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Expression of Human Papilloma Virus in Oral Sub mucous Fibrosis Patients- A PCR Based Assay Study

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Introduction

Oral submucous fibrosis is a chronic disease of oral mucosa characterized by inflammation and progressive fibrosis of the lamina propria and deeper connective tissues, followed by stiffening of oral mucosa resulting in difficulty in opening the mouth.[1] The pathogenesis of OSMF is multifactorial. Epidemiological data indicate that areca nut is the main etiological factor for OSMF. Other etiological factors are chillies, lime, tobacco, nutritional deficiencies, immunological disorders, viruses and collagen disorders.[2]

Human papilloma viruses (HPVs) are small DNA viruses of papillomaviridae that infect various human epithelial tissues exclusively. More than 130 HPV types are identified which are classified into low or high – risk groups according to their potential for oncogenesis.[3] Several studies have shown that HPV is associated with increased risk of oral cancer, independent of exposure to tobacco and alcohol. This association is valid for HPV 16 and 18 which are generally considered high-risk types because they have been detected in oral dysplastic lesions and oral cancers. The role of HPV as an etiologic agent in cancer was first recognized in the uterine cervix and is now well established.[4]

The first evidence of HPV in OSCC was shown in 1977, numerous studies have been conducted in supporting the role of HPV as a causative agent for OSCC and premalignant conditions.[5] There is an increasing evidence of a causal association between high risk HPV types mainly HPV 16/18 and OSMF.[6]

A vast number of studies have been conducted to show the correlation of HPV with oral cancer as an etiologic agent with oncogenic potential but very few studies have been conducted to show the correlation between HPV and OSMF. Although many studies have shown positivity of HPV in oral malignant lesions, only few researchers have compared HPV in normal mucosa and OSMF. The present study was taken up for determining the expression of HPV-16 in normal oral mucosa and OSMF through polymerase chain reaction assay (PCR) technique.

Materials And Methods

Sample Size and method of collecting data: The patients selected for the present study are with the following parameters.

Group I: Ten patients without any chewing or smoking habits and with clinically normal appearing oral mucosa served as control group.

Group II: Forty patients with clinically diagnosed and histopathologically confirmed cases of OSMF. The paraffin embedded tissue biopsies of both the control and OSMF patients were stained with Haemotoxylin and eosin for confirmatory diagnosis.

Inclusion criteria: Different stages of clinically diagnosed and histopathologically confirmed OSMF cases of both genders.

Exclusion criteria: Patients with any lesions in the oral mucosa and patients with any systemic diseases are excluded.

Methodology

A total of 50 cases were included in the study, a thorough case history was taken to rule out any systemic diseases and other mucosal lesions. Then out of 50 cases, 10 cases were selected for control group and 40 clinically diagnosed cases were of four different grades of OSMF, 10 cases in each grade.

Biopsy specimens were obtained from lesional region. The specimen were formalin fixed and processed to paraffin blocks. Sectioning of the paraffin tissue sample was done and staining was done for histopathological confirmation of OSMF. After confirmatory diagnosis, paraffin embedded OSMF blocks were sectioned.

4 µm thick 6-8 sections were sectioned and placed in Eppendorf's tubes (PCR bottles). Purified DNA was extracted from formalin fixed paraffin embedded (FFPE) oral biopsied tissue by using DNA extraction kit. Once the presence of DNA was confirmed in samples, PCR amplification was performed.

DNA Extraction Protocol (From paraffin embedded tissue samples) using DNA extraction kit

Thin sections of 4µm thickness were made from the FFPE tissue blocks. The tissue sections were subjected to dehydration by adding 1ml of Xylene and kept for 30 min. The samples were centrifuged at 10,000 rpm and a supernatant was discarded. The tissue sections were subjected for dehydration once again by adding 1ml of xylene for 30 mins and was also centrifuged subsequently the overlying supernatant was discarded.

1ml of absolute alcohol (ethnanol) was added and kept for 30 min. The sample mixture was centrifuged at 10,000 rpm and the supernatant was discarded. Ethanol was added again to the sample mixture and kept for 30 mins and was centrifuged and the supernatant was discarded.

1ml of PBS (Phoshate buffer saline) was added and kept for 30 min centrifuged and the supernatant was discarded and the sample pellet gets suspended at the bottom of the tube. Thus the dehydration process was completed.

The pellet was then suspended in 500µl T.E.buffer in Eppendorff's tube and Vortexed. They were then centrifuged at 10,000 rpm for 5 min and the supernatant was discarded. The procedure of washing with fresh TE buffer was repeated for 2-3 times.

Supernatant was discarded and 50 µl L1 (Lysis buffer I) was added and then vortexed for 1 minute and kept for 5 min. 50 µl L2 (Lysis buffer II) was added along with 10 µl Proteinase –K(10mg/ml) and vortexed vigorously. It was then kept in water bath at 60˚C for 2 hrs and then kept in boiling water bath for 10 minutes for enzyme deactivation. The supernatant containing DNA was taken to fresh tube and Stored at -20^{0}C.

Composition of reagents

TE Buffer

1M Tris Buffer	: 0.5ml
0.5M EDTA	: 100 µl
Distilled water	: made to 50ml.

L1 (Lysis Buffer I)

1M Tris buffer	: 500 µl
Triton X-100	: 500 µl
0.5M EDTA	: 100 µl
Distilled Water	: Made to 50 ml

L2 (Lysis II Buffer)

Tris HCL : 50mM (pH 8.0)

KCL : 50mM

MgCl2 : 2.5mM

Tween 20 : 0.45 %

Nodient P-40 : 0.45%

PCR reagents and procedures

PCR Master Mix

HPV-16 specific Primer

Molecular grade water

Template (DNA extracts)

AMPLIQON RED 2X Mastermix was used which contain following reagents

Tris-HCL pH 8.5, $(NH_4)_2SO_4$, 3mM $MgCl_2$, 0.2% Tween 20

0.4mM of each dNTP

0.2 units/µl Ampliqon Taq DNA Polymerase

Inert red dye and stabilizer

HPV-16 Primer

Forward primer: 5'- TCA AAA GCC ACT GTG TCC TG -3'

Reverse primer: 5' - CGT GTT CTT GAT GAT CTG CA -3'

A premixture was prepared and aliquoted into each tube. The premix contains following components in a final volume of 20 µl/aliquot.

PCR master mix was gently vortexed and briefly centrifuged after thawing. A thin walled PCR tube was placed on ice and the following components were added for each 50 µl reaction.

RED 2X Mastermix : 10 µl

HPV-16(Forward primer) : 0.8 µl (20 pmole)

HPV-16 (Reverse primer) : 0.8µl (20 pmole)

Template DNA : 2 µl (< 1 µg/ reaction)

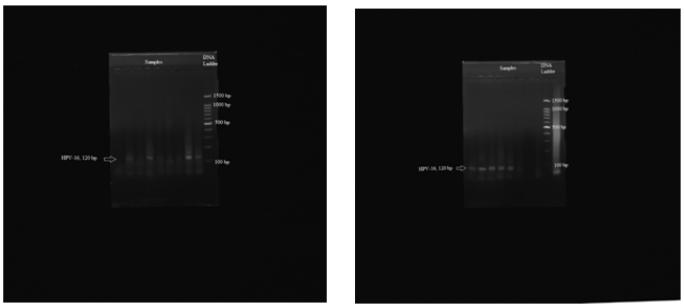
Water: Added to made final volume to 20 µl

Samples were gently vortexed and spinned down. The tubes were placed in conventional thermal cycler.

The PCR conditions were as follows, initial denaturation was done at 95^0 C for 5 minutes, followed by denaturation done at 95^0 C for 1 minute, annealing done at 53^0c for 1 minute and an extension was done at 72^0C for 2 minutes. 40 cycles were done for denaturation followed by annealing and extension. After these 40 cycles, a final extension was done by keeping the temperature of 72^0C for 5 minutes and stored at 4^0C.

Denaturation	(95^0 C, 1 min)	40 cycles
Annealing	(53^0 C, 1 min)	
Extension	(72^0 C, 2 min).	

The amplified products were run on 2% Agarose gel electrophoresis for detection of HPV-16 specific bands. Amplicon size of 120 base pair corresponds to HPV-16. Rest other bands were considered as non-specific.



Statistical analysis: In the study, the Chi-square test was used to investigate the difference in HPV prevalence between OSMF and control group. In the analysis, statistical significance was achieved when the p value of all test was $p<0.05$. The statistical package for social science (SPSS for Windows, Version 16.0; SPSS INC., USA) was used for statistically analysing the clinical

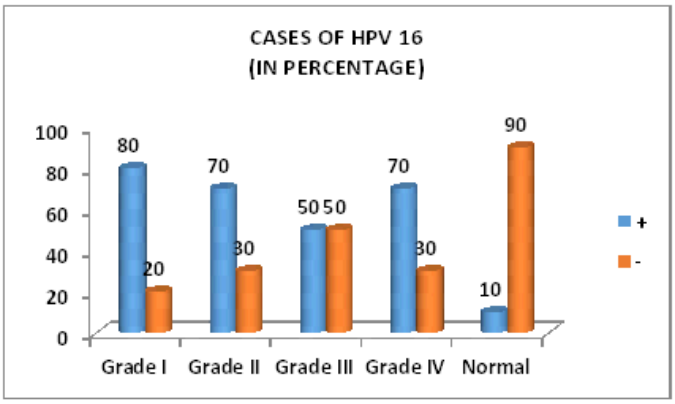
variables. In analysis, probability values less than $p<0.05$ were regarded as significant. The prevalence of the OSMF was found to be 62.5%.

Results: 50 cases were included in the study, of which 40 cases were OSMF patients, 10 patients in each grade of OSMF and 10 cases were of control group. On analysing the HR-HPV 16 results in the patients, Table 1 summarizes the results of HPV DNA detection in normal and OSMF group and Graph 1 summarizes comparison between OSMF and control group. In 40 cases of OSMF, 27 (67.5%) subjects showed positivity while 13 (32.5%) subjects were negative. In Grade 1 OSMF 8(80%) subjects were positive out of 10 cases. Similarly in grade 2 OSMF 7(70%) subjects were positive, in grade 3 OSMF 5(50%) cases were positive and grade 4 OSMF 7(70%) cases were positive for HPV out of 10 cases each. In 10 cases of control group, 1(10%) case was positive while 9(90%) cases were negative for HR-HPV 16. HPV positivity was found to be more in Grade 1 OSMF. The study showed a significant difference between the OSMF and the control group.

Table 1 : Distribution Of Cases For HPV-16 According To Osmf Grades And Comparsion By Chi Square Test

OSMF	+		-	
	Number	**Percentage**	**Number**	**Percentage**
Grade I (10 cases)	8	80	2	20
Grade II (10 cases)	7	70	3	30
Grade III (10 cases)	5	50	5	50
Grade IV(10 cases)	7	70	3	30
Normal(10 cases)	1	10	9	90
CHI SQUARE VALUE=12.7(P VALUE=0.013<0.05) SHOWS SIGNIFICANT DIFFERENCE				

Graph 1



Discussion: Oral sub mucous fibrosis is a commonly seen premalignant condition of oral cavity in clinical practice. It occurs most commonly in South Asian and African countries. The chewing habit of areca nut with or without any tobacco and use of various commercial products containing areca nut were significant factors for OSMF.[7]

In India, about 5 million people suffer from OSMF. A marked male predominance in the study was noted by **Schwartz et al.**[8] In our study male predominance was also observed. Out of 40 cases of OSMF, 39 cases were of males and one case was of female.

Kumar et al[9] conducted a hospital based study on patients of OSMF in Chennai and he observed that maximum number of cases belonged to 21-40 years with mean age of 30 years and most of them were in middle class or low socioeconomic status. In our present study, maximum number of cases belonged to 21-31 years of age group with mean age of 28 years and most of them were of low or middle socioeconomic status.

Jalouli J[10] conducted a study on OSMF tissues of Indian, African and Sweden patients by PCR assay method. He concluded that exact role of HPV infection in development of oral cancer is a major risk particularly in tumours that involve the lymphatic tissue in the lingual and palatine tonsil. In biopsies from Indian patients with the premalignant lesion like OSMF, they observed a high

prevalence of HPV (91%) and of these 55% were high risk HPV type 16. Positivity for HR-HPV in his study indicates that ethnicity may play a role alone or in combination with the betel quid habit that paws the road to viral infection. Our study results are in accordance with the study by **Jalouli J[10]** which reports high prevalence of HPV-16 in OSMF cases.

Chen et al[11] reported the positivity of HPV 16 to be 52.6% in OSMF by PCR method and concluded that HPV may be an independent risk factor in transformation of OSMF to OSCC. In our study, the positivity of HPV 16 in OSMF cases was detected by PCR assay as 62.5% and it can be suggested that virus may play an etiological role in carcinogenesis in the oral cavity.

Gonsalez et al[12] conducted a study for HPV 16 detection by PCR assay. HPV 16 was not detected in series of control samples. In our control group samples, only one sample was found to be positive for HR-HPV 16. Our study is consistent with the results by **Gonsalez et al[12]** and also with reports of **Kujan et al[13]** , **Chaudhary et al[14], Giovanelli et al[15], Ostwald et al[16], Bouda et al[17]** that revealed a very low HPV positivity in the control group.

The HPV-16 frequency of occurrence obtained in our study in OSMF samples (62.5%) is consistent with some previous study data by **Chen et al[11], Gonzalez et al[12]** and **Chaudhary et al[14],** in which HPV positivity was atleast 50% or higher than the average obtained analysing reports. In our study, HR-HPV-16 detection in OSMF patients is undoubtedly greater than in the normal controls.

Conclusion

Chewing habits, alcohol consumption and virus infections are considered to be the main etiological factors of oral cancer. HPV may play a role in the carcinogenesis of head and neck malignancies. The prevalence of HPV in premalignant and malignant lesions has been confirmed by many molecular techniques and among them PCR is the most sensitive method. The high prevalence of HPV DNA in OSMF may be due to the ethnicity alone or in combination with betel quid chewing habit. The high detection rate of HPV 16 in OSMF is found to be in Grade 1, it can be concluded that HPV detection by PCR can be used as a reliable biomarker for early diagnosis of HPV in OSMF that could help us prevent carcinogenesis by using various therapeutical measures like antiviral therapy and HPV vaccinations etc. Our study data shows a significant rise in detection of HPV 16 in OSMF compared with healthy individuals. The findings of our study supports that HPV 16 plays an etiological role in transformation of OSMF and in oral cancer development. Evidence has showed that strong epidemiological data would provide additional support for a causal association between HR-HPV and oral lesions. Further studies with larger sample size are needed for PCR assay in HR-HPV 16 detection to be a reliable diagnostic and screening marker in cases of oral precancer and to find the prognostic value in HPV infection as a biomarker for early diagnosis of oral mucosal malignant disease especially relating to the design of a chemo-preventive approach.

Summary: The present study comprised of 50 samples. 10 cases of normal mucosa tissue, 40 cases of OSMF. These OSMF were further divided based on their histopathological grading. 10 cases in each grade of OSMF were included in the study. 10 cases in Grade 1, 10 cases in grade 2, 10 cases in grade 3 and 10 cases in grade 4 of OSMF. Maximum number of cases belonged to 21-31 years of age group and most of them were in the low or middle socioeconomic status.

In our study, 40 cases of OSMF and 10 cases of control group were taken. Out of 40 OSMF patients, 27 (62.5%) patients were reported positive for HR-HPV while 13 (37.5%) cases were found negative. On the other hand, in

control group, 1 (10%) subjects was found positive while 9 (90%) were negative. Hence the reliability of this technique is apparent in this study.

- A statistically significant result was obtained using Pearson Chi-Square test when control group was compared with OSMF groups.

The present study suggests HPV 16 may play as an etiological role in transformation of OSMF to carcinoma either as an independent factor or with other factors like carcinogens, radiations etc.

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