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A correlative study of potentially malignant and malignant lesions of oral cavity with reference to high-risk Human papilloma virus status

KEYWORDS

OSMF, OED, OSCC, Cytology, Histology, HR-HPV DNA, Hybrid Capture II.

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ABSTRACT

Background: *The incidence of potentially malignant lesions especially oral submucous fibrosis (OSMF) and malignant lesions- oral squamous cell carcinoma (OSCC) has increased many folds in North India especially in younger generation, which is of great concern. Therefore this hospital based cross-sectional study was undertaken to correlate various histological findings regarding grades of OSMF with or without oral epithelial dysplasia (OED) and OSCC with high-risk Human papilloma virus (HR-HPV) status.*

Methods: *This study includes 125 cases of OSMF along with 135 healthy controls. Samples were processed for cytology, histopathology and detection of HR-HPV DNA by the second-generation Hybrid Capture-II (HC-II).*

Results: *Of the total 125 cases studied, 92 (73.6%) were of OSMF and 33 (26.4%) of OSCC, along with 135 healthy controls. The HR-HPV positivity in OSMF in toto was 32.6% while in OSCC it was 66.6%. It was observed that out of 92 cases of OSMF, 35 (38%) revealed dysplasia and 57 (62%) were nondysplastic lesion, the HR-HPV positivity was 54.3% (19) and 11 (19.3%) respectively. In 135 healthy controls 19 (14.1%) showed HR-HPV positivity. The difference between the HR-HPV status in control and non-dysplastic OSMF was not significant (p=0.5380). The difference between OSMF in toto and control group for HR-HPV positivity was just significant (p=0.038933). When the control group for HR-HPV positivity was compared with OSMF-dysplastic group (p=0.000423) and between dysplastic and non-dysplastic (p=0.00051) group both were highly significant. Degree of dysplasia increased with higher grades of OSMF more with HR-HPV positivity (r = 0.68). A significant association was found in HR-HPV positivity and malignancy (p=0.04). However the difference between OSMF dysplastic lesion and malignancy was insignificant (p=0.598409).*

Conclusion:

It is concluded that there is a significant association of HR-HPV positivity in OSMF with increasing grades of OED and OSCC, thus HR-HPV positivity may be recognized as an additional carcinogenic factor which has synergistic effect on the progression of potentially malignant lesion (OSMF) to dysplasia and neoplasia.

Background:

In the struggle to control cancer, knowledge of many phases of the disease process as well as the ability to recognize it in premalignant or early stages of the disease is required [1-4]. Head and neck cancer (HNC) is the sixth most common cancer worldwide, 48% are located in oral cavity and more than 90% cases are of squamous cell carcinoma (SCC) which are preceded by potentially malignant lesions- oral sub mucous fibrosis (OSMF) [5] being commonest. OSMF is a chronic, progressive, scaring disease with an insidious course, [6] typically affecting the buccal mucosa, lips, retro molar areas, the soft palate and presents as whitish-yellow discoloration of the mucosa rarely, involves larynx [7,8]. About 5 million people suffer from this disease [9] predominantly of Southeast Asian origin [10]; some cases are also reported in other parts of the world [11]. Its incidence has increased manifold in India specially in younger generation [12], due to the increased popularity of chewing commercially freeze-dried products such as Pan masala, Dohra, Gutka and Mava all having high concentrations of Areca nut with or without tobacco. Many other factors like, malnutrition, vitamin deficiency, genetic background, exposure to chemical or physical carcinogen and viral infections etc [13, 14, and 15] also effect progression of lesion, clinical staging, histological grading of OSMF, dysplasia and neoplasia. Viruses, specially HPV, a non enveloped double stranded DNA virus having tropism for epithelial cells can cause epithelial aberrations [15, 16, 17].There are reports of HPV being detected in oral mucosa, increased with increasing degree of epithelial dysplasia [18]. It is the most prevalent, sexually transmitted infection worldwide [19, 20], not having any correlation to various addiction habits [21]. The infection may occur frequently on normal oral mucosa [22], and majority infections may be transient rather than persistent [15].

The International Agency of Cancer Research (IARC) has considered 15 different HPVs as high Risk (HR) types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82), involved in human carcinogenesis [23, 24]. Literature reveals that HR-HPV may be a risk factor for many malignancies [15, 23-27] including carcinoma of cervix uteri [24-26]. This association is independent of the betel quid and tobacco chewing- two established causal factors for oral precancers [13, 28-30]. HNC are not related either to HPV or addictions (tobacco/alcohol) and are placed in an idiopathic group, but the proportion of idiopathic to HPV and tobacco, induced remains undetermined [31]. Its role in progression of potentially malignant lesions of the oral cavity to malignancy is still a matter of research and discussion [15, 22, 32]. Therefore, this hospital based cross-sectional study was undertaken to correlate various histological findings regarding grades of OSMF with or without dysplasia and malignancy with HR-HPV status.

Material and Method:

The study included 125 cases of OSMF along with 135 matched healthy controls, from outpatient department of Otorhinoloaryngology and various departments of Swaroop Rani Nehru Hospital of Moti Lal Nehru Medical College, Allahabad, U.P, North India, as well as from the private sector of the city; this study was approved by institutional ethical committee. After informed consent, a detailed demographic data including the patient's age, sex, religion, educational, marital, socioeconomic status, occupation, habitat (rural/urban), food habits (vegetarian/ nonvegetarian), intake of chillies or spicy food and addiction habits were obtained in a pretested proforma. Relevant clinical history regarding burning sensation, difficulty in eating, opening of the mouth (clinical staging of OSMF), and examination of each patient was done to assess the site, size and extent of lesions.

The oral brush technique was used for collection of cytology samples with the help of brush sampling instrument (Medical Packaging Corporation Omarillo, CA.USA), to take a complete transepithelial sample with minimum discomfort to the patient and maximum output of cells.

Histopathology samples were collected by punch biopsy as per standard protocol and the tissue was processed by paraffin

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embedding; 2-3 micrometer thick sections were cut and stained by haematoxylin and Eosin (H and E). Before making the diagnosis, the grading criteria of OSMF was made which were circulated among two pathologists to avoid subjective error, according to criteria proposed by Pindborg and Sirsat 1966 [33]. OED was categorized by the World Health Organization as slight/mild, moderate, and severe/carcinoma in situ, according to the presence and severity of cell atypia and other structural aspects of the epithelium [34].

Detection of HR-HPV DNA was done by a second-generation HC-II assay kit (Digene Corporation-FDA approved). Samples were collected from the suspicious lesions of the oral cavity by a soft brush provided in the kit by gentle rolling strokes over the affected area as per the manufacturer's instructions. Samples collection tubes were stored at 40C till the HC-II test was performed. Detection of HR-HPV DNA was carried out in the presence of a probe which consists of the 13 HR-HPV types (16, 18,31,33,35,39,45,51, 52,56,58,59 and 68). This technique is a nucleic acid hybridization assay with signal amplification that utilizes micro-plate chemiluminiscent detection. The emitted light was measured as relative light unit (RLU) in a luminometer (DML 2000, Digene®). The mean of three positive controls (PC)/RLU of sample were taken as an estimate of approximate viral load. Cut off value (RLU of specimen/mean RLU of PC) was 1.0 pecogram/ml of sample. Cut-off ratio of 0 to 0.99 was negative for HR-HPV; Cut-off ratio greater than 1.0 was positive for HR-HPV.

Statistical analysis:
Data obtained from the analysis were finally transported to the excel sheet and the average value of each parameter was calculated. Chi square test was applied for each parameter to judge the statistical significance. SPSS Chicago, IL statistical programme was used for this purpose; P-values<0.050 were considered to be statistically significant.

Result:
Of the total 125 cases studied, 92 (73.6%) were of OSMF and 33 (26.4%) of OSCC, along with 135 healthy controls. The HR-HPV positivity in OSMF in toto was 32.6% while in OSCC it was 66.6%. It was observed, that out of 92 cases of OSMF 35 (38%) revealed dysplastic lesion (14 of mild, 12 moderate, and 9 of severe grade); and 19 (54.3%) were positive and 16 (45.7%) were negative for HR-HPV. While in the non dysplastic group the HPV positivity was only 19.3% (11).

In 135 cases of the healthy control group 19 (14.1%) showed HPV positivity. The difference of HR-HPV positive status between the control group and the nondysplastic OSMF was insignificant (p=0.5380). When the control group was compared with OSMF group for HPV positivity, including both nondysplastic and dysplastic cases it was just significant (p=0.038933). The difference between HPV positivity in the dysplastic and nondysplastic group was also highly significant (p=0.00051). When the HPV positivity was compared between control and OSMF-dysplastic group, it was highly significant (p=0.000423). The degree of dysplasia increased with higher grades of OSMF, more with HPV positivity (r=0.68). In the malignant group there was significant association in HPV positivity and malignancy (p=0.04) but there was no difference between malignant and premalignant dysplastic group (p=0.598409). (Table1)

On histology epithelial thickness did not show any consistent result with grades of OSMF as it was either hyperplastic or atrophic in all grades of OSMF; however all cases of grade IV showed atrophy (least thickness). Initially, the sub epithelium showed vascular proliferation and dilatation with round cell infiltration in matrix with minimal fibrosis; later, congestion and inflammatory cells decreased but fibrosis increased. In HPV negative cases koilocytes were not seen (Fig.1 & 2).

The number of koilocytes per low power field (Fig.3-8) were found to gradually increase in HPV positive cases with increase in grades of OSMF (p=0.001). The oedema, mononuclear cell infiltration, increased vascularity, dilatation and minimal fibrosis as above was followed by dense collagenisation in sub epithelial zone, koilocytosis and localization in basal layer. Presence and number of koilocytes in both cytology and biopsy preparations showed close association with HPV positivity and viral load with increasing grades of epithelial aberration (p=0.001) in the form of epithelial hyperplasia, dysplasia and neoplasia in higher grades of OSMF.

Discussion:
In previous study conducted by us in 1989, in the non malignant lesions of oral cavity the occurrence of OSMF was found 50%. A gross and significant rise was seen in 2010 being 73.8% with shift from lower to middle socioeconomic status and illiterate to educated class, at an earlier age group (mean age 30 years) [12]. Thus implicating tobacco and betel quid chewing, the most frequently observed addictions in oral lesions as one of the important risk factor [35]. This change is already reported by us due to availability of small attractive and inexpensive scathes of betel quid substitutes widely available along with aggressive advertisement, style and family trend particularly in North India [30]. It causes interaction of collagen related genes and susceptibility to betel quid induced OSMF and also alters the mutations of ki-ras oncogene [9,35,36]. Genetic predisposition and environmental factors-addiction-Areca nut alone or in combinations lead to various grades of OSMF (1, 2, 3, 4) due to seepage of alkaloids (mainly Arecoline, Cu, etc) through epithelium to sub epithelial zone- the matrix. Changes in the matrix due to various irritants as above causes release of interleukins (IL) and other chemical mediators of inflammation and activation of lymphocytes and macrophages [9,35].

Recent advances in the molecular study of the mechanisms have revealed that reactive oxygen and nitrogen species, harmful endogenous genotoxic substances, produced by inflammatory cells are largely involved in the carcinogenic process [37]. The activation and increase in mast cells, leads to degradation of connective tissue providing space for neovascularisation, vascular changes and fibrosis [38-40]. This causes imbalance between matrix metalloproteinases (MMPs) and tissue inhibitor metalloproteinases (TIMPs), leading to higher grades of OSMF [41-43]. It is associated with epithelial changes in the form of basal cell hyperplasia followed by atrophy and ulceration with more seepage of chemicals in matrix, leading to higher grades of OSMF with epithelial cell dysplasia and finally neoplasia. (Figure 9)

Changes in premalignant and malignant lesions of oral cavity is a highly complex and multifactorial process, that takes place when epithelial cells are effected by several genetic changes that alter normal functions of oncogenes and tumor suppressor genes [15,31]. This may increase the production of growth factors, the number of receptors on the cell surface, increase transcription factors or intracellular signal messengers, leading to hyperplasia and dysplasia. These lesions are characterized by increase in mitosis, cellular pleomorphism, hyperchromasia, prominent nucleoli, loss of cellular cohesiveness with or without individual cell keratinisation in the spinous layer, followed by loss of cell cohesion and finally infiltration in adjacent tissue-invasive carcinoma[34, 45, 46].

Cancer prevalence in young is of great concern [10, 12] which is preceded by OSMF. There has been a 60% increase in the number of under 40-year old with tongue cancer, over the past 30 years [42, 45]. All patients of oropharyngeal cancer do not give history of exposure to the carcinogens usually, implicated in this disease like- areca nut, tobacco, alcohol etc alone or in combinations; then other factors like genetic predisposition, immunodeficiency, diet-nutritional status and viral infections have been considered [13, 14, 15]. These cases

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show higher aggressiveness and worse prognosis [18, 45].

In the present study, healthy control group showed 14.1% HPV positivity. Other authors also reported 15.38% HPV in saliva, in particular in young asymptomatic subjects. [47] There are contradictory reports that normal buccal mucosa of healthy volunteers contained HPV DNA only in 1%, even other group of authors reported the HPV negativity in normal oral mucosa [48,49]. There are reports that, it may occur frequently on normal oral mucosa and majority of infections may be transient rather than persistent [15].

HR-HPV positivity in OSMF in toto was 32.6% and in dysplastic lesions of OSMF it was 54.8% and 45.2% were negative for HR-HPV. In non dysplastic group the HPV positivity was only 19.3 %. A highly significant association was found with HR-HPV positivity with dysplasia and neoplasia in this potentially malignant lesion- OSMF (Table-1).

The significant difference was found between healthy controls and potentially malignant OSMF in toto in HR-HPV status. HR-HPV association was highly significant between dysplastic potentially malignant lesion and healthy controls. While the difference between control and nondysplastic group was insignificant.

The HR-HPV relation between dysplastic and nondysplastic OSMF was highly significant, indicating definite role of HPV in progression of nondysplastic lesion to dysplastic lesion in OSMF-a potentially malignant lesion, so much so that the status of HR-HPV between dysplastic and malignant lesion was not significant and significant association of HPV positivity was seen in malignant lesion. Many studies also reported role of HPV in oral carcinogenesis and clinical implications, other than OSMF [50-52].

In OSCC HPV positivity was 66.6%, other study also revealed the similar incidence of HPV (61.5%) [48]. Recently, the authors investigated the prevalence of HPV along with other oncogenic viruses in OSCC from eight different countries from different ethnic groups, continents and with different socioeconomic backgrounds and the HPV positivity was 35%, it was highest in Sudan (65%) [53]

The oncogenic viruses including HR-HPV represent important factors that may affect cell cycle regulation [45,54-57]; the transformation by HR-HPV depends on oncoproteins E6 and E7 which bind with p53 and pRb proteins and affects their ability to stimulate DNA repair and apoptosis [58-64].

Higher expression of transcription factor NF-kB and its activation sequences in oral cancer that arises from OED lesions was reported; however they suggested further research to confirm and explain their findings [65]. In other report, authors predicted definite progression of potentially malignant lesion to malignancy [66].

The histological and cytological findings both for epithelial atypia and presence of koilocytes ran parallel to each other as compared with the HR-HPV positivity, which was statistically significant. The similar observation has been found in other reports also, but the authors suggested specialized techniques like DNA hybridization to confirm HPV positivity [67]. Koilocytosis is reported as a good predictor of HPV infection [12, 30], however recently other authors stated contradictory results [68].

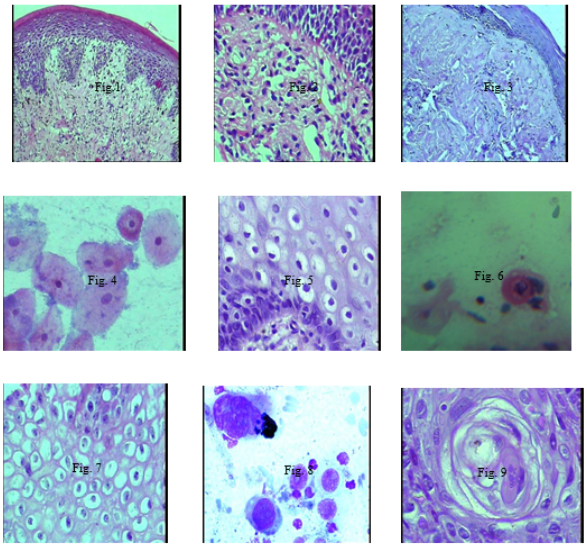
Presence and number of koilocytes in both cytology and biopsy preparations showed close association with HPV positivity with increasing grades of epithelial aberration in OSMF, which was found statistically significant. Degree of dysplasia increased with higher grades of OSMF more with HR-HPV positivity (r = 0.68).

Although HPV positivity showed no relation to addictions as already reported by us [21], the association was independent of the influence of the betel quid and tobacco chewing- two established causal factors for oral precancers [28-30]. When HPV positivity was clubbed with addictions, there was a significant association with occurrence of dysplasia and neoplasia with the positive HR-HPV status. It indicates that the virus may remain silent (latent infection or asymptomatic), unless it is provoked by some irritating factor that makes transcriptionally active HPV, like chemical mediators released due to chronic inflammation induced by irritants /addictions -areca nut, tobacco, spicy food etc . This may enhance oral carcinogenesis more in genetically predisposed, immunodefficient and malnourished individuals (Figure 9).

Conclusion:

Since there is a significant association of HR-HPV positivity in OSMF with increasing grades of OED and OSCC of oral cavity; thus HR-HPV positivity may be recognized as an additional carcinogenic factor which has synergistic effect on the progression of potentially malignant lesion (OSMF) to dysplasia and neoplasia.

As HR-HPV (16 and 18) is involved in oral malignancy and infection is sexually transmitted, public at large should be made aware of the mode of infection and implementation of vaccine program for HR-HPV (16 and 18) before exposure, which may prove to be beneficial in preventing not only carcinoma cervix uteri but possibly also OSCC. Simultaneously HR-HPV positive cases should be advised to maintain good nutritional status, oral hygiene and avoid intake of any irritant like spicy food or addiction, to prevent above synergism. It can help to control this global health problem by prevention, early diagnosis and curative services Therefore in cancer control and immunization programmes along with health education, screening and treatment for any suspicious lesion in oral cavity and HPV vaccination should also be included as in the developed countries.



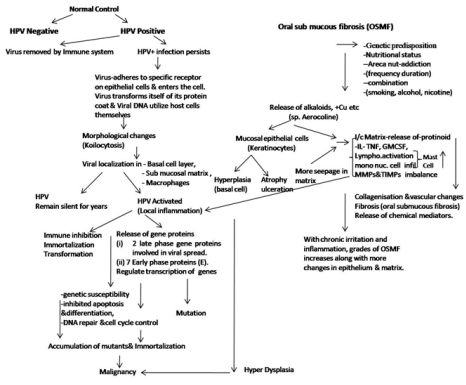


Fig. 10

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Table 1: HPV status in different grades of OSMF (1-4 grades)-dysplasia (OED), OSCC and healthy control group.

Table Legends:

HPV Status	Potentially Malignant Lesion(OSMF)						Malignant Lesion(OSCC)				Total Study Group	Healthy Control Group
	Dysplastic(OED)				Non Dysplastic	Total	W.D.S.C.C.	M.D.S.C.C.	P.D.S.C.C.	Total		
	Mild	Mod.	Sever	Sub Total								
HPV+ OSMF grade	6 3,2, 1,0.	7 2,4,1, 0	6 0,0, 3,3	19 (54.3 %)	11 (19.3%) 5,3,2,0	30 (32. 6%)	17	3	00	20 (66. 6%)		19 (14. 1%)
HPV- OSMF grade	8 5,3, 0,0,	5 3,1,1, 0	3 0,2, 1,0	16 45.7 %	46 (80.7) 12,13,11, 10	62 (67. 3%)	8	4	1	13 (33. 4%)		116 (85. 9%)
Total	14	12	9	35 (38%)	57 (62%)	92 (73. 6%)	25	7	1	33 (26. 4%)	125	135
P-Value				***0. 0004	** 0.54	*0.0 38				### 0.5 984		
				# 0. 0005					## 0.0 4			

* HPV status between healthy control and OSMF

** HPV status between healthy control and non dysplastic OSMF

*** HPV status between healthy control and dysplastic OSMF

\# HPV status between dysplastic and nondysplastic OSMF

HPV status and OSCC with OSMF

HPV status between OSCC and dysplastic OSMF

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