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Human Papillomavirus in Oral Leukoplakia, Verrucous Carcinoma, Squamous Cell Carcinoma, and Normal Mucous Membrane

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ABSTRACT

Objectives: Squamous cell carcinoma (SCC) is the most common oral malignancy, and verrucous carcinoma (VC) is a less invasive type of SCC. Leukoplakia (LP) is the most frequent premalignant lesion in the oral cavity. The human papillomavirus (HPV) has been recognized as one of the etiologic factors of these conditions. The association of anogenital and cervical cancers with HPV particularly its high-risk subtypes (HPV HR) has been demonstrated. The purpose of our study was to investigate the hypothetical association between HPV and the mentioned oral cavity lesions. ***Methods:*** One hundred and seventy-three samples (114 SCCs, 21 VCs, 20 LPs) and 18 normal mucosa samples (as a control group) were retrieved from the Department of Oral and Maxillofacial Pathology of Mashhad Dental School, Iran. The association of HPV genotypes in LP, VC, and SCC was compared to normal oral mucosa using the polymerase chain reaction. ***Results:*** The results showed the absence of HPV in normal mucosa and LP lesions. In three samples of VC (14.3%), we observed the presence of HPV HR (types 16 and 18). All VCs were present in the mandibular ridge of females aged over 65 years old. No statistically significant correlation between HPV and VC was observed (p=0.230). Additionally, 15 (13.1%) SCCs showed HPV positivity, but this was not significant (p=0.830). The prevalence of SCC was higher on the tongue with the dominant presence of less carcinogenic species of HPV (types 6 and 11). A statistically significant association was not observed between HPV and SCC or VC in the oral cavity. ***Conclusions:*** More studies are necessary to better understand the relationship between HPV and malignant/premalignant oral cavity lesions.

Oral cancer is the eleventh most common cancer worldwide. The highest incidence and mortality rates are registered in developing countries. Squamous cell carcinoma (SCC) is the most common type of oral cancer accounting for 94% of all oral malignancies. In the United States, slightly more than 5,300 individuals die of this disease each year.[1,2]

We previously studied the expression of human papillomavirus (HPV) in leukoplakia (LP) and verrucous carcinoma (VC), which have similar clinical features but different therapeutic and prognostic perspective. In this study, we added oral squamous cell carcinoma (OSCC) as it is the most common cancer of the oral cavity. Therefore, by increasing the sample size and power of the study we obtained results that are more comprehensive for clinical purposes.[3]

Ackerman first described VC in 1948.[4] It is the least invasive type of SCC and can be found in the oral cavity as well as other parts of the body. It is more commonly seen in men over 55 years old.[1]

According to the World Health Organization (WHO) definition, LP is a white plaque that is not pathologically or clinically related to any other lesion. Proliferative verrucous leukoplakia (PVL) is a specific type of LP with the highest malignant transformation rate. It is one of the differential diagnoses of VC.

Similarities in the clinical and histopathological manifestations of VC, PVL, and some types of SCC, especially papillary SCC, are observed. As the prognostic and therapeutic measures for these entities are different, differentiating them is important.[1]

Different etiological factors have been proposed for the development of the lesions mentioned. Among these factors, one of the most well known

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Table 1: Sequences of beta globin region and HPV genotype-specific primers used in the PCR assays.

Target gene	Sequence
GH20	F:GAAGAGCCAAGGACAGGTAC
PCO4	R:CAACTTCATCCACGTTCACC
GP5+	F:TTTGTTACTGTGGTAGATACTAC
GP6+	R:GAAAAATAAACTGTAAATCATATTC
MY09	F:CGTCCMARRGGAWACTGATC
MY11	R:GCMCAGGGWCATAAYAATGG
HPV 6	F:TAG TGG GCC TAT GGC TCGTC
	R:TCC ATT AGC CTC CAC GGG TG
HPV 11	F:GGA ATA CAT GCG CCA TGT GG
	R:CGA GCA GAC GTC CGT CCT CG
HPV 16- E6	F:CAG GAC CCA CAG GAG CGA CC
	R:ATC GAC CGG TCC ACC GAC CC
HPV 18 –E6	F:GCT TTG AGG ATC CAA CAC GG
	R:TGC AGC ACG AAT GGC ACT GG
HPV 31-E6/E7	F:GAA ATT GCA TGA ACT AAG CTC G
	R:CAC ATA TAC CTT TGT TTG TCA A

W= A+T, R= A+G, Y= C+T, M= A+C.
HPV: human papillomavirus; PCR: polymerase chain reaction.

is HPV.[1] More than 130 HPV genotypes have been recognized which are divided into high risk (HPV HR) and low risk (HPV LR) based on their malignant transformation rate. HPV HR has been found in 81% of subjects with normal oral mucosa.[1] HPV is a double-stranded DNA virus belonging to the Papovavirus family. Despite multiple studies, there is no clear association between HPV and oral cancers. On the contrary, its association with uterine cervix and anogenital cancers has been established.[1,5]

Overexpression of some HPV genes such as E6 and E7 promote tumor growth and malignant transformation (especially in HR subtypes). Binding of E6 and E7 to tumor suppressor genes P53 and pRb results in disruption of the cell cycle and cellular immortality. HPV genotypes may also be found in the oral cavity as the normal flora of the human body.[6-8]

The association of HPV with head and neck cancers was first investigated in 1960.[9] Loning first proposed the etiologic role of this virus in 1985,[10] but the available data regarding the role of HPV in malignant transformation is extremely controversial. The range of prevalence reported in different studies is 0% to 100%.[6] There are various techniques to code the viral genome, including the polymerase chain reaction (PCR), in situ hybridization (ISH), and Southern blot (SB) analysis, with different sensitivities.[6]

Our study was accomplished to detect the existence of the HPV LR (types 6 and 11) and the HPV HR (types 16, 18, and 31) in the mentioned oral lesions, using PCR. The studied groups included LP as the most common premalignant lesion, SCC as the most common oral cavity cancer, VC as the least invasive form of SCC, and normal mucosa as a control group. Determining the association between HPV and malignant/premalignant lesions could be useful for understanding the etiology of these lesions and the potential treatment options.

METHODS

In this retrospective case-control study, we analyzed paraffin-embedded biopsies of 155 patients with LP, SCC, and VC obtained from 2001 to 2011. The samples were retrieved from the Department of Oral and Maxillofacial Pathology of Mashhad Dental School, Iran. Diagnosis of LP, SCC, and VC was based on histological examination of hematoxylin and eosin stained tissue sections, which was performed blindly by two pathologists. Blocks with a sufficient amount of tissue (114 SCC, 21 VC, and 20 LP) were selected for the experiment.

For the control group, we used non-neoplastic, reactive lesions of connective tissue (like fibrotic lesions and mucoceles) with normal-appearing epithelium. In total, 18 control samples were collected

which were mostly obtained from the mucosal area of the lower lip and cheek. To extract DNA and run PCR, paraffin blocks were sliced thinly under ideal conditions[11] and placed in Eppendorf microtubes.

Xylene (Xylol), 1 ml, was added to the tubes to remove the paraffin wax. To digest the tissue we used 20 mmol Proteinase K. After separating the DNA strands, the temperature was raised to 95 °C to annihilate Proteinase K from the samples. They were subsequently centrifuged, and the DNA in the sediment were lyophilized and dissolved in Tris and EDTA buffer solution and then kept at 4 °C for PCR. It is worth mentioning that for a higher purity of the DNA phenol, buffered saturated, and phenol/chloroform/isoamyl alcohol (at a ratio of 25:24:1) were also added.

The extracted DNA was analyzed by PCR for beta globulin genes using the GH20 and PCO4 primers. The genes that were amplified with these primers selected and used to detect the presence of HPV using the MY09/MY11 and GP5+/GP6+ primers that were designed for L1 portion of HPV genome.[12,13] The other primers [Table 1] were used for HPV genotyping (HPV 6, 11, 16, 18, and 31) as described previously.[14]

In total, a volume of 50 µl PCR mixture containing 200–700 ng of DNA, 10 mM Tris-Hcl (pH 8.3), 50 mM potassium chloride (KCl), 2.5 mM magnesium chloride ($MgCl_2$), one unit Taq DNA polymerase (Cinnagen, Iran), 0.2 mM of each dNTP, and 50 pmol of each primer were prepared in 0.2 ml microtubes. HeLa cells DNA and distilled water were used as template in positive and negative control tubes, respectively. Subsequently, the PCR amplified fragments were electrophoresed in 2% agarose gel and visualized using a DNA Green viewer dye (Pars Tous, Iran).

RESULTS

In our study, a total number 173 samples were included. Of these, 114 were SCCs, 21 were VCs, 20 were LPs, and 18 were normal mucosal membranes. The SCC samples consisted of 58 male and 56 female subjects. Patients were aged from 19–85 years old. The average age was 58.6±15.4 years.

The VC group consisted of 10 male patients with an average age of 59.6±11 years and 11 female patients with an average age of 59.0±11.4 years. The LP group consisted of 15 male subjects and five female subjects with an average age of 61.6±15.6 and 62.8±3.7 years, respectively. Of the 18 normal mucosa samples, seven were from male subjects and 11 from females (average age of 59.6±12.3 and 55.5±13.7 years, respectively). The case and control group were adjusted for age and sex.

DNA extraction and PCR were performed to determine various HPV HR genomes (types 16, 18, and 31) and HPV LR (types 6 and 11) in the studied groups. In total, 18 cases (10.4%) were positive for HPV. All positive samples were VC and SCC.

Of 114 SCC samples, 15 (13.1%) were positive for HPV 6 and 11. The most common site of SCC was the tongue (64.9%). Anatomical distribution of SCC lesions and their HPV status are presented in Table 2.

Out of 99 HPV-negative patients, 50 patients (50.5%) were male. There were 15 HPV-positive

Table 2: The anatomical distribution of SCC lesions and their HPV status.

Location (SCC)	HPV					
	Negative		Positive		Total	
	Number	Percentage	Number	Percentage	Number	Percentage
Tongue	64	64.6	10	66.7	74	64.9
Buccal mucosa	18	18.2	0	0.0	18	15.8
Mandibular ridge	9	9.1	3	20.0	12	10.5
Palate	2	2.0	0	0.0	2	1.8
Mouth floor	2	2.0	1	6.7	3	2.6
Lip	2	2.0	0	0.0	2	1.8
Nasopharynx	1	1.0	1	6.7	2	1.8
Oropharynx	1	1.0	0	0.0	1	0.9
Total	**99**	**100.0**	**15**	**100.0**	**114**	**100.0**

SCC: squamous cell carcinoma; HPV: human papillomavirus.

Table 3: Gender distribution in HPV-positive patients with squamous cell carcinoma.

Gender	HPV					
	Negative		Positive		Total	
	Number	Percentage	Number	Percentage	Number	Percentage
Male	50	50.5	8	53.3	58	50.9
Female	49	49.5	7	46.7	56	49.1
Total	**99**	**100**	**15**	**100.0**	**114**	**100.0**

HPV: human papillomavirus.

Table 4: The relative anatomical distribution of lesion in patients with squamous cell carcinoma.

Lesion location	HPV					
	Negative		Positive		Total	
	Number	Percentage	Number	Percentage	Number	Percentage
Tongue	64	64.6	10	66.7	74	64.9
Oral cavity except tongue	35	35.4	5	33.3	40	35.1
Total	**99**	**100.0**	**15**	**100.0**	**114**	**100.0**

HPV: human papillomavirus.

patients, eight (53.3%) of which were male. The K-2 test was used to evaluate the association between sex and HPV positivity. No statistically significant difference was observed (X^2=0.042; p=0.838) [Table 3].

Out of 99 HPV-negative patients, 64 (64.6%) had SCC of the tongue. Whereas this number was 10 out of 15 (66.7%) in HPV-positive patients. According to the K-2 test no significant association between the lesion location and HPV infection was shown (X^2=0.023; p=0.879). HPV positivity was detected in three patients with alveolar ridge SCC, one with mouth floor SCC, and one with nasopharyngeal SCC [Table 2 and 4]. Common anatomical sites of VCs were the mandibular vestibule, lower lip-cheek mucosa, and the gingiva. LP was prevalent in the cheek mucosa, tongue, upper lip, and the mouth floor. Control samples were mostly obtained from lower lip and cheek mucosa.

A total of 15 SCC cases (13.3%) and three VC cases (14.3%) were positive for HPV. There was no statistically significant difference in the expression of HPV in the groups (p=0.830 for SCC and p=0.230 for VC). The Fisher's exact test did not show any association between sex or age and HPV expression (p=0.138 and p=0.124, respectively). It is important to note that all three VC samples contained HPV HR (types 16 and 18). They were observed in female patients over 65 years old in the mandibular vestibule. Despite positive HPV expression in VC samples, there was no statistically significant difference between the VC and control group (p=0.230) [Table5].

DISCUSSION

Oral cancer is one of the most frequent malignancies. SCC is the most common type of oral cancer accounting for 94% of all oral malignancies. The low-grade type of SCC is called the VC and is not

Table 5: Distribution rate of HPV expression in LP, VC, and NM (control group).

Results	Group					
	VC		LP		NM	
	Male	Female	Male	Female	Male	Female
HPV+	0	3	0	0	0	0
HPV-	8	10	14	5	8	10
Total	**8**	**13**	**14**	**5**	**8**	**10**

HPV: human papillomavirus; VC: verrucous carcinoma; LP: leukoplakia; NM: normal mucosa.

exclusive to the oral cavity and can be seen in other parts of the body.[1,15]

The most frequent sites of SCC are the tongue and mouth floor. The buccal mucosa is the most common site for VC and has a higher prevalence in those over 55 years old.[1] LP is known as the most common premalignant oral cavity lesion with histopathological features similar to VC.[1]

Among the multiple causes proposed as the etiology of VC, SCC, and LP,[16,17] HPV has been the focus of various studies. HPV is a double-stranded epitheliotropic DNA virus. More than 100 types of HPV have been recognized, but only a few of them are active in the oral cavity (types 1, 2, 4, 6, 7, 11, 13, 16, 18, 30, 32, 57).[5] HPV types are classified either as high (types 16, 18, 31, 33, 39, 45, 51, 52, 54 and 56) or low risk (types 6, 11, 13, 16, 18, 30, 32 and 57).[1,6,8]

The role of HPV in the prognosis and carcinogenesis of anogenital cancer has been demonstrated. However, there is no clear evidence to support its carcinogenic effect in the oral cavity. Several studies have investigated the role of HPV in oral carcinogenesis over the last three decades.[10,11] Meta-analytic reviews have also been conducted to investigate the association between HPV and SCC.[16,17] Using PCR and in situ hybridization (ISH) the data showed 22–28% association.

Multiple studies showed various expression of this virus in different geographical areas. Southern blot and PCR-ISH techniques demonstrated 19.2–50.0% and 41.5–100.0% association, respectively. The DNA microarray technique did not show any association.[18-21] In our study, 13.1% of all SCCs were positive for HPV, which shows lower association than meta-analysis and universal reviews.

We investigated the possible association of HPV with SCC and VC, as a less aggressive form of SCC. Both of these conditions are in the differential diagnosis of papillary SCC and LP, especially the PVL subtype.

HPV association with VC in this study was about 14.3% (p=0.230). This finding was also found in another study.[22] Some studies reported a higher expression of HPV in VC and a possible association between them.[17,23]

The consistent and specific association of HPV HR, especially 16 and 18, with VC has been shown in several studies using PCR/ISH technique.[1] This is consistent with the results of our study and some others.[24] Although the absence of HPV 16 has been reported in VC samples by Fujita et al.[25] Limited reports that showed higher HPV association with LP showed higher expression of HPV in normal mucosa.[6,23] Szarka et al,[26] did not demonstrate any association between LP and HPV which is consistent with other studies.[19,27]

Some studies showed higher expression of HPV HR in LP samples.[16,28] Although Lou et al,[23] reported the opposite in their study. The expression of the virus was reported between 0–100% with PCR,[24,27] 0–73% with ISH,[6,29] and 80–100% using SB analysis.[10,19] HPV expression in normal mucosa was reported between 1–60% in various studies.[30] In our study, there was no correlation between the HR and LR subtypes of HPV.

CONCLUSION

As multiple factors can be involved in neoplastic transformation, evaluating the possible role of HPV has been the focus of many studies. In the Iranian population, we found no significant association between HPV and malignant transformation. We observed the absence of HPV in normal mucosa and LP lesions, whereas 13.1% of SCCs and 14.3% of VCs contained HPV DNA. The presence of HPV HR (16 and 18) was detected in VCs while SCC samples demonstrated HPV LR (6 and 11). The role of HPV in malignant transformation has always been under discussion and requires larger studies.

Disclosure

The authors declared no conflicts of interest. No funding was received for this study.

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