

High-risk human papillomavirus in oral cavity squamous cell carcinoma

HPV and oral cavity tumors

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Conflict of interests

None of the authors declare any conflicts of interest.

Abstract

Human papillomavirus (HPV) is a known risk factor for certain head and neck cancers. Tumors of head and neck region are heterogeneous in nature with different incidences, mortalities and prognosis for different subsites. Unlike oropharynx, where data favors inclusion of HPV status in disease management, role of HPV in oral cavity squamous cell carcinoma (OSCC) is not well understood. The prevalence of HPV in OSCC, although considered lower than oropharynx, vary greatly based on the choice of HPV assay and patient geography. Additionally, data on HPV +ve OSCC is scarce and there is less agreement on HPV being a good prognostic factor in OSCC. Here with 153 OSCC patients, using multiple analytes and assays, we show that a high prevalence (33-58%) of HPV16/18 DNA did not correlate with an equally high prevalence of transcriptionally active viral genomes (E6/E7 RNA prevalence 15%) in tumors. Only 6% of the tumors showed the presence of both HPV DNA and HPV16/18 E6/E7 RNA and none with both p16 and HPV RNA. Most tumors with relatively high-copy HPV DNA and/or HPV E6/E7 mRNA, 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 E6/E7 RNA, either alone or in combinations, did not correlate with patient survival. Using genome-wide methylation data, 9 HPV-associated genes stratified the HPV +ve from the HPV -ve tumor groups with high confidence ($p<0.008$) when relatively high-copy number of HPV DNA and/or HPV E6/E7 RNA were considered to define HPV positivity and not HPV DNA alone irrespective of their copy number ($p<0.2$). Taken together, we conclude that tests measuring HPV DNA alone without viral load and/or viral RNA may not be a true measures of HPV infection in oral cavity tumors and therefore are not informative.

Keywords: HPV, OSCC, HPV DNA, HPV RNA, copy number, survival, somatic mutations, methylation.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide with an incidence of 550,000 cases annually [1]. Oral cavity squamous cell carcinoma (OSCC) constitute a majority of HNSCC including tumors of oral/anterior tongue and buccal mucosa [2]. The major known risk factors of OSCC are tobacco products, alcohol, and infection with human papillomavirus (HPV) [3]. Unlike oropharyngeal tumors, where the HPV incidence is reported to be very high, (upto 90%) [4,5], the incidence of HPV in OSCC varies widely (from none to 74%) depending on the detection methodology used and the geography of the patient cohort [6]. Additionally, unlike oropharyngeal tumors [7-11], the role of HPV in disease prognosis and response to therapy in patients with OSCC is less equivocal. Among the high risk HPV types, infection with HPV16 or HPV18 has been epidemiologically linked with head and neck malignancies [12]. Presence of HPV DNA is routinely used as a measure of HPV infection in tumors. However, there is a considerable variation in the sensitivity of DNA-based assays, which leads to differences in reporting HPV DNA prevalence among different cohorts. On the otherhand, the presence of HPV E6/E7 RNA and/or their associated protein products remain as gold standard tests defining HPV positivity.

Large-scale sequencing studies have demonstrated that the majority of HPV-negative tumors harbor mutations in *TP53* and *CASP8* and a large proportion of HPV-positive tumors in *PIK3CA* [13-15]. Additionally, past studies have identified specific mutations in potential drug targets like *FGFR2/3* and lack of *EGFR* aberrations in HPV-positive patients [15] and the role of *CASP8* in HPV-negative cell line and patients [13,16]. Despite the wealth of information, which HPV tests are accurate and whether HPV plays an important factor in oral cavity tumor stratification and treatment remain to be answered.

In the present study, we addressed the following questions on HPV in a cohort of 153 patients with oral cavity tumors. Does sensitivity matter in the detection of HPV DNA in tumors? Does

presence of tumor p16 and HPV DNA correlate with that of HPV E6/E7 RNA? Is high-copy HPV DNA a true reflection of HPV infection in oral cavity tumors? Are p16 protein, HPV DNA, HPV E6/E7 RNA, individually or together, linked with patient survival? And finally, do somatic mutations and gene methylation at 5-Cytosine residues distinguish the HPV +ve from the HPV -ve tumors?

Materials and Methods

Patient cohort

Informed consent was obtained voluntarily from each patient enrolled in the study. Ethical approval (NHH/MEC-CL/2014/197; NHH/MEC-CL-2014/224) was obtained from the Institutional Ethics Committees of the Mazumdar Shaw Medical Centre. Patients diagnosed and treated at the cancer clinic of the Mazumdar Shaw Medical Centre for oral cavity tumors were subjected to a screening procedure before being enrolled in the study. Only those tumors where the histological sections confirmed the presence of squamous cell carcinoma and with at least 70% tumor cells in the section were used in the current study. One hundred and fifty three patients underwent staging according to AJCC criteria, and curative intent treatment as per NCCN guideline involving surgery with or without post-operative adjuvant radiation or chemo-radiation at the Mazumdar Shaw Medical Centre were accrued for the study (Supplementary Table 1). Post-treatment surveillance was carried out by clinical and radiographic examinations as per the NCCN guidelines. All the tissues were frozen immediately in liquid nitrogen and stored at -80°C until further use.

Cell cultures

Cell lines (UM-SCC47, from Dr. Thomas Carey, University of Michigan, USA [17]; Hep2, National Centre for Cell Science, Pune, India; UPCI:SCC29B and UPCI:SCC040, from Dr. Susanne Gollin, University of Pittsburg, USA [18]) were maintained in Dulbecco's Modified Eagles' Media (DMEM) supplemented with 10% FBS, 1X MEM non-essential amino acids

solution and 1X penicillin/streptomycin mixture (Gibco) and grown in incubators at 37°C with 5% CO₂.

Immunohistochemistry (IHC)

For p16 immunohistochemistry (IHC) staining was carried out using primary antibody from Biogenex, USA (cat: AM540-5M) and using the poly-HRP detection system (Cat: QD400-60KE, BioGenex, Fremont, CA, USA) following manufacturers' instructions. Sections of cervical cancer were used as the positive control.

DNA extraction

The genomic DNA from tumor tissues and cell lines were extracted using DNeasy Blood and Tissue extraction kit using the spin column method with an intermediate RNase digestion following manufacturer's instructions (Qiagen, USA). The DNA was quantified using Qubit 2.0 fluorometer (Invitrogen, USA) and stored at -20°C until further use. Total 300ng of genomic DNA (unless specified otherwise) from tumors was used for the detection of HPV using PCR, qPCR and digital droplet PCR (ddPCR) methods.

Detection of HPV DNA with PCR

PCR primers were designed to amplify either consensus or type-specific regions of the virus (Figure 1A). Five sets of primers, including 4 HPV consensus (GP5-6; CP-I/II; MY09/11 and PGMY09/11) and 1 type-specific (HPV16-L1) primers as described in the literature [19], in the PCR assays. Additionally, we designed two sets of new type-specific primers (HPV16-E6 and HPV18-L1) to detect HPV DNA. The primer sequences and the amplification conditions used are provided in Supplementary Table 2.

Detection of HPV DNA with qPCR

For qPCR, E6 and E7 regions from HPV16 and HPV18 respectively were cloned in pUC19 vector. The sequences for the recombinant plasmids were confirmed by Sanger sequencing. The qPCR was carried out using KAPA Probe Fast qPCR master mix Universal (2X) (cat: KK4701, KAPA Biosystems, USA). The primers and probes were designed (Supplementary Table 2) within the cloned regions of the HPV16 and HPV18 plasmids and standard quantitative PCR was performed. The standard curves were generated using serial dilutions (from 10 to 100,000 copies) of HPV16 and HPV18 cloned pUC19 plasmids. Genomic DNA from positive and negative control cell lines were used as positive and negative controls for HPV18 and HPV16 respectively (Supplementary Figure 1). All amplification reactions were carried out in triplicates, using nuclease free water (cat: AM9932, Ambion, USA) as negative control. The analysis for each sample was done by using the absolute quantification using standard curve generated with serial dilutions of cloned plasmids. Tumors and cell lines samples were counted as positive for those having *Ct* values 3 times away from the standard deviation from the negative controls.

Detection of HPV DNA using droplet digital PCR (ddPCR)

Digital PCR was performed using the QX100 droplet digital PCR system (Bio-Rad, USA) using primers and probes as provided in Supplementary Table 2. The reaction mix consisted of 10 µl of 2× ddPCR Supermix without dUTP (cat: 1863024; Bio-Rad, USA), 450 nM of both forward and reverse primers of HPV16 and 250 nM probe and 300ng of genomic DNA in a final volume of 20 µl. The entire 20 µl reaction was loaded into a droplet cartridge, followed by 70 µl of droplet generation oil and droplets were generated using droplet generator. The droplets were transferred into a 96-well PCR plate (PCR96-FLT-C, Axygen, USA) and sealed using an aluminium foil. Further, the PCR amplification was carried out on in a thermal cycler (C1000, Bio-Rad, USA). After amplification, the plate was transferred to a Bio-Rad droplet reader. The droplets per well were measured and normalized in every reaction using the QuantaSoft V.6 software (Bio-Rad, USA). All the samples were processed in triplicates. Tumors and cell lines samples were

counted as positive for those having droplet numbers 3 times away from the standard deviation from the negative controls.

HPV DNA copy number

We deduced the HPV absolute copy number from the qPCR standard curves using cloned HPV16/18 (Figure 2A,D). We considered a tumor or cell line to have relatively high-copy of HPV DNA when the copy number for HPV16 and HPV18 DNA were more than 3.3×10^2 and 3.3×10^3 per microgram of tumor DNA respectively. In order to minimize the effect of tumor cellularity, ploidy and heterogeneity, we expressed the HPV copy number as copies per microgram of tumor DNA used in the reaction.

Detection of HPV16/18 E6/ E7 RNA

The total RNA was extracted from 25mg of tumor tissues using the RNeasy mini kit from Qiagen (cat: 74104) following manufacturer's instructions. The residual DNA was digested on column during extraction using RNase free DNase set from Qiagen (cat: 79254). Total 500ng of RNA was subjected to cDNA synthesis using Takara's Prime Script first strand cDNA synthesis kit (cat: 6110A). The qPCR was carried out using KAPA SYBR Fast qPCR master mix Universal (cat: KK4601, KAPA Biosystems, USA). The GAPDH was amplified and used as internal control. The primer sequences and the amplification conditions are provided in Supplementary Table 2. All amplification reactions were carried out in triplicates, using nuclease free water (cat: AM9932, Ambion, USA) as no template control and cDNA from UPCI:SCC29B and UPCI:SCC40 cell lines as negative controls. Complementary DNA from cell lines UM-SCC47 and Hep2 were used as positive controls for E6 and E7 amplifications respectively. Data was normalized using relative quantification method ($2^{-\Delta\Delta Ct}$) [20]. Positive tumors were counted as ones that have *Ct* values 3 times from the standard deviation of the negative control.

Integration status of HPV genome

We performed HPV16E2 PCR [21,22] to determine whether HPV has integrated into tumor genomes or remained as episomal DNA in tumors those were positive for HPV DNA and RNA. Genomic DNA from the early passage of W12 cell line (gift from Prof. Sharmila Sengupta, NIBMG, Kalyani, India) and p3796 pGEX-16E2 (wt) (Addgene plasmid # 24123) were used as positive controls for E2 PCR. In addition, genomic DNA from UPCI;SCC29B and PCI;SCC040 cell lines were used as negative controls. The primer sequences and the amplification conditions are provided in Supplementary Table 2. The PCR amplicons were resolved on 2% agarose gel and visualized with a Gel Doc system (G-box, Syngene, UK). Tumors with no amplification were considered to have HPV integrated into host genomes.

Mutation analysis by Sanger sequencing

The mutation data on tumors for *TP53*, *CASP8* and *RASAI* were retrieved from the previously published data [16].

Statistical Analysis

The Chi square test was used to analyse significance of different clinical parameters of patients. The relationship between tumor HPV status and survival in patients was examined by Kaplan-Meier analysis and Cox proportional hazards regression with overall survival (OS) and disease-free survival (DFS) with logrank test for determining significance (p values <0.05).

Whole genome methylation and statistical data analyses

Production of whole genome methylation data using Illumina Infinium Methylation450 BeadChip, chip scanning and the data pre-processing are described previously [23]. Three tumors in HPV +ve group (relatively with high-copy HPV DNA and/or E6/E7 RNA) and 18 tumors in HPV -ve group (HPV DNA -ve and HPV RNA -ve) were considered for further analysis. Differential methylation for probes was estimated as $\Delta\beta$ values ($\text{Tumor}_\beta - \text{Normal}_\beta$) resulting in values ranging from -1 (hypo-methylation) to 1 (hyper-methylation). Using a supervised clustering approach,

differentially methylated probes that discriminated HPV +ve and HPV -ve patients samples were identified. Hyper-methylated probes in the HPV +ve group that were hypo-methylated in the HPV -ve group, and *vice versa*, along with other probes with Db values that differed by at least 0.5 between the two groups, with a sample frequency of at least 60% were considered further. Finally, the list of genes consisting of average Db values across tumors for multiple probes (where present) was drawn. Neighborhood interaction network for these genes was inferred using PCViz from PathwayCommons (<http://www.pathwaycommons.org/pcviz/#neighborhood/>). The genes and their interacting partners were mapped to the Viral Carcinogenesis pathway from KEGG (hsa05203), and mapped genes were linked back to respective genes found using supervised clustering. For the linked genes, an unpaired *t*-test was performed to determine the significance of difference between the HPV +ve and HPV -ve tumor groups. The same was performed for the HPV-associated genes between the DNA-based (irrespective of the DNA copy number) HPV +ve and HPV -ve tumor groups.

Results

p16 expression

The tumors (n = 56) were first confirmed by H and E staining of the paraffin tissue sections followed by the immunohistochemical staining with the surrogate marker p16. Supplementary Figure 2 shows the representative p16 +ve and -ve staining in oral cavity tumor sections (OSCC) along with the cervical tissue section (+ve control). Fifty one percent (28/55) of the oral cavity tumors studied showed positive p16 staining (Table 1).

Incidence of HPV DNA

We used three different molecular techniques (PCR, quantitative PCR and digital PCR) to detect HPV DNA in oral cavity tumors. We tested 5 sets of primers published in the literature and 2 newly designed ones in the amplification reactions (Figure 1A). The primers were either consensus (GP5+6⁺, MY09/11, CPI-II and PGMY09/11) or type-specific (HPV16L1, HPV16E6 and

HPV18L1). We used genomic DNA from UMSCC-47 and Hep2 cell lines to detect HPV16 and HPV18 respectively at various dilutions to determine relative efficiency, sensitivity and accuracy of amplification (Figure 1B). Primers described in the literature (GP5⁺6⁺, MY09/11, CPI-II or PGMY09/11, HPV16L1) could only detect HPV DNA when 0.03ng or higher amount of genomic DNA from the cell line UMSCC-47/Hep2 was used (Figure 1B). However, the newly designed type specific primers (HPV16E6 and HPV18L1) were able to detect HPV DNA with as low as 0.0003ng and 0.003ng genomic DNA for HPV16 and HPV18 respectively (Figure 1B). In order to check the efficiency of the newly designed primers in relation to absolute copy numbers of HPV genome, we used serial diluted HPV cloned plasmids DNA. As shown in Supplementary Figure 3, efficient amplification with 100 copies of HPV genome or higher could be detected. Once we optimized the conditions and primers for all the amplification reactions, we performed PCR in oral cavity tumors ($n = 70$). In our cohort, 58% (41/70) of the tumors were HPV DNA +ve for any of the PCR (Table 1, Supplementary Figure 4). Figure 1C shows the efficiency of the consensus and type specific primers in a set of representative oral cavity tumors. Widely used primers from the literature (MY09/11, PGMY09/11, GP5⁺6⁺ and HPV16L1) yielded either least or moderate (CPI-II) sensitivity of detection while the newly designed HPV16E6 and HPV18L1 primers showed optimum sensitivity of detection (Figure 1C). We observed inhibition of amplification reactions at high concentration of tumor genomic DNA with positive cell line spike-in experiment (Supplementary Figure 5) and therefore avoided in the reactions. Additionally, an increase in amplification cycles also did not aid in the detection of HPV DNA in PCR as shown in Supplementary Figure 6.

Next, we used quantitative PCR using Taqman chemistry to detect HPV DNA. We performed serial dilution using HPV16 E6 and HPV18 L1 cloned plasmids to obtain standard curves (Figure 2 A,D). We used increasing amount of genomic DNA from negative control cell lines to demonstrate specific amplification of HPV DNA (Supplementary Figure 1). Genomic DNA from HPV +ve and -ve cell lines were used to plot the baseline. Tumors were counted as HPV

DNA positive for those having *Ct* values 3 times away from the standard deviation from the negative controls (Figure 2 B,E). Results from qPCR indicated that 33% (35/106) of tumors were positive for HPV DNA (Table 1). Although, we found high incidence for HPV16 (30%; 32/106) than HPV18 (18%; 19/106) type, the HPV18 positive tumors had very high copy numbers of viral DNA as reflected in their *Ct* values (Figure 2 C,F). The prevalence of HPV DNA in oral tongue tumors was higher (44%) compared to that of buccal mucosa (4%).

Finally, we used one of the most sensitive methods, digital PCR, to detect HPV DNA. Digital PCR is recently shown successfully to detect HPV DNA in oropharyngeal tumors in highly specific manner [24]. We performed droplet digital PCR (ddPCR) with serially diluted plasmid HPV16E6 clones to generate the standard curve (Figure 2G). The genomic DNA from positive- (UM-SCC47) and negative control cell lines (Hep2) for HPV16 along with a no template control (NTC) were used to plot the baseline (Figure 2H). Digital PCR results indicated that 43% (59/136) of oral cavity tumors were positive for HPV16 DNA (Table 1). Moreover, HPV16 infection was more prevalent in oral tongue tumors (55%) compared to buccal mucosal ones (17%) (Table 1).

HPV E6 and E7 RNA expression

The viral mRNA, E6 and E7 (for both HPV16 and HPV18), were measured by qPCR in oral cavity tumors and from cell lines (UMSCC-47 and Hep2). Compared to the cell lines, tumors showed very low levels of expression of E6 and E7 mRNA (Figure 3). Unlike HPV DNA, only 15% of the tumors showed expression of E6 and/or E7 RNA and 6% of the tumors showed the presence of both HPV DNA (with all the 3 assays) and transcriptionally active HPV genomes (Table 1). Similar to DNA, oral tongue tumors had higher number (17%) of HPV E6/E7 RNA compared to the buccal mucosa (9%, Table 1). In our cohort, younger patients (≤ 40 yrs age) were significantly more HPV RNA +ve than the older patients when analyzed by *Chi-square* analysis ($p = 0.029$).

When combining the results from all the assays (p16 IHC, HPV DNA and HPV RNA), we found 0-48% of the tumors were positive in various assay combinations with PCR + ddPCR yielding the highest (48%) and p16 IHC + RNA the lowest (0%) numbers (Table 1). We found that 22% (23/106) of the tumors had relatively high-copy of HPV DNA and/or HPV E6/E7 mRNA.

Integration of HPV DNA in the host tumor genomes

We performed HPV16 E2 PCR to understand what percent of HPV DNA is integrated into the host tumor genomes and found that HPV is episomal in 18 % (n = 40) of the oral cavity tumors and in the majority of cases, HPV DNA was integrated into the host genome (Table 1). We used a positive control (genomic DNA from W12 cell line) to demonstrate the accuracy of the E2 PCR (Supplementary Figure 7).

Linking tumor attributes, somatic mutations and HPV with survival

We performed Kaplan Meir survival analysis with various tumor attributes that revealed significant association between tumor differentiation ($p=0.03$) and clinical stage ($p=0.0003$) with survival (Supplementary Figure 8 A-D). None of the other tumor attributes showed significant association with survival (Supplementary Figure 8 E-J). Expression of p16 in oral cavity tumors did not stratify patients based on both overall and disease-free survival (Supplementary Figure 9 A,B). HPV DNA status alone in any of the DNA-based assays or in combination did not correlate with survival (Supplementary Figure 9 C-L), except when measured with ddPCR for overall survival (Supplementary Figure 9 I, $p=0.03$). We found no correlation between HPV E6/E7 mRNA and with both overall ($p=0.7$) and disease-free ($p=0.3$) survival (Supplementary Figure 9 M,N).

Furhter, we investigated whether somatic mutations in significantly mutated genes in OSCC have a role to play in HPV DNA +ve tumors and patient survival. We looked at 3 genes (*TP53*, *CASP8*, *RASA1*) shown to be significantly mutated in oral cavity tumors [13,16,25] and tested

whether the mutation in any of the genes, alone or in combination, with relatively high HPV DNA copy and/or HPV E6/E7 mRNA is linked with survival. As shown in Figure 4A, we did not find any significant association with this group of tumors with both overall- ($p = 0.45$) and disease-free ($p = 0.68$) survival. Ninety five percent of the HPV +ve tumors in the group were wild-type for *TP53*, and *CASP1* gene and 85% of the HPV +ve tumors for *RASAI*. (Figure 4B). Somatic mutations in a group with relatively high-copy of HPV DNA, either alone or in combinations, were not associated with both overall and disease-free survival (Supplementary Figure 10 A-F).

Linking methylation to HPV

Supervised clustering of the first group of patients (a group defined as one with high-copy number HPV DNA and/or E6/E7 RNA) resulted in a list of 60 genes, out of which 9 (*FERMT3*, *GIT2*, *HK3*, *PRKCZ*, *ZCCHC8*, *IRF5*, *IFFO1*, *ARID3A*, *HOXA2*) were mapped to the HPV pathway (Figure 4C). Methylation of those genes are involved in downstream control of the expression of different target genes. For example, *ZCCHC8* methylation is linked with the expression of *RBI*, *PRKCZ* methylation controls-state-change-of *DLG1*, methylation in *ARID3A*, *IRF5*, *IFFO1* and *HOXA2* are linked with the expression of *TP53*, and *FERMT3*, *HK3* and *GIT2* genes control the expression of AP1 (*JUN*) (Figure 4C). All the genes, except for *HOXA2*, were significantly hypermethylated in the HPV +ve group of tumors compared to the HPV -ve group (Figure 4C). The linked 4 genes, obtained from the 9 significantly methylated genes, were mapped to the pathways involving HPV E6 and E7 proteins (Figure 4D). To test significance, we performed unpaired *t*-tests between the two groups, group 1 that has relatively high-copy HPV DNA and/or HPV RNA and group 2 that were negative for both HPV DNA and HPV RNA. All of the 8 hyper-methylated genes showed very significance ($p < 0.00001$) and for *HOXA2* that was hypomethylated the p-value was 0.007. However, when the patients were grouped based on HPV DNA positivity alone (irrespective of their copy number), most of these 9 HPV-linked genes did not show significant association (Supplementary Table 3).

Discussion

HPV plays an important role in the prognosis of patients with oropharyngeal tumors [26,27]. Unlike oropharynx, HPV incidence and its role in disease prognosis in oral cavity tumors is not well established. Past results on HPV DNA incidence in oral cavity tumors were widely variable (from very low to very high) depending on the assay sensitivity, analyte and patient cohort used [28]. The accuracy of the HPV tests employed is important and HPV positivity need to be answered in order to make treatment decisions in patients with head and neck tumors confidently [29]. There are fewer studies that used multiple analytes (protein, DNA and RNA) and multiple 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 the present study, we attempted to do this in 153 patients with oral cavity tumors.

While p16 expression, as measured by immunohistochemistry (IHC), is a commonly used proxy for HPV in HNSCC, its expression is not specific in HPV associated tumors [27]. Although, numerous studies correlated p16 expression with HPV [30-32], p16 IHC has shortcomings, especially when correlating its expression with patient survival. Limitations, such as, variation in staining intensities [33,34], nonspecific binding of antibodies and the lack of scoring and interpretive criteria used for p16 staining [35] make the method less reliable. Unlike some of the earlier studies [36,37], we could not find any correlation (either positive or negative) between p16 expression with both HPV DNA and survival (Supplementary Figure 9 A,B). Like previous reports [30,38], we found p16 expression not to be an effective surrogate marker for HPV detection in oral cavity tumors (there was no tumor that expressed both p16 and had detectable HPV RNA) (Table 1). A poor correlation between p16 IHC and HPV *in situ* hybridization (ISH) is reported in the past [39]. In our study, we did not perform HPV ISH, which could have provided additional information on p16 positivity and HPV prevalence.

Unlike antibody-based methods, nucleic acids-based methods detect HPV with high sensitivity and therefore used widely [40]. Meta-analysis of 5478 oral cavity tumors suggested the

overall HPV DNA prevalence to be 24.2% with 11% tumors being positive for both HPV DNA and E6/E7 RNA [41]. India has one of the highest incidence rates of oral cavity cancers with a significant difference in the trend of incidence between oropharyngeal and oral cavity cancer [42]. Previously, PCR coupled with MassArray is shown to provide high sensitivity of detection with low amount of input genomic DNA [5]. From our results, we found 38% and 13% of the tumors to be positive and negative in all 3 DNA-based assays (PCR, qPCR and ddPCR) respectively. Overall, the prevalence (33-58%) of HPV DNA was dependent on the type of assay used with PCR yielding the highest prevalence over the more sensitive methods like qPCR and ddPCR assays (Table 2A). This was possibly due to the use of consensus primers in PCR but not in qPCR and ddPCR, in addition to the type-specific ones, resulting in the detection of non-HPV16/18 subtypes. As expected, digital PCR, being the most-sensitive method out of the three DNA-based assays, resulted in more number of tumors being HPV16-DNA positive resulting in the detection of very low copy viral genomes in tumor samples. We wanted to test whether the presence of HPV DNA alone reflects a biological role of HPV in tumors. Based on several levels of evidence as noted below, we conclude that the presence of low copy HPV DNA alone may not be a reflection of functionally active HPV. First, in the tumors positive in HPV DNA, we found only a fraction (15%) with HPV E6/E7 RNA. Second, only 6% of tumors were positive for both the presence of HPV genome and E6/E7 RNA. Third, almost all the tumors with relatively high-copy HPV genome and/or HPV RNA had wild type *TP53* and *CASP8* genes, which was not the case with tumors with the low-copy HPV DNA. Both *TP53* and *CASP8* are known to be wild-type primarily in HPV positive tumors [13] and in our study too we found this corresponds to the tumors with high-copy HPV genome and/or transcriptionally active genome only (Table 2B). From all of the evidence above, we believe that the presence of HPV DNA in the tumor tissues might have been a result of highly sensitive assays used in our study and might suggest either the presence of passenger HPV genomes coming from adjacent normal cells, as earlier reported [43,44] and/or be a reflection of inactive or passenger virus in oral cavity tumors. Although very few (n=3), we can not explain why some tumors in our study with HPV E6/E7 RNA did not show the presence of HPV DNA. It is possible that the

genomic DNA for those tumors were degraded and therefore, could not serve as ideal templates for DNA-based assays. An additional factor could be the presence of inhibitors for DNA-based assays in those tumors. Although HPV RNA is considered to be gold standard analyte to test for HPV in tumors, RNA is a difficult analyte to handle and is more labile than DNA. Additionally, studying RNA in archival samples may pose additional set of challenges.

We did not find any significant correlation between p16, HPV-DNA and/or HPV RNA and disease outcome (Supplementary Figure 9). Even the tumors with relatively high-copy HPV genomes and/or E6/E7 RNA did not support the role of HPV in patient survival (Figure 4A). There are several interesting aspects to our results that need further studies and analysis. First, like cervical tumors [41] but unlike oropharyngeal tumors [13 14 15 16], we did not find HPV to be a predictor of survival. It is possible that HPV has a different mechanism of action in oral cavity tumors. An indirect support to linking high HPV genome copy number with HPV positivity in our study comes from the fact that we did not find significance in HPV-linked genes stratifying HPV +ve from HPV -ve group when HPV DNA irrespective of their copy number is taken into account in defining the HPV positivity (Figure 4C-D). It is possible that the presence of high-copy HPV genome in those tumors does not correlate with the presence of biologically active virus. Further studies may help answer this question. Till we have a good answer on high-copy number HPV DNA, HPV RNA may be the best analyte to test for HPV positivity in oral cavity tumors.

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Author contributions

VP and BP designed the study, performed data analysis and wrote the manuscript. VP carried out all the molecular assays and cell culture experiments; JB, UC and MP assisted in conducting molecular assays; NMK performed methylation data analysis; AS, GS and BLJ conducted p16 IHC studies, GS assisted in cell culture and maintenance of cell lines; AS, VK and MAK collected patient samples, collected follow up information and provided clinical attributes. BP provided the overall study supervision.

Abbreviations

HPV, human papillomavirus; HNSCC, head and neck squamous cell carcinoma; OSCC, oral cavity squamous cell carcinoma; IHC, immunohistochemistry; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; ddPCR, droplet digital polymerase chain reaction

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Table 1: Summary of HPV prevalence by all assays. DNA PCR was performed with consensus or HPV16/18 type-specific primers; DNA qPCR with Taqman assays for HPV16/18; DNA ddPCR with HPV16 primers; RNA qPCR with SYBR based assay to detect HPV16/18 E6/E7 mRNA. The primer and probe sequences along with the amplification conditions are provided in Supplementary Table 2.

Analyte/Assay	Detection Method	HPV Positivity (%)		
		Oral tongue	Buccal mucosa	Oral cavity (both subsites)
Protein	Immunohistochemistry	51% (28/55)	NA	51% (28/55)
DNA	PCR	59% (39/66)	50% (2/4)	58% (41/70)
	qPCR	44% (34/78)	4% (1/28)	33% (35/106)
	ddPCR	55% (52/95)	17% (7/41)	43% (59/136)
RNA	qPCR	17% (05/30)	9% (1/11)	15% (06/41)
Combinations	PCR + qPCR	38% (23/60)		
	PCR + ddPCR	48% (29/60)		
	qPCR + ddPCR	27% (27/99)		
	PCR + qPCR + ddPCR (all 3 methods, 3/3 method)	38% (20/53)		
	p16 + 3/3 Methods	19% (7/36)		
	p16 + RNA	0% (0/16)		
	RNA + 3/3 methods	6% (1/17)		
Final HPV positivity	Relative high-copy HPV DNA and/or HPV E6/E7 RNA positive	22% (23/106)		
Integrated/episomal	HPV16E6 PCR followed with E2-PCR	18% (7/38)	0% (0/2)	18% (07/40)

540

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Table 2: Summary of the current study on prevalence (A) and tumors with somatic mutations in key genes in tumors with relatively high-copy HPV DNA and/or HPV E6/E7 mRNA. BM: buccal mucosa, OT: oral tongue, ●: detected, ○: not-detected, Y: high-copy present, N: high-copy absent, L: low copy present, -: not done, M: mutant, WT: wild -type.

Sample Code	Protein	DNA					RNA	Integration Status
	p16 IHC	PCR	qPCR	ddPCR	+ve in 3/3	DNA high-copy	E6/E7 RNA	E2 PCR
BM1	-	-	○	○	-	N	-	-
BM10	-	-	-	○	-	-	-	-
BM11	-	-	-	○	-	-	-	-
BM12	-	-	○	○	-	N	-	-
BM13	-	-	○	○	-	N	○	-
BM14	-	-	○	○	-	N	○	-
BM15	-	-	-	○	-	-	-	-
BM16	-	-	-	●	-	-	-	-
BM17	-	-	○	○	-	N	-	-
BM18	-	-	○	●	-	N	-	-
BM19	-	-	○	○	-	N	-	-
BM2	-	-	-	●	-	-	-	-
BM20	-	-	○	○	-	N	-	-
BM21	-	-	○	●	-	N	-	-
BM22	-	-	○	○	-	N	○	-
BM23	-	-	○	○	-	N	-	-
BM24	-	-	○	○	-	N	○	-
BM25	-	-	○	○	-	N	-	-
BM26	-	-	○	●	-	N	○	-
BM27	-	-	-	○	-	-	-	-
BM28	-	-	-	●	-	-	-	-
BM29	-	○	○	○	○	N	-	-
BM3	-	-	○	○	-	N	-	-
BM30	-	-	○	○	-	N	-	-
BM31	-	-	-	○	-	-	-	-
BM32	-	-	-	○	-	-	-	-
BM33	-	-	-	○	-	-	-	-
BM34	-	-	-	○	-	-	-	-
BM35	-	●	○	○	○	N	-	○
BM36	-	○	○	○	○	N	-	-
BM37	-	-	○	○	-	N	-	-
BM38	-	-	○	○	-	N	○	-
BM39	-	-	○	○	-	N	○	-
BM4	-	-	○	○	-	N	○	-
BM40	-	-	○	○	-	N	-	-
BM41	-	-	-	○	-	-	-	-
BM5	-	●	●	○	○	Y	-	○
BM6	-	-	○	○	-	N	●	-
BM7	-	-	-	○	-	-	-	-
BM8	-	-	○	○	-	N	○	-
BM9	-	-	○	●	-	N	○	-
OT10	●	●	○	●	○	N	-	○
OT100	-	●	○	●	○	N	○	○
OT101	-	-	○	○	-	N	●	-
OT102	-	-	-	●	-	-	-	-
OT103	-	-	○	○	-	N	-	-
OT104	-	-	-	●	-	-	-	-
OT105	-	-	-	○	-	-	-	-
OT106	-	-	○	○	-	N	○	-
OT107	-	-	-	●	-	-	-	-
OT108	-	-	-	○	-	-	-	-
OT109	-	-	○	○	-	N	-	-
OT11	●	●	-	○	-	-	○	○
OT110	-	-	○	○	-	N	●	-
OT111	-	-	-	○	-	-	-	-
OT112	-	●	○	●	○	N	-	○
OT113	-	-	-	○	-	-	-	-
OT114	●	●	●	●	●	Y	-	-
OT115	○	●	-	-	-	-	-	○
OT116	-	-	○	●	-	N	-	-
OT12	●	●	-	-	-	-	-	○
OT13	○	●	-	-	-	-	-	○
OT14	○	●	●	●	●	Y	○	●
OT15	○	-	-	-	-	-	-	-
OT16	○	●	●	●	●	L	-	-
OT17	○	●	●	-	-	L	●	○
OT18	○	●	○	-	-	N	-	○
OT19	○	○	○	○	○	N	-	-
OT2	●	○	○	○	○	N	-	-
OT20	○	●	●	○	○	L	-	○
OT21	-	○	○	-	-	N	○	-
OT22	-	○	-	○	-	-	-	-
OT23	○	-	-	-	-	-	-	-
OT24	●	●	○	○	○	N	○	○
OT25	●	●	-	●	-	-	○	●
OT26	○	○	○	○	○	N	-	-
OT27	-	○	-	○	-	-	○	-
OT28	○	○	○	-	-	N	-	-
OT29	●	○	○	●	○	N	-	○
OT3	●	●	○	○	○	N	○	○
OT30	●	●	●	●	●	L	-	●
OT31	●	○	●	●	○	-	-	-
OT32	○	○	●	●	○	Y	-	○
OT33	●	○	●	○	○	L	○	-
OT34	●	○	-	●	-	-	-	-
OT35	-	-	-	-	-	-	-	-
OT36	-	○	●	-	-	Y	-	-
OT37	-	○	○	-	-	N	-	-
OT38	○	○	●	●	○	L	-	-

OT39	●	-	-	-	-	-	-	-
OT4	○	○	○	●	○	N	-	●
OT40	●	○	-	●	-	N	○	-
OT41	○	○	○	○	○	N	-	-
OT42	○	●	●	●	●	L	○	○
OT43	●	○	○	○	○	N	○	○
OT44	●	●	●	●	●	L	○	○
OT45	-	○	-	○	-	-	-	-
OT46	-	●	●	●	●	L	-	●
OT48	○	●	●	●	●	L	-	-
OT5	●	-	-	-	-	-	-	-
OT50	-	○	○	●	○	N	-	-
OT51	●	○	●	-	-	L	-	-
OT52	○	-	-	-	-	-	-	-
OT53	●	●	○	○	○	N	-	○
OT54	●	●	●	●	●	L	-	○
OT55	●	-	○	●	-	N	○	○
OT56	-	-	○	○	-	N	-	-
OT57	-	-	-	-	-	-	-	-
OT58	-	-	●	○	-	Y	-	-
OT59	-	-	-	○	-	-	-	-
OT6	●	○	●	○	○	-	-	-
OT60	-	-	○	○	-	N	○	-
OT61	○	●	●	●	●	Y	●	○
OT62	-	●	○	●	○	N	○	○
OT63	-	-	-	○	-	-	-	-
OT64	-	●	●	●	●	L	○	○
OT65	○	●	●	●	●	Y	-	●
OT66	-	-	○	●	-	N	-	-
OT67	○	●	●	●	●	Y	○	○
OT68	-	-	●	●	-	Y	-	-
OT69	●	●	●	●	●	Y	-	-
OT7	●	●	○	●	○	N	-	-
OT70	-	-	○	○	-	N	-	○
OT71	-	-	○	○	-	N	-	-
OT72	-	-	○	●	-	N	●	-
OT73	-	-	-	●	-	-	-	-
OT74	-	-	-	○	-	-	-	-
OT75	-	●	●	●	●	Y	○	○
OT76	-	●	●	●	●	Y	-	●
OT77	●	●	●	●	●	L	○	○
OT78	○	○	○	●	○	N	○	-
OT79	-	-	-	○	-	-	-	-
OT80	-	○	●	●	○	Y	○	-
OT81	●	●	●	●	●	Y	-	-
OT82	○	●	●	●	●	Y	-	○
OT83	○	●	○	●	○	N	-	○
OT84	○	-	○	○	-	N	-	-
OT85	-	●	○	●	○	N	-	○
OT86	-	-	○	○	-	N	-	-
OT87	-	-	○	○	-	N	○	-
OT88	-	○	●	●	○	Y	○	○
OT89	-	-	-	○	-	-	-	-
OT9	●	●	○	●	○	N	-	-
OT90	-	-	○	○	-	N	-	-
OT91	○	-	○	○	-	N	-	-
OT92	-	○	●	●	○	L	-	-
OT93	-	-	-	●	-	-	-	-
OT94	-	-	-	○	-	-	-	-
OT95	-	●	●	●	●	Y	-	-
OT96	-	-	-	○	-	-	-	-
OT97	-	-	○	○	-	N	-	-
OT98	-	-	-	●	-	-	-	-
OT99	-	-	○	○	-	N	-	-

B

Sample Code	DNA	RNA	Somatic mutation		
	High-copy	E6/E7 RNA	TP53	CASP8	RASA1
BM5	Y	-	NA	NA	NA
BM6	N	●	NA	NA	NA
OT101	N	●	M	NA	WT
OT110	N	●	WT	M	WT
OT114	Y	-	WT	WT	WT
OT14	Y	○	WT	WT	WT
OT17	L	●	WT	WT	WT
OT32	Y	-	WT	M	WT
OT36	Y	-	WT	WT	M
OT58	Y	-	NA	NA	NA
OT61	Y	●	WT	WT	WT
OT65	Y	-	WT	WT	WT
OT67	Y	○	WT	WT	WT
OT68	Y	-	WT	WT	WT
OT69	Y	-	WT	WT	WT
OT72	N	●	WT	WT	WT
OT75	Y	○	WT	WT	WT
OT76	Y	-	WT	M	WT
OT80	Y	○	WT	WT	WT
OT81	Y	-	WT	WT	WT
OT82	Y	-	NA	WT	NA
OT88	Y	○	WT	WT	WT
OT95	Y	-	WT	WT	WT

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Figure legends.

Figure 1A: Primer and probes locations used in the study to detect HPV DNA and HPV E6/E7 RNA.

Figure 2: HPV qPCR (A-F) and digital PCR (G-I). Standard curves were obtained using cloned HPV16/18 plasmids (A,D,G), positive and negative cell line DNA (B,E,H) were used to count DNA in tumors (C,F,I).

Figure 3: HPV16/18 E6/E7 mRNA expression using qPCR.

Figure 4: Kaplan Meier survival plots linking tumors with high-copy HPV DNA and/or HPV RNA status (A), somatic mutations in 3 frequently mutated genes in tumors (B), clustering of nine methylated genes stratifying HPV positive from negative group of tumors (C) and HPV-associated pathways (D) in HPV positive tumors.

Supplementary Figures and Tables legends:

Supplementary Table 1: Clinical details of patients (n = 153) used in the study.

Supplementary Table 2: Primer and probe sequences along with amplicon size and the conditions of amplification reactions used for nucleic acid based detection.

Supplementary Table 3: Significance (*p* values) from unpaired *t*-tests between HPV positive and negative tumors in two groups based on HPV DNA copy number.

Supplementary Figure 1: Genomic DNA from cell lines were used as controls for HPV16 (A,B) and HPV18 (C,D) PCR.

Supplementary Figure 2: Representative images for p16 immunohistochemical staining (IHC) in –ve OSCC, +ve OSCC and cervical cancer (+ve control) samples.

Supplementary Figure 3: Amplification efficiency as measured by serially diluted HPV16/18 cloned plasmids.

Supplementary Figure 4: PCR gel images with OSCC tumor DNA.

585 Supplementary Figure 5: Inhibition of amplification reactions at high concentration of tumor
586 genomic DNA with spike-in experiment.

587
588 Supplementary Figure 6: The effect of amplification cycle on PCR.

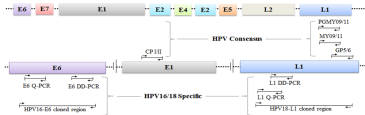
589
590 Supplementary Figure 7: HPV E2PCR to test viral integration into host genomes.

591
592 Supplementary Figure 8: KM survival analysis plots with tumor attributes. Tumor differentiation
593 (A), stage (B-D), habits (E-H), age (I), and nodal status (J).

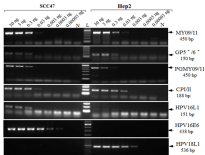
594
595 Supplementary Figure 9: KM survival analysis plots with p16 (A,B), HPV DNA- PCR (C,D), qPCR
596 (E-H), ddPCR(I,J), positive in 3/3 DNA-based method (K,L), and E6/E7 mRNA (M,N).

597
598 Supplementary Figure 10: KM survival analysis plots in various somatic mutational backgrounds
599 (A-F).

A



B



C

