DETECTION OF HUMAN PAPILLOMAVIRUS DNA SEQUENCES IN ORAL LESIONS USING POLYMERASE CHAIN REACTION

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Abstract- The purpose of the present study was to estimate the frequency of HPV DNA in four groups of oral lesions, including oral squamous cell carcinoma. Sixty paraffin-embedded oral tissue samples were examined for the presence of HPV DNAs using the PCR technique. These specimens were obtained from patients with oral squamous cell carcinoma (OSCC), leukoplakia, oral lichen planus (OLP), and pyogenic granuloma (PG). Consensus primers for L1 region (MY09 and MY11) and specific primers were used for detection of HPV DNA sequences in this study. we detected HPV DNA in 60% (9 out of 15) of OSCCs, 26.7% (4 out of 15) of leukoplakia, 13.3% (2 out of 15) of OLPs, and 6.7% (1 out of 15) of PGs. Statistical analysis showed that the prevalence of HPV in OSCC was significantly higher than other groups ($P < 0.05$). The frequency of HPV-16 and 18 detection in OSCC samples were 40% and 20%, respectively. The prevalence of these high risk HPVs was significantly higher in OSCC group ($P < 0.05$). The results of the present study show a successive increase of detection rate of HPV-16 and 18 DNAs from low level in samples of pyogenic granuloma and non-premalignant or questionably premalignant lesions of OLP to premalignant leukoplakia and to OSCC.
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Key words: Oral squamous cell carcinoma, Leukoplakia, Lichen planus, Human papillomavirus, Polymerase chain reaction

INTRODUCTION

Squamous cell carcinoma is a malignant tumor common to the skin, mouth, and other body cavities covered by a squamous epithelium. Oral squamous cell carcinoma (OSCC) is the sixth most common cancer of the body and is one of the 10 most common causes of death worldwide (1, 2). Cancers of the oral cavity and oropharynx account for approximately 3% of all new cancers diagnosed annually in the United States (about 30000 cases per year) (2). However, the incidence of OSCC varies in different geographic locations. OSCC accounts for up to 40% of all malignancies in India and South East Asia (1). There is substantial evidence that OSCC is usually etiologically linked with tobacco and/or alcohol or betel use (2). However, differences have been observed in tobacco smoking and alcohol drinking habits in OSCC patients. There are clearly patients who develop OSCC in the absence of

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exposure to these agents and in the absence of any obvious predisposing genetic defect (3). Since carcinogenesis is a multi-step process and transition from normal mucosa to invasive SCC takes a long time, it is possible that several other factors, such as dietary factors and viruses (especially human papillomavirus) play a role in the development of OSCC. Papillomaviruses are small, non-enveloped, icosahedral DNA viruses of about 55 nm in diameter. Virus particles contain a circular, double-stranded, covalently closed DNA of about 8000 base pairs in length, which is complexed by cellular histones and packaged in a protein coat (4). It is believed that human papillomaviruses (HPV) are capable of inducing hyperplastic, papillomatous, and verrucous squamous epithelial lesions in the skin and at various mucosal sites, including oral cavity (5).

More than 100 human papillomaviruses have been identified so far, some of which are carcinogenic, while the majority generally cause benign epithelial lesions (6). In the oral cavity, 24 types have been shown to be associated with benign lesions and at least 12 types with malignant lesions, among them HPV-16 and 18 play especially important roles in the development of OSCCs (7). Although HPVs have been implicated in the OSCC, but the field has been controversial. Many groups have identified HPV in patients with OSCC, but the results of case series and case control studies have not been consistent (3). The aim of the present study was to determine the presence of HPV-16 and 18 in four groups of oral lesions, including OSCC, using the PCR technique.

MATERIALS AND METHODS

Specimens

Sixty formalin-fixed and paraffin-embedded tissue samples that had been examined at the Department of Oral Pathology, School of Dentistry, Kerman University of Medical Sciences from 1995 to 2003, were used. Microscopic slides of all samples were reviewed by a second pathologist to confirm the histologic diagnosis. These specimens were obtained from patients with oral squamous cell carcinoma (OSCC), leukoplakia, oral lichen planus (OLP), or pyogenic granuloma (PG) (15 specimens in each group) and were chosen on a random basis. Table 1 shows the age and sex distribution of the patients in each group.

Extraction of DNA and PCR amplification

Extraction of DNA and PCR amplification was done at Department of Virology, Tehran University of Medical Sciences.

DNA was extracted from 5 μM paraffin sections, avoiding any cross contamination between samples (using separate disposable stuffs such as gloves, blade and tubes).

The first section of each specimens plus gloves and blade were discarded and new blade and gloves were used for main sectioning.

Sections were deparaffinized and placed in 200 μl of digestion buffer (50 mM Tris, PH 8.5, 1 mM EDTA, 0.5% Tween 20) containing 200 μg/ml proteinase K and incubated overnight at 37°C (8). The lysate was extracted twice with phenol: chloroform (1:1), and precipitated by ethanol. The DNA was dissolved in 100 μl TE buffer. The extracted DNA was stored at 4°C until to be tested. DNA quality was evaluated in all specimens by PCR using primers PCO3/PCO4 (PCO3: 5′ ACA CAA CTG TGT TCA CTA GC /PCO4: 5′ CAA CTT CAT CCA CGT TCA CC), that amplifies a 110 base pair product from human β-globin gene. HPV consensus primers, MY09 and MY11 which cover a broad spectrum of HPV types, were used in the PCR assay to amplify an approximately 450 bp fragment from the open reading frame L1 region (ORF) (9).

Table 1. Age and sex distribution of the study population

Group	Age Range (Year)	Mean Age	Sex Male	Female
Oral squamous cell carcinoma	27-74	51	11/15 (73.3%)	4/15 (26.7%)
Leukoplakia	31-67	45.9	12/15 (80%)	3/15 (20%)
Oral lichen planus	25-67	43.2	6/15 (40%)	9/15 (60%)
Pyogenic granuloma	4-65	32.2	7/15 (46.7%)	8/15 (53.3%)

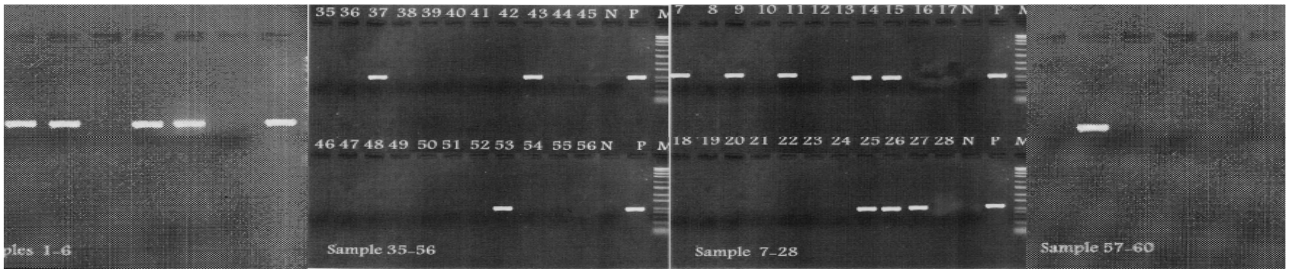

Fig. 1. Detection of 450 bp L1 PCR product, on 1.5% agarose gel electrophoresis.

The sequences of the primers are MY09: 5' CGT CCM ARR GGA WAC TGA TC 3' and MY11: 5' GCM CAG GGW CAT AAY AAT GG 3'. PCR amplification carried out in a DNA thermal cycler and involved an initial denaturation, followed by 38 cycles at 95°C for 60 sec, 55 °C for 60 sec, and 72°C for 120 sec with an additional final cycle at 95°C for 60 sec, 55°C for 60 sec, and 72 °C for 5 min. Each PCR experiment was run in parallel with a negative control and a DNA sample known to be positive for HPV. Extracted DNA from HeLa cell line was used as HPV positive control. No DNA was added for negative controls. The material of positive HPV samples was used for determination of HPV types, including HPV-16 and 18 among others, using PCR method. Specific primers were used at this stage. The sequences of primers used for detection of HPV-16 and 18 were HPV-16-T7: 5' TTA ATA CGA CTC ACT ATA GGG TCA TTT GTT GGG GTA ACC AA 3', HPV-16-2: 5' TAG GTC TGC AGA AAA CTT TTC 3', HPV-18-T7: 5' TTA ATA CGA CTC ACT ATA GGG TTT GTT GGG GCA ATC AGG TA 3' and HPV18-2: 5′ TAA GTC TAA AGA AAA CTT TTC 3′. PCR was done as described above.

Statistical analysis

The statistical analysis included X^2 and Fisher exact tests. *P* values < 0.05 were considered significant.

RESULTS

Sixty oral mucosal specimens with benign or malignant epithelial changes were analyzed for HPV DNA by the PCR.

L1 PCR showed that 9 (60%) of the 15 OSCC samples were positive for HPV DNA (Fig. 1). HPV DNA was identified in 26.7% of leukoplakia, 13.3% of OLP, and 6.7% of PG specimens as indicated by a 450 bp L1 PCR product, on 1.5% agarose gel electrophoresis (Tables 2 and 3). The frequency of HPV DNA detection in OSCC was significantly higher than leukoplakia, OLP, and PG ($P < 0.05$). The results for HPV DNA typing by PCR showed that 6 (40%) of the 15 OSCC specimens were positive for HPV-16 DNA and 3 (20%) of these samples were positive for HPV-18 DNA. HPV-16 DNA was found with the type specific primers in 13.3% (2/15) of leukoplakia. Neither HPV-16 DNA nor HPV-18 DNA was identified in OLP and PG specimens. Statistical analysis showed that the frequency of HPV-16 and 18 DNA detection in OSCC as compared with leukoplakia, OLP, and PG was significantly higher ($P < 0.05$). There was no statistically significant association between HPV status and gender, age, or tumor location in OSCC cases.

Table 2. HPV types in biopsy specimens of four different oral lesions

		HPV Types			
Group	**PCR+**	**6**	**11**	**16**	**18**
OSCC	9/15 (60%)	-	-	6 (40%)	3 (20%)
Leukoplakia	4/15 (26.7%)	-	2 (13.3%)	2 (13.3%)	-
OLP	2/15 (13.3%)	2 (13.3%)	-	-	-
PG	1/15 (6.7%)	-	1 (6.7%)	-	-

Abbreviations: OSCC, oral squamous cell carcinoma; OLP, oral lichen planus; PG, pyogenic granuloma.

Table 3. Clinical data and HPV type in the study population

No.	Histologic diagnosis	Age	Sex	Location	Detected HPV
1	OSCC	27	M	Gingiva	-
2	OSCC	58	M	Alveolar ridge	16
3	OSCC	68	M	Hard palate	16
4	OSCC	65	M	Tongue	-
5	OSCC	40	M	Buccal mucosa	18
6	OSCC	55	F	Gingiva	16
7	OSCC	73	M	Lip	18
8	OSCC	31	M	Gingiva	-
9	OSCC	35	M	Gingiva	16
10	OSCC	46	M	Lip	-
11	OSCC	53	F	Alveolar ridge	16
12	OSCC	52	F	Lip	-
13	OSCC	37	M	Buccal mucosa	-
14	OSCC	52	F	Alveolar ridge	16
15	OSCC	74	M	Lip	18
16	Non-dysplastic leukoplakia	67	F	Alveolar ridge	-
17	Non-dysplastic leukoplakia	31	M	Gingiva	-
18	Mild dysplastic leukoplakia	49	M	Buccal mucosa	-
19	Non-dysplastic leukoplakia	48	M	Buccal mucosa	-
20	Moderate dysplastic leukoplakia	31	M	Buccal mucosa	-
21	Non-dysplastic leukoplakia	57	M	Buccal mucosa	-
22	Non-dysplastic leukoplakia	44	M	Buccal mucosa	-
23	Non-dysplastic leukoplakia	48	M	Gingiva	-
24	Non-dysplastic leukoplakia	39	F	Buccal mucosa	-
25	Non-dysplastic leukoplakia	50	M	Gingiva	11
26	Non-dysplastic leukoplakia	35	F	Gingiva	16
27	Non-dysplastic leukoplakia	54	M	Buccal mucosa	16
28	Non-dysplastic leukoplakia	50	M	Mouth floor	-
29	Non-dysplastic leukoplakia	43	M	Buccal mucosa	-
30	Non-dysplastic leukoplakia	43	M	Buccal mucosa	11
31	Lichen planus	30	M	Buccal mucosa	-
32	Lichen planus	50	F	Gingiva	-
33	Lichen planus	39	M	Buccal mucosa	-
34	Lichen planus	36	M	Buccal mucosa	-
35	Lichen planus	28	M	Buccal mucosa	-
36	Lichen planus	39	F	Buccal mucosa	-
37	Lichen planus	48	F	Gingiva	6
38	Lichen planus	38	M	Buccal mucosa	-
39	Lichen planus	67	F	Buccal mucosa	-
40	Lichen planus	49	F	Buccal mucosa	-
41	Lichen planus	37	F	Buccal mucosa	-
42	Lichen planus with dysplasia	60	F	Buccal mucosa	-
43	Lichen planus	43	F	Buccal mucosa	6
44	Lichen planus	25	M	Buccal mucosa	-
45	Lichen planus	60	F	Buccal mucosa	-
46	Pyogenic granuloma	17	F	Gingiva	-
47	Pyogenic granuloma	19	F	Gingiva	-
48	Pyogenic granuloma	15	M	Lip	-
49	Pyogenic granuloma	64	M	Gingiva	-
50	Pyogenic granuloma	50	F	Tongue	-
51	Pyogenic granuloma	65	F	Gingiva	-
52	Pyogenic granuloma	35	M	Gingiva	-
53	Pyogenic granuloma	47	F	Gingiva	-
54	Pyogenic granuloma	32	F	Gingiva	-
55	Pyogenic granuloma	9	F	Gingiva	-
56	Pyogenic granuloma	15	M	Gingiva	-
57	Pyogenic granuloma	50	M	Gingiva	-
58	Pyogenic granuloma	4	M	Gingiva	11
59	Pyogenic granuloma	53	F	Gingiva	-
60	Pyogenic granuloma	9	M	Gingiva	-

Abbreviations: OSCC, oral squamous cell carcinoma; M, male; F, female.

DISCUSSION

Many HPVs are accepted to be involved in carcinogenesis. These viruses have been identified in various human epithelial neoplasms, including malignant and premalignant lesions of uterine cervix, vulva, penis, conjunctiva, and upper aerodigestive tract (10-12).

Human papillomaviruses have the ability to immortalize or transform human fibroblasts and keratinocytes *in vitro* (13). It is believed that HPVs may promote cellular transformation through inactivation of pRb and p53 tumor suppressor functions and transactivation of a family of serine/threonine protein kinase cyclin-dependent kinase (CDK) complexes that control cell division (4). HPV DNAs have been detected in OSCC by numerous investigators (3). However, it is unclear whether the virus is casually associated with OSCC. In the present study, we compared the frequency of HPV DNA detection in OSCC with three groups of oral lesions: 1) leukoplakia, the most common oral premalignant lesion, 2) oral lichen planus, a common chronic mucocutaneous disease with a questionable or low potential for malignant transformation, and 3) pyogenic granuloma, a reactive hyperplastic lesion that basically originates from the cellular elements of the connective tissue with a nearly normal epithelial covering.

We analyzed 60 oral tissue specimens to detect HPV DNA by the PCR. Human papillomavirus was present in 60% of OSCC samples. The prevalence of HPV DNA in OSCC was significantly higher than leukoplakia (26.7%), OLP (13.3%), and PG (6.7%). In our study, the frequency of HPV-16 (40%) and HPV-18 (20%) infection in OSCC was significantly higher when compared with leukoplakia, OLP, and PG. The percentage of HPV DNA-positive OSCC in the present study is consistent with findings of other investigators who identified HPV DNA in 46.7% to 66.7% of OSCCs (14-16). However, the rate of HPV detection in OSCC has varied widely in reports from 0 to 94% (3). The inconsistency in results may be explained by small sample size in some studies, and the presence of HPV DNA below the sensitivity of the assay. Recently, data from 94 reports (overall 4680 OSCC specimens) were included in meta-analysis (17). Based on this study, the frequency of HPV DNA detection in OSCC, benign leukoplakia, and normal oral mucosa is 46.5%, 22.2%, and 10%, respectively and the difference is statistically significant. The probability of detecting high-risk HPVs in OSCCs was 2.8 times greater than that of low-risk HPVs in this study.

The results of the present study show a successive increase of detection rate of HPV-16 and 18 DNAs from low level in pyogenic granuloma and non-premalignant or questionably premalignant lesions of OLP to premalignant leukoplakia and to OSCC. This finding is consistent with the results of a recent study in which HPV DNA has been identified in 43.2% of OSCC, 22.2% of leukoplakia, and 15.4% of lichen planus cases (18).

In this study, the prevalence of HPV-16 (40%) infection in OSSC samples was higher than HPV-18 (20%). This finding is in agreement with studies by other investigators (19, 20) and shows that HPV-18 plays a less significant role in the development of OSCC. In our study, there was no significant correlation between presence of HPV DNA and sites, gender, or age in the OSCC cases, probably because of the small number of our cases. Similar findings have been reported by some investigators (16, 19). However, Paz *et al.* in their study on 167 head and neck SCC found that HPV-associated SCC had site specificity with the viral DNA frequently found in tumors in Waldeyer's tonsillar ring (12). Also, a significant association between age and presence of HPVs in OSCC has been shown by Cruz *et al.* (21). The prevalence of HPVs in patients older than 60 years (29.4%) was lower than that in patients below this age (77.8%).

In summary, the present study confirms frequent finding of HPV DNA in OSCC and a significant association between "high-risk" HPV-16 and 18 and OSCC as compared with leukoplakia, oral lichen planus, and pyogenic granuloma.

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Conflict of interests

We have no conflict of interests.

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