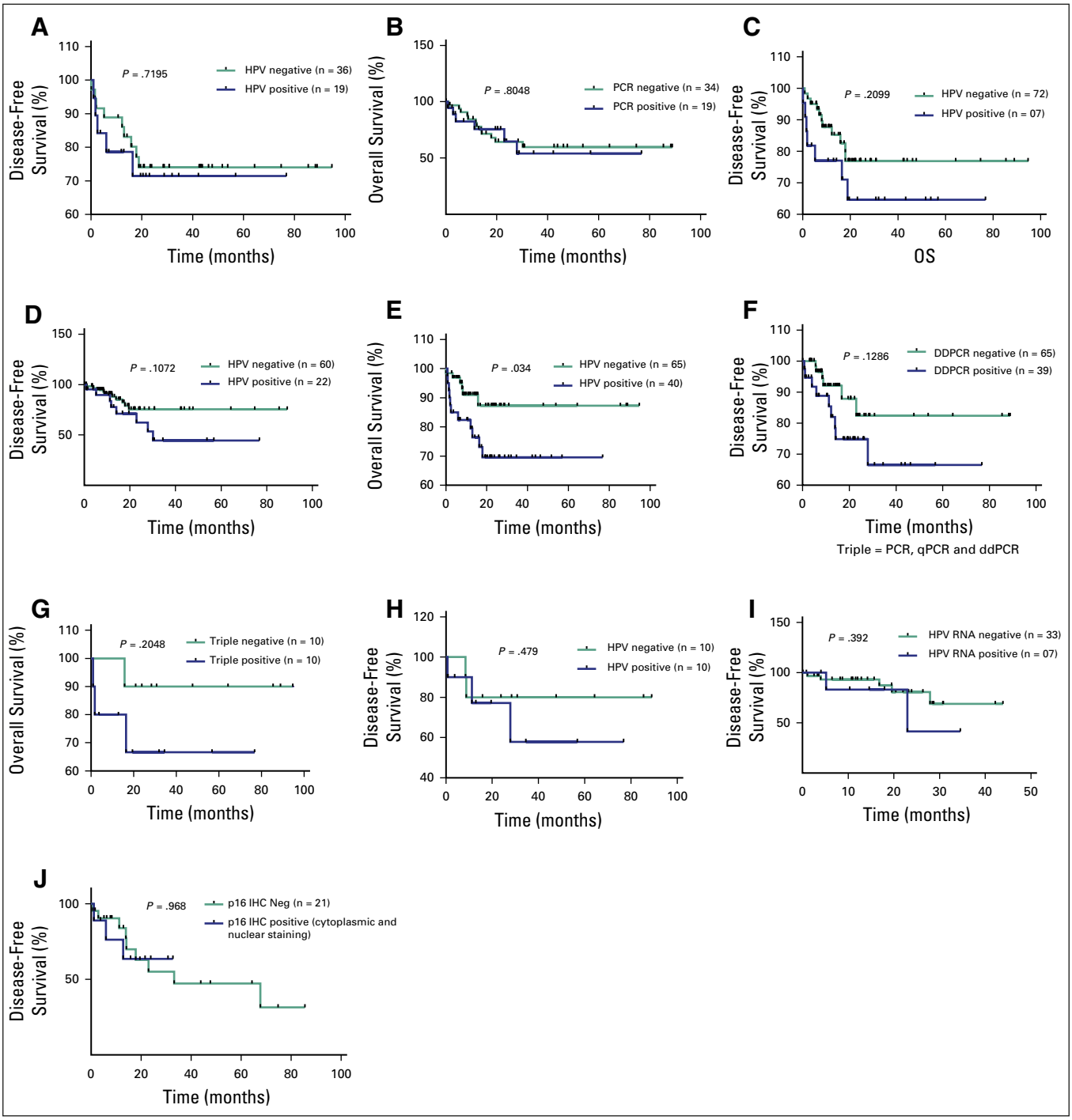


Fig A8. Kaplan-Meier survival analysis with (A-H) human papillomavirus (HPV) DNA and (I-J) HPV RNA. (A) Overall survival (OS) with DNA polymerase chain reaction (PCR). (B) Disease-free survival (DFS) with DNA PCR. (C) OS with DNA qPCR. (D) DFS with DNA qPCR. (E) OS with DNA ddPCR. (F) DFS with DNA ddPCR. (G) OS with HPV positive in PCR+qPCR+ddPCR vs HPV negative in PCR+qPCR+ddPCR. (H) DFS with HPV positive in PCR+qPCR+ddPCR vs HPV negative in PCR+qPCR+ddPCR. (I) DFS with HPV RNA, and (J) DFS with p16 IHC.

Fig A9. Kaplan-Meier survival analysis of tumors with (A-B) high copy number human papillomavirus (HPV) DNA and/or HPV RNA and (C-D) HPV-negative tumors with mutations in significant genes. DFS, disease-free survival; Mut, mutation; OS, overall survival; WT, wild-type.

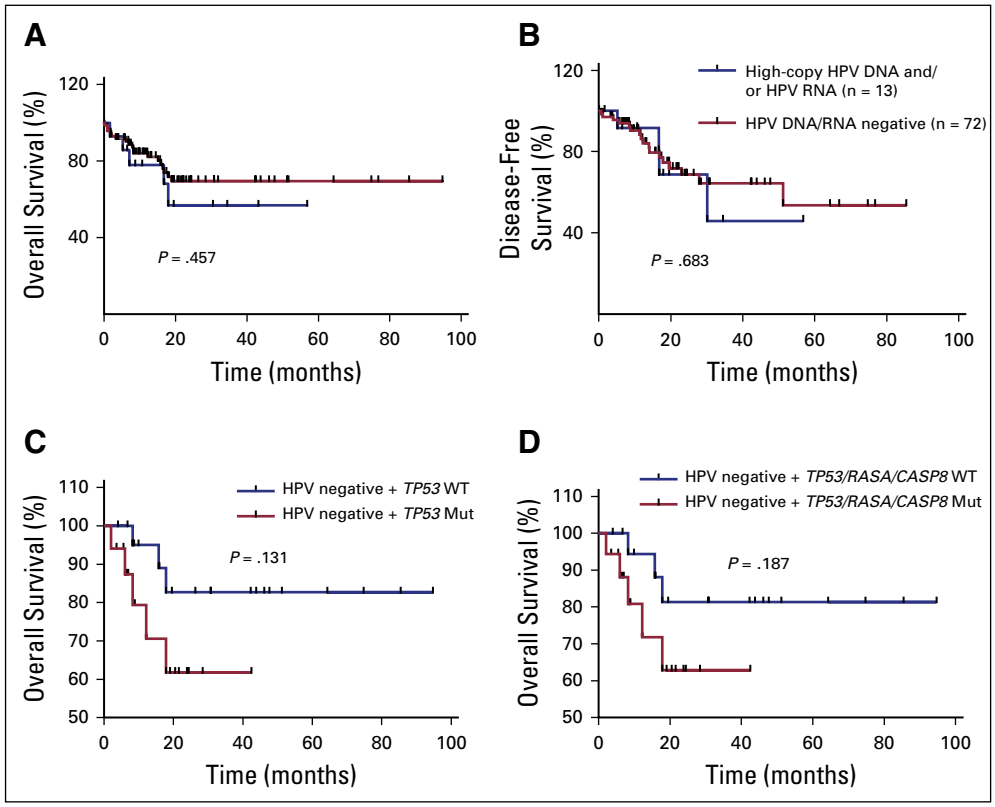


Fig A10. Mutational frequency in tumors with mutations in three commonly mutated (Mut) genes. WT, wild-type.

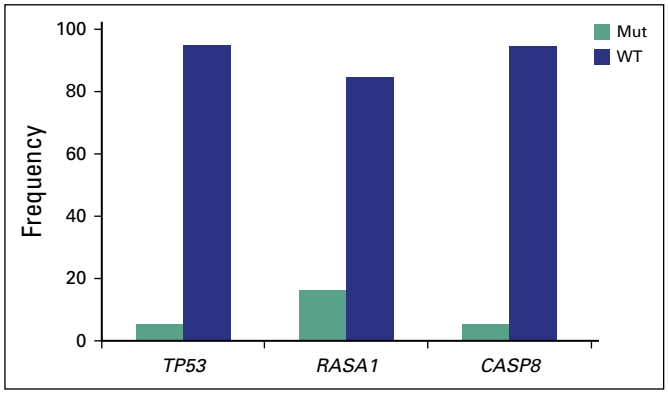


Table A1. Primer and Probe Sequences Used in the Study With Amplicon Size and Conditions for Amplification Reactions

Assay and Primer	Sequence	Domain	Region (bp)	Amplicon Size (bp)	PCR Conditions	Reference if any
DNA						
PCR						
HPV16L1	5′ TGC TAG TGC TTA TGC AGC AA 3′ 3′ATT TAC TGC AAC ATT GGT AC 5′	L1	6030-6180	151	94°C, 3 min; 94°C, 60 sec; 55°C, 60 sec; 72°C, 60 sec; 40 cycles; 72°C, 2 min and 4°C hold	Pool of 11F and 9R primers from Gravitt PE, et al: J Clin Microbiol 38:357-361, 2000 and Karlsen F, et al: J Clin Microbiol 34:2095-2100, 1996)
$GP5^{+}6^{+}$	5′ TTT GTT ACT GTG GTA GAT AC 3′ 3′ GAA AAA TAA ACT GTA AAT CA 5′	L1	6624-6746	150	94°C, 5 min; 94°C, 60 sec; 57.8°C, 60 sec; 72°C, 30 sec; 40 cycles; 72°C, 7 min and 4°C hold	
MY09/11	5′ CGT CCM ARR GGA WAC TGA TC 3′ 5′ GCM CAG GGW CAT AAY AAT GG 3′	L1	6602-7034	450	94°C, 5 min; 94°C, 60 sec; 57.8°C, 60 sec; 72°C, 60 sec; 40 cycles; 72°C, 7 min and 4°C hold	

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Table A1. Primer and Probe Sequences Used in the Study With Amplicon Size and Conditions for Amplification Reactions (Continued)

Assay and Primer	Sequence	Domain	Region (bp)	Amplicon Size (bp)	PCR Conditions	Reference if any	
CP I-II	5′ TTA TCW TAT GCC CAY TGT ACC AT 3′ 3′ ATG TTA ATW SAG CCW CCA AAA TT 5′	E1	1777-1942	188	94°C, 5 min; 94°C, 60 sec; 61.7°C, 60 sec; 72°C, 30 sec; 40 cycles; 72°C, 7 min and 4°C hold		
PGMY09/11	Pool of 11F and 9R primers from Gravitt PE, et al: J Clin Microbiol 38:357-361, 2000	L1	6602-7034	450	94°C, 5 min; 94°C, 60 sec; 57.8°C, 60 sec; 72°C, 60 sec; 40 cycles; 72°C, 7 min and 4°C hold		
HPV16E6 primer for PCR	5′ CAG GAG CGA CCC AGA AAG TT 3′ 3′ CAG CTG GGT TTC TCT ACG TGT 5′	E6	119-556	438	94°C, 3 min; 94°C, 30 sec; 53°C, 30 sec; 72°C, 30 sec; 40 cycles; 72°C, 2 min and 4°C hold		Newly designed used for PCR

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Table A1. Primer and Probe Sequences Used in the Study With Amplicon Size and Conditions for Amplification Reactions (Continued)

Assay and Primer	Sequence	Domain	Region (bp)	Amplicon Size (bp)	PCR Conditions	Reference if any	
HPV18L1 primer for PCR	5′ TCG CGT CCT TTA TCA CAG GGC GA 3′ 3′ TGC CCA GGT ACA GGA GAC TGT G 5′	L1	6141-6676	536	94°C, 3 min; 94°C, 40 sec; 55°C, 40 sec; 72°C, 30 sec; 40 cycles; 72°C, 2 min and 4°C hold		
qPCR							
HPV16E6 cloning primer	5′ CAG GAG CGA CCC AGA AAG TT 3′ 3′ CAG CTG GGT TTC TCT ACG TGT 5′	E6	119-556	438	As described above		Used for cloning HPV16E6 region in PUC19 plasmid
HPV16E6	5′ GCA CAG AGC TGC AAA CAA CT 3′ 3′ GCA TAA ATC CCG AAA AGC AA 5′ probe-ATTAGAATGTGTGTACTGCAAGCA-FAM-BHQ	E6	150-256	107	95°C, 3 min; 95°C, 30 sec; 55°C, 30 sec; 72°C, 30 sec; 40 cycles followed with dissociation curve		

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Table A1. Primer and Probe Sequences Used in the Study With Amplicon Size and Conditions for Amplification Reactions (Continued)

Assay and Primer	Sequence	Domain	Region (bp)	Amplicon Size (bp)	PCR Conditions	Reference if any	
HPV18L1 cloning primer	5′ TCG CGT CCT TTA TCA CAG GGC GA 3′ 3′ TGC CCA GGT ACA GGA GAC TGT G 5′	L1	6141-6676	536	As described above		Used for cloning HPV18L1 region in PUC19 plasmid)
HPV18L1	5′ TGA CAC TGT GCC TCA ATC CT 3′ 3′ AGA GCC ACT TGG AGA GGG AG 5′ Probe-TGCCTGCTTCACCTGGCAGC-VIC-BHQ	L1	6416-6506	91	95°C, 3 min; 95°C, 30 sec; 60°C, 30 sec; 72°C, 30 sec; 40 cycles followed with dissociation curve		
ddPCR							
HPV16E6	5′ ACT GTC AAA AGC CAC TGT GT 3′ 3′ GCT GGG TTT CTC TAC GTG TT 5′ Probe-AGGGGTCGGTGGACCGGTCGATGT-FAM-BHQ	E6	417-554	138	95°C, 10 min; 95°C, 15 sec; 55°C, 20 sec; 40 cycles; 95°C, 10 min		

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Table A1. Primer and Probe Sequences Used in the Study With Amplicon Size and Conditions for Amplification Reactions (Continued)

Assay and Primer	Sequence	Domain	Region (bp)	Amplicon Size (bp)	PCR Conditions	Reference if any
RNA						
E6						
HPV16 _E6_ RTPCR using SYBR chemistry	GCACCAAAAGAGAACTGCAATGTT AGTCATATACCTCACGTCGCAGTA	E6	85-108 197-236	152	95°C, 3 min; 95°C, 3 sec; 60°C, 30 sec; 40 cycles followed with dissociation curve	Cattani[17]
HPV18_E6_ RTPCR using SYBR chemistry	CTATAGAGGCCAGTGCCATTCG TTATACTTGTGTTTCTCTGCGTCG	E6	503-524 558-581	79	Same as above	Cattani[17]
E7 RT-PCR (						
HPV16_E7_ RTPCR using SYBR chemistry	CAAGTGTGACTCTACGCTTCGG GTGGCCCATTAACAGGTCTTCCAA	E7	738-759 796-818	81	Same as above	Cattani[17]
HPV18_E7_ RTPCR using SYBR chemistry	TAATCATCAACATTTACCAGCCCG CGTCTGCTGAGCTTTCTACTACTA	E7	721-744 810-833	113	Same as above	Cattani[17]
GAPDH						
	CTGCACCACCAACTGCTTAG TTCTGGGTGGCAGTGATG	NA	7537-7641	105	Same as above	Szostek S, et al: Folia Biol (Krakow) 62:73-78, 2014

NOTE. All the primers were aligned or designed using NC_001526.4 and NC_001357.1 sequences from the National Center for Biotechnology Information for human papillomavirus16 (HPV16) and HPV18, respectively. Sanger sequencing for the mutation study was performed as described in Krishnan et al.[29]
Abbreviations: ddPCR, droplet digital polymerase chain reaction (PCR); GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NA, not available; qPCR, quantitative PCR; RT-PCR, real-time PCR.

Table A2. Summary of Tumor HPV Status in Individual Tumors Used in This Study

	Protein	DNA				
Sample Code	p16 IHC	PCR	qPCR	ddPCR	In all 3 DNA-Based Assays	E6/E7 RNA
BM1	ND	ND	–	–	ND	ND
BM10	ND	ND	ND	–	ND	ND
BM11	ND	ND	ND	–	ND	ND
BM12	ND	ND	–	–	ND	ND
BM13	ND	ND	–	–	ND	–
BM14	ND	ND	–	–	ND	–
BM15	ND	ND	ND	–	ND	ND
BM16	ND	ND	ND	+	ND	ND
BM17	ND	ND	–	–	ND	ND
BM18	ND	ND	–	+	ND	ND
BM19	ND	ND	–	–	ND	ND
BM2	ND	ND	ND	+	ND	ND
BM20	ND	ND	–	–	ND	ND
BM21	ND	ND	–	+	ND	ND
BM22	ND	ND	–	–	ND	–
BM23	ND	ND	–	–	ND	ND
BM24	ND	ND	–	–	ND	–
BM25	ND	ND	–	–	ND	ND
BM26	ND	ND	–	+	ND	–
BM27	ND	ND	ND	–	ND	ND
BM28	ND	ND	ND	+	ND	ND
BM29	ND	–	–	–	–	ND
BM3	ND	ND	–	–	ND	ND
BM30	ND	ND	–	–	ND	ND
BM31	ND	ND	ND	–	ND	ND
BM32	ND	ND	ND	–	ND	ND
BM33	ND	ND	ND	–	ND	ND
BM34	ND	ND	ND	–	ND	ND
BM35	ND	+	–	–	–	ND
BM36	ND	–	–	–	–	ND
BM37	ND	ND	–	–	ND	ND
BM38	ND	ND	–	–	ND	–
BM39	ND	ND	–	–	ND	–
BM4	ND	ND	–	–	ND	–
BM40	ND	ND	–	–	ND	ND
BM41	ND	ND	ND	–	ND	ND
BM5	ND	+	+	–	–	ND
BM6	ND	ND	–	–	ND	+
BM7	ND	ND	ND	–	ND	ND
BM8	ND	ND	–	–	ND	–

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Table A2. Summary of Tumor HPV Status in Individual Tumors Used in This Study (Continued)

	Protein	DNA				
Sample Code	p16 IHC	PCR	qPCR	ddPCR	In all 3 DNA-Based Assays	E6/E7 RNA
BM9	ND	ND	–	+	ND	–
OT10	–	+	–	+	–	ND
OT100	ND	+	–	+	–	–
OT101	ND	ND	–	–	ND	+
OT102	ND	ND	ND	+	ND	ND
OT103	ND	ND	–	–	ND	ND
OT104	ND	ND	ND	+	ND	ND
OT105	ND	ND	ND	–	ND	ND
OT106	ND	ND	–	–	ND	–
OT107	ND	ND	ND	+	ND	ND
OT108	ND	ND	ND	–	ND	ND
OT109	ND	ND	–	–	ND	ND
OT11	–	+	ND	–	ND	–
OT110	ND	ND	–	–	ND	+
OT111	ND	ND	ND	–	ND	ND
OT112	ND	+	–	+	–	ND
OT113	ND	ND	ND	–	ND	ND
OT115	–	+	ND	ND	ND	ND
OT12	–	+	ND	ND	ND	ND
OT116	ND	ND	–	+	ND	ND
OT13	–	+	ND	ND	ND	ND
OT14	–	+	+	+	+	–
OT15	–	ND	ND	ND	ND	ND
OT16	–	+	+	+	+	ND
OT17	–	+	+	ND	ND	+
OT18	–	+	–	ND	ND	ND
OT19	–	–	–	–	–	ND
OT2	–	–	–	–	–	ND
OT20	–	+	+	–	–	ND
OT23	–	ND	ND	ND	ND	ND
OT21	ND	–	–	ND	ND	–
OT22	ND	–	ND	–	ND	ND
OT25	–	+	ND	+	ND	–
OT26	–	–	–	–	–	ND
OT28	–	–	–	ND	ND	ND
OT3	–	+	–	–	–	–
OT27	ND	–	ND	–	ND	–
OT31	–	–	+	+	–	ND

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Table A2. Summary of Tumor HPV Status in Individual Tumors Used in This Study (Continued)

	Protein	DNA				
Sample Code	p16 IHC	PCR	qPCR	ddPCR	In all 3 DNA-Based Assays	E6/E7 RNA
OT32	–	–	+	+	–	ND
OT33	–	–	+	–	–	–
OT38	–	–	+	+	–	ND
OT4	–	–	–	+	–	ND
OT41	–	–	–	–	–	ND
OT42	–	+	+	+	+	–
OT43	–	–	–	–	–	–
OT35	ND	ND	ND	ND	ND	ND
OT36	ND	–	+	ND	ND	ND
OT37	ND	–	–	ND	ND	ND
OT44	–	+	+	+	+	–
OT48	–	+	+	+	+	ND
OT51	–	–	+	ND	ND	ND
OT52	–	ND	ND	ND	ND	ND
OT54	–	+	+	+	+	ND
OT55	–	ND	–	+	ND	–
OT6	–	–	+	–	–	ND
OT61	–	+	+	+	+	+
OT45	ND	–	ND	–	ND	ND
OT46	ND	+	+	+	+	ND
OT65	–	+	+	+	+	ND
OT67	–	+	+	+	+	–
OT50	ND	–	–	+	–	ND
OT69	–	+	+	+	+	ND
OT7	–	+	–	+	–	ND
OT77	–	+	+	+	+	–
OT78	–	–	–	+	–	–
OT81	–	+	+	+	+	ND
OT56	ND	ND	–	–	ND	ND
OT57	ND	ND	ND	ND	ND	ND
OT58	ND	ND	+	–	ND	ND
OT59	ND	ND	ND	–	ND	ND
OT82	–	+	+	+	+	ND
OT60	ND	ND	–	–	ND	–
OT83	–	+	–	+	–	ND
OT62	ND	+	–	+	–	–
OT63	ND	ND	ND	–	ND	ND
OT64	ND	+	+	+	+	–

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Table A2. Summary of Tumor HPV Status in Individual Tumors Used in This Study (Continued)

	Protein	DNA				
Sample Code	p16 IHC	PCR	qPCR	ddPCR	In all 3 DNA-Based Assays	E6/E7 RNA
OT84	–	ND	–	–	ND	ND
OT66	ND	ND	–	+	ND	ND
OT91	–	ND	–	–	ND	ND
OT68	ND	ND	+	+	ND	ND
OT9	+	+	–	+	–	ND
OT114	+	+	+	+	+	ND
OT70	ND	ND	–	–	ND	ND
OT71	ND	ND	–	–	ND	ND
OT72	ND	ND	–	+	ND	+
OT73	ND	ND	ND	+	ND	ND
OT74	ND	ND	ND	–	ND	ND
OT75	ND	+	+	+	+	–
OT76	ND	+	+	+	+	ND
OT24	+	+	–	–	–	–
OT29	+	–	–	+	–	ND
OT79	ND	ND	ND	–	ND	ND
OT80	ND	–	+	+	–	–
OT30	+	+	+	+	+	ND
OT34	+	–	ND	+	ND	ND
OT39	+	ND	ND	ND	ND	ND
OT40	+	–	ND	+	ND	–
OT85	ND	+	–	+	–	ND
OT86	ND	ND	–	–	ND	ND
OT87	ND	ND	–	–	ND	–
OT88	ND	–	+	+	–	–
OT89	ND	ND	ND	–	ND	ND
OT5	+	ND	ND	ND	ND	ND
OT90	ND	ND	–	–	ND	ND
OT53	+	+	–	–	–	ND
OT92	ND	–	+	+	–	ND
OT93	ND	ND	ND	+	ND	ND
OT94	ND	ND	ND	–	ND	ND
OT95	ND	+	+	+	+	ND
OT96	ND	ND	ND	–	ND	ND
OT97	ND	ND	–	–	ND	ND
OT98	ND	ND	ND	+	ND	ND
OT99	ND	ND	–	–	ND	ND

Abbreviations: (+), positive; (–), negative; BM, buccal mucosa; ddPCR, droplet digital polymerase chain reaction; HPV, human papillomavirus; IHC, immunohistochemistry; ND, not done; OT, oral tongue; qPCR, quantitative PCR.

Table A3. *P* Values From Unpaired *t* Tests Measuring Significance in Differences Between Differential Methylation in Nine HPV-Associated Genes Between HPV-Positive and HPV-Negative Groups

Gene	Group 1 HPV positive *v* HPV negative	Group 2 HPV positive *v* HPV negative
FERMT3	< .00001	.0346
GIT2	< .00001	.1052
HK3	< .00001	.0574
PRKCZ	< .00001	.052
ZCCHC8	< .00001	.0504
IRF5	< .00001	.083
IFFO1	< .00001	.0608
ARID3A	< .00001	.0654
HOXA2	.0074	.1788

NOTE. Group 1: when high-copy and/or HPV E6/E7 RNA is taken into consideration to define HPV positivity. Group 2: when HPV DNA only, irrespective of copy number, is taken into consideration to define HPV positivity.

Table A4. Literature Survey of HPV Studies in Oral Cavity Tumors

					Method/Marker of HPV Detection									Prevalence		Linked With Outcome		
Sr. No.	First Author	Cohort	Subsite	Patient No. (n)	HPV DNA by PCR/ qPCR/ RFLP/ sequencing	HPV Genotyping INNO-LiPA	PCR-Mass Array	DNA-PCR-Dot Blot	DNA by ISH	HPV RNA by q RT-PCR (E2/E6/E7)	p16 IHC	E6/E7 IHC	E6/E7 Antibody-ELISA	HPV DNA	HPV Subtype	p16	HPV DNA	p16
1	Huang (2014)[18]	Taiwan	Oral cavity	312	+	–	–	–	–	–	–	–	–	16.6	HPV16	NA	High HPV16/18 E7 viral load identified a small subgroup of patients at high-risk of 5-year distant metastases.	NA 0
2	Lee (2012)[a]	Taiwan	Oral cavity	333	+	–	–	+	–	–	–	–	–	21.3	HPV16	NA	HPV16 infection in patients with advanced oral cavity cancer is related to an increased risk of distant metastases and poor survival.	NA 0
3	Gracía (2014)[b]	Spain	Oral tongue	64	+	+	–	–	–	–	–	–	–	26.2	HPV56	NA	Mortality showed a statistically significant correlation, being higher in patients with high-risk HPV.	NA 0
4	Lee (2015)[c]	Taiwan	Oral cavity	1002	–	–	–	+	–	–	–	–	–	19	HPV16	NA	HPV infections are common in Taiwanese patients with OSCC and predict 5-year OS; 5-year OS rate of HPV-positive patients was significantly lower than that for HPV-negative patients.	NA 0
5	Lee (2013)[d]	Taiwan	Oral cavity	410	–	–	–	+	–	–	–	–	–	21.2	HPV16	NA	Low-risk HPV infection was a predictor of poor 2-year DFS, disease-specific survival, and OS in the subgroups of patients with OSCC with poor differentiation and pN2 lymph node metastases.	NA 0

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Table A4. Literature Survey of HPV Studies in Oral Cavity Tumors (Continued)

					Method/Marker of HPV Detection									Prevalence			Linked With Outcome		
Sr. No.	First Author	Cohort	Subsite	Patient No. (n)	HPV DNA by PCR/ qPCR/RFLP/ sequencing	HPV Genotyping INNO-LiPA	PCR-Mass Array	DNA-PCR-Dot Blot	DNA by ISH	HPV RNA by q RT-PCR (E2/ E6/E7)	p16 IHC	E6/E7 IHC	E6/E7 Antibody-ELISA	HPV DNA	HPV Subtype	p16	HPV DNA	p16	
6	Ringström (2002)[e]	United States	Oral cavity and others[f]	41	+	–	–	–	–	–	–	–	–	5	HPV16	NA	HPV-positive younger group with less alcohol consumption habit had better clinical outcome than HPV-negative group.	NA	1
8	Smith (2008)[g]	United States	Oral cavity and others[f]	170	+	–	–	–	–	–	+	–	–	15	HPV16	25	High-risk HPV is a positive predictor of outcome.	p16 is a positive predictor of outcome.	1
9	Smith (2010)[h]	United States	Oral cavity and others[f]	21	–	–	–	+	–	–	+	–	+	15.8 E6/ E7	?	?	Two distinct patient groups with HNC with HPV DNA–positive tumors distinguishable by E6 and/or E7 antibody status. Differences in antibody status were associated with distinct risk factors and clinical outcomes.		?
10	Smith (2008)[i]	United States	Oral cavity and other[f]	166	+	–	–	+	–	–	–	–	–	16	HPV16	NA	Joint assessment of p53/HPV status provided different HRs for each clinical outcome (p53 overexpression = 48%); p53/HPV provides a better indicator of prognosis.	NA	1
11	Zhao (2009)[j]	China	Oral cavity	52	+	–	–	–	–	–	–	–	–	40.4	HPV16	NA	HPV was significantly correlated with better survival for patients with OSCC.	NA	1
15	Ramshankar (2014)[k]	India	Oral tongue	167	+	–	–	–	–	–	–	–	–	52	HPV16	15.3	HPV16 DNA was not a significant predictor for DFS and disease outcome.	p16 was found to be a significant predictor of poorer outcome with increased risk of death and recurrence.	?

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Table A4. Literature Survey of HPV Studies in Oral Cavity Tumors (Continued)

				Method/Marker of HPV Detection									Prevalence			Linked With Outcome		
Sr. No.	First Author	Cohort	Subsite	Patient No. (n)	HPV DNA by PCR/ qPCR/RFLP/ sequencing	HPV Genotyping INNO-LiPA	PCR-Mass Array	DNA-PCR-Dot Blot	DNA by ISH	HPV RNA by q RT-PCR (E2/E6/E7)	p16 IHC	E6/E7 IHC	E6/E7 Antibody-ELISA	HPV DNA	HPV Subtype	p16	HPV DNA	p16	
16	Chung (2014)[l]	United States	Oral cavity and others[f]	89/80	–	–	–	–	+	–	+	–	–	14.6 (89)	HPV 16	26.3 (80)	There was no significant difference in PFS or OS between HPV ISH–positive and –negative patients. Moreover, patients with p16-positive OPSCC have better PFS and OS than patients with p16-positive non-OPSCC, but patients with p16-negative OPSCC and non-OPSCC have similar outcomes.	Patients with p16-positive tumors had significantly longer PFS (P = .04) and OS (P = .01) than patients with p16-negative tumors.	?
12	Vietía (2014)[m]	Venezuela	Oral cavity and others[f]	25	–	+	–	–	–	–	–	–	–	35.4	HPV16	NA	HPV positivity in SCC is mainly associated with high-risk HPV.	No correlation	2
17	Gröbe (2013)[n]	Germany	Oral cavity and others[f]	222	+	–	–	–	–	–	+	–	–	6.9	?	?	No statistically significant correlation to recurrence-free survival of HPV-positive patients or OS could be observed.		?
7	Duncan (2013)[o]	United States	Oral cavity	81	+	–	–	–	–	–	+	–	–	8.6	HPV16	8 to 27	Statistical correlation among HPV PCR positivity, 3+ staining (p16 IHC), and younger age, but not with survival.		2
12	Elanago (2011)[p]	India	Oral cavity	60	+	–	–	–	+	–	+	+	–	50	HPV16	33	No statistically significant difference in the survival rate among patients with respect to different clinical and pathologic variables.	No correlation	2

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Table A4. Literature Survey of HPV Studies in Oral Cavity Tumors (Continued)

					Method/Marker of HPV Detection									Prevalence			Linked With Outcome		
Sr. No.	First Author	Cohort	Subsite	Patient No. (n)	HPV DNA by PCR/ qPCR/RFLP/ sequencing	HPV Genotyping INNO-LiPA	PCR-Mass Array	DNA-PCR-Dot Blot	DNA by ISH	HPV RNA by q RT-PCR (E2/ E6/E7)	p16 IHC	E6/E7 IHC	E6/E7 Antibody-ELISA	HPV DNA	HPV Subtype	p16	HPV DNA	p16	
14	Stephen (2014)[38]	United States	Oral cavity and others[f]	20	+	–	–	–	–	–	+	–	–	50	HPV16	20	The prognostic effects of HPV16 and p16 alone were analyzed for individual non-oropharyngeal sites (oral cavity, larynx, hypopharynx). but were not statistically significant.	Both HPV16 and p16 showed positive correlation when all sites were combined.	1
18	Kouketsu (2015)[q]	Japan	Oral cavity	174	+	–	–	–	+	–	+	–	–	7.4	HPV16	13.7		No information	3
19	Walline[7]	United States	Oral cavity and other[f]	108	+	–	+	–	+	–	+	–	–	26	HPV16	18.9		No information	3
20	Lingen (2013)[r]	United States	Oral cavity	409	–	+	–	–	–	+	+	–	–	5.9				No information	3
21	Chaudhary (2013)[s]	India	Oral submucous fibrosis and oral cavity	222	+	–	–	–	–	–	–	+	–	37.83	HPV16			No information	3
22	Rivero (2006)[t]	Brazil	Oral cavity	40	+	–	–	–	–	–	–	–	–	0				No information	3
23	Pannone (2012)[u]	Italy	Oral cavity		+	–	–	–	+	–	+	–	–	11				No information	3
24	Smith (2004)[v]	United States	Oral cavity and others[f]	193	+	–	–	–	–	–	–	–	–	13.3	HPV 16		HPV is not statistically significant in oral cavity cancer compared to OPSCC.	No information	3
27	Herrero (2003)[w]	France	Oral cavity and others[f]	766	+	–	–	–	–	–	–	–	+	3.9	HPV16	NA	NA	NA	3
25	Kurose (2004)[x]	Japan	Oral cavity	662	+	–	–	–	–	–	–	–	–	0.6	HPV71 and HPV12	NA	NA	NA	3
26	Rice (2000)[y]	London, United Kingdom	Oral cavity	267	+	–	–	–	–	+	–	–	–	51.7	NA	NA	NA	NA	3

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Table A4. Literature Survey of HPV Studies in Oral Cavity Tumors (Continued)

				Method/Marker of HPV Detection									Prevalence			Linked With Outcome		
Sr. No.	First Author	Cohort	Subsite	Patient No. (n)	HPV DNA by PCR/ qPCR/RFLP/ sequencing	HPV Genotyping INNO-LiPA	PCR-Mass Array	DNA-PCR-Dot Blot	DNA by ISH	HPV RNA by q RT-PCR (E2/E6/E7)	p16 IHC	E6/E7 IHC	E6/E7 Antibody-ELISA	HPV DNA	HPV Subtype	p16	HPV DNA	p16	
28	Lohaus (2014)[z]	Germany	Oral cavity and others[f]	60	+	–	–	–	–	–	+	–	–	12	HPV16	18.3	HPV16 DNA status correlated with oropharyngeal carcinoma but not in the oral cavity.	Overexpression of p16 showed a significant association with distant metastases (HR, 0.31; $P = .02$) andon OS.	2
29	Chandarana (2013)[aa]	Canada	Oral cavity and others[f]	49	–	–	–	–	–	–	+	–	–	NA	NA	13	NA	Patients with OCSCCs showed no association between biomarkers and outcome.	2
30	Huang (2012)[bb]	Taiwan	Oral cavity	103	–	+	–	–	–	–	–	–	–	30.1	HPV16	NA	HPV infection was not associated with tumor aggressiveness, risk exposure, or treatment outcome.	NA	2
31	Duray (2012)[cc]	Belgium	Oral cavity	162	+	–	–	–	+	–	+	–	–	44	HPV16	53	High-risk HPV positivity was associated with shorter DFS in our series of 147 patients with OSCC (negative correlation).	Statistical analyses did not show any impact of p16 expression on disease-free survival0	
32	Rautava (2012)[dd]	Finland	Oral cavity and others	37	–	+	–	–	–	–	–	–	–	41	HPV16	NA	HPV-positive and -negative HNSCC similar survival. Patients with low-risk HPVs who were treated with radiotherapy had a poor prognosis.		0

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Table A4. Literature Survey of HPV Studies in Oral Cavity Tumors (Continued)

					Method/Marker of HPV Detection									Prevalence			Linked With Outcome	
Sr. No.	First Author	Cohort	Subsite	Patient No. (n)	HPV DNA by PCR/ qPCR/RFLP/ sequencing	HPV Genotyping INNO-LiPA	PCR-Mass Array	DNA-PCR-Dot Blot	DNA by ISH	HPV RNA by q RT-PCR (E2/ E6/E7)	p16 IHC	E6/E7 IHC	E6/E7 Antibody-ELISA	HPV DNA	HPV Subtype	p16	HPV DNA	p16
33	Chen (2012)[43]	Taiwan	Oral cavity	65		–	–	–	+	–	+	–	–	37	HPV11	42	Related to a better outcome with longer survival and bears a causally associated relationship different from other carcinogenic mechanisms.	
34	Reyes (2015)[ee]	Chile	Oral cavity	80	+	–	–	–	–	+	+	–	–	11	HPV16 and HPV18	NA	No association with the presence of HPV.	2
35	Quintero (2013)[ff]	Colombia	Oral cavity and others	175	+	–	–	–	–	–	–	–	–	23.9	HPV 16 and HPV19	NA	?	
36	Patil (2014)[gg]	India	Oral cavity	30	–	–	–	–	–	–	+	–	–	NA	NA	86.66	Association between HPV and OSCC.	
37	Gichki (2012)[hh]	Pakistan	Normal oral cavity	192	+	–	–	–	–	–	–	–	–	24.5			Association between the presence of HPV and smoking.	
38	Hwang (2012)[ii]	Taiwan	Oral papillary and verrucous lesions	31	+	–	–	–	+	–	–	–	–	28.3	HPVL1		HPV infection was independently associated with malignant transformation and disease-specific survival.	

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Table A4. Literature Survey of HPV Studies in Oral Cavity Tumors (Continued)

					Method/Marker of HPV Detection									Prevalence			Linked With Outcome	
Sr. No.	First Author	Cohort	Subsite	Patient No. (n)	HPV DNA by PCR/ qPCR/RFLP/ sequencing	HPV Genotyping INNO-LiPA	PCR-Mass Array	DNA-PCR-Dot Blot	DNA by ISH	HPV RNA by q RT-PCR (E2/ E6/E7)	p16 IHC	E6/E7 IHC	E6/E7 Antibody-ELISA	HPV DNA	HPV Subtype	p16	HPV DNA	p16
39	Terai (1999)[53]	Japan	Normal cavity	37	+	+	–	–	–	–	–	–	–	81.1	HPV5 subtypes			

NOTE. In the last column, 0 represents the study that shows HPV as a negative indicator of outcome; 1 represents the study that shows HPV as a positive indicator of outcome; 2 represents the study that shows HPV as no indicator of outcome; 3 represents the study that shows no information on HPV as indicator of outcome.

Abbreviations: (–), negative; (+), positive; DFS, disease-free survival; ELISA, enzyme-linked immunosorbent assay; HNC, head and neck cancer; HNSCC, head and neck squamous cell carcinoma; HPV, human papillomavirus; HR, hazard ratio; IHC, immunohistochemistry; INNO-LiPA, INNO line probe assay; ISH, in situ hybridization; NA, not available; OP, oropharyngeal; OPSCC, oropharyngeal squamous cell carcinoma; OS, overall survival; OSCC, oral cavity squamous cell carcinoma; PCR, polymerase chain reaction; PFS, progression-free survival; qPCR, quantitative polymerase chain reaction; qRT-PCR, quantitative real-time PCR; RFLP, restriction fragment length polymorphism; SCC, squamous cell carcinoma; seq, sequence.

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