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Molecular detection of human papillomavirus DNA in dysplastic and non-dysplastic oral lichen planus patients

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***Abstract*---**Introduction: Oral Lichen Planus (OLP) is a chronic inflammatory disease of cell-mediated dysregulation characterized by relapses and remissions. The aetiology of OLP has been extensively studied for decades. The pathogenesis is unclear with a debatable malignant transformation. The pathogenesis and malignant transformation of OLP may be affected by viruses such as Hepatitis C Virus (HCV), Human Papillomavirus (HPV), Herpes Simplex Virus (HSV), and stress. HPV has been proved to be an etiological agent in oropharyngeal cancers and non-tobacco-associated leukoplakia. The role of human papillomavirus in the pathogenesis of OLP and its malignant transformation has to be studied extensively. Aim: This study aims to detect the presence of HPV DNA in the biopsy samples of dysplastic and non-dysplastic OLP and thus determine the role the virus played in the malignant transformation of OLP. Materials and Methods: Biopsy samples comprising 250 OLP tissues were collected. The DNA was extracted from the fixed tissue by using the Cetyltrimethylammonium bromide (CTAB) method. Polymerase Chain Reaction (PCR) was performed using primers to amplify the HPV E6 gene. Results: Hundred and three out of 250 (41.2%) OLP cases were positive for HPV DNA. Compared HPV prevalence in dysplastic and non-dysplastic OLP significant relation was obtained between HPV

and dysplastic OLP. (P<0.01). Conclusion: This study confirmed the presence of high-risk HPV 16 and HPV 18 DNA in OLP. The study showed a significant difference in the expression of HPV in dysplastic and nondysplastic OLP.

***Keywords*---**Human Papillomavirus, Oral Lichen Planus, PCR, dysplasia.

Introduction

OLP (Oral Lichen Planus) is an inflammatory autoimmune disease with a white reticular appearance that is sometimes accompanied by atrophic or erosive lesions.[1,2]It is considered a highly prevalent disorder that may develop in up to 2% of the general population. The aetiology is hypothesized to be factors such as diabetes, stress, trauma, and hypersensitivity to metals and medications. The aetiology of OLP has recently been linked to viruses such as HPV(Human papillomavirus) and human herpes virus.[3]OLP is considered an oral potentially malignant disorder (OPMD). The mechanism of malignant transformation from OLP is not clearly understood, it could probably be a multifactorial mechanism. The malignant transformation rate of OLP is controversial due to the restrictive diagnosis criteria.[4,5]Microscopically hyperkeratosis, basal layer liquefaction of the epithelium, and a strong infiltration of a band of lymphocytes are present.[6,7]However, many authors consider lichenoid dysplasia (dysplasia seen in Oral Lichenoid lesions or OLPs)as an early stage of a malignant transformation from OLP. [8,9]

Human papillomavirus (HPV) is a small, epitheliotropic, non-enveloped DNA virus.[10]The HPV genome consists of 7200 to 8000 base pairs of closed-circular double-stranded DNA, containing up to 10 open reading frames.[11]HPV causes a wide spectrum of diseases affecting the cutaneous and mucosal areas of the body, ranging from benign common warts to invasive carcinoma.[11]HPV infections have been reported in several body sites, including the anogenital tract, urethra, skin, larynx, tracheobronchial mucosa, nasal cavity, paranasal sinus, and oral cavity.[12,13]Predominantly Human papillomavirus (HPV) is proven as the major etiological agent in uterine and cervical cancer. [14,15] Recent studies have shown an increase in presence of HPV-associated oropharyngeal cancers in non-tobacco and alcohol users. HPV has been isolated from proliferative verrucous Leukoplakia which has a 95% chance of malignant transformation.

Existing data suggest that in OLP, auto-cytotoxic CD8+ T-cells are activated and replace the apoptotic keratinocytes.[3,5] The reaction of these specific CD8+ T cells is similar to what occurs during a viral infection, in which a virus can act as a cytoplasmic antigen.CD8+ T-cells cause the apoptosis of virally infected cells.[9] Steroids that are commonly used to treat OLP decrease the number of surrounding immune cells, including lymphocytes and monocytes, and decline the secretion levels of cytokines, such as tumor necrosis factor-alpha and interleukins 1 and 6 (IL-1, IL-6).[11] Furthermore, some cytokines, such as IL-1 and tumor necrosis factor-alpha, could selectively suppress HPV transcription. Therefore, extracellular HPV could more easily interfere with immune cell

networks from antigen-presenting cells to effector T-cells to activate immune evasion and promote to further persist in the epithelium and accomplish its replication cycle. This persistent viral infection may cause immune dysregulation and favour the slow progression from infection to chronic dysplasia, and, eventually, cancer.[11,12]

This indicates that oral mucosal viral infections may play a role in the pathogenesis of OLP and its malignant transformation.[16,17,18] So, our study aims to investigate the role of HPV in dysplastic and non-dysplastic OLP.

Materials and Methods

Collection of biopsy specimens

The study sample consists of 250 histopathologically confirmed cases of oral lichen planus. The OLP biopsy specimens were obtained from the Department of Oral Medicine & Radiology, from patients clinically diagnosed with reticular and erosive types of OLP. Written informed consent was taken from all the participants and the study was approved by the Institutional Review Board. The samples were collected in a biopsy bottle containing DNA stabilizer solution. The specimens were divided into two parts, one for histopathologic examination to confirm the clinical diagnosis and the other part for identification and typing of HPV DNA. The OLP specimens were examined by two experienced oral pathologists. After examination of Haematoxylin and Eosin-stained sections, a consensus diagnosis was reached in all the cases. When the typical criteria for OLP were fulfilled without any histopathological signs of dysplasia, the specimen was included in the group "OLP without dysplasia." If the OLP specimen had any dysplastic changes (mild to severe), it was included in the group "OLP with dysplasia."

The portion of the tissue subjected to HPV DNA analysis was immediately transferred to a deep freezer (-80^{0}C) and stored until the tissues were taken for further processing. For DNA extraction, specimens that were proven to be OLP positive based on histological assessment were chosen. 250 tissue samples in a DNA stabilizer were provided for HPV detection and typing of HPV-16, and HPV-18 using a conventional single Polymerase chain reaction (PCR) assay.

Deoxyribonucleic acid extraction

Genomic DNA extraction was done from the tissue sample using the Cetyltrimethylammonium bromide (CTAB) method. The chemicals and reagents implemented to extract the genomic DNA from the samples provided were 1)Extraction Buffer which included(100mM Tris HCL,100mM EDTA,1.4M NaCl,1% CTAB(N-Cetyl-N,N,N-trimethyl-ammonium Bromide),Proteinase K – 0.03µg/ml, Lysozyme – 0.67µg/ml(e & f were added freshly at the time of use) 2)20% SDS w/v 3)Chloroform: Isoamyl alcohol (24:1) 4)Isopropanol 5)Ethyl alcohol 70% v/v. The tissue sample stored at -20^{O}C were transferred to a sterile mortar and pestle and grounded to a fine powder using liquid N2. 675µl of extraction buffer was added and incubated at 37 ^{O}C for 30 min. To the above 75µl of SDS was added and incubated at 65 ^{O}C for 2 hours followed by centrifugation at 10000 rpm for 10 min at 4^{O}C. The supernatant was collected in a sterile

microcentrifuge tube. Equal volumes of Chloroform and Isoamyl alcohol were added and centrifuged at 10000 rpm for 10 min at 4^{O}C. The aqueous phase was transferred to a sterile microcentrifuge tube. .0.6 volumes of isopropanol were added and incubated at room temperature for 1 hour. After incubation, it was then centrifuged at 10000 rpm for 10 min. The pellet was washed with 500µl of 70% Ethanol and centrifuged at 10000 rpm for 10 min at room temperature. The pellet was air-dried and dissolved in 20µl of sterile water.

The quantity and quality of the isolated DNA were checked in a UV-VIS spectrophotometer (Vivaspec Biophotometer, Germany). 50 times dilution is obtained from the stock by mixing 1µl DNA with 49-µl sterile distilled water. The A260/A280 OD (Optical Density) ratio was recorded to check the purity of DNA preparation. The quality of DNA at 260/280 OD (Optical Density) was estimated ranging from 1.32 to 2.02 µg/mL, indicating a good concentration of DNA.PCR was done with GAPDH primers to check the quality of DNA(Product Size:496bp); GAPDHF: TTCTGGGGACTGGCTTTCC; GAPDHR: AAAGTGGTCGTTGAGGGCAA. Bands were observed in all 250 samples. A No Template Control is included in the last lane to rule out contamination. The DNA isolated was loaded into a 1.2% Agarose gel electrophoresis to visualize the bands and their quality.

Single polymerase chain reaction assay

A single polymerase chain reaction assay was used to detect target HPV DNA and typing of HPV-16 and HPV-18. HPV detection was done by PGMY09/11 primers (200 bp HPV-18 and 500 bp HPV-16). The PCR was carried out using primers for the E6 region: HPV16F:5'GTCAAAAGCCACTGTGTCCT3',HPV16R:5'CCATCCATTACATCCCGTAC3 ;,HPV18F:5'CCGAGCACGACAGGAACGCT3',HPV18R:5'TCGTTTTCTTCCTCTGAGT CGCTT3'. Each PCR reaction mixture contained 2 µL of 10 times PCR buffer with $MgCl_2$(1.5mM), 2 µL of dNTP mix(2.5mM), 2 µL of oligonucleotide primer *F* (10picomoles/µL), 2µL of oligonucleotide primer R (10picomoles/µL),10.70 µL of H_2 O, 1 µL of template DNA(50ng/µL), 0.30 µL of Taq-polymerase (5 U) making the total amount of each reaction to 20.0 µL. Thermocycling conditions were as follows: Initial denaturation at 94°C for 3 minutes, 35 cycles of denaturation at 94°C for 50 seconds, annealing at 50°C for 40 seconds, extension at 72°C for 1.30 minutes and final extension at 72°C for 40 seconds. The PCR product was loaded in 1.2% agarose gel to visualize the bands.

Statistical analysis

The data obtained were analyzed using the Statistical Packages for Social Sciences (IBM SPSS Statistics, SPSS South Asia Pvt Ltd., Bangalore, for Windows, Version 20.0). Categorical variables were expressed as frequency (percentage). Prevalence of HPV in OLP was expressed using 95 % CI. A Chi-square test was used to find the association of HPV with dysplastic and non-dysplastic OLP. A Fischer exact test was used to find the association of HPV in dysplastic and non-dysplastic OLP with selected characteristics of OLP. For all statistical interpretations, $p<0.05$ was considered the threshold for statistical significance.

Results

103 samples (41.2%) out of 250 specimens were positive for HPV in PCR analysis. All the study subjects were between the age of 18 and 56 years, with 120 males and 130 females. Out of 250 specimens,14 were dysplastic OLP samples and 236 were non-dysplastic OLP samples. HPV was detected in 91 of 236 (38.6%) OLP specimens without dysplasia and in 12 of the dysplastic OLP samples (85.7%). (Table 1) In the HPV type-specific PCR assay, the frequency of positivity for HPV-16 is 25.6% and for HPV-18 genomes was 15.6% (64/250 and 39/250 respectively). The study sample included a 120:130 male to female ratio, with HPV positive rates of 83.3% (5/6) in males in dysplastic and 25.4 % (29/114) in non-dysplastic OLP. The HPV positivity in females is 87.5% (7/8) and 50.8 % (62/122) in dysplastic and nondysplastic OLP specimens respectively (Table 2). The frequencies of HPV positivity in reticular OLP were 66.7% (2/3) and 19% (27/142), in dysplastic and non-dysplastic OLP respectively. The frequencies of HPV positivity in erosive OLP were 90.9% (10/11) and 68.1% (64/94), in dysplastic and non-dysplastic OLP respectively (Table 3). According to the OLP site, HPV positivity rates were 87.5% (7/5) in the buccal mucosa of dysplastic OLP specimens and 50.7% (72/142) in non-dysplastic OLP specimens (Table 4). The frequencies of HPV positivity in labial mucosa were 83.3% (5/6) and 20.2% (19/94), in dysplastic and non-dysplastic OLP respectively. Compared between buccal mucosa (p=0.002) and labial mucosa (p=0.013) in dysplastic and non-dysplastic OLP significant relation was observed between site and dysplastic OLP. A significant relation was observed between the male gender and dysplastic OLP. However, no significant difference was found between the percentage of HPV positivity in dysplastic and non-dysplastic OLP specimens in relation to the female gender, and type of lesion(P>0.005).

Table 1 Association of HPV with Dysplastic/Non-dysplastic OLP

		Negative		Positive		χ^2	P
		Count	Percent	Count	Percent		
D/ND	Dysplasia(D)	2	14.3	12	85.7	12.13	p<0.01
	N Dysplasia(ND)	145	61.4	91	38.6		

Table 2 Association of HPV in OLP with D/ND based on gender

Gender		Negative		Positive		p
		Count	Percent	Count	Percent	
Male	Dysplasia	1	16.7	5	83.3	0.007
	N Dysplasia	85	74.6	29	25.4	
Female	Dysplasia	1	12.5	7	87.5	0.066
	N Dysplasia	60	49.2	62	50.8	

Table 3 Association of HPV in OLP with D/ND based on type of OLP

Type of OLP		Negative		Positive		p
		Count	Percent	Count	Percent	
Reticular	Dysplasia	1	33.3	2	66.7	0.102
	N Dysplasia	115	81.0	27	19.0	
	Dysplasia	1	9.1	10	90.9	

Table 4 Association of HPV in OLP with D/ND based on site pf OLP

Site of OLP		Negative		Positive		P
		Count	Percent	Count	Percent	
Buccal	Dysplasia	1	12.5	7	87.5	0.044
	N Dysplasia	70	49.3	72	50.7	
Labial	Dysplasia	1	16.7	5	83.3	0.003
	N Dysplasia	75	79.8	19	20.2	

Discussion

OLP affects 1.27% of the global population with prevalence varying according to geographic location. [11] The prevalence of OLP is 2.6 % in the Indian population. [7] The relationship between HPV infection and OLP is still controversial. The role of HPV in the development of dysplasia and its subsequent malignancy is not still clear as most of the studies and meta-analyses show discrepant results. [11]

There are more than 150 types of HPV, which are circular double-stranded DNA molecules. Papillomavirus infection occurs when the epithelium becomes damaged, allowing the virus to enter the basal layer. As a result of the above-mentioned mechanism of infection, HPV and OLP may be related. Among HPV proteins, the oncoproteins E6 and E7 of high-risk HPVs (HR-HPVs) interact with host cell proteins in different degrees to interfere with normal epithelial differentiation and apoptosis by promoting cellular proliferation, DNA synthesis, and inhibiting cell cycle regulators. [12,13]

In this study, we investigated HPV DNA in both dysplastic and non-dysplastic tissue specimens from patients diagnosed with OLP. In the present study, we found a prevalence of 85.7% in dysplastic OLP and 38.6% in non-dysplastic OLP. A statistically significant relation (P<0.01)) was found in the association between HPV and dysplastic OLP.

HPV is an established risk factor in oral cancer development. [14] Previously, in a review article by Sand and Jalouli, they described that HPV-16 produces two oncoproteins, E6 and E7, which are necessary for viral replication. According to the authors, the continued and aberrant interactions between E7 and pRB and E6 and p53 leads to genomic instability and mutational events. [19] The high affinity of HR-HPV proteins for tumour suppressor gene and their tendency to stay in an abraded epithelium may result in dysplasia and subsequent malignant transformation of erosive OLP.

In our study, the prevalence of HPV DNA in OLP patients with dysplasia (85.7%) was higher than in OLP patients without dysplasia (38.6%). Sahebjamiee et al 2015 in their study from Iran detected HPV DNA in 11 of 40 (27.5%) cases of OLP. Furthermore, the prevalence of HPV-16 in OLP patients with dysplasia (43%) was significantly higher than in OLP patients without dysplasia (0%) ($P = 0.0035$). [20]

Contrary to this study Armayones et al in a Spanish cohort detected no evidence of association of HPV in dysplastic and non-dysplastic OLP. [21] The authors studied the association of HPV in a total of 83 cases. From those, 7 and 34 cases were OLP that progressed or not to invasive cancer during follow-up, whereas 24 and 18 cases were dysplasia that progressed or not to invasive cancer during follow-up, respectively. HPV DNA was detected in one of the OLP cases. The authors concluded their study by suggesting that HPV is unlikely to play a significant role in oral carcinogenesis in the Spanish population.[21]

The prevalence of HPV varies markedly among geographically diverse populations. Sand et al. found the HPV genome in five of 22 (27.3%) OLP lesions. [19] Oswald et al reported the presence of HPV 16 and 18 in 9.4% of OLP cases. The authors concluded that a successive increase in the detection rate of HPV 16 and 18 were noted from OLP to Oral Carcinoma. [22] Giovannelli et al. reported the presence of HPV 16, 18, 33 and 35 in 22.4% of cases. [23] Two studies conducted by Pol et al., and Debanth et al., from India, showed a strong association between OLP and HPV. [24,25] Increased risk of HPV infection was reported in separate studies by Campisi and Furrer et al. [26,27]

Mattila et al studied the role of HPV in atrophic OLP with regard to DNA content and repair, proliferation activity, apoptosis, cell adhesion and lymphocyte infiltration.[28]The authors observed that static cytometry is a sensitive aid in the detection of OLPs with malignant potential by detecting changes in DNA content and cellular proliferation associated with HPV infections. They concluded that the proliferation of suprabasal epithelial cells in HPV-infected cells is attributed to the expression of the viral oncogenes, E6 and E7 and the activity of these genes allows the minimum number of infected cells to increase that subsequently go on to produce infectious virions.[28]

Szarka et al. on HPV DNA in non-erosive /atrophic OLP (Non-EA-OLP) and erosive/ atrophic OLP (EA-OLP), showed a higher prevalence in EA-OLP than Non-EA-OLP (22.4%).[30] HPV prevalence differed significantly between OLP lesions associated with higher malignancy risk versus OLP lesions with lower malignancy risk (42.6% vs. 22.4%) in this Eastern Hungarian population. The above authors have explored the interaction mechanism between HPV and OLP. One explanation was the damaged oral mucosal epithelium might promote a higher rate of HPV infections within erosive lesions, likely leading to differences in associations between HPV and clinical types. Another explanation for the upregulation of HPV replication in OLP is the immunosuppression through chronic use of steroid drugs. [29,30]

Jontell M. et al in their study examined the prevalence of HPV - 6,16 in OLPe (erosive oral lichen planus) and they concluded that 65% of OLP cases were HPV positive and may represent one of the risk factors for oral squamous cell

carcinoma in erosive oral lichen planus.[31] The authors supported the hypothesis of viral aetiology probably leads to cell-mediated immunity in the pathogenesis of the OLP which might indirectly result in the disruption of the epithelial lining. In addition, the authors also emphasized trauma plays an essential role in the introduction of HPV into the basal cell layer of the epithelium in the initiation of the infection. [31]

The present study showed HPV positivity in 41.2% of samples of OLP indicating a definite presence of HPV in oral lichen planus. The frequencies of HPV 16 and HPV 18 positivity were 25.6% (64/250) and 15.6% (39/250), respectively. Given the p-value gained by the Chi-square test, a significant relation was observed between HPV infection in dysplastic OLP($p<0.01$).Compared between males ($p=0.007$) and females ($p=0.066$), erosive($p=0.168$) and reticular($p=0.102$), labial($p=0.003$) and buccal mucosa($p=0.044$) between dysplastic and non-dysplastic OLP significant relation was observed between males and dysplastic OLP. All erosive OLP showed HPV DNA positivity in both dysplastic and non-dysplastic OLP because atrophic-erosive types of OLP lesions always provide an environment for the entry of the virus into the basal layer through micro-abrasions and subsequent multiplication. This might be one of the reasons for the presence of HPV in more malignant potential atrophic- erosive OLP. However, no significant difference in HPV infection rate was evident when comparing the AE (atrophic-erosive) and non-AE OLP variants of OLP.

However, there are few current investigations of HPV as a risk factor for dysplasia in OLP and its subsequent malignant transformation. [11,20,28,30,31]A progressive increase in HPV prevalence is found from lesions with low malignant potential to those with high.[22] The increased prevalence of high-risk HPV 16 and HPV 18 in OLP may be due to the favourable environment the lesions provide. These favourable environments may be the micro-abrasions in atrophic-erosive OLP to the use of immunosuppressive agents in the management of OLP. The chronic use of high-potency topical steroids may be associated with the reactivation of latent HPV.[11,31]

Our preliminary study to analyze the HPV DNA in OLP patients showed a possible higher occurrence of HPV in dysplastic OLP samples compared to non-dysplastic OLP samples. However, a significant relation was found between HPV and dysplastic OLP. In the present study, we used PCR, which is a sensitive method, for the detection of HPV. More prospective cohort studies including more samples of dysplastic OLP that had transferred to Squamous cell carcinoma (SCC) are needed to establish a conclusive association of HPV DNA in dysplastic OLP.

Limitation

The major limitation of the current study is the small sample size of dysplastic OLP. The study should be conducted with a greater number of dysplastic OLP cases to confirm the hypothesis of the relationship between HPV and dysplastic OLP. This low prevalence limited our ability to analyse various factors associated with HPV infection. In the present analysis HPV prevalence in clinically normal sites was not analyzed. Thus, future studies comparing HPV infection in normally and pathologically oral sites are required to determine whether HPV is a factor

that predisposes to the pathogenesis of OLP and its malignant transformation.

Conclusion

In the current study, HPV was detected in 12 out of 14 cases and 91 out of 236 cases in dysplastic and non-dysplastic oral lichen planus respectively. The high-risk types HPV 16 and 18 present more in erosive lichen planus in dysplastic and non-dysplastic OLP in the present study augments the previous theory that ulceration is frequent in erosive OLP making it more susceptible to HPV infection. Adequate long-term follow-up of erosive OLP is essential taking into consideration the premalignant potential of OLP and the increased presence of high-risk genotypes 16 and 18. Further studies are needed to assess the potential role of HPV and specifically HPV-16 and HPV 18, in dysplastic OLP lesions in the Indian population.

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