



The Role of Human Papilloma Virus in the Development of Oral Squamous Cell Carcinoma in Pakistani Population



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Abstract

Oral cancer is one of the most prevalent cancers in Pakistan and the Pakistani population seems to be especially susceptible to the development of oral cancer. Human Papilloma virus has already been implicated in the etiology of cervical cancer, and strong evidence suggests its close association with the oral cancer. The aim of our study was to define the role of Human Papilloma virus in the development of oral squamous cell carcinoma patients in Pakistani population. Ninety-five patients with oral squamous cell carcinoma samples were recruited for this study. HPV infectivity was determined histopathological by observing Koilocytosis and molecularly by conducting PCR using HPV consensus primers. HPV genotyping was then carried out using the HPV genotyping PCR assay technique. The results of HPV prevalence and genotyping were evaluated in relation to various clinico-pathological parameters Statistical Package for the utilizing social sciences version 12.0 for windows (SPSS Inc, Chicago, IL). HPV was detected in 78% cases, out of whom 74% were associated with HPV16, 4% with HPV 18, 15% were co infected with HPV 16 and 18, and 7% were positive for HPV by the general primer and could not be type specified. This study finds a significant association between type 16 HPV prevalence and oral squamous cell carcinoma with male patients showing high co-relations.

Keywords: Oral cancer; Human papilloma virus HPV; Genotyping; Polymerase chain reaction PCR; Oral squamous cell carcinoma OSCC; Histopathology; Koilocytes; Oncogenic virus

Abbreviations: GP: General Primer for L1 HPV; TS: Type-Specific primers for L1 HPV; BG: primers for β -Globin gene; BP: Base Pairs

Introduction

Pakistan is one of the high-risk countries for oral cancer development, where oral cancer is the most prevalent cancer in men and may account for up to 30% of all new cases of cancer per year, compared to 3% in the UK and 6% in France [1]. Many known etiological factors for oral cancer development, like Pan, Betel Nut, Areca nuts and smoking are very commonly used in Pakistan, however not all the people exposed to these factors develop cancer. Furthermore, many patients not exposed to any of these factors develop cancer. This indicates the presence of additional risk factors, including Human Papilloma Virus that can make these people more susceptible to cancer.

More recently research has associated certain genotypes of HPV to the Papillary SCC of the Head and Neck [2], to OSCC [3], to the upper aerodigestive tract tumours [4,5], not to mention malignant and pre-malignant lesions of the uterine cervix [6], ulva [7], penis [8], and conjunctiva [9]. However, the exact mechanism of the interaction of HPV and the significance of its presence in the development of carcinogenesis is still not clear. Discrepancies in the prevalence of HPV in OSCC have been observed, geographically, in the more recent studies, creating further confusion. Certain

studies investigating role of HPV in PSCC, a subset of SCC have reported prevalence of transcriptionally active HPV in almost 54% of cases², while some others report a failure of PCR studies to show transcriptionally active HPV [10].

The aim of this study was to detect the prevalence of HPV in the OSCC patients of the Pakistani origin and to determine the most prevalent genotype of HPV associated with OSCC in Pakistani patients.

Methods and Materials

FFPE tissue samples of oral squamous cell carcinoma

This study is based on samples retrospectively collected from 72 different oral cancer patients diagnosed with oral squamous cell carcinoma OSCC during the period of 2005-2010. Inclusion criteria were complete clinicopathologic data and availability of enough paraffin- embedded tumour material. The clinico-pathological data consisted of age, gender, geographical location of the patient, site of lesion and histopathological grade. OSCC samples were formalin fixed paraffin embedded. A total of 72 blocks of paraffin-embedded tissue including samples diagnosed

as oral squamous cell carcinoma stages I, II and III, verrucous carcinoma, focal dysplasias, and fibroepithelial hyperplasias were retrieved from the archive of Pakistan Institute of Medical sciences (PIMS), and KRL hospital Islamabad, from year 2005 to 2010. Samples were taken from different lesion sites including maxilla, mandible, tongue, floor of the mouth, labial and buccal mucosa, naso-pharynx and vocal cords.

Fresh tissue samples of oral squamous cell carcinoma

The second group of samples consisted of 23 fresh samples in which around 6 were fresh unfixed biopsy specimen while 17 were fresh endoscopic samples obtained from ENT department CMH, Rawalpindi. The samples collected were stored at 4 °C in Diagnostic Laboratory, NCVI, NUST, Islamabad until they were ready for further processing.

Histopathological examination

Representative tissue sections of 3 µm thickness were cut on a microtome and the slides were kept at 60°C for 30 minutes. Sections were stained with Harri’s Haematoxylin and observed under the microscope. Histopathological diagnosis was confirmed and histopathological evaluation for staging was carried out.

Genomic DNA extraction

Genomic DNA Extraction from Formalin Fixed Paraffin Embedded (FFPE) Tissue Biopsies: Using a standard microtome with disposable blades, sections of 20µm thicknesses was obtained from the block with the microtome blade properly cleaned in between every sample to avoid contamination. Each of the sections was placed in a 1.5ml eppendorf tube. Blocks with the similar amount of tissue and the same section thickness were used

for all DNA isolation methods. DNA was extracted from formalin-fixed paraffin blocks by Xylene-ethanol method. Cut section from each case was deparaffinized and DNA was extracted by adding one ml of xylene to each 20µm section. Tubes were agitated for 30 min at room temperature and spun at 13000rpm for 10min. The supernatant was removed carefully, one ml fresher xylene was added, and the step repeated. Two identical washes with 100% ethanol were performed followed by spinning at 13000rpm for 10 minutes. Tissue pellets were air dried at 37°C. Tissue digestion was done by adding 400µl of the tissue digestion buffer (Tris-Cl (100mM, pH = 7.5), Tween-20 (0.05%)) to each tube containing tissue pellet. Then, 20µl of 500µg/ml of Proteinase K (Invitrogen) was added, agitated, and tubes were left overnight at 56 °C in water-bath. Digestion buffer was inactivated by heating for 10 minutes in boiling water and the cell debris was spun down. The supernatant containing the DNA was transferred to a new DNA tube and was used directly in PCR. The extracted DNA was frozen at -20 °C until use.

Genomic DNA extraction from fresh biopsy/ endoscopy tissue samples: Fresh biopsy specimen was homogenized in glass homogenizer. Homogenized tissue was then placed in 1.5ml eppendorf tube to which 400µl of digestion buffer [Tris- Cl (100 mM, pH = 7.5), Tween-20 (0.05%)] was added. Then 5µl of 20µg/µl Proteinase K (Invitrogen) was added to it, and tubes left to agitate overnight at 56 °C in a water-bath. Digestion buffer was inactivated by heating for 10 minutes in boiling water and the cell debris was spun down. The supernatant containing the DNA was transferred to a new DNA tube and was used directly in PCR. The extracted DNA was frozen at -20 °C until use.

Testing for DNA quality and quantity

Table 1: Primers used for PCR amplification.

Sequence	Target Gene	Annealing temperature (°C)	Amplimer Length(bp)	
BG-F	GTGCTCGGTGCCTTTAGTGA	β-globin	56	130
BG-R	CAGGGTGAGTCTATGGGACG			
GP5	TTTGTACTGTGGTAGATAC	L1	45	155
GP6	GAAAAATAAAGTAAATCA			
TS16F	GGTCGGTGGACCGGTCGATG	E6	54	96
TS16R	GCAATGTAGGTGTATCTCCA			
TS18F	CCTTGGACGTAAATTTTGG	L1 type18	58	160
TS18R	CACGCACACGCTTGGCAGGT			

DNA concentration was measured by spectrophotometer (Eppendorf Biophotometer plus, Germany). Optical density was measured at 260 and 280nm. The ratio of absorbance at 260/280 was used to determine quality of DNA. A ratio above 1.0 was unacceptable range. Successful DNA extraction was assessed by PCR amplification of a 130bp fragment of the β-globin gene. Sequences of primers are given in Table 1.

HPV detection PCR

HPV detection PCR was performed using primers published elsewhere (Baay MF, et al, 1996). The sequence for the primers

GP5 and GP 6 is given in the Table 1 along with the length of respective amplimer and target gene. Polymerase chain reaction round was performed in 0.2ml tubes (Axygen , USA) with 5µl of sample DNA dilution in 25µl of reaction mixture, using 0.4µl of MBI Fermentas, UK Taq polymerase (2U/µl), 2.4µl MgCl2 (25 mM), 3.0µl of (10X) PCR Buffer, 1.0µl DNTPs (2mM), 2.0µl each of both Forward and reverse primers (10 pmol/µl) and 9.2 µl dH2O (nuclease free). The PCR was performed on Applied Biosystem (ABI Veriti, Singapore) 96 well thermocycler. The PCR thermal profile was 94 °C for 4 minutes, and 35 cycles of 94 °C for 30

seconds, 45 °C for 30 seconds, 72 °C for 30 seconds, and 7 minutes final extension at 72 °C.

HPV 16 and 18 type specific PCR

All samples were further genotyped for type 16 and type 18, by carrying out the type specific PCR published elsewhere [11]. The sequence for the primers for type 16 and 18 are given in the Table 1, along with the length of respective amplicon and target gene. Polymerase chain reaction round was performed in 0.2ml tubes (Axygen, USA) with 5µl of sample DNA dilution in 20µl of reaction mixture for both reactions, using 0.4µl of MBI Fermentas, UK Taq polymerase, 2.3µl MgCl₂ (25 mM), 2.0µl of (10X) PCR Buffer, 1.0µl DNTPs (2mM), 1.0µl each of both Forward and reverse primers (10pmol/µl) and 7.3µl dH₂O (nuclease free). The PCR was performed on Applied Biosystem (ABI Veriti, Singapore) 96 well thermocycler. The PCR thermal profile for both reactions was 94 °C for 4 minutes, and 35 cycles of 94°C for 30 seconds, annealing temperature for type 16/18 was 54/61 °C for 30 seconds, 72 °C for 30 seconds, and 7 minutes final extension at 72 °C.

Statistical analysis

Data were processed by SPSS statistical software program version 12.0. The correlations were analysed by running cross tabulation tests. Cross tabulations were analysed using chi-square test or Fisher's exact test and Pearson co-relation test where applicable. P value measures the significance of an association and is significant when it is less than or equal to 5% (0.05).

Results

Demographics of the study population:

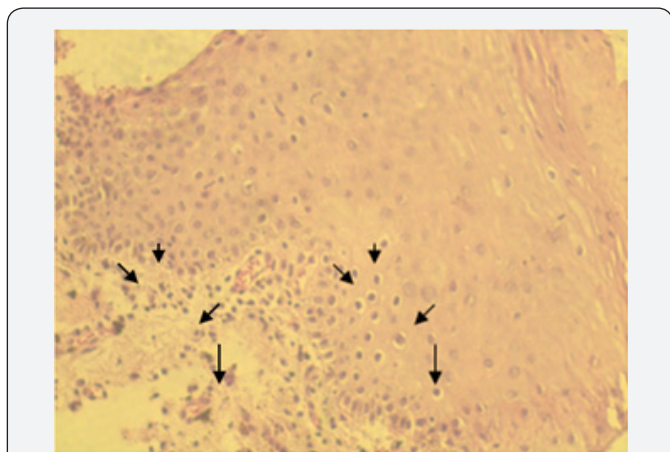


Figure 1: Photomicrograph of H & E stained well differentiated squamous cell carcinoma infected with HPV. The morphological signs of HPV infection in the form of koilocytotic can be seen. Koilocytes (arrow heads) are shown to be arising from the basal cells of oral epithelium and are moving towards the upper layers, as the HPV gene expression is associated with the degree of differentiation. Magnification×20.

The study population consisted of a total of 95 patients admitted to the dental ward of PIMS, Islamabad, Maxillo-facial Surgery ward of KRL hospital, Islamabad and ENT ward of CMH, Rawalpindi. The demographic parameters included in our study were age, gender, geographic location and specimen site. Patients

ages ranged from 16 years to 86 (mean age=51 years; ±SD=14). 87% of the patients were above 35 years of age (n=83). 55% of the patients were males (n=52; ±SD=0.500) while around 45% (n=43; ±SD=0.500) were females. A total of 86% of males were >35 years of age (n=45) and about 89% of the female patient population was >35 years (n=38). There were 80 (83%) cases of squamous cell carcinoma, 3 (3.2%) cases of oral submucous fibrosis which is a pre-malignant condition and 12 (13%) cases of fibroepithelial polyps. The most common anatomic site of involvement was mandible including the tongue area with 40% (n= 38) of the cases occurring here. The next most common site was the nasopharynx and vocal cords with 23.2% (n=22) of the patients having carcinoma of the oral cavity that had metastasized to the neck. Around 20% (n=19) of the cases involved either the buccal or the labial mucosa with 4% (n=4) showing gingival involvement, while maxillary and palatal involvement was found in 12.4% (n=12) of the cases.

Histopathology

The histopathological evaluation was carried out and the squamous cell carcinomas were graded. We had a total of 49 (52%) cases of well differentiated squamous cell carcinomas designated as histopathological stage I cancer. 25 (26%) cases were of moderately differentiated squamous cell carcinoma graded as stage II cancer while 4 (4%) cases were of the poorly differentiated variety graded as stage III cancer. All the samples were also observed for the presence of koilocytes (Figure 1) to determine the efficacy of morphological assays in the identification of HPV infection. Out of the 95 samples about 50 (53%) were positive for the presence of koilocytes.

Detection of HPV by GP PCR

Out of a total of 95 oral squamous cell carcinoma samples 72 were formalin fixed and paraffin embedded, 6 were fresh biopsy specimen unfixed and 17 were endoscopic specimen. Out of these 95, 51 (54%) were positive for HPV when GP PCR was done (Figure 2 & 3). However, it was observed during genotyping PCR's that some of the samples previously determined to be negative by the GP PCR were positive for type 16 HPV. Therefore, the total infected samples were calculated to be 74 (78%) (Figure 4). The sensitivity and specificity of the GP PCR for HPV detection were also determined and was found to be 74% and 81% respectively. The following formulas were utilized to determine the sensitivity and specificity.

SENSITIVITY = Those that are test positive / All with disease= 74%

SPECIFICITY= Those that are test negative / All without disease= 81%

Cross tabulation analysis showed that out of 43 females 29 (67%) were infected. Similarly, out of 52 males 45 (87%) were infected. Sex difference among the patients was significantly correlated to HPV infectivity as determined by the Pearson chi square test (P=0.02). HPV was detected in 37(82%) out of 45 stage I well differentiated squamous cell carcinomas, 21 (84%) out of 25 stage II moderately differentiated carcinomas, 3 (75%) out of 4 stage III poorly differentiated carcinomas. The single sample

of verrocus carcinoma was positive for HPV as well as 12 (75%) out of 16 fibro-epithelial proliferations. All in all, no significant

association was found between age groups, site of involvement and histological differentiation.

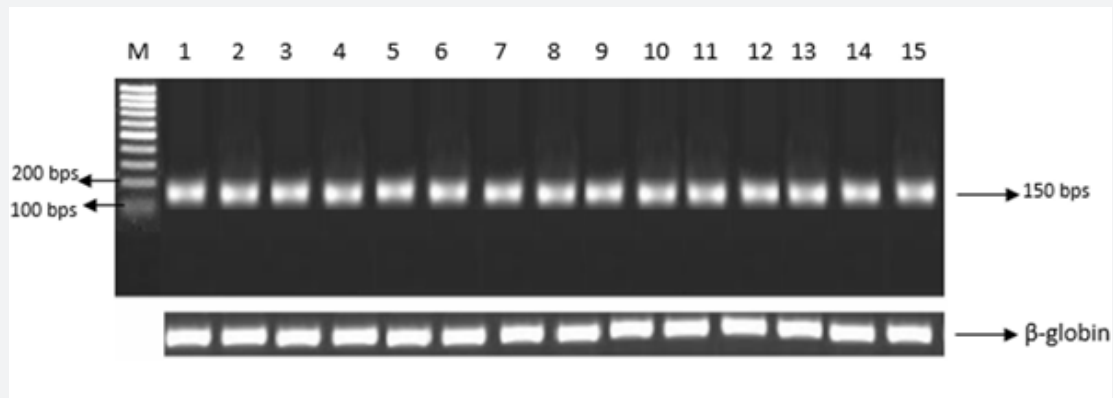


Figure 2: PCR amplification of HPV using general primers. The amplified products were separated on 2.0 % agarose gel. Lane-M: 100bp ladder molecular size marker; Lane-1: Positive control. Lanes; 2,3,4,5,6,7,8,9,10,11,12,13,14,15: (150 bp product); β -globin gene is shown as a PCR DNA control. (Lower panel).

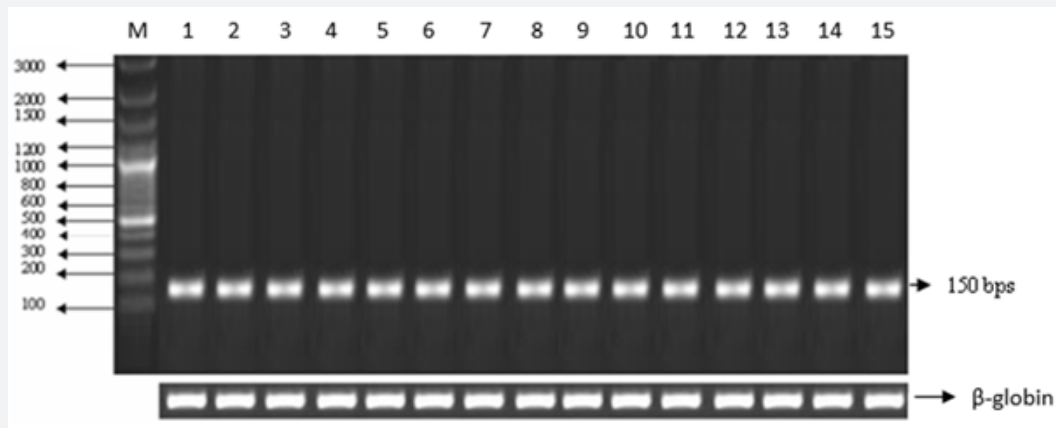


Figure 3: PCR amplification of HPV using general primers. The amplified products were separated on 2.0 % agarose gel. Lane-M: 100bp ladder molecular size marker; Lanes; 16,17,18,19,20,21,22,23,24,25,26,27,28,29,30: (150 bp product); β -globin gene is shown as a PCR DNA control. (Lower panel).

PERCENTAGE INFECTIVITY WITH HPV

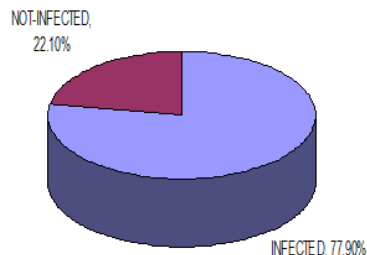


Figure 4: Total Infectivity levels of HPV as determined by compilation of results of both HPV GP PCR assay plus the genotyping assays.

Genotyping for type 16 and 18:

Out of the total 74 infected patients about 55 (74%) were infected with HPV type 16 (Figure 5), 3 (4%) were infected with HPV type 18 (Figure 6), 11 (15%) had HPV type 16 and 18 co-infection and 5 (7%) were positive for HPV by GP PCR but could not be type specified (Figure 7).

Discussion

OSCC is normally considered to be linked with tobacco, alcohol and betel/areca nut chewing, however a percentage of patients not genetically predisposed to developing cancer, manage to develop oral carcinogenesis in the absence of all these agents [12-14]. This fact indicates the presence of another etiological agent which can cause cancer independent of these factors. The evidence of presence of viral nucleic acids in OSCC tissue was presented for the first time in 1982, 1983 by Scully C et al. [15]. A meta-analysis by Miller CS & Johnstone BM performed in 2001 revealed HPV to have been significantly associated with oral cancer as well as

pre-malignant lesions [16]. The role of HPV in the development of squamous cell carcinoma of the cervix is a well-established fact. Although anatomically distantly located the squamous epithelia of both the cervix and oral cavity have remarkable similarities. The

HPV high risk types found in association with cervical cancer like type 16, 18, 31 and 33 have also found to be associated with the cancerous lesions of the oral cavity as well as the naso-pharynx [17,18].

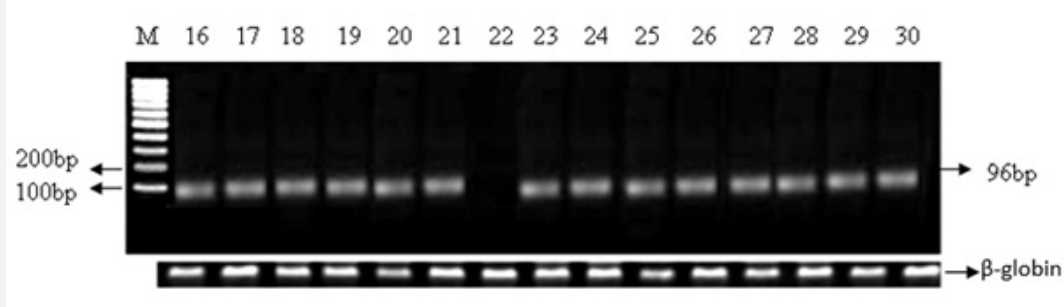


Figure 5: Genotyping for HPV type 16. The amplified products (fragment sized 96bp) were separated on 2.0 % agarose gel. Lane-M: 100bp ladder molecular size marker; Lanes 16,17,18,19,20,21,22,23,24,25,26,27,28,29,30. (96 bp product); β -globin gene is shown as a PCR DNA control. (Lower Panel).

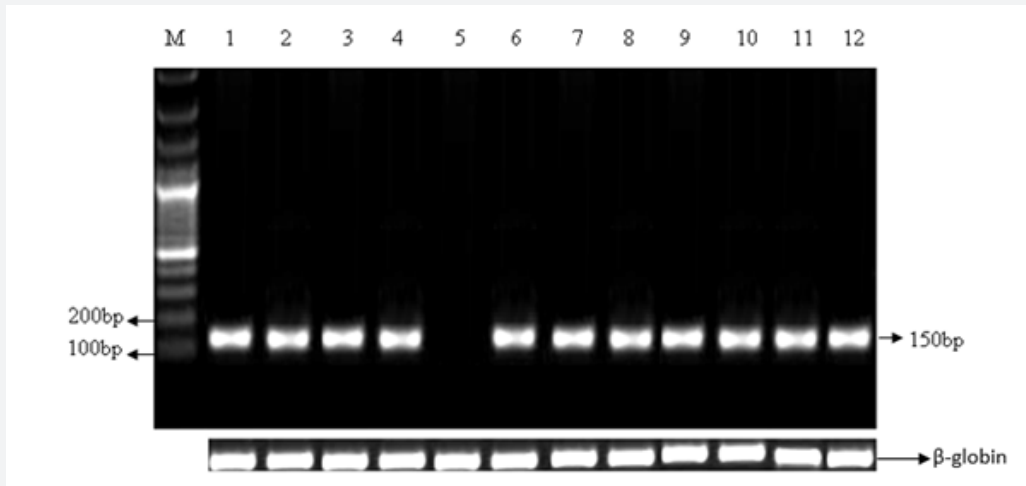


Figure 6: Genotyping for HPV type 18. The amplified products (fragment sized 155bp) were separated on 2.0 % agarose gel. Lane-M: 100bp ladder molecular size marker; Lane 1: Positive control (human cervical cancer sample previously shown to be positive for type 18). Lanes 2,3,4,6,7,8,9,10,11,12 positive for type 18. (155 bp product) Lane 5: negative for type 18; β -globin gene is shown as a PCR DNA control. (Lower Panel).

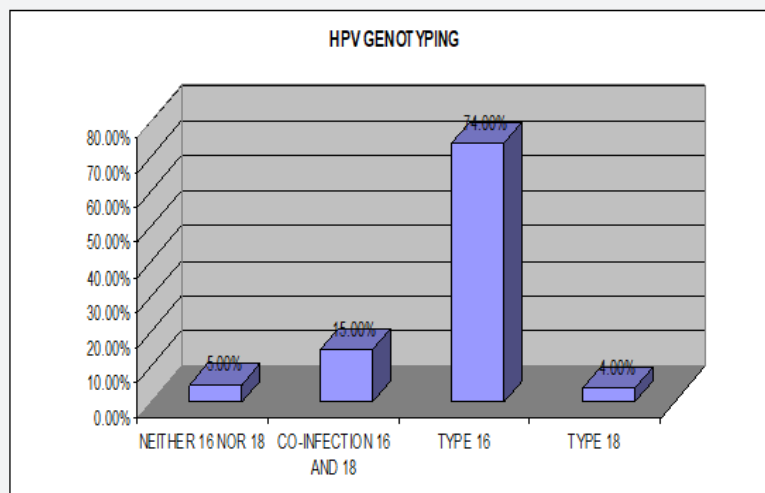


Figure 7: HPV type distribution in study population.

Table 2: Comparative analysis of HPV infectivity derived from HPV GP PCR assays and the Genotyping PCR assays.

	GP PCR		Total HPV infectivity	
	Frequency	Percentage	Frequency	Percentage
Positive	51	53.7%	74	77.9
Negative	44	46.3	21	22.1

In our study, two methods were employed to determine the presence of HPV. The first one was the histopathological evaluation, in which the morphological changes occurring in the HPV infected squamous cell were observed. This was especially emphasized as more than one third of Pakistani population lives below the poverty line [19], and most of them cannot afford to have PCR analysis done to determine HPV infection. We had a total of 95 samples which were histopathological analysed for the presence of koilocytes, which are HPV infected squamous cells with pyknotic nuclei and a clear halo around the cytoplasm (Figure 1). 50 samples tested positive for the presence of koilocytes out of 95, which constitutes about 53% of the total samples. All these 50 samples were later tested positive for HPV infection using GP PCR assays (Table 2).

Histopathological grading of the OSCC samples was also carried out in order to determine the severity of the cancerous process. The histopathological parameters that were observed were the degree of differentiation, cellular atypia, presence of keratin pearls and individual cell keratinizations and degree of dysplasia. The

Table 3: HPV genotype distribution in different types of OSCC.

Diagnosis		Genotype				Total
		Not Present	Both 16, 18	Type 16	type 18	
	Fibroepithelial polyp/SQUAMOUS PAPILOMA	3		6		9
	Focal dysplasia			1		1
	hyperplasia with chronic infection			3		3
	Keratinized squamous cell carcinoma	15	5	27	2	49
	Moderately differentiated/non Kerat. Squamous cell carcinoma	6	4	14	1	25
	Undifferentiated squamous cell carcinoma	1	1	2		4
	Oral submucos fibrosis	1	1	1		3
	Verrocus Carcinoma			1		1
Total	26	11	55	3	95	

GP (general primers) based PCR assay was then performed for the detection of HPV. This assay uses primers that have already been reported in the literature [11]. In our study the GP PCR revealed 51 samples (54%) to be positive for HPV infection while the remaining 44 were HPV free. However, some of the samples that were negative for HPV as tested by GP PCR were determined to be HPV type 16 positive, when genotyped for type specific HPV. In order to reduce human error, this genotyping PCR was repeated thrice in duplicates for the samples that were proven to be negative by GP PCR. The samples positive for type 16 were HPV positive, so a total percentage of infectivity was derived after compiling the data derived from both GP PCR based assay and genotyping assay. The compiled results showed the HPV infected cases to be a total of 74 (78%) of the 95 OSCC samples (Figure

7), (Tables 2 & 3), which lies within the range of 25%-75% HPV infectivity levels as have been reported by other studies [21,22]. This discrepancy maybe due to mutational changes in the HPV genome in the area previously considered to be highly conserved or the designated area might be comparatively less well conserved in Pakistani origin HPV, as the studies utilizing them have all been based on Caucasian population derived HPV.

Out of these 95 samples 72 were formalin fixed and paraffin embedded, 6 were fresh biopsy specimen unfixed and 17 were endoscopic specimen. In this study group patients ages ranged from 16 years to 86, 87% of the patients were above 35 years of age. The most common anatomic site of involvement was mandible including the tongue area followed by nasopharynx and vocal cords with 23.2% (n=22) of the patients having carcinoma of the oral cavity that had metastasized to the neck. Around 20% (n=19) of the cases involved either the buccal or the labial mucosa with 4% (n=4) showing gingival involvement, while maxillary and palatal involvement was found in 12.4% (n=12) of the cases.

When HPV infection and its gender distribution cross tabulations were carried out a very significant association was observed. Out of the 43 females 29 (67%) were infected with HPV. Similarly, out of 52 males 45 (87%) were infected. Sex difference among the patients was significantly co-related to HPV infectivity as determined by the Pearson chi square test (P=0.02), which

showed a male predilection for HPV infection. This finding may prove to be significant in terms of including anti-HPV vaccination in vaccination regimens for the males, to protect against oral cancer.

Out of the total 74 infected patients about 55 (74%) were infected with HPV type 16, 3 (4%) were infected with HPV type 18, 11 (15%) had HPV type 16 and 18 co-infection and 5 (7%) were positive for HPV by GP PCR but could not be type specified (Figure 7). Type 16 emerged as the most prevalent type of HPV in our OSCC patient population. Type 18 was rare; however, co-infection was observed in 11 cases. All the cases positive for HPV type 18 were malignant, it was not found in the fibro-epithelial polyps that were included in our study. The previous studies have also reported HPV type 16 prevalence over the other types of HPV in OSCC patients' population [23-25]. Different HPV types have also been shown to be variably prevalent because of the geographic differences.

Co-infection was not observed in the HPV infected benign proliferative lesions, the only lesions co-infected were malignant, which may indicate a more aggressive pattern of disease in the case of prevalence of the two high risk types together (Table 3). However, a larger co-infected population group needs to be studied in order to derive statistically significant association between co-infection and aggressiveness of the disease. The 5 samples that could not be genotyped for both type 16 and 18 must be infected with some other type of HPV. Cross tabulations of genotyping and sex, age of patients and primary tumour site did not show significant association.

Conclusion

- a. HPV is very prevalent in OSCC patients of Pakistani origin, the prevalence being 78%. Therefore, a significant association exists, and HPV could be the leading cause of oral cancer.
- b. HPV type 16 is the most prevalent type of HPV found in association with OSCC patients. Based on this study, serious efforts must be devoted to containing the viral induced oral squamous cell carcinoma.

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