High-risk human papillomavirus in oral cavity squamous cell carcinoma

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Running Head: HPV in oral cavity tumors.

ABSTRACT

Purpose: The prevalence of human papillomavirus (HPV) in oral cavity squamous cell carcinoma (OSCC) varies significantly based on assay sensitivity and patient geography. Accurate detection is essential to understand the role of HPV in disease prognosis and management of patients with OSCC.

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

Results: High prevalence (33-58%) 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 RNA positive. Most tumors with relatively high-copy 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 combinations, did not correlate with patient survival. Nine HPV-associated genes stratified the virus +ve from the –ve tumor group with high confidence ($p<0.008$) when HPV DNA copy number and/or HPV RNA were considered to define HPV positivity and not HPV DNA alone irrespective of their copy number ($p < 0.2$).

Conclusions: In OSCC, the presence of both HPV RNA and p16 are rare. HPV DNA alone is not an accurate measure of HPV positivity and therefore not informative. Moreover, HPV DNA, RNA or p16 don't correlate with outcome.

Keywords: Human papillomavirus (HPV), HPV DNA, HPV RNA, oral cavity tumors, copy number, survival, somatic mutation, DNA methylation

49 **INTRODUCTION**

50 Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide
51 with an incidence of 550,000 cases annually[1]. Oral cavity squamous cell carcinoma (OSCC)
52 constitute a majority of HNSCC including tumors of the oral/anterior tongue and buccal mucosa[2].
53 The major known risk factors of OSCC are tobacco products, alcohol, and infection with human
54 papillomavirus (HPV) [3]. Unlike oropharyngeal tumors, where HPV incidence with HPV DNA,
55 RNA, and HPV-proteins is reported to be very high, (up to 90%) [4, 5], the prevalence of HPV in
56 OSCC varies widely (from none to 74%) depending on the choice of analyte, detection
57 methodology and the geography of the patient cohort [6]. Additionally, unlike oropharyngeal tumors [7-
58 11], the role of HPV in disease prognosis and response to therapy in patients with OSCC is equivocal.
59 Despite the fact that HPV RNA is shown to function as better screening and patient management
60 tool [12], the presence of HPV DNA is routinely used as a measure of HPV infection in tumors.
61 However, there is a considerable variation in the sensitivity of DNA-based assays. Although widely
62 used, HPV DNA results does not always match with HPV RNA, especially in OSCC. Therefore, in
63 tumors, presence of HPV E6/E7 RNA and/or their protein products are considered as gold standard
64 tests defining HPV positivity.

65 HPV16 and HPV18 subtypes have been epidemiologically linked with head and neck
66 carcinoma [13]. High-risk HPV16 and HPV18 are the most predominant subtypes in oral cavity
67 tumors of Indian patients while the other subtypes (HPV33, HPV6, and HPV11) are rare [14, 15]. HPV
68 E6 interacts with p53 to promote its degradation via ubiquitin pathway while E7 forms a complex
69 with Rb leading to its functional inactivation and dysregulation of the cell cycle [16]. In some HPV-
70 related tumors, E6- and E7-mediated inactivation of p53 and Rb result in the accumulation of p16
71 protein [17] while in others p16 expression does not directly correlate with HPV positivity [18]. Large-
72 scale sequencing studies have demonstrated that the majority of HPV-negative tumors harbor
73 mutations in *TP53* and *CASP8* and a significant proportion of HPV-positive tumors in *PIK3CA* [19-21].
74 Additionally, past studies have identified specific mutations in potential drug targets like *FGFR2/3*
75 and lack of *EGFR* aberrations in HPV-positive patients [21] and the role of *CASP8* in HPV-negative
76 cell line and patients [19, 22]. Despite the wealth of information, accuracy of different HPV tests and

77 whether HPV plays an important factor in oral cavity tumor stratification and treatment remain to
78 be answered.
79 In the present study, we addressed the following questions on HPV in a cohort of 153 patients
80 with oral cavity tumors. Does sensitivity matter in the detection of HPV DNA in tumors? Does the
81 presence of tumor p16 and HPV DNA correlate with HPV E6/E7 RNA? Does the presence of high-
82 copy HPV DNA accurately reflect HPV positivity? Are p16 protein, HPV DNA, HPV E6/E7 RNA,
83 individually or together, linked with patient survival? And finally, do somatic mutations and DNA
84 methylation at 5-Cytosine residues distinguish the HPV +ve from the HPV –ve tumors?
85
86 METHODS
87 *Patient cohort*
88 Tumor samples from patients with OSCC (Buccal mucosa, BM, including from upper and
89 lower gingivobuccal sulcus and retromolar trigone and Oral tongue, OT) were accumulated
90 consecutively. Informed consent was obtained voluntarily from each patient enrolled in the study
91 and ethical was obtained from the Institutional Ethics Committees.All the tissues were frozen
92 immediately in liquid nitrogen and stored at -80^{0}C until further use. Only those tumors with
93 squamous cell carcinoma and at least 70% tumor cells and confirmed diagnosis were included in the
94 current study. Patients (n = 153) underwent staging according to AJCC criteria, and curative intent
95 treatment as per NCCN guidelines involving surgery with or without postoperative adjuvant
96 radiation or chemo-radiation at the Mazumdar Shaw Medical Centre were accrued for the study
97 (Table 1). Patients who received prior investigational therapy, CT, surgery or RT within four weeks
98 of initiating the investigation were excluded from the current study. Post-treatment surveillance was
99 carried out by clinical and radiographic examinations as per the NCCN guidelines.
100
101 *Cell cultures*
102 Cell lines (UM-SCC47, from Dr.Thomas Carey, University of Michigan, USA [23]; Hep2,
103 National Centre for Cell Science, Pune, India; UPCI:SCC29B and UPCI:SCC040, from Dr.
104 Susanne Gollin, University of Pittsburg, USA [24]) were maintained in Dulbecco's Modified Eagles'

105 Media (DMEM) supplemented with 10% FBS, 1X MEM non-essential amino acids solution and 1X
106 penicillin/streptomycin mixture (Gibco) and grown in incubators at 37^0C with 5% CO2.
107
108 *Immunohistochemistry (IHC)*
109 For p16 immunohistochemistry (IHC) staining was carried out using FFPE tissue blocks and
110 with primary antibody from BioGenex, USA (cat: AM540-5M; Anti-p16(INK4), Clone G175-405
111 in the NordiQC list) and using the poly-HRP detection system (Cat: QD400-60KE, BioGenex,
112 Fremont, CA, USA) following manufacturers' instructions. Sections of cervical cancer were used as
113 the positive control.The presence of p16 protein was scored based on the proportion of
114 immunopositive cells with both cytoplasmic and nuclear staining, irrespective of staining intensity
115 (weak/moderate/strong). The scoring pattern was: 0 - no immunopositive cells, 1 - <10%
116 immunopositive cells; 2 – 10-40% immunopositive cells and 3 - >40% immunopositive cells.
117 Tumors with a score of 3 were considered to be p16 positive.
118
119 *DNA extraction*
120 The genomic DNA from tumor tissues and cell lines were extracted using DNeasy Blood and
121 Tissue extraction kit using the spin column method with an intermediate RNase digestion following
122 manufacturer's instructions (Qiagen, USA). The DNA was quantified using Qubit 2.0 fluorometer
123 (Invitrogen, USA). Total 300ng of genomic DNA (unless specified otherwise) from tumors was
124 used for the detection of HPV using PCR, qPCR, and digital droplet PCR (ddPCR) methods.
125
126 *Detection of HPV DNA with PCR*
127 We tested five sets of primers published in the literature and two newly designed ones in the
128 amplification reactions (Figure 1). PCR primers were either consensus or type-specific regions of
129 the virus (Figure 1). Five sets of primers, including 4 HPV consensus (GP5-6; CP-I/II; MY09/11
130 and PGMY09/11) and one type-specific (HPV16-L1) primers as described in the literature [25], in the
131 PCR assays. Additionally, we designed two sets of new type-specific primers (HPV16-E6 and
132 HPV18-L1) to detect HPV DNA. We used genomic DNA from UMSCC-47 and Hep2 cell lines to

133 detect HPV16 and HPV18 respectively at various dilutions to determine relative efficiency,
134 sensitivity, and accuracy of amplification (Figure 2B). The primer sequences and the amplification
135 conditions used are provided in Supplementary Table 1.
136
137 *Detection of HPV DNA with qPCR*
138 For qPCR, E6 and E7 regions from HPV16 and HPV18 respectively were cloned in pUC19
139 vector, and Sanger sequencing confirmed their sequences. The qPCR was carried out using KAPA
140 Probe Fast qPCR master mix Universal (2X) (cat: KK4701, KAPA Biosystems, USA). The primers
141 and probes were designed (Supplementary Table 1) within the cloned regions of the HPV16, and
142 HPV18 plasmids and standard quantitative PCR was performed. The standard curves were
143 generated using serial dilutions (from 10 to 100,000 copies) of HPV16, and HPV18 cloned pUC19
144 plasmids. Genomic DNA from positive and negative control cell lines was used as positive and
145 negative controls for HPV18 and HPV16 respectively (Supplementary Figure 1). All amplification
146 reactions were carried out in triplicates, using nuclease-free water (cat: AM9932, Ambion, USA) as
147 a negative control. The analysis for each sample was performed by using the absolute quantification
148 using standard curve generated with serial dilutions of cloned plasmids. Tumors and cell lines
149 samples were counted as positive for those having Ct values three times away from the standard
150 deviation from the mean of negative controls.
151
152 *Detection of HPV DNA using droplet digital PCR (ddPCR)*
153 Digital PCR was performed using the QX100 ddPCR system (Bio-Rad, USA) using primers
154 and probes as provided in Supplementary Table 1. The reaction mix consisted of 10 µl of 2× ddPCR
155 Supermix without dUTP (cat: 1863024; Bio-Rad, USA), 450 nM of both forward and reverse
156 primers of HPV16 and 250 nM probe and 300ng of genomic DNA in a final volume of 20 µl. The
157 droplets were generated following manufacturer's standard instructions and were measured and
158 normalized in every reaction using the Quanta soft V.6 software (Bio-Rad, USA). All the samples
159 were processed in triplicates. Tumors and cell lines samples were counted as positive for those

160 having droplet numbers three times away from the standard deviation from the mean of negative
161 controls.
162
163 *HPV DNA copy number*
164 We deduced the HPV absolute copy number from the qPCR standard curves using cloned
165 HPV16/18 (Figure 3A). We considered a tumor or cell line to have relatively high-copy of HPV
166 DNA when the copy number for HPV16 and HPV18 DNA were more than 3.3×10^2 and 3.3×10^3
167 per microgram of tumor DNA respectively. To minimize the effect of tumor cellularity, ploidy, and
168 heterogeneity, we expressed the HPV copy number as copies per microgram of tumor DNA used in
169 the reaction.
170
171 *Detection of HPV16/18 E6/ E7 RNA*
172 The total RNA was extracted from 25mg of tumor tissues using the RNeasy mini kit from
173 Qiagen (cat: 74104) following manufacturer's instructions. The residual DNA was digested on the
174 column during extraction using RNase free DNase set from Qiagen (cat: 79254). RNA (500ng) was
175 subjected to cDNA synthesis using Takara's Prime Script first strand cDNA synthesis kit (cat:
176 6110A). The qPCR was carried out using KAPA SYBR Fast qPCR master mix Universal (cat:
177 KK4601, KAPA Biosystems, USA) with GAPDH as an internal control. The primer sequences and
178 the amplification conditions are provided in Supplementary Table 1. All amplification reactions
179 were carried out in triplicates, using nuclease-free water (cat: AM9932, Ambion, USA) as no
180 template control, cDNA from UPCI:SCC29B and UPCI:SCC40 cell lines as negative controls and
181 cDNA from cell lines UM-SCC47 and Hep2 as positive controls for E6 and E7 amplifications
182 respectively. Data were normalized using relative quantification method ($2^{\Delta\Delta Ct}$) [26]. Positive tumors
183 were counted as ones that have Ct values three times from the standard deviation of the mean of
184 negative control.
185
186 *Mutation analysis by Sanger sequencing*

187 The mutation data on tumors for *TP53*, *CASP8*, and *RASA1* were retrieved from the
188 previously published data [22].
189
190 *Statistical Analysis*
191 The Chi-square test was used to the significance of different clinical parameters of patients.
192 The relationship between tumor HPV status and survival in patients was examined by Kaplan-Meier
193 analysis with overall survival (OS) and disease-free survival (DFS) with log-rank test for
194 determining significance (p values <0.05) Disease-free survival was calculated as the time between
195 surgery and relapse/recurrence (as an event) of the disease or the last follow update in the case there
196 is no recurrence. The median follow up time of the patients is 15 months. The KM curves were
197 plotted using Graphpad Prism 5.0 software.
198
199 *Whole genome methylation and statistical data analyses*
200 Production of whole genome methylation data using Illumina Infinium Methylation450
201 BeadChip, chip scanning and the data pre-processing are described previously [27]. Three tumors in
202 HPV +ve group (relatively with high-copy HPV DNA and/or E6/E7 RNA) and 18 tumors in HPV -
203 ve group (HPV DNA –ve and HPV RNA –ve) were considered for further analysis. Differential
204 methylation for probes was estimated as $\Delta\beta$ values (Tumorβ - Normalβ) resulting in values ranging
205 from -1 (hypo-methylation) to 1 (hypermethylation). Using a supervised clustering approach,
206 differentially methylated probes that discriminated HPV +ve and HPV -ve patient's samples were
207 identified. Hyper-methylated probes in the HPV +ve group that were hypo-methylated in the HPV -
208 ve group, and *vice versa*, along with other probes with $\Delta\beta$ values that differed by at least 0.5
209 between the two groups, with a sample frequency of at least 60% were considered further. Finally,
210 the list of genes consisting of average $\Delta\beta$ values across tumors for multiple probes (where present)
211 was drawn. Neighbourhood interaction network for these genes was inferred using PCViz from
212 PathwayCommons (http://www.pathwaycommons.org/pcviz/#neighborhood/). The genes and their
213 interacting partners were mapped to the Viral Carcinogenesis pathway from KEGG (hsa05203), and
214 mapped genes were linked back to respective genes found using supervised clustering. For the

215 linked genes, an unpaired t-test was performed to determine the significance of the difference
216 between the HPV +ve and HPV -ve tumor groups. The same was performed for the HPV-associated
217 genes between the DNA-based (irrespective of the DNA copy number) HPV +ve and HPV -ve
218 tumor groups.
219
220 RESULTS
221 *p16 expression*
222 For the p16 immunohistochemical study, the tumors (n = 55) were first confirmed by H and E
223 staining of the paraffin tissue sections followed by the immunohistochemical staining with the
224 surrogate marker p16. Figure 2A shows the representative p16 +ve and –ve staining in oral cavity
225 tumor sections (OSCC) along with the cervical tissue section (+ve control). As indicated
226 previously, we considered cells with cytoplasmic + nuclear staining for p16 to be counted as overall
227 p16 positive. In our study, 18% of the tumors were p16 positive (Table 2).
228
229 *Incidence of HPV DNA*
230 *Detection of HPV using PCR:*
231 The efficacy of HPV16/18 DNA detection using various consensus or type-specific primers is
232 shown in Figure 2B. The primers described in the literature (GP5+6+, MY09/11, CPI-II or
233 PGMY09/11, HPV16L1) could only detect HPV DNA when 0.03ng or higher amount of genomic
234 DNA from the cell line UMSCC-47/Hep2 was used (Figure 2B). However, the newly designed
235 type-specific primers (HPV16E6 and HPV18L1) were able to detect HPV DNA with as low as
236 0.0003ng and 0.003ng genomic DNA for HPV16 and HPV18 respectively (Figure 2B). To check
237 the efficiency of the newly designed primers with absolute copy numbers of HPV genome, we used
238 serial diluted HPV cloned plasmids DNA. As shown in Supplementary Figure 2, efficient
239 amplification with 100 copies of HPV genome or higher could be detected. Once we optimized the
240 conditions and primers for all the amplification reactions, we performed PCR in oral cavity tumors
241 (n = 70). In our cohort, 58% (41/70) of the tumors were HPV DNA +ve for any of the PCR (Table
242 2, Supplementary Figure 3). Figure 2C shows the efficiency of the consensus and type-specific

243 primers in a set of representative oral cavity tumors. Widely used primers from the literature
244 (MY09/11, PGMY09/11, GP5+6+ and HPV16L1) yielded either least or moderate (CPI-II)
245 sensitivity of detection while the newly designed HPV16E6 and HPV18L1 primers showed the
246 optimum sensitivity of detection (Figure 2B). We observed inhibition of amplification reactions at a
247 high concentration of tumor genomic DNA with positive cell line spike-in experiment
248 (Supplementary Figure 4) and therefore avoided in the reactions. Additionally, an increase in
249 amplification cycles also did not aid in the detection of HPV DNA in PCR as shown in
250 Supplementary Figure 5.
251
252 *Detection of HPV using real-time PCR:*
253 Next, we used quantitative PCR using Taqman chemistry to detect HPV DNA. We performed
254 serial dilution using HPV16 E6, and HPV18 L1 cloned plasmids to obtain standard curves (Figure 3
255 I, IV). We used an increasing amount of genomic DNA from negative control cell lines to
256 demonstrate specific amplification of HPV DNA (Supplementary Figure 1). Genomic DNA from
257 HPV +ve and –ve cell lines were used to plot the baseline. The real-time PCR was performed on
258 tumors of the oral cavity (*n*=106) and were counted as HPV DNA positive for those having Ct
259 values three times away from the standard deviation from the mean of negative controls (Figure 3
260 II, V). Results from qPCR indicated that 33% (35/106) of tumors were positive for HPV DNA
261 (Table 2). Although we found high incidence for HPV16 (30%; 32/106) than HPV18 (18%; 19/106)
262 type, the HPV18 positive tumors had very high copy numbers of viral DNA as reflected in their Ct
263 values (Figure 3A III, VI). The prevalence of HPV DNA in oral tongue tumors was higher (44%)
264 compared to that of buccal mucosa (4%).
265
266 *Detection of HPV using droplet digital PCR:*
267 Finally, we used one of the most sensitive methods, digital PCR, to detect HPV DNA. Digital
268 PCR was recently shown to successfully to detect HPV DNA in oropharyngeal tumors in highly
269 specific manner [30]. We performed droplet digital PCR (ddPCR) with serially diluted plasmid
270 HPV16E6 clones to generate the standard curve (Figure 3B, I). The genomic DNA from positive-

271 (UM-SCC47) and negative control cell lines (Hep2) for HPV16 along with a no template control
272 (NTC) were used to plot the baseline (Figure 3B, II; Supplementary Figure 6). The digital PCR
273 performed on the oral cavity tumors results indicated that 43% (59/136) of oral cavity tumors were
274 positive for HPV16 DNA (Figure 3B, III and Table 2). Moreover, HPV16 infection was more
275 prevalent in the oral tongue tumors (55%) compared to the buccal mucosal ones (17%) (Table 2).
276
277 *HPV E6 and E7 RNA expression*
278 The viral mRNA, E6, and E7 (for both HPV16 and HPV18) were measured by qPCR in oral
279 cavity tumors and from cell lines (UMSCC-47 and Hep2). Compared to the cell lines, tumors
280 showed very low levels of expression of E6 and E7 mRNA (Figure 3C). Unlike HPV DNA, only
281 15% of the tumors showed expression of E6 and/or E7 RNA and 6% of the tumors confirmed the
282 presence of both HPV DNA (with all the three assays) and transcriptionally active HPV genomes
283 (Table 2). Similar to DNA, oral tongue tumors had a higher number (17%) of HPV E6/E7 RNA
284 compared to the buccal mucosa (9%, Table 2). In our cohort, younger patients (≤40yrs age) were
285 significantly more HPV RNA +ve than the older patients when analyzed by Chi-square analysis (p
286 $= 0.029$). When combining the results from all the assays (p16 IHC, HPV DNA, and HPV RNA),
287 we found 6-48% of the tumors were positive in various assay combinations with PCR (Table 2).
288 We found that 22% (23/106) of the tumors had relatively high-copy of HPV DNA and/or HPV
289 E6/E7 mRNA.
290
291 *Linking tumor attributes, somatic mutations, and HPV with survival*
292 We performed Kaplan Meir survival analysis with various tumor attributes that revealed
293 significant association between tumor differentiation (p=0.03) and clinical stage (p=0.0003) with
294 overall survival (Figure 4A)**.** None of the other tumor attributes showed significant association with
295 survival (Supplementary Figure 7 A-F). In patients with oral cavity tumors, p16, HPV DNA and
296 HPV RNA did not correlate with the either overall or disease-free survival of (Figure 4A,
297 Supplementary Figure 8). HPV DNA status alone measured by any of the DNA-based assays alone
298 or in combination did not correlate with survival (Supplementary Figure 8), except when measured

299 with ddPCR for overall survival (Supplementary Figure 8E, p=0.03). We tested whether tumors
300 with relatively high HPV DNA copy and/or HPV E6/E7 mRNA, is linked with survival. As shown
301 in Supplementary Figure 9 A,B, we did not find any significant association with this group of
302 tumors with both overall- (p = 0.45) and disease-free (p = 0.68) survival.
303
304 Further, we investigated whether somatic mutations in significantly mutated genes in OSCC
305 have a role to play in HPV DNA +ve tumors and patient survival. We looked at three genes (*TP53*,
306 *CASP8*, and *RASA1*) shown to be significantly mutated in oral cavity tumors [19, 22, 31]. Ninety-five
307 percent of the HPV +ve tumors in the group were wild-type for *TP53* and *CASP1* genes and 85% of
308 the HPV +ve tumors for *RASA1* gene. (Supplementary Figure 10). We tested whether the mutation
309 in any of the genes, alone or in combination in HPV –ve tumor group is linked with survival. As
310 shown in Supplementary Figure 9 C,D, we did not find any significant association with this group
311 of tumors with survival.
312
313 *Linking methylation to HPV*
314 Supervised clustering of the first group of patients (a group defined as one with high-copy
315 number HPV DNA and/or E6/E7 RNA) resulted in a list of 60 genes, out of which 9 (*FERMT3*,
316 *GIT2*, *HK3*, *PRKCZ*, *ZCCHC8*, *IRF5*, *IFFO1*, *ARID3A*, *HOXA2*) were mapped to the HPV pathway
317 (Figure 4B). Methylation of those genes is involved in the downstream control of the expression of
318 different target genes. For example, *ZCCHC8* methylation is linked with the expression of RB1,
319 *PRKCZ* methylation controls-state-change-of *DLG1*, methylation in *ARID3A*, *IRF5*, *IFFO1*, and
320 *HOXA2* are connected with the expression of *TP53*, and *FERMT3*, *HK3*, and *GIT2* genes control
321 the expression of *AP1* (*JUN*) (Figure 4B). All the genes, except for *HOXA2*, were significantly
322 hypermethylated in the HPV +ve group of tumors compared to the HPV –ve group (Figure 4B).
323 The linked four genes, obtained from the nine significantly methylated genes, were mapped to the
324 pathways involving HPV E6 and E7 proteins (Figure 4C). To test significance, we performed
325 unpaired t-tests between the two groups, group 1 that has relatively high-copy HPV DNA and/or
326 HPV RNA and group 2 that were negative for both HPV DNA and HPV RNA. All of the eight

327 hypermethylated genes showed very significance ($p < 0.00001$) and for *HOXA2* that was
328 hypomethylated the *p*-value was 0.007 (Supplementary Table 2). However, when the patients were
329 grouped based on HPV DNA positivity alone (irrespective of their copy number), most of these 9
330 HPV-linked genes did not show significant association.
331
332 **DISCUSSION**
333 HPV plays a vital role in the prognosis of patients with oropharyngeal tumors [32, 33]. Unlike
334 oropharynx, HPV incidence and its role in disease prognosis in oral cavity tumors are not well
335 established. Past results on HPV DNA incidence in oral cavity tumors were widely variable (from
336 very low to very high) depending on the assay sensitivity, analyte and patient cohort used [34]. We
337 performed literature survery and found that the rate of incidence, the role of HPV in disease
338 outcome varies significantly in different groups (Supplementary Table 3). The accuracy of the HPV
339 tests employed is, and HPV positivity needs to be answered to make treatment decisions in patients
340 with head and neck tumors confidently [35]. There are fewer studies that used multiple analytes
341 (protein, DNA, and RNA) and various molecular tests (IHC, PCR, qPCR and digital PCR) to
342 establish HPV positivity in oral cavity tumors and that correlated HPV with tumor attributes
343 (including somatic mutations and methylation) and survival. In the present study, we attempted to
344 do this in 153 patients with oral cavity tumors.
345
346 While p16 expression, as measured by immunohistochemistry (IHC), is a commonly used
347 proxy for HPV in HNSCC, its expression is not specific in HPV associated tumors [33]. Although a
348 number of past studies correlated p16 expression with HPV [36-38], p16 IHC has shortcomings,
349 especially when relating its expression with patient survival. Limitations, such as variation in
350 staining intensities [39, 40], nonspecific binding of antibodies and the lack of scoring and interpretive
351 criteria used for p16 staining [41] make the method less reliable. In our study, although we found an
352 unusually high number (51%) of tumor cells showing immunopositive staining, only a small
353 percentage (18%) had both cytoplasmic+nuclear staining, an accurate reflection of HPV positivity
354 as described earlier [28, 29]. Unlike some of the previous studies [24, 42], we could not find any correlation

355 (either positive or negative) between p16 expression and survival (Figure 4). Like previous reports
356 [36, 43], we found p16 expression not to be a useful surrogate marker for HPV in oral cavity tumors. A
357 weak correlation between p16 IHC and HPV *in situ* hybridization (ISH) is reported in the past [44].
358 Unlike antibody-based methods, nucleic acids-based methods detect HPV with high
359 sensitivity and therefore used widely [45]. Meta-analysis of 5478 oral cavity tumors suggested the
360 overall HPV DNA prevalence to be 24.2% with 11% tumors being positive for both HPV DNA and
361 E6/E7 RNA [46]. India has one of the highest incidence rates of oral cavity cancers with a significant
362 difference in the trend of incidence between oropharyngeal and oral cavity cancer [47]. Previously,
363 PCR coupled with Mass Array is shown to provide high sensitivity of detection with a low amount
364 of input genomic DNA [48]. From our results, we found 38% tumors to be positive, and 13% were
365 negative in all three DNA-based assays (PCR, qPCR, and ddPCR) respectively. Overall, the
366 prevalence (33-58%) of HPV DNA was dependent on the type of test used with PCR yielding the
367 highest incidence over the more sensitive methods like qPCR and ddPCR assays (Table 2). This
368 was possibly due to the use of consensus primers in PCR but not in qPCR and ddPCR, in addition
369 to the type-specific ones, resulting in the detection of non-HPV16/18 subtypes. As expected, digital
370 PCR, being the most-sensitive method out of the three DNA-based assays, resulted in more number
371 of tumors being HPV16-DNA positive resulting in the detection of very low copy viral genomes in
372 tumor samples. Based on several levels of evidence as noted below, we conclude that the presence
373 of low copy HPV DNA alone may not be a reflection of functionally active HPV. First, in the
374 tumors positive for HPV DNA, we found only a fraction (15%) with HPV E6/E7 RNA. Second,
375 only 6% of tumors were positive for both the presence of HPV genome and E6/E7 RNA. Third,
376 almost all the tumors with relatively high-copy HPV genome and/or HPV RNA had wild-type *TP53*
377 and *CASP8* genes, which was not the case with tumors with the low-copy HPV DNA. Both *TP53*
378 and *CASP8* are known to be wild-type primarily in HPV positive tumors 19, and in our study, we
379 also found this corresponds to the tumors with high-copy HPV genome and/or transcriptionally
380 active genome only (Table 2). We believe that the high prevalence of HPV DNA in the tumor
381 tissues might have been a result of highly sensitive assays used in our study and might suggest
382 either the presence of passenger HPV genomes coming from adjacent normal cells, as earlier

383 reported [48, 49] and/or might have been a reflection of inactive or passenger virus in oral cavity
384 tumors. Although very few (*n*=3), we cannot explain why some tumors in our study with HPV
385 E6/E7 RNA did not show the presence of HPV DNA. It is possible that the genomic DNA for those
386 tumors were degraded and therefore, could not serve as ideal templates for DNA-based assays. An
387 additional factor could be the presence of inhibitors for DNA-based assays in those tumors.
388 Although HPV RNA is considered to be gold standard analyte to test for HPV in tumors, RNA is a
389 difficult analyte to handle and is more labile than DNA. Additionally, studying RNA in archival
390 samples may pose an additional set of challenges.
391
392 The fact that there were only two tumors, which were p16 positive and HPV RNA negative, a
393 definitive conclusion on the lack of correlation between p16 and HPV RNA can't be made from our
394 study. Similarly, there were two tumors, which were positive for HPV RNA and negative for p16.
395 In HNSCC, p16 is often mutated/silenced resulting in its loss of expression. This could have led to
396 the lack of p16 expression in those two tumors. We did not find any significant correlation between
397 p16, HPV-DNA and/or HPV RNA and disease outcome (Figure 4A and Supplementary Figure 8).
398 Even the tumors with relatively high-copy HPV genomes and/or E6/E7 RNA did not support the
399 role of HPV in patient survival (Supplementary Figure 9 A, B). These aspects need further studies
400 and analysis.
401
402 There are several limitations of our study. Not all the tumors were assayed with all the
403 analytes, making the sample number different for different methods. We could not perform
404 additional survival analyses in the tumors that were HPV RNA positive given the small sample size.
405 In our study, we did not perform HPV ISH, which could have provided additional information on
406 p16 positivity and HPV prevalence. It is possible that HPV has a different mechanism of action in
407 oral cavity tumors. Indirect support to linking high HPV genome copy number with HPV positivity
408 in our study comes from the fact that we did not find significance in HPV-linked genes stratifying
409 HPV +ve from HPV –ve group when HPV DNA irrespective of their copy number is taken into
410 account in defining the HPV positivity (Figure 4B). It is possible that the presence of high-copy

411 HPV genome in those tumors does not correlate with the presence of the biologically active virus.
412 Further studies may help answer this question.
413

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419

420 **CONFLICT OF INTEREST**

421 None of the authors declare any conflicts of interest.
422

423 **AUTHOR CONTRIBUTIONS**

424 Study design: Vinayak Palve and Binay Panda
425 Data analysis: Vinayak Palve, Neeraja M Krishnan and Binay Panda
426 Manuscript writing: Vinayak Palve and Binay Panda
427 Conducting experiments: Vinayak Palve, Jamir Bagwan, Udita Chandola, Manisha Pareek, Amritha
428 Suresh, Gangotri Siddappa and Bonne L James
429 Collection of patient samples: Amritha Suresh, Vikram Kekatpure and Moni A Kuriakose
430 Overall study supervision: Binay Panda
431

432 **ABBREVIATIONS**

433 HPV, human papillomavirus; HNSCC, head and neck squamous cell carcinoma; OSCC, oral cavity
434 squamous cell carcinoma; IHC, immunohistochemistry; PCR, polymerase chain reaction; qPCR,
435 quantitative polymerase chain reaction; ddPCR, droplet digital polymerase chain reaction
436

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605 **Table 1:** Patient details and characteristics of tumor tissues used in the study.
606

Characteristics		**Number**
Primary site		
	Buccal mucosa	41
	Oral tongue	112
Gender		
	Male	114
	Female	39
Age		
	>40	112
	<=40	40
	NA	1
Risk habits		
	Alcohol	4
	Chewing	43
	Smoking	6
	Alcohol+ Chewing	14
	Smoking + Alcohol	16
	Smoking + Chewing	9
	Smoking + Alcohol+ Chewing	10
	No habits	44
	NA	7
Tumor classification/stage		
	I+II	43
	III+IV	109
	NA	1
Differentiation		
	Well	48
	Moderate	73
	Poor	20
	NA	12
Abbreviations: NA = Not Available		

607

608 **Table 2.** Summary of HPV assays in oral cavity tumors. p16 was measured by the presence of
609 immuopositive cells with both nulcear and cytoplasmic staining in IHC, PCR results indicate
610 presence of any HPV subtype with consensus primers or HPV16/18 type specific primers, qPCR
611 and ddPCR results indicate Taqman assay results with primers and probes for HPV 16/18 and
612 HPV16 respectively and HPV RNA results indicate presence of E6 and/or E7 mRNA for
613 HPV16/HPV18.

614
615

Detection	**Method**	**% Positivity**		
		Oral tongue	**Buccal mucosa**	**Together (Oral cavity)**
p16 IHC	p16	18% (10/55)	NA	18% (10/55)
DNA based	PC R	59% (39/66)	50% (2/4)	58% (41/70)
	qPCR	44% (34/78)	3.6% (1/28)	33% (35/106)
	ddPCR	55% (52/95)	17% (7/41)	43% (59/136)
RNA based	qPCR	17% (5/30)	9% (1/11)	15% (6/41)
Combinations	PCR + qPCR	38% (23/60)		
	PCR + ddPCR	48.3%(29/60)		
	qPCR + ddPCR	27.2% (27/99)		
	PCR +qPCR + ddPCR	37.7% (20/53)		
	p16 + 3/3 Methods	5.5% (2/36)		
	RNA + DNA	6% (1/17)		

616

617

618 **Figure legends**

619 **Figure 1:** HPV genome organization and locations of different primer/probes used in the study to
620 detect HPV DNA (either consensus or type specific) and RNA. The numbers represent the
621 corresponding nucleotide number in the HPV genome.

622

623 **Figure 2:** p16 and HPV DNA in OSCC. Representative images of immunohistochemical staining
624 of p16 in positive, negative OSCC tissue sections and cervical tissue as positive control (A), the
625 relative PCR amplification efficiency and sensitivityof consensus /type specific primers for
626 detection of HPV using HPV16 or HPV18 individual positive control cell lines (UMSCC-47 and
627 Hep2) (B) and representative HPV DNA PCR in oral cavity tumors with both consensus and type-
628 specific primers (C).

629

630 **Figure 3:** Detection of HPV DNA and RNA. HPV16/18 assays using qPCR (A I-VI) and digital
631 PCR (B I-III) in OSCCS is shown. Standard curves were obtained using cloned HPV16/18 plasmids
632 (A-I,IV, B -I) and data was subsequently obtained using the positive (UM-SCC-47 and Hep2) and
633 negative (UPCI:SCC29B & UPCI:SCC40) cell line DNA (A-II,V, B-II) to count HPV DNA in oral
634 cavity tumors (A-III,VI, B-III). Figure 3C indicates HPV16 (top panel) and HPV18 (bottom panel)
635 E6/E7 mRNA expression in tumors using qPCR..

636

637 **Figure 4:** Kaplan Meier survival plots. Survival plots linking tumors with various attributes like
638 grade, stage, p16 IHC, HPV DNA and HPV RNA (A) is shown. Figure 4B and 4C depict clustering
639 of nine methylated genes stratifying HPV positive from negative group of tumors (B) and HPV-
640 associated pathways (C) in HPV positive tumors.

641

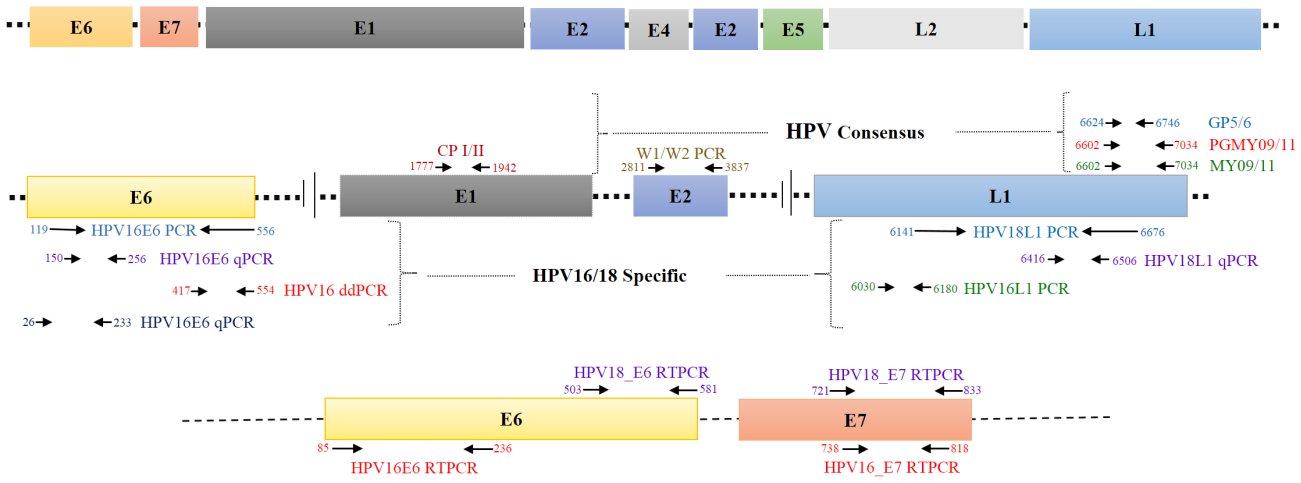
E6
E7
E1
E2
E4
E2
E5
L2
L1
HPV Consensus
6624
6746
GP5/6
6602
7034
PGMY09/11
6602
7034
MY09/11
CP I/II
1777
1942
W1/W2 PCR
2811
3837
E6
E1
E2
L1
119
HPV16E6 PCR
556
150
256
HPV16E6 qPCR
417
554
HPV16 ddPCR
26
233
HPV16E6 qPCR
HPV16/18 Specific
6141
HPV18L1 PCR
6676
6416
6506
HPV18L1 qPCR
6030
6180
HPV16L1 PCR
HPV18_E6 RTPCR
503
581
HPV18_E7 RTPCR
721
833
E6
E7
85
236
HPV16E6 RTPCR
738
818
HPV16_E7 RTPCR

Figure 1

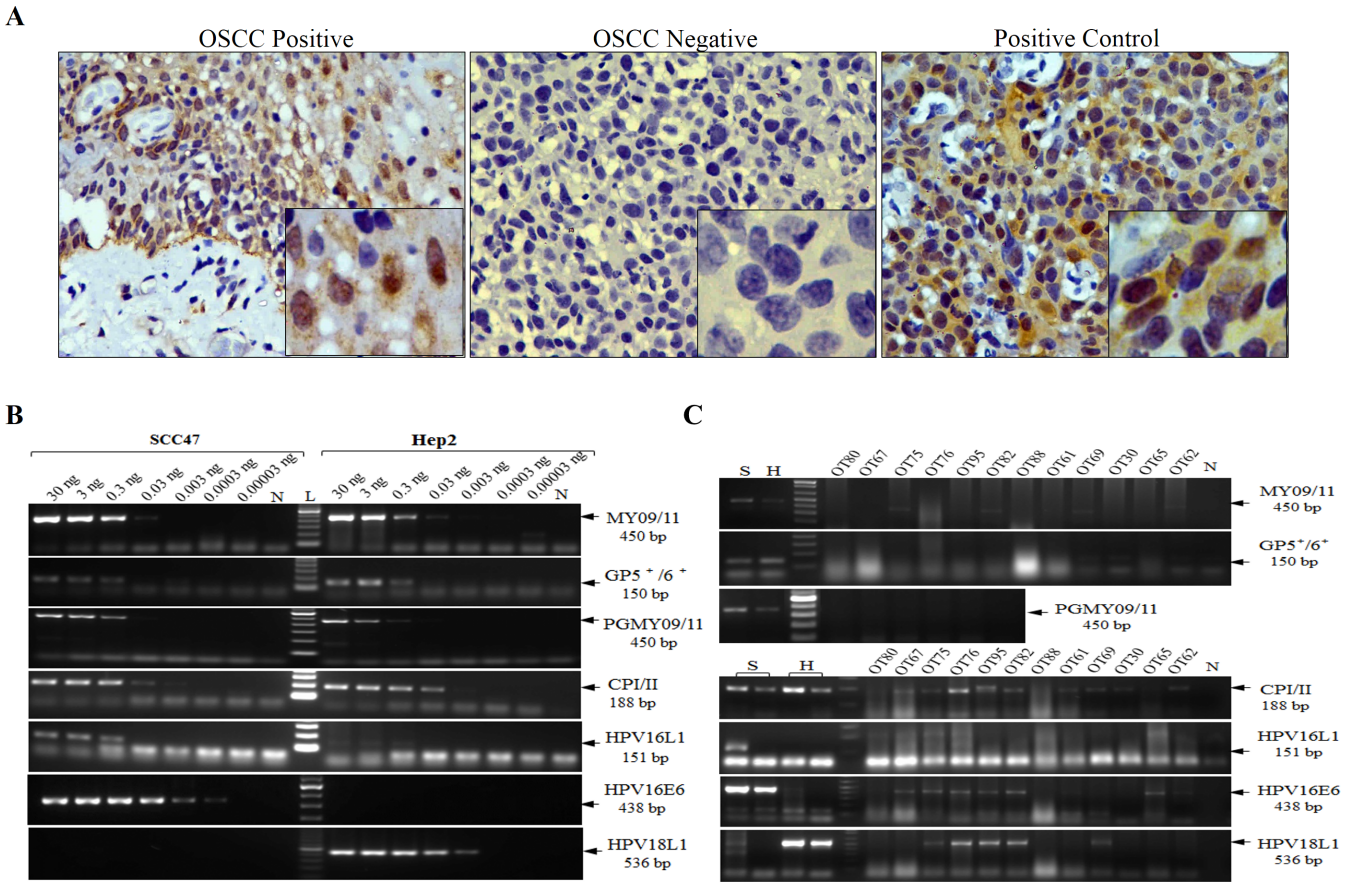
A
OSCC Positive
OSCC Negative
Positive Control
B
SCC47
Hep2
30 ng
3 ng
0.3 ng
0.03 ng
0.003 ng
0.0003 ng
0.00003 ng
N
L
MY09/11
450 bp
GP5 + /6 +
150 bp
PGMY09/11
450 bp
CPI/II
188 bp
HPV16L1
151 bp
HPV16E6
438 bp
HPV18L1
536 bp
C
S
H
OT80
OT67
OT75
OT76
OT95
OT82
OT88
OT61
OT69
OT30
OT65
OT62
N
MY09/11
450 bp
GP5+/6+
150 bp
PGMY09/11
450 bp
CPI/II
188 bp
HPV16L1
151 bp
HPV16E6
438 bp
HPV18L1
536 bp

Figure 2

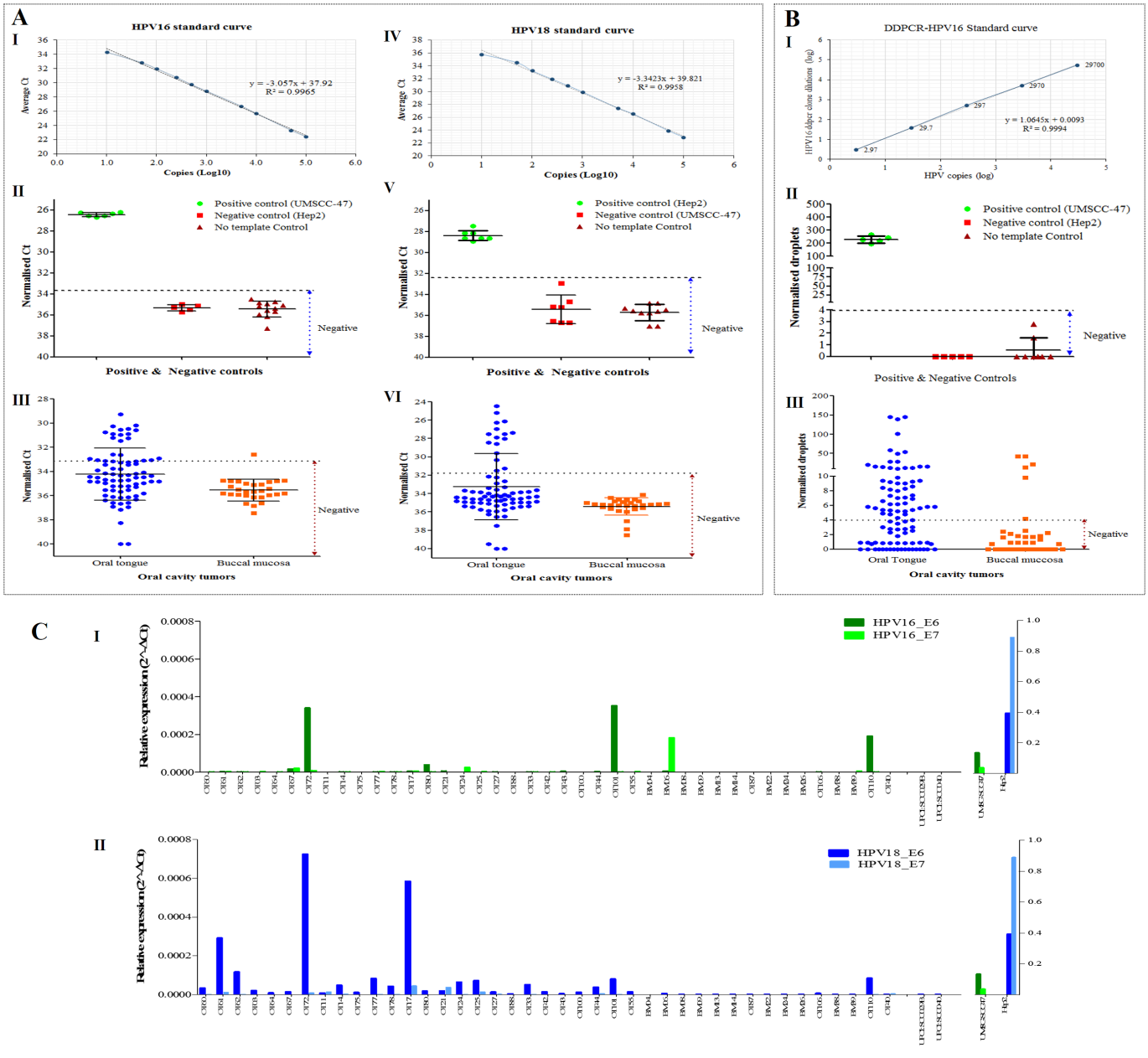
A
I
HPV16 standard curve
y = -3.057x + 37.92
R² = 0.9965
Average Ct
Copies (Log10)
IV
HPV18 standard curve
y = -3.3423x + 39.821
R² = 0.9958
Average Ct
Copies (Log10)
II
Positive control (UMSCC-47)
Negative control (Hep2)
No template Control
Normalised Ct
Negative
Positive & Negative controls
V
Positive control (Hep2)
Negative control (UMSCC-47)
No template Control
Normalised Ct
Negative
Positive & Negative controls
III
Normalised Ct
Negative
Oral tongue
Buccal mucosa
Oral cavity tumors
VI
Normalised Ct
Negative
Oral tongue
Buccal mucosa
Oral cavity tumors
B
I
DDPCR-HPV16 Standard curve
HPV16 dilper clone dilutions (log)
y = 1.0645x + 0.0093
R² = 0.9994
HPV copies (log)
II
Positive control (UMSCC-47)
Negative control (Hep2)
No template Control
Normalised droplets
Negative
Positive & Negative Controls
III
Normalised droplets
Negative
Oral Tongue
Buccal muccosa
Oral cavity tumors
C
I
HPV16_E6
HPV16_E7
Relative expression (2^-ΔCt)
II
HPV18_E6
HPV18_E7
Relative expression (2^-ΔCt)

Figure 3

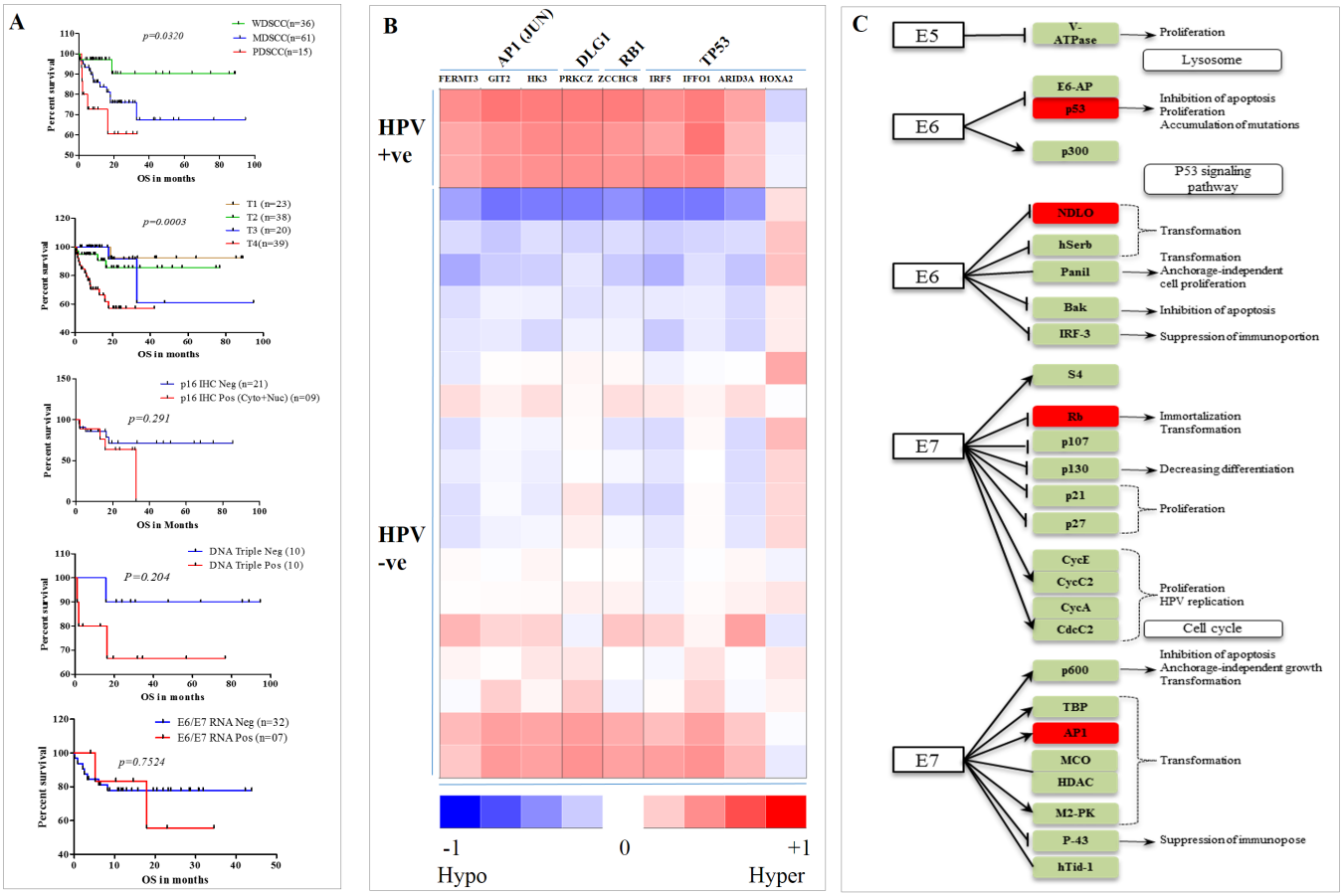
A
p=0.0320
WDSCC(n=36)
MDSCC(n=61)
PDSCC(n=15)
Percent survival
OS in months
p=0.0003
T1 (n=23)
T2 (n=38)
T3 (n=20)
T4(n=39)
p16 IHC Neg (n=21)
p16 IHC Pos (Cyto+Nuc) (n=09)
p=0.291
OS in Months
P=0.204
DNA Triple Neg (10)
DNA Triple Pos (10)
E6/E7 RNA Neg (n=32)
E6/E7 RNA Pos (n=07)
p=0.7524
B
AP1 (JUN)
DLG1
RB1
TP53
FERMT3
GIT2
HK3
PRKCZ
ZCCHC8
IRF5
IFFO1
ARID3A
HOXA2
HPV +ve
HPV -ve
-1
0
+1
Hypo
Hyper
C
E5
V-ATPase
Proliferation
Lysosome
E6
E6-AP
p53
p300
Inhibition of apoptosis
Proliferation
Accumulation of mutations
P53 signaling pathway
NDLO
hSerb
Panil
Bak
IRF-3
Transformation
Transformation
Anchorage-independent cell proliferation
Inhibition of apoptosis
Suppression of immunoportion
E7
S4
Rb
p107
p130
p21
p27
CycE
CycC2
CycA
CdcC2
Immortalization
Transformation
Decreasing differentiation
Proliferation
Proliferation
HPV replication
Cell cycle
p600
TBP
AP1
MCO
HDAC
M2-PK
P-43
hTid-1
Inhibition of apoptosis
Anchorage-independent growth
Transformation
Transformation
Suppression of immunopose

Figure 4