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Original Research Article

Detection of High Risk Human Papilloma Virus (HPV) Genotypes 16/18 in Oral Lesions of Tobacco Chewers in Pakistan

Abstract

Background

The human papillomavirus (HPV) has evolved as a new culprit of malignant and pre malignant oral lesions. The objective of this study was to find out the frequency of HPV and its high risk genotypes in different lesions of oral cavity.

Methods

From 492 subjects (421 males and 71 females), 20ml of oral rinse sample was collected after an informed consent. Normal subjects with no chewing habits (250) including 135 males and 115 females were also taken from same setting. Gentle brushings over the lesions with the help of dental floss brush was done which was left in the oral rinse and stored at 4°C until DNA extraction. DNA was extracted and PCR was performed using HPV consensus primers Gp5+/Gp6+ and HPV 16, 18 specific primers for genotyping. Categorical data was calculated as frequencies and percentages.

Results

Oral pre-malignant lesions were present in 421 (86%) males and 72(14%) females having leukoplakia(173), erythroplakia(60), submucous fibrosis(192) and L/E (67). Total number of HPV positives were 128 (26%), having 13% HPV 16 and 11% HPV 18 whereas, 76% had other genotypes. Submucous fibrosis was most frequent lesion (185) with 82(46%) HPV positive. Out of total 128 HPV patients 92% were males and 8% were females. All controls were found HPV negative.

Conclusion

Frequency of HPV was found high (26%) in oral lesions with HPV16/18 as 13% and 11% respectively. The patients with submucous fibrosis are at greater risk of having HPV. Other HPV genotypes causing premalignant lesions require further investigation.

Key Words: HPV, oral rinse, pre malignant oral lesions, submucous fibrosis

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Background:

Although oral lesions have diverse causes, but in our population it can largely be ascribed to exposure to chewable tobacco products (Gutka,areca nut, betel quid, naswar etc) as they are well-established risk factors of many cancers of the oral cavity (Secretan B, 2009). In Pakistan, 8.5 to 10 times increase in the risk of oral cancers have been reported since the increase in popularity of chewable tobacco (Merchant A, 2000). Several studies have shown a link between the use of chewable tobacco and oral submucosal fibrosis, oral cavity cancers, leukoplakias and other head and neck malignancies (Merchant A, 2000; Gupta PC R. C., 2004; Nair U B. H., 2004) It has been estimated that 58% of the total worldwide head and neck cancers occur alone in South and Southeast Asia (Nair U B. H., 2004). In Pakistan, 40% of the adolescent and adult population of squatter settlement in Karachi has been reported using at least one product of chewable tobacco on a daily basis (Khawaja MR, 2005) . Abrasions caused by corrosive material (Gupta PC R. C., 2004)in chewable tobacco as well as continuous exposure give opportunity to infectious agents to the development of oral lesion (Secretan B, 2009). Human Papilloma Virus (HPV) seems to be associated with a subset of oral benign proliferative and malignant lesions, head and neck carcinoma, notably carcinoma of the oropharynx, tonsils and tongue (Gypsyamber D'Souza, 2007). The common term oral potentially malignant disorders' (OPMD) has been suggested for oral precancers, including both oral precancerous lesions (e.g. leukoplakia, erythroplakia, and oral proliferative verrucous leukoplakia) and oral precancerous conditions (e.g.lichen planus and submucous fibrosis) by Warnakulasuriya et al, 2007 and van der Waal, 2009 (Warnakulasuriya S, 2007 ; Van der Waal I, 2008).

In chewable tobacco users ulceration, leukoplakia, erythroplakia, rough mucosa Abrasions like white patches, swelling, sub-mucosal fibrosis (SMF), pain, trismus, cuts, sepsis are due to continuous exposure of oral mucosa to these tobacco preparations, making the mucosal surface more vulnerable to viruses like HPV (Saeeda Baig, 2012). After gaining entry into the cells of the epithelial basal layer, replication of the virus occurs in the nuclei of the infected cells, and the production of mature virions in the suprabasal epithelial cell layers (Del Mistro A, 2001). HPV infection is highly transmissible, has a variable incubation period that can culminate in latent infection with low HPV DNA copy-number in basal cells insufficient to support

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transmissibility in subclinical infection that is active but without clinical signs; or in clinical infection leading to benign, potentially malignant or malignant epithelial lesions (Zil-a-Rubab, 2013).

Human papillomavirus is one of the most important human carcinogens, causing greater than 14,500 cases of oropharyngeal disorders per year. Viruses like HPV, target p53 and pRb tumor suppressor pathways resulting in cell cycle alteration are implicated in 10–20% of all cancers (Talbot SJ, 2004; Sellers WR, 1997). The high-risk types of HPV, 16/18 are strongly associated to squamous cell carcinomas and its frequency is progressing in precancerous lesions of increasing grades (Koutsky LA, 2002). Approximately the cause of 25% to 50% of oropharynx cancers is HPV with HPV16 being the source for more than 90% infection (DM, 2006; Bouvard V, 2009).

The increasing trend of tobacco chewing combined with rise in oral disparities was the basis for designing this study**.** The objective of this study was to detect the presence of HPV and its genotypes 16, 18 in different lesions of oral cavity.

Materials and Methods

Clinical Data and Sample Collection

Oral rinse samples (20 ml) were collected from 492 subjects (421 males and 71 females) aged 18 years and above with known oral lesions after an informed consent. Lesions included leukoplakia*,* erythroplakia and submucous fibrosis. Age matched subjects (250) including 135 males and 115 females were also taken from same setting who were non-chewable tobacco users and had no oral lesions.

Sample Collection

The cases of leukoplakia*,* erythroplakia, submucous fibrosis were taken through setting up camps in different regions of Karachi, Pakistan. from April 2013 to May 2014 in a random manner after obtaining informed consent from the subjects and approval from the Ziauddin University ethical review committee. Comprehensive demographic data of each patient, including the patient's age, sex, addiction habits was obtained. All patients were using some form of chewable tobacco at the time of sample collection. A 15-20 ml of oral rinse sample was collected from both 250 normal subjects and 492 subjects (421 males and 71 females) with

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leukoplakia, erythroplakia, submucous fibrosis and oral squamous cell carcinoma after an informed consent. All persons were aged 18 and above. Gentle brushings from the lesions were taken from subjects with the help of a brush at the other end of dental floss and the oral rinse was stored at 4°C until DNA extraction.

DNA Extraction and PCR

DNA was extracted with Omni-Pure™ DNA Purification System kit. 20 ml of oral rinse was centrifuged for 15 minutes at 10,000 rpm. Supernatant was discarded, leaving 100 µl at the bottom containing cells. This was transferred to 1.5 ml Eppendorf tube containing 900 µl cell suspension solutions. After spinning at 12000 rpm for 2 minutes, supernatant was discarded and 300 ul of cell lysis solution was added and incubated at 65^0C for 10 minutes. Protein precipitation solution was added and it was vortexed for 20 seconds and centrifuged for 5 minutes at 12,000 rpm. Supernatant was transferred to a fresh tube with already added 300 ul 0f 100% isopropanol.Contents were mixed by inversion (30-40 times). DNA pellet was retrieved after centrifugation at 4°C at 10,000 rpm for 10 minutes. Supernatant was discarded and pellet was dried and reconstituted with 50 µl of reconstitution buffer.

DNA was amplified in a conventional thermocycler (BIOFLUX). HPV positive controls from known HPV positive cervical carcinoma tissue samples, human ß-globin gene and a blank were used with every reaction.

ß-globin

The PCR amplification was carried out in a volume of 25 µl containing 12.5 µl of master mix (Promega) containing as final concentrations 10 mM Tris-HCl (pH 8.8 at 25°C), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM deoxynucleoside triphosphates (dNTPs), and 1 unit of Taq DNA polymerase (Promega) and 10 µl DNA (1 µg). One µmol of primers PCO3 and PCO4, The first DNA denaturation was performed for 5 minutes at 95°C; and then 35 cycles of PCR consisting of denaturation for 60 seconds at 95°C, annealing for 60 seconds at 51°C, and extension for 2 minute at 72°C were run, followed by a final extension for 10 minutes at 72°C.

HPV GP5+/GP6

The PCR amplification was carried out in a volume of 25 µl containing 12.5 µl of master mix (Promega) containing as final concentrations 10 mM Tris-HCl (pH 8.8 at 25°C), 50 mM KCl, 1.5

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mM MgCl2, 0.2 mM deoxynucleoside triphosphates (dNTPs), and 1 unit of Taq DNA polymerase (Promega) and 10 µl DNA (1 µg).One µmol of primers Gp5+ and Gp6+, The first DNA denaturation was performed for 3 minutes at 94°C; and then 35 cycles of PCR consisting of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 55°C, and extension for 1 minute at 72°C were run, followed by a final extension for 5 minutes at 72°C.

HPV 16 and HPV 18

The PCR amplification was carried out in a volume of 25 µl containing 12.5 µl of master mix (Promega) containing as final concentrations 10 mM Tris-HCl (pH 8.8 at 25°C), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM deoxynucleoside triphosphates (dNTPs), 1 unit of Taq DNA polymerase (Promega) , 10 µl DNA (1 µg) and One µmol of HPV 16 ,1 8 specific primers each. The first DNA denaturation was performed for 2 minutes at 94°C; and then 35 cycles of PCR consisting of denaturation for 30 seconds at 95°C, annealing for 30 seconds at 51°C, and extension for 30 seconds at 74°C were run, followed by a final extension for 5 minutes at 74°C.

Statistical Analysis

It was a Descriptive Cross sectional study. Analyses were conducted with SPSS16.0 .Categorical data (gender, oral lesions, HPVstatus) was presented as frequency and percentages.

Results

Four ninety two cases (average age 33.6 years) were incorporated in this study, suffering from potentially malignant disorder of the oral cavity such as 173 cases of Leukoplakia (86% males and 14% females), 192 SMF patients (88% males and 76; 12% females), 60 cases of erythroplakia (72% males and 76; 28% females). 67 patients had leukoplakia and erythroplakia both (90% males,10% females) **Table 1**

Out of total 128 HPV positive subjects, 92% were males and 8% were females HPV 18 was present in 11% of subjects and HPV 16 in 13%, whereas, other genotypes in 76% of subjects. **(Table 2, 3)**

According to **Table 2**, total 82 cases of SMF (91% males, 9% females) were HPV DNA positivite .Out of 26 leukoplakia subjects, 100% were males while among 9 HPV positive cases of erythroplakia, 78 % were males and 22 % were females, while in 67 cases of combined

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leukoplakia and erythroplakia, 11 patients (91% males, 9% females) were HPV positive. All 250 subjects with no history of oral lesions and tobacco chewing habits were found HPV negative. These samples were again tested by the type specific HPV 16 and HPV 18 with the help of PCR. **(Table 3).** It is noteworthy that out of total 128 HPV positive cases in all pre malignant lesions, 98 patients were having the HPV genotype other than prevalent HPV 16 (13%) and HPV 18(11%).

Discussion

Despite high prevalence of oral lesions among chewable tobacco users in Pakistan the exploration link with HPV and its oncogenic genotypes has not been explored and established though it is a well known pathogen of mucous membranes which is leading precancerous lesions to oral squamous cell carcinoma. Chewable tobacco products are considered the risk factors for pre malignant and malignant disorders such as leukoplakia, erythroplakia and submucous fibrosis of the oral cavity (Raúl Fernando Venezuela Ángel Talavera, 2013). This study initiates that missing link that is causing a high prevalence of oral cancer among tobacco chewers and leading Karachi south (Pakistan) in oral cancer worldwide. According to one of the largest survey by Bhurgri in Karachi, Pakistan, the hazardous factors of oral cancer in the developed countries are smoking, alcohol and oral sex (Bhurgri, 2005). In Pakistan generally, alcohol is a noteworthy exclusion as a risk factor and also oral sex is sinful and immoral act according to religion. However, tobacco, areca nut, betal quid chewing, paan masala, and poor diet are popular among the masses and may be associated with the disease. Areca nut, betal quid, gutka, naswar and poor diet are added risk factors of oral lesions in Asia (Raheem Dhanani, 2010; Dongre A, 2008; Arif Valliani, 2012). A rising inclination in chewing habits is observed in the people of Karachi in recent days (Ali NS, 2009; Mazahir S, 2006).

Currently, the information regarding probable role of HPV in potentially premalignant oral lesions currently is scarce. There is one distinctive thing in our study that we used oral rinse of both patients and normal subjects for HPV DNA analysis in spite of oral tissues biopsy used in other studies (Mravak-Stipetic´ M, 2013; Ravi Mehrotra, 2010; Li-Li Gan, 2014) . Genotyping for particular HPV types was performed on all oral rinse samples to increase the HPV detection sensitivity. HPV 16 and 18 types preferred for genotyping due to their abundance in cervical carcinoma in the world (De Sanjose S, 2010) .

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Similar studies of HPV detection were done to detect association of HPV in OSCC, SMF and other potentially malignant lesions such as oral leukoplakia, oral lichen ruber planus, and epithelial dysplasia (Syrja¨nen S, 2011) .In a review evaluating the literature prior to 1998, 890 leukoplakic and keratotic lesions had been tested for HPV DNA, of which 25.4% were HPV-positive (Syrja¨ nen S, 2000). In recent days, Szarka et al detected HPV more frequently in lesions than in controls ($P < 0.001$ in all comparisons). HPV prevalence increased gradually with increasing severity of the lesions; 32.8%, and 40.9%, in oral lichen planus (OLP) and oral leukoplakia (OL) respectively (Szarka K, 2009). The present study has followed the same pattern providing the further insight into the frequency of HPV and its important genotypes 16 and 18 in cases of oral potentially malignant disorders.

Of the 492 patients finally included in this study, 421(86%) were males and 71(14%) were females. Similar studies from the neighbouring countries reported a marked male predominance (Ravi Mehrotra, 2010; Mehrotra R, 2008).The frequency of HPV in oral lesions was higher in men than women and this is in accordance with other studies reported in the literature (Mravak-Stipetic´ M, 2013; Herrero R, 2003).

Among potentially malignant lesions, SMF had the highest number of HPV positive cases compared to leukoplakia and erythroplakia in this study. Surprisingly, the frequency of malignant genotypes 16 and 18 was less prevalent in potentially malignant oral disorders, such as out of 26 HPV positive leukoplakia patients only one each was of HPV16 and 18. Thus, it seems that strains other than HPV 16/18 were the cause not typed in this study. In literature high risk genotypes are less commonly found in premalignant oral lesions (Ali Yousif Babiker, 2013; Raúl Fernando Venezuela, 2013). The same pattern was observed in this study where 92% of leukoplakia and 74% of submucous fibrosis was due to HPV genotype other than HPV 16/18. SMF being declared as the notorious premalignant lesion worldwide displayed the same pattern in our study showing high frequency of malignant genotypes HPV 16/18 (table 4) compared to other oral lesions (Syrja¨nen S, 2011; Ho PS, 2007).

Although we cannot rule out an imperative role for HPV in the induction of OSCC, we postulated a mechanism of HPV entry into the squamous epithelium in one of the reviews by Rubab et al in which we hypothesized that the chewable tobacco also plays a pivotal role by causing abrasions and desquamation of epithelial cells making penetration of HPV easy and

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comfortable to the squamous epithelium (Rubab et al., 2013). An invitro study on cell cycle profiles of HPV-positive and HPV-negative oral keratinocytes and oral HPV-negative fibroblasts, exposed to smokeless tobacco extract (STE) showed that tobacco products may enhance the effects of HPV 16 and the risk of DNA aneuploidy leading to malignant alteration in the genome (Merne et al., 2014).

Doctors, GPs, Oncologists and specialists in general agree and attribute the ever rising incidence of oral cancer in Pakistan to the use of precarious products of chewable tobacco. The prolonged consumption of such materials first causes preliminary stages of mouth cancer like sub-mucous fibrosis, leukoplakia and trismus leading to an incurable state of cancer.

Still the question remains regarding the source of HPV in oral mucosa. Many researchers agree that sexual transmission is the primary source then what is the source of HPV in genital area. Future research should target this issue with epidemiological studies targeting a sound mode of treatment and preventive strategies.

Conclusion

Frequency of HPV was found high (26%) in oral lesions with HPV16/18 as 13% and 11% respectively. The patients with submucous fibrosis are at greater risk of having HPV. Other HPV genotypes causing premalignant lesions require further investigation.

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Legends

1. **Distribution of Oral Lesions According to Gender**
2. **Distribution of HPV in Oral Lesions According to Gender**
3. **Distribution of HPV 16 & 18 in Oral Lesions According to Gender**

Table 1

Distribution of Oral Lesions According to Gender

Oral Lesions	Male n (%) 421(86)	Female n (%) 71(14)	Total Numbers of Cases n=492
Leukoplakia	149 (86)	24(14)	173
Erythroplakia	43(72)	17(28)	60
SMF*	169(88)	23(12)	192
L/E**	(60)90	07(10)	67

SMF * - submucous fibrosis

L/E** - leukoplakia/erythroplakia

Table 2

Distribution of HPV in Oral Lesions According to Gender

Oral Lesions	Total number of HPV n=128	HPV	
		Male n (%) 118(92)	Female n (%) 10(8)

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Leukoplakia	26	26(100)	0
Erythroplakia	09	07(78)	02(22)
SMF	82	75(91)	7(9)
L/E	11	10(91)	01(9)

Table 3

Distribution of HPV 16 & 18 in Oral Lesions According to Gender

Oral Lesions	**Total number of HPV Positive Cases n=128**	**HPV 16 n(%) 16(13)**		**HPV 18 n(%) 14(11)**		**OTHER Genotypes n(%) 98(76)**
		Male n(%)	**Female n(%)**	**Male n(%)**	**Female n(%)**	**n(%)**
Leukoplakia	26	01(4)	0(0)	01(04)	0(0)	24(92)
Erythroplakia	09	02(22)	0(0)	04(45)	0(0)	03(33)
SMF	82	11(14)	01(01)	08(10)	01(01)	61(74)
L/E	11	01(9)	0 (0)	0 (0)	0(0)	10(91)