Prevalence and Expression of Human Papillomavirus in 53 Patients with Oral Tongue Squamous Cell Carcinoma

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Abstract. *Background/Aim: Human papillomavirus (HPV) infection has been considered a potential risk factor for the development of oral tongue squamous cell carcinoma (SCC). The purpose of the present study was to investigate HPV infection and high-risk HPV E6/E7 mRNA expression in SCC of the oral tongue in 53 Greek patients. Materials and Methods: Fifty-three biopsies were collected from patients with SCC of the oral tongue and tested for HPV DNA and E6/E7 mRNA expression. Results: The overall prevalence of HPV DNA was 11.3% (6/53), while high-risk HPV DNA was found in 7.5% (4/53) of SCC of the oral tongue. E6/E7 mRNA expression was observed in 9.4% (5/53) samples examined. HPV 16 was the commonest genotype identified by both DNA and RNA assays. A total of 28.3% (15/53) of the patients were non-smokers and non-drinkers. HPV infection was strongly associated with abstinence from tobacco and alcohol (p=0.013). Conclusion: The presence of high-risk HPV E6/E7 mRNA expression suggests that HPV may be implicated in the pathogenesis of SCC of the oral tongue.*

HPV infection with high-risk (hr) types has an established role in the pathogenesis of a subset of squamous cell carcinoma (SCC) of the oropharynx (1). In contrast, detection rates of hrHPV in SCC of the oral cavity demonstrate a great variability (2, 3), even in young patients (<45 years old) with no exposure to traditional risk factors, such as tobacco usage and alcohol consumption. A notable increase of SCC of the oral cavity in young patients has been reported (4, 5).

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The majority of HPV-associated SCCs of the oropharynx are characterized by expression of hrHPV-type oncoproteins E6/E7 (6), which de-regulate cell division and differentiation, due to persistent HPV infection and subsequent viral integration. Interestingly, HPV integration and expression of E6/E7 oncogenes is a less common event in SCC of the oral cavity, indicating that other mechanisms may be implicated in malignant transformation of oral mucosa (5, 7, 8).

Both the oropharynx and the oral cavity include portions of the tongue as one of their anatomical sites. Indeed, the anterior two-thirds of the tongue or the oral tongue belong to the oral cavity, while the posterior third or the base of tongue is a part of the oropharynx.

hrHPV, especially type 16, is frequently detected in SCC of the base of the tongue indicating its predilection to non-keratinized locations of the oropharynx with inflammatory activity (9). However, the same does not necessarily apply to SCC of the oral tongue, which is the commonest subsite of SCC of the oral cavity. Many investigators have reported HPV infection rates in SCC of the oral tongue ranging from 0% to 100% (9-15). The remarkable difference in HPV prevalence among studies does not allow for firm conclusions to be drawn regarding the role of HPV in the development of SCC of the oral tongue.

The purpose of the current study was to investigate HPV infection and hrHPV E6/E7 mRNA expression in patients with SCC of the oral tongue.

Materials and Methods

Biopsies were collected from 53 patients who subsequently underwent surgical treatment between May 2012 and May 2013 and sent for routine histopathological analysis. A part of the tumorous tissue was kept in liquid storage medium (Thin-Prep PreservCyt Solution; Hologic, Inc. Ltd. West Sussex, UK) in 4˚C. All samples had histologically-confirmed, non-metastatic, moderately-to-poorly differentiated (grade II-III) SCC of the oral tongue and were analyzed for the presence of HPV DNA and E6/E7 mRNA. Patients were eligible if they had not received any

previous treatment. Ethical approval was granted by the Ethics Committee of St. Savvas Regional Anticancer Oncology Hospital of Athens (approval number 6937/448) and all participants provided written, informed consent.

Extraction of nucleic acids. Total nucleic acids were extracted using the QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. DNA quality test was carried out using Human Globin, Beta, Primer set kit (Maxim Biotech, Inc., South San Francisco, CA, USA) according to the manufacturer's instructions. To assess RNA integrity, 5 μg of RNA per sample were separated on 1% formaldehyde-agarose gel.

HPV detection and genotyping. The PapilloCheck® HPV genotyping assay (Greiner Bio-One GmbH, Frickenhausen, Germany) was used. This technology is based on a DNA chip for the type-specific identification of 24 types of HPV (hr: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82; probable hr: 53 and 66; and low-risk (lr): 6, 11, 40, 42, 43, 44/55, 70). Nucleic acids were extracted from oral tongue biopsies preserved in ThinPrep. E1-based polymerase chain reaction (PCR) was performed according the manufacturer's guidelines. For each sample, 19.8 μl PapilloCheck® MasterMix, 0.2 μl HotStarTaq DNA polymerase (5 U/μl) and 5 μl DNA from the oral tongue sample were mixed. Hybridization followed by mixing 30 μl of the PapilloCheck® Hybridization buffer in a fresh reaction tube with 5 μl of the PCR product at room temperature and transferring 25 μl of the hybridization mix into each compartment of the chip. The chip was incubated for 15 min at room temperature in a humid atmosphere, then washed in three washing solutions (30 sec, 1 min and 30 sec, respectively), centrifuged and scanned on a CheckScanner™ (Greiner Bio-One GmbH).

HPV E6/E7 mRNA expression. Real-time nucleic acid sequence-based amplification (NASBA) and detection assay NucliSENS® EasyQ® HPV (BioMérieux Hellas, Athens, Greece) was performed for the qualitative detection of E6/E7 oncoproteins of five hrHPV types (16, 18, 31, 33 and 45). The NucliSENS EasyQ HPV assay was performed according to the manufacturer's instructions (BioMérieux). Firstly, three pre-mixes were made by adding reagent sphere diluent [Tris-HCl, 45% dimethyl sulfoxide (DMSO)] to reagent spheres (nucleotides, dithiothreitol and $MgCl_2$). To each pre-mix U1A/HPV 16, HPV 33/45, or HPV 18/31 primer and molecular beacon mixes, KCl stock solution and nucleic acid sequence-based amplification (NASBA) water were added. Secondly, 10 μl of this pre-mix were distributed to each well in a reaction plate and the addition of 5 μl RNA followed. The plates were incubated for 4 min at 65˚C to destabilize secondary structures of RNA, followed by cooling to 41˚C. The reaction was started by addition of enzymes (AMV-RT, RNase H, T7 RNA polymerase, and bovine serum albumin) and measured in real time using the Lambda FL 600 fluorescence reader (Bio-Tek, Winooski, VT, USA) at 41˚C for 2 hours and 30 minutes.

Statistical analysis. Data were analyzed using SAS v9.0. (SAS Institute Inc. NC, USA). Absolute and relative frequencies were used to present HPV positivity. Chi-squared tests were performed to assess statistical significance of any differences in prevalence. 2×2 contingency tables and Fisher's exact test was performed to estimate *p*-values. *p*-Values of less than 0.05 were considered statistically significant.

Results

A toted of 53 SCCs of the oral tongue were analyzed for the presence of HPV DNA and hrHPV E6/E7 mRNA expression. The patients' age ranged from 19 to 75 years (mean age=51 years). HPV DNA was detected in 11.3% (6/53) of SCC of the oral tongue; 7.5% of the samples (4/53) harbored the hrHPV type 16 (5.7%, 3/53) or 18 (1.9%, 1/53), while 3.8% (2/53) were infected with lrHPV type 6.

The presence of HPV was not associated with younger patient age (p=0.184) but it was more common in female compared to male patients (p=0.036). HPV infection was significantly more common in the non-smoking and non-drinking patient group compared to patients who used tobacco and alcohol (p=0.013) (Table I).

Overall, 9.4% (5/53) of oral tongue samples were positive for hrHPV E6/E7 mRNA expression. E6/E7 mRNA from HPV 16 was found in 7.5% (4/53) and HPV 18 in 1.9% (1/53) of cases. Patients' characteristics, with HPV DNA-positive oral tongue SCC, as well as the expression pattern of hrHPV are summarized in Table II.

Discussion

Multiple studies have confirmed the etiological role of HPV infection in the onset of tonsillar and base of tongue SCC worldwide (9, 11, 16-19). However, a similar link has not been clearly identified in cancer of the oral tongue because of the great variation in HPV prevalence found in this subsite of SCC of the oral cavity in different studies. The goal of the present study was to detect HPV DNA, as well as hrHPV E6/E7 mRNA expression, in Greek patients with SCC of the oral tongue.

The results of the current study showed that 11.3% of the oral tongue samples were positive for HPV DNA. hrHPV infection was observed in 7.5% of SCCs of the oral tongue, a percentage which is in the range reported by previous data. In a study conducted in Greece, it was found that only 3% of SCCs of the oral tongue were hrHPV-positive (20), similar to other reports worldwide, using general and type-specific PCR methods (9, 13). In contrast, a much higher frequency of hrHPV infection in SCC of the oral tongue has been observed in other studies, ranging from 25 to 74%, using either commercial PCR kits for the detection of HPV or genotyping DNA chips, reverse line blot hybridization and P16 immunohistochemistry (15, 21-24).

The large discrepancy in hrHPV infection rates among studies is possibly attributed not only to the different methods for HPV detection, but also to the different sample size, the sample type and storage conditions (*i.e.* formalin-fixed or fresh biopsies) and geographic variation (8, 25-27).

According to the results of the present study, HPV 16 and HPV 18 were the only hr types detected in HPV DNA-positive oral tongue cases, similar to other reports (9, 15, 22-

Table I. *Association of patients' characteristics with human papilloma virus (HPV) status.*

Characteristics	N (%) (n=53)	HPV DNA (+) (n=6)	HPV DNA (–) (n=47)	*p*-Value
Gender				0.036
Male	39 (73.6%)	2 (5.1%)	37 (94.9%)	
Female	14 (26.4%)	4 (28.6%)	10 (71.4%)	
Age				0.184
<45 years	20 (37.8%)	4 (20%)	16 (80%)	
>45 years	33 (62.2%)	2 (6.1%)	31 (93.9%)	
Risk factors				0.013
Tobacco and alcohol use	29 (55%)	1 (3.4%)	28 (96.6%)	
No tobacco and no alcohol use	15 (28.3%)	5 (33.3%)	10 (66.7%)	

Table II. *Patients' characteristics and E6/E7 mRNA status of human papilloma virus (HPV)-infected squamous cell carcinoma of the oral tongue.*

Gender	Age (years)	Tobacco	Alcohol	DNA test	mRNA test
Female	39	No	No	HPV 16	HPV 16
Male	67	No	No	HPV 16	HPV 16
Male	73	Moderate (26 packets per year)	Social	HPV 16	HPV 16
Female	34	No	No	HPV 18	HPV 18
Female	43	No	No	HPV 6	HPV 16
Female	25	No	No	HPV 6	Negative

24). It is important to note that 3.8% of SCCs of the oral tongue harbored the lrHPV 6, which is in agreement with other studies indicating the presence of lrHPV types in oral cavity SCC (28-30). However, the detection of lrHPV in oral cancer alone does not prove their possible implication in carcinogenesis, and therefore these types may infect the tumors and act as 'by-standers' rather than drivers of cancer development (28-34).

It has been established that HPV-mediated carcinogenesis is based on the expression of viral oncoproteins E6 and E7, which has been correlated to the de-regulation of cell cycle in infected cells and maintenance of the transformed status (28, 35). In addition, HPV-infected head and neck carcinomas that express E6/E7 mRNA demonstrate different genetic patterns compared to those caused by environmental factors in relation to early markers of head and neck oncogenesis and differentially expressed genes (36). Therefore, E6/E7 mRNA expression is a necessary step for the onset of HPV-infected head and neck carcinomas and viral DNA alone is likely to have no biological significance in the pathogenesis of these types of cancer (37-40).

Relatively few studies have investigated the E6/E7 mRNA expression from hrHPV types in oral cavity cancer (28, 37, 41-44). In this series, mRNA expression of E6 and E7 oncoproteins from HPV 16 or HPV 18 was found in 9.4% (5/53) of SCCs of the oral tongue, demonstrating the potential role of these hrHPVs in cancer onset. Recent studies have reported on high E6/E7 mRNA expression from hrHPV in SCC of the oral tongue, particularly HPV 16 (15, 23, 24, 41).

In the present study, two cases were positive for lrHPV 6 by the DNA assay, while no E6/E7 mRNA expression of HPV 6 was detected by the RNA assay. These are not discrepant cases as the RNA test used detects only hrHPV types 16, 18, 31, 33 and 45.

Interestingly, in one patient of the present study, HPV 16 was detected only through E6/E7 mRNA expression. It has been suggested that viral DNA integration into the host's genome often occurs in the E1 or E2 region (45-47), leading to loss of binding sites for the primers of the DNA assay used.

Many investigators have observed a considerable increase in incidence of SCC of the oral tongue among patients younger than 45 years of age during the past decade, without being able to accurately identify the causal factors (5, 48-51). The results of the current study are in line with this fact in that 37.8% of patients with SCC of the oral tongue were less than 45 years old. However, it has been reported that HPV infection is probably not associated with younger patient age, corroborating with the results of the current study (22, 24).

What is interesting is that in this series, HPV infection was found to be strongly related to abstinence from tobacco and alcohol consumption, demonstrating that HPV may independently induce carcinogenesis. This is contrary to what has been observed in other studies, where no HPV infection was detected in non-smokers and non-drinkers (2, 52).

In summary, the present study revealed that 9.4% (5/53) of SCCs of the oral tongue were positive for hrHPV E6/E7 mRNA expression, suggesting that HPV may be implicated in the pathogenesis of oral tongue cancer. Further investigation of a greater number of SCC case with longer follow-up is required to better-assess the etiological role of HPV and its clinical significance in this sub-site of SCC of the oral cavity.

Conflicts of Interest

The Authors declare that there are no conflicts of interests.

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