RESEARCH COMMUNICATION

Detection of Human Papillomavirus in Normal Oral Cavity in a Group of Pakistani Subjects using Real-Time PCR

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Abstract

Since there is evidence that human papillomavirus (HPV) may play some role in oral carcinogenesis, we investigated the presence of HPV in a group of Pakistani subjects with normal oral cavity using real-time PCR analysis. Two-hundred patients attending the Dental Department, Sandaman Provincial Hospital, Balochistan, Pakistan, were recruited. After interview, oral epithelial cells were collected by scraping and subjected to DNA extraction. The HPV-positive DNA samples were further analyzed using primer sets specific for HPV-16 and -18. It was found that out of 200 DNA samples, 192 were PCR-positive for the *β-globin* gene and these were subsequently examined for the presence of HPV DNA. Among these, 47 (24.5%) were HPV-positive with the virus copy number ranged between 0.43-32 copies per 1 *μ*g of total DNA (9-99 copies per PCR reaction). There were 4 and 11 samples containing HPV-16 and -18, respectively. Additionally, one sample harbored both types of HPV. Among the investigated clinical parameters, smoking habit was associated with the presence of HPV (p = 0.001) while others indicated no significant association. The prevalence of HPV in normal oral cavity in our Pakistani subjects appears to be comparable to other studies. However, the association between the presence of HPV and smoking warrants further investigations whether both of these factors can cooperate in inducing oral cancer in this group of patients.

Keywords: Human papilllomavirus - oral epithelial cell - normal oral cavity - real-time PCR - Pakistani

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Introduction

Oral cancer is ranked as the eighth common cancer worldwide with epidemiologic variations and the third common in the South-Central Asia (Moore et al., 2009). Pakistan, a republic in South Central Asia, shares international geographical boundaries and cultural similarities with India, Iran, Afghanistan, China and the Soviet Central Asian Republics. This cross-cultural heritage is reflected in the occurrence of cancers in the country. In some areas of Pakistan, oral cancer was ranked the second most common cancer of all malignancies in both male and female with the highest incidence among oral cancers in the world (Moore et al., 2009). The age-standardized incidence rates per 100,000 population (ASIRs) for oral cancer reported in Karachi, a large city in Pakistan, were 17.1 and 16.5 in males and females, respectively (Bhurgri et al., 2003). This high incidence of oral cancer is identical to the high risk geographical zones of India, suggesting that life-style characteristics retained by the migrants are one of the major determinants of the disease.

While the oral squamous cell carcinoma (OSCC) has been found to be associated with tobacco, alcohol abuse and betel nut chewing habit, recent reports suggest that human papillomavirus (HPV) may also contribute to the etiology of this malignant neoplasm. HPV is a small DNA tumor virus which plays an important role in a variety of proliferative diseases. More than 100 HPV types have been identified and categorized into low-risk and high-risk groups according to their association with malignancies (Chang et al., 1991). HPV-16 and -18 are the predominant high-risk types associated with several malignancies including cervical and oral cancers. They commonly affect the younger age-groups, with a predisposition for infection with these types in males (Jayaprakash et al., 2011).

In oral cavity, the detection rate of HPV varies considerably among the published data ranging from 0-100%, depending upon the status of the disease, tissue origin, detection techniques, and other environmental risk factors such as geographic regions (Esquenazi et al., 2010). HPV-16 was detected more often in the normal oral cavity than HPV-18 (Kojima et al., 2003). Because in some studies, the prevalence of HPV in normal oral cavity was lower than in oral dysplastic and cancerous lesions, HPV is regarded as a risk factor in oral carcinogenesis. For

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example, in one study, HPV was identified with increasing frequency: 13.5% in normal oral mucosa, 14.8% in benign leukoplakia, 18.5% in intraepithelial neoplasia, 26.2% in squamous cell carcinoma, and 27.0% in verrucous carcinoma (Miller & White, 1996). This result suggests that HPV may play some role in oral carcinogenesis.

As viral replication often precedes the development of virus-associated pathology, the feasibility to identify HPV in persons 'at risk' for HPV-associated diseases may be an informative prognostic indicator. Not only low-risk types of HPV impact negatively on quality of life but the causal relationship between high-risk types of HPV and malignancy also indicates the significance of early detection when it is suspected that a lesion may harbor such HPV. To date, there have been no reports demonstrating the presence of HPV in normal oral mucosa of Pakistanis. Therefore, we set out this cross sectional study to determine the prevalence and types of HPV, especially HPV-16 and HPV-18, in normal oral epithelial cells and examine its association with risk factors of oral cancer. The fundamental data may instigate a better HPV prevention and treatment program in Pakistan and may be helpful for the decision to treat the lesion before it progresses to a more severe form of disease.

Materials and Methods

Ethical consideration

The study protocol was revised and approved by the committee on human rights to human experimentation, Mahidol University, Thailand (MU-IRB 2010/115.2004). The specimen collection was performed under the permission of the head of the Dental Department, Sandeman Provincial Hospital. All details about the volunteers and their identity were anonymous. Each subject was given both verbal and written information about the nature of the study and a written informed consent was received. All individuals were allowed to depart the study at any time during the experiments.

Study population

A group of 200 subjects attending the Dental Department, Sandeman Provincial Hospital, Quetta, Balochistan, Pakistan were recruited. These subjects were between 18-60 years old and willing to provide informed consent. They had no serious systemic diseases, such as leukemia, hemophilia, uncontrolled diabetes mellitus or serious cardiac problems. Demographic parameters including age, gender, smoking and betel quid chewing habit were recorded. According to the Islamic laws, alcohol consumption was not investigated in this study.

Specimen collection and DNA preparation

Oral epithelial cells were collected by a soft-bristled toothbrush from buccal mucosa, hard palate and tongue, and suspended in 4 ml of lysis buffer containing 0.5% sodium dodecyl sulfate, 1 mM EDTA, and 10 mM Tris. DNA from each sample was prepared using the QIAamp DNA Mini Kit (Qiagen®, Hilden, Germany) according to the manufacturer's protocol. The extracted DNA was eluted in 200 µl of the eluting buffer (provided by the kit) and kept at -20°C until further use. The concentration and purity of the prepared DNA were spectrophotometrically measured at 260 nm and 280 nm (GENESYS 10uv, model 10-S, MA, USA). HeLa cells were kindly provided by Assistant Professor Kongthawat Chairatvit, Faculty of Dentistry as well as Associate Professor Songsak Petmitr, Department of Tropical Nutrition and Food Science, Faculty of Tropical Medicine, Mahidol University, Thailand. DNA from the HeLa cells was similarly prepared as the oral epithelial cells and employed as the HPV-18 positive control. Additionally, SiHa genomic DNA, which was employed as the HPV-16 positive control, was kindly provided by Associate Professor Mathurose Ponglikitmonkol, Faculty of Science, Mahidol University, Thailand.

Real-time PCR

To investigate the presence and quantity of HPV, a real-time PCR was performed in duplicate using the KAPA SYBR FAST qPCR Kit (Kapabiosystems®, MA, U.S.A) with the primers shown in Table 1. The β-globin primers, KM 29 and KM 138, were employed to confirm successful DNA extraction from each sample. All β-globin-positive samples were subjected to real-time PCR using the HPV1003 and HPV1004 primers, which were reported to detect the L1 region of HPV-1, -6, -8, -11, -13, -16, -18, -30, -31, -32 and -33 (Kojima et al., 2003) (Table 1). Since the copy number of HPV-18 in each HeLa cell was approximately 40 copies per genome, serial 10-fold dilutions from $1x10^5$ viral copies/μl were employed to enumerate the copy numbers of HPV in

Table 1. List of Primers used in this Study

Primer	Target region	Thermocycling	Sequence	Size
HPV 1003	L1 HPV region	50X (95°C (3s), 53.9°C (30s))	TTTGTTACTGTGGTAGATA	150 bp
HPV 1004			GAAAAATAAACTGTAAATC	
KM 29	β-globin region	50X (95°C (3s), 56.3°C (30s))	GGTTGGCCAATCTACTCCCAGG	262 bp
KM138			TGGTCTCCTTAAACCTGTCTTG	
HPV 16-outer F	E6 HPV region	35X (95°C (30s), 53°C (30s), 72°C (30s))	ATGACTTTGCTTTTCGGGAT	223 bp
HPV 16-outer R			CTTTGCTTTTCTTCAGGACA	
HPV 16-inner F	E6 HPV region	35X (95°C (30s), 57°C (30s), 72°C (30s))	GAGATGGGAATCCATATGCTG	125 bp
HPV 16-inner R			CAACGGTTTGTTGTATTGCTG	
HPV 18-outer F	E7 HPV region	35X (95°C (30s), 61°C (30s), 72°C (30s))	TGAAATTCCGGTTGACCTTC	191 bp
HPV 18-outer R			GGTCGTCTGCTGAGCTTTCT	
HPV 18-inner F	E7 HPV region	35X (95°C (30s), 62°C (30s), 72°C (30s))	ATGTCACGAGCAATTAAGC	137 bp
HPV 18-inner R			TTCTGGCTTCACACTTACAACA	

the samples. Each real-time PCR mixture was prepared in a total volume of 10 μl containing 0.8 μl of each 2.5 μM of either primer pairs, 5 μl of 2X Master mix, 1 μl of investigated DNA and 2.4 μl of sterile distilled water. The real-time PCR products were verified using 1.5 % agarose gel electrophoresis at 80 volt for 30 min. The gel was stained with 0.5 μg/ml ethidium bromide and analyzed under the UV transilluminator (Geldoc, Bio-Rad®, CA, USA).

Nested PCR analysis

Nested PCR was employed to detect HPV-16 and -18 in the HPV positive samples using the HPV-16 and HPV-18 inner and outer primers (Table 1). The employed primers showed no cross-reactivity with other sequences of other organisms in the GenBank using the BLAST search. The PCR mixture was prepared in a total volume of 10 μl. PCR products from the first PCR analysis using outer primers were employed as templates for the second amplification using inner primers. For HPV-16 amplification, the PCR mixture contained 1 μl of each 2.5 μM primer, 2 μl of 5X Green GoTaq™ reaction buffer (Promega®, WI, USA), 1 μl of 25 mM $MgCl_2$, 1 μl of the investigated DNA, 0.2 μl of 10 mM dNTPs, 1 unit of *i*-Taq® DNA polymerase (iNtRON Biotechnology, Gyeonggi-do, Korea) and sterile distilled water to the total volume of 10 μl. Preparation of the PCR mixture for the detection of HPV-18 was similar to that of HPV-16, except that 0.2 μl, instead of 1 μl, of 25 mM $MgCl_2$ was used.

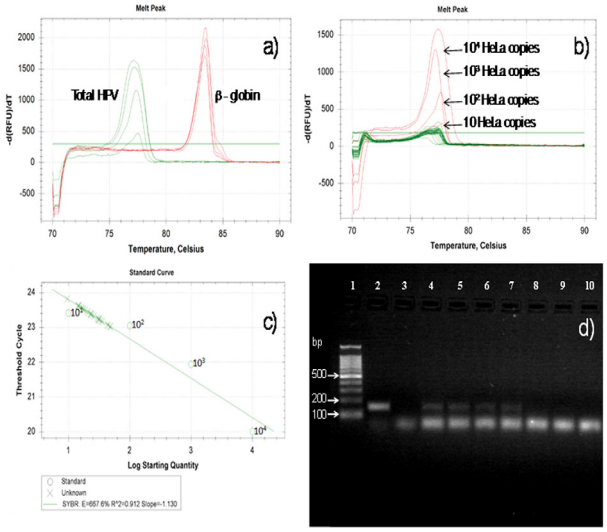

Figure 1. Enumeration of HPV from Clinical Samples by the Real Time PCR. a) Bio-Rad Precision Melt Analysis of total HPV and β-globin PCR products. The green and the red lines represent the derivative melt curve plots of total HPV and β-globin, respectively. In the negative first derivative plot, the melting temperatures of the amplicons were at the inflection point of the plot or the melt peak. b) Bio-Rad Precision Melt Analysis of total HPV PCR products. The pink and the green lines represented the melt peak of the amplicons from the serial ten-fold dilutions from the 10^4 HeLa copies and some investigated samples, respectively. c) Log copy numbers of total HPV versus threshold c(t) value of some investigated samples. The correlation coefficient was 0.912. Standards = O, Samples = X. d) Agarose gel electrophoresis of the PCR products amplified by the HPV 1003 and HPV 1004 primers. The expected amplicon size was 150 basepairs. Lane 1, 100-bp molecular marker; Lane 2, The HeLa DNA (positive control); Lane 3, Sterile distilled water (negative control); Lanes 4-7, HPV positive samples and Lanes 8-10, HPV negative samples.

Table 2. Association between the Presence of HPV and Investigated Clinical Parameters by the Pearson χ^2 Test

Variable	Categories	HPV+ (%)	HPV- (%)	Total	P value
Age:	16–19	0 (00.0)	6 (100)	6	0.227
	20–29	9 (16.1)	44 (83.9)	53	
	30–39	16 (26.3)	45 (73.7)	61	
	40–49	12 (29.2)	29 (70.8)	41	
	50–59	10 (35.7)	18 (64.3)	28	
	60–69	0 (00.0)	3 (100)	3	
Sex:	Male	33 (27.5)	87 (72.5)	120	0.279
	Female	14 (19.5)	58 (80.5)	72	
Smoking:					
	Non-smoker	14 (13.7)	88 (86.2)	102	0.001
	Former smoker	20 (37.7)	33 (62.3)	53	
	Current smoker	13 (35.1)	24 (64.9)	37	
Betel quid chewing;					
	Non chewer	33 (21.9)	118 (78.1)	151	0.266
	Former chewer	8 (34.8)	15 (65.2)	23	
	Current chewer	6 (33.3)	12 (66.7)	18	

Data analysis

Statistical analysis was performed using the SPSS version 18.0 (SPSS Inc., Chicago, IL, USA; licensed for Mahidol University). The distribution of socio-demographic factors was demonstrated using the descriptive statistics such as number, percentages and means. The Pearson χ^2 test was employed to determine an association between the independent variables and HPV. The p-value of less than 0.05 was considered statistically significant. The melting curves were normalized using each appropriate temperature ranges for all types of HPV prior to background fluorescence subtraction.

Results

Patient characteristics

The characteristics of the patients in this study are presented in Table 2. The average age of the patients was 40 years old and the majority of the patients were male. One hundred and two subjects (53.1%) were non-smokers, while 53 (27.6 %) were former smokers and 37 (19.3%) were current smokers. For betel quid chewing habit, 151 (78.6%) volunteers were non-chewers whereas 23 (12%) were former chewers and 18 (9.4%) were current chewers. In this study, alcohol drinking was not determined as it is prohibited according to the Islamic laws.

Enumeration of viral copy number by real-time PCR

Of all 200 samples, 192 samples were positive for β-globin and therefore used for the detection of HPV DNA. Using HeLa DNA as a positive control, Figure 1a showed the melt curve analysis of the HPV and the β-globin real-time PCR products. Real-time amplification from the DNA samples revealed that out of 192 samples, 47 (24.5%) samples were positive for HPV. Viral load in each sample was estimated from a plot of log quantity versus threshold cycle or c(t) of the serial dilutions (Figure 1b and c). The copy number of HPV ranged from 9-99 viral

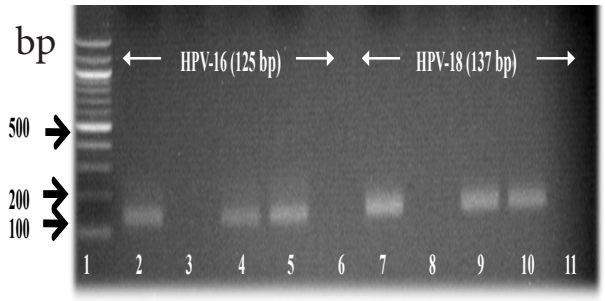

Figure 2. Gel Electrophoresis of HPV-16 and -18 PCR Products from Nested PCR Analysis. Lane 1 represents 100-bp DNA ladder. Lanes 2-6 represent the detection of HPV-16 using nested PCR; lane 2, positive control using SiHa DNA; lane 3, negative control using sterile distilled water; lanes 4 and 5, positive samples; lane 6, negative sample. Lanes 7-11 represent the detection of HPV-18 using nested PCR; lane 7, positive control using HeLa DNA; lane 8, negative control using sterile distilled water; lanes 9 and 10, positive samples and lane 11, negative sample.

copies per reaction with the average of 24.5 copies per reaction. This translated to the viral copy number of 0.43-32 copies (average 6.1 copies) per μg of genomic DNA. By agarose gel electrophoresis, the 150-bp amplicons were obtained from all 47 HPV real-time PCR positive samples. Figure 1d showed the 150-bp positive PCR products from the positive control and the four positive samples, which were derived from the PCR using the HPV1003 and HPV1004 primers.

Detection of HPV-16 and -18

Since the presence of HPV-16 and/or -18 is important in oral carcinogenesis, the presence of these 2 types of HPV was further determined using nested PCR analysis. Of all 47 HPV-positive samples, 4 samples contained HPV-16 whereas 11 samples carried HPV-18 (Figure 2). Among these, one sample harbored both types of HPV.

Presence of HPV-16 and -18 and associated risk factors of oral cancer

HPV was more prevalent in current smokers combined with former smokers than in non-smokers (p=0.001). Using the Pearson χ^2 test, we found that smoking was significantly associated with the presence of HPV. In addition, out of 4 HPV-16 positive samples, one subject was a former smoker. Out of 11 HPV-18 positive samples, 3 subjects were current smokers and 3 subjects were former smokers.

No association between other investigated variables and the presence of HPV was found (Table 2).

Discussion

Oral cavity is a reservoir for HPV, which has been implicated in the pathogenesis of several diseases. In healthy individuals, HPV may be present at a low level, but it is capable of amplification in appropriate environments especially when the host defense deteriorates. Since the presence of HPV is associated with oral carcinogenesis and the high incidence of oral cancer in Pakistanis has been reported, we investigated the prevalence of HPV and its relation to risk factors for oral cancers in 200 volunteers with normal oral cavity who attended the dental clinic in Quetta, Bolochistan, Pakistan.

The presence of HPV in normal oral cavity has been reported in several studies and the prevalence varies considerably. For example, using PCR analysis, no HPV was found in a study of 100 young volunteers from Brazil (Esquenazi et al., 2010), whereas a high prevalence of HPV in the normal oral cavity (81%) was found in Japanese adults (Terai et al., 1999). The prevalence rate of HPV in normal oral cavity in our study (24.5%) is comparable to other studies in that approximately 25% of the subjects were positive for HPV, using PCR analysis (Miller & White, 1996; Sosorbaram et al., 2006).

Several methods can be used to detect HPV and different techniques with varying sensitivity and specificity could potentially influence the detection rate of HPV. In situ hybridization, immunohistochemistry and Southern blotting were utilized in the early period of HPV detection and were less sensitive than PCR (Yeudall & Campo, 1991; Frazer et al., 1993; Kay et al., 2002; Boy et al., 2006). In our study, quantitative PCR was used for the detection of HPV which is sensitive and could detect as low as 9 viral copies per reaction. Using quantitative PCR, a high prevalence of HPV has been reported in the normal oral cavity of adults. HPV DNA was detected in 30 of 37 (81.1%) specimens and their copy numbers per cell were 10-, 10-4 and 10-3 (Terai et al., 1999). Multiple HPV types were present in 17 of 30 (56.7%) specimens and 2 specimens contained HPV-16, -18 and other types. Using real-time PCR analysis, Szarka et al. demonstrated that the HPV prevalence increased gradually with the severity of the lesions (32.8, 40.9 and 47.7% in oral lichen planus (OLP), oral leukoplakia (OL) and OSCC, respectively) (Szarka et al., 2009). Similarly, the average HPV copy numbers by the real-time PCR were 5.2 X 10^2 (range: 10-840), 6.8 X 10^3 (range 10-51,000), 7.2 X 10^3 (range 10-27,000) and 2.4 X 10^5 (range 90-130,000) per μg of total DNA in oral controls, OLP, OL and OSCC patients, respectively (18). In comparison with our study, the study by Szarka et al. demonstrated higher viral copy number in normal control (5.2 X 10^2 copy per μg of total DNA) compared to our study (6.1 copy per μg of total DNA).

The low HPV copy numbers detected in normal oral mucosa found in this study suggested that persistent or transient HPV infection of the mucosa of the oral cavity occurs frequently. Another reason for the low copy of HPV may arise from the collection method of the sample. Although scrapes were used and samples were kept refrigerated to allow for better detection of HPV DNA (Hoffmann et al., 1998) this method extracts only superficial epithelial cells that are infected in subclinical or clinical infection. Thus, no viruses from latent infection in the basal or suprabasal layer of cells were removed. The low copy number in our study is less likely due to the poor DNA quality since all of the β-globin- positive samples were used. Thus the integrity of the DNA should not contribute to this low copy number. The low HPV loads in normal epithelial cells in this study suggested that oral mucosa may be a suitable reservoir for such virus. Although there is no strong evidence that the number

of HPV is involved in the progression of the disease, previous reports about the linkage between HPV and the development of oral cancer and viral amplification under an appropriate environment may remind us that it would be important to identify potential factors that can induce such transformation (Swan et al., 1999).

According to further real-time PCR analysis of HPV-16 and -18, the prevalence of HPV-16 positive samples was lower than that of the HPV-18 positive ones in this study. This is in contrast with the reports in subjects with oral cancer, suggesting that there may be other factors that favor HPV-16 survival in such environment (Smith et al., 2004). Both HPV-16 and HPV-18 were found in one positive sample. A few reports also demonstrated that more than one type of HPV was detected in the oral cavity (Terai et al., 1999; Sosorbaram et al., 2006). Taken together, our data suggested that the nature of HPV infection may resemble other types of infection in that more than one strains of the same organism are able to reside in the oral cavity.

The prevalence of HPV in this study was higher in male than female. This is in agreement with the findings of previous studies as male are exposed to more HPV transmission risk factors (Reiter et al., 2010). It is still unclear whether the presence of HPV is age-dependent. Our data revealed the HPV incidence increased with age between 20-59 years old. This may partly result from race and geographical region. Moreover, variations in tobacco and betel nut chewing habit have a strong impact on the highly variable prevalence of cancers of the mouth and oropharynx around the world and HPV seems to contribute to the risks related to tobacco and betel nut chewing. Our data were similar to the previous findings in that low prevalence of HPV was found in patients with history of betel nut chewing habit (Khovidhunkit et al., 2008). The numbers of HPV positive samples were higher in the non-chewer than the chewer group and there was no statistical significance in the influence of betel quid chewing on the presence of HPV.

In this study, 72.8% of the HPV positive patients were accustomed to smoking. Smoking and HPV are both distinct risk factors for head and neck cancer. However, the interaction between these 2 risk factors in the development of head and neck SCC (HNSCC) remains inconclusive. Some authors have observed no association, whereas other studies demonstrated an additive or synergistic association. According to a literature review, 3 categories of this association could be obtained (Sinha et al., 2012). The first ones included studies with evidence that HPV is associated with an increased risk of HNSCC in non-smokers. The second included studies with no difference in HPV-related HNSCC prevalence between smokers and non-smokers. The last ones included the studies with evidence of and additive or synergistic effect between smoking and HPV-related HNSCC.

It has been postulated that smoking may suppress the immune response thus facilitating persistence of HPV infection. This step is important for the development of HPV-related cancers. HPV E6 and E7 could inactivate 2 tumor suppressor proteins namely, p53 and pRB, respectively (Münger et al., 1992). Hence in a person with HPV infection, the 2 tumor suppressor pathways which are crucial for the inhibition of carcinogenesis are compromised. Smoking, in addition, could induce DNA damages which may further induce aberrations of the cells and, in the absence of functional p53 and pRB, may lead to carcinogenesis. It has also been suggested that the carcinogenic potential of HPV increases with viral integration with host DNA (Spence et al., 2005). The process of integration occurs at fragile sites or hot spots of DNA breakage, and there is evidence that tobacco smoking induces DNA breaks in human cells (Luo et al., 2004). Hence, an increased frequency of HPV integration in smokers may increase the risk of carcinogenesis in the presence of HPV infection. Taken together, it is logical to believe that HPV and smoking may have a synergistic effect on oral carcinogenesis in a particular group of patients. In our study, HPV-16 and -18 were presence in 14 subjects. Of these, 7 were accustomed to smoking. HPV-16 was observed in a former smoker and HPV-18 was revealed in 3 former and 3 current smokers. Stringent follow-up should be considered in this group of patients with HPV-positive in addition to smoking habit.

In summary, using the real-time PCR, the prevalence of HPV in normal oral epithelial cells in Pakistani subjects was 25%, and the prevalence rates of HPV-16 and HPV-18 were 2% and 6%, respectively. The copy numbers of HPV were comparatively low implying subclinical or latent HPV infection. More than one type of HPV were simultaneously detected in normal oral cavity. Additionally, smoking habit appeared to be related to the presence of HPV. Therefore, stringent surveillance for oral cancer should be considered in this high-risk group of HPV-positive smokers.

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