

Etiopathogenesis of Oral Lichen Planus and Epstein - Barr Virus in North Karnataka, India

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Abstract: Oral Lichen planus (OLP) is a disease of adulthood, and children are rarely affected. It may involve various mucosal surfaces either independently or concurrently (oral, skin, and oral and skin lesions). Prevalence of skin LP in general population is 0.9% -1.2% and prevalence of oral LP is reported between 0.1% and 2.2% (1). The relative risk for oral lichen planus was highest (13.7) among those who smoked and chewed tobacco. The etiopathogenesis appears to be complex, with interactions between genetic, environmental, lifestyle factors, and interestingly with new associations such as with liver disease have emerged. Viral infections have recently been linked with OLP. Herpes Simplex virus-1 (HSV-1), Cytomegalovirus (CMV), Human Herpes virus-6 (HHV-6), Epstein- Barr virus (EBV), Human Papilloma virus (HPV) and Hepatitis C virus (HCV) are virus types that have been studied in the etiopathogenesis of OLP(2). EBV is associated with infectious mononucleosis, oral hairy leukoplakia (nonmalignant disorders), Burkitt lymphoma and nasopharyngeal carcinoma (NPC) (malignant disorders). The purpose of this study is to screen Epstein-Barr Virus (EBV) genotypes and to detect its role in the pathogenesis of Oral Lichen Planus (OLP) using tissue samples. Samples consisted of 80 cases of OLP and 30 age and sex matched controls. The genomic DNA isolated from the tissue samples were electrophoresed on a 0.8% agarose gel. The PCR based detection of EBV sequences in the clinical samples did not produce any amplification using primers F-EBV (TC-70) and R-EBV (TC-72). This investigation showed no EBV positivity in OLP and control subjects but this result cannot reject the role of EBV in oral lesions such as OLP, therefore, further studies need to be done for arriving at definitive conclusions.

Key words: Oral Lichen planus, Epstein-Barr Virus, PCR

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I. Introduction

Head and neck squamous cell carcinoma (HNSCC) is the 6th most common cancer. Overall 57.5% global head and neck cancer occur in Asia especially in India (3). Oral Lichen Planus (OLP) undergoing malignant transformation rates 0% to 9%(4), then OLP would be the major source of oral cancer in many parts of the world. OLP is a disease of adulthood, and children are rarely affected. It is usually observed in or stressed nervous, 'highly strung' people (Shaler 1983). It may manifest anywhere in the oral cavity. The buccal mucosa, tongue, and gingiva are the most common sites, whereas palatal lesions are uncommon. They are usually symmetrical and bilateral lesions or multiple lesions in the mouth are common. Andreasen (1968) divided oral lichen planus into six types: reticular, papular, plaque-like, erosive, atrophic, and bullous. The reticular, papular, and plaque-like forms are usually painless and appear clinically as white keratotic lesions. The erosive, atrophic, and bullous forms are often associated with a burning sensation and in many cases can cause severe pain (5).

DIFFERENT TYPES OF LICHEN PLANUS SEEN IN ORAL CAVITY



RETICULAR LICHEN PLANUS



EROSIVE LICHEN PLANUS



ATROPHIC LICHEN PLANUS



PLAQUE TYPE LICHEN PLANUS

The overall prevalence of oral lichen planus among Indians was 1.5%; it is highest (3.7%) in those people with mixed oral habits and lowest (0.3%) in non- users of tobacco. The annual age-adjusted incidence rate was 2.1 and 2.5 per 1000 among men and women, respectively. It is highest (8.2 per 1000) among men who smoked as well as chewed tobacco; among women it was highest (4.5 per 1000) in chewers. The relative risk for oral lichen planus was highest (13.7) among those who smoked and chewed tobacco. There seems to be a slightly higher incidence of oral squamous cell carcinoma in patients with oral lichen planus than in the general population. The actual overall frequency of malignant transformation is low, varying between 0.3% to 3%. The forms that more commonly undergo malignant transformation are the erosive and atrophic forms(5). Lichen planus is probably of multifactorial origin, possibly induced by drugs or dental materials, psychological factors, infective agents, often idiopathic. The etiopathogenesis appears to be complex, with interactions between genetic, environmental, lifestyle factors, and interesting with new associations such as with liver disease have emerged. Viral infections have recently been linked with OLP. Herpes Simplex virus-1 (HSV-1), Cytomegalovirus (CMV), Human Herpes virus-6 (HHV-6), Epstein- Barr virus (EBV), Human Papilloma virus (HPV) and Hepatitis C virus (HCV) are virus types that have been studied in the etiopathogenesis of OLP(2).

The Epstein-Barr virus (EBV), unlike other members of the human herpes virus family, was the first human tumor virus identified. Two EBV subtypes have been identified, i.e., EBV-1 and EBV-2, differing in the genes coding for nuclear proteins EBNA-LP, 2,-3B, and -3C, with differences in the predicted amino acid sequence of between 28% and 47% (6).The human herpes virus family consists of three subfamilies, i.e., alpha, beta, and gamma. EBV belongs to the gamma subfamily, which is split into two genera, lymphocryptovirus and rhadinovirus. EBV is the prototype for the lymphocryptovirus because it latently infects B-lymphocytes(7). Like other herpes viruses, EBV has a toroid-shaped protein core, wrapped with DNA; a nucleocapsid with 162 capsomeres; a protein tegument between the nucleocapsid and the envelope; and an outer envelope with external

glycoprotein spikes. The EBV stores its genome in the form of a linear, doublestranded, 172 kbp DNA molecule(8). Earlier studies on EBV prevalence in normal oral mucosa showed different results which apparently depended on methods of sample collection. Oral smears, scrapings and throat washings seem to give a higher EBV prevalence, with 20% to 90% EBV positivity in adults. High EBV prevalence in OLP in some studies might be due to a decrease in the immune defense, locally or generally. Studies on immune-compromised patients seem to support this theory, because they show a higher prevalence of EBV, even in clinically normal oral mucosa(9).

EBV is able to encode a protein homologue to IL-10, which can suppress cellular immune responses, and in this context of local immunosuppression, the infection of epithelial cells by "high risk" HPV types might be facilitated. The presence of both viruses EBV and HPV seen in 30.6% of the oral squamous cell carcinoma (OSCC) samples. It is likely that the ability of EBV to transform oral epithelial cells to neoplasm is dependent on the level of immunodepression of the patients and in severely immunosuppressed patients, EBV might play a direct role in the cancer development and/or progression(10).

Three theories for the presence of EBV DNA in oral premalignant and malignant lesions have proposed by Horiuch et al : [i] EBV infection may be involved in the carcinogenesis of oral squamous cell epithelium; [ii] EBV easily infects squamous cell carcinoma cells [iii] EBV exists in cancer cells as a passenger(11).

The polymerase chain reaction (PCR) assay is a technique that offers several advantages over other methods. It requires only a small quantity of biological material and can detect the viral presence in "early" infections. PCR detection of HPV, EBV, and HSV is highly sensitive and specific, and can supplement the detection of clinical manifestations of virus-associated oral lesions(12).

Hence, the purpose of this study is to screen Epstein-Barr Virus (EBV) genotypes and to detect its role in the pathogenesis of Oral Lichen Planus(OLP) using tissue samples. The efficacy of tissue usage in the isolation of virus using PCR based detection method will be assessed. A better understanding of genetic correlation of the virus and the disease will help in prevention and better treatment prognosis in future.

II. Materials and Methods

Sampling: Ethical clearance from Institutional Review Board (IRB) of SDMCDS was obtained from prior to commencement of the study. Clinically and histopathologically diagnosed cases of OLP presenting to SDMCDS enrolled in the study. 2-5 mm³ of tissue sample was collected in individual autoclaved, nuclease free 5 ml vials containing 2 ml of Ambion'sRNAlater[®], an aqueous, non-toxic tissue storage reagent that rapidly permeates tissues to stabilize and protect cellular DNA and RNA. The vials were number coded. After each collection, the vials containing the samples were transported in ice cold conditions to the laboratory. Once in the laboratory the vials were kept in freezers at -20°C. Raji cell line which contains DNA sequences of EBV was obtained from NCCS, Pune. The cell lines were maintained and cultured at research laboratory of Maratha Mandal Dental College Belgavi. The cell pallet of cultured cells was used for DNA isolation. Along with the sample collection the details of patients' clinicopathological and demographic details viz. age, family history of cancer, residence (urban or rural), occupation, income, etc. were collected in a predesigned data collection sheet (Annexure II) from the case records.

EBV sequence Detection by PCR: The detection of EBV sequences in the clinical samples was first carried out by employing PCR amplification based detection. The steps involved in this process are as follows;

1) DNA Isolation and quantification: Genomic DNA was isolated from the clinical samples and Raji cells using DNeasy[®] Blood & Tissue Kit (Cat. no. 69504, Qiagen), The isolated DNA was stored at -20°C until further quantification and PCR assays. Agarose gel (0.8%) electrophoresis was done for visual inspection of the isolated genomic DNA of both the clinical samples and Rajicell pallet. The quantification was done on a Micro-volume spectrophotometer (Quawell Technology).

2) Primer Design: The sequence of EBV that was selected for PCR based detection should be highly specific and should not have homology to any of the human or other viral sequences. Primers mentioned in earlier reports of PCR based detection of EBV sequences in human cancer tissues were used in this work. The isolated genomic DNA was checked for PCR amplification using primers (GAPDH-F and GAPDH-R) for GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) gene, a housekeeping gene. The details of the primers are given in the table (Table 1). The primers were checked for specificity using online tools like Primer-BLAST and UCSC In-SilicoPCR.

Table 1: Details of primers used for PCR based detection of EBV

Primer	Sequence (5'-3')	Product Size	Tm (°C)
F-EBV (TC-70)	CTTGGAGACAGGCTTAACCAGACTCA	265	60°C
R-EBV (TC-72)	CCATGGCTGCACCGATGAAAGTTAT		
GAPDH -F	CGACCACTTTGTCAAGCTCA	332	64°C
GAPDH -R	AGGGGTCTACATGGCAACTG		

3) PCR amplification: PCR assays were conducted with utmost care to rule out any possibility of cross contamination resulting in false results. PCR mixture preparation of the isolated genomic DNA from the samples was carried out in a separate laboratory. The components of PCR mixture were checked on regular bases. All the consumables used in these assays were sterile and nuclease free. PCR amplification was carried out in a 20 µl reaction volume containing 0.5 µl of genomic DNA (75 ng/µl to 150 ng/µl), 0.5 µl of each primer (5 pmol), 0.4 µl of dNTP (10 pmol), 0.2 µlTaq DNA polymerases (3 units/ µl), 4 µlTaq Buffer (5X) (BioRad, USA) and total volume was adjusted to 20 µl using molecular biology grade water. Amplification was carried out in Mastercycler gradient (Eppendorf, Germany) under the following conditions: an initial denaturation at 98°C for 10 sec, followed by 35 cycles at 98°C for 10 sec (cycle denaturation), primer annealing temperature was set depending on the annealing temperature of each primer set (Table 1) for 10 sec, 72°C for 15 sec (primer extension) and a final extension at 72°C for 5 min.

4) Agarose Gel Electrophoresis: The PCR amplicons were subjected to agarose gel electrophoresis using submarine electrophoresis system (Bio-Rad Laboratories, Inc.). Agarose gel (4% w/v) was prepared in 1X TAE, melted in microwave oven and ethidium bromide was added to a final concentration of 0.5µg/mL. The molten gel was poured in a gel casting tray with comb and allowed to solidify at 10°C for 20 min. 10µL of each PCR product was mixed with 2µL of 6X loading dye and loaded into the well. Along with the products 100 bp DNA molecular weight marker (NEB) was run. Electrophoresis was carried out at 100 Volts/cm, for 1 hr. Following electrophoresis the gel profile was visualized under UV illumination at 254nm and photographed in a gel documentation system (VilberLourmat).

III. Results

Samples consisted of 80 cases of OLP and 30 age and sex matched controls. Using the DNA of reference Raji cell line, which contains DNA sequences of EBV, the PCR protocol was standardized for detection of viral sequences in clinical samples. Specific amplification of the 265-bp region is detected in as little as 1 pg of Raji cell DNA, corresponding to 0.1 Raji cell or five copies of EBV. DNA from none of the OLP patient showed specific amplification of this EBV gene. The PCR based detection of EBV sequences in the clinical samples did not produce any amplification using primers F-EBV (TC-70) and R-EBV (TC-72) (Fig. 2).



Fig. 2: Detection of EBV

Line M: 100 bp DNA ladder;PC: Positive Control (DNA of Raji cells);1 to 27: 9 OLP to 18 OLP

The size of PCR products using F-EBV (TC-70) and R-EBV (TC-72) primers was 265 bp and the size of PCR products of internal control (by primers GAPDH-F and GAPDH-R) was 332 bp. All OLP and control specimens were positive for GAPDH and thus included in the study. However none of the specimens are positive for EBV sequences.

IV. Discussion:

The association between EBV and premalignant and malignant disorders has been studied for the oral region. Some authors consider OLP to be premalignant lesions, but the premalignant potential of OLP remains controversial (4, 13, 14). Regarding the possible premalignant potential of OLP, earlier investigators have reported a correlation between degree of dysplasia in leukoplakias and EBV prevalence (11, 15, 16). In any cases, the premalignant potential of OLP cannot be ruled out.

Pedersen (17) observed specific EBV DNA in some OLP specimens and suggested that EBV may be involved in the pathogenesis of some oral lesions. In another study, investigators found 26/1% EBV positivity in OLP as compared with 7/3% in control subjects (18). But whether the EBV infection is involved in the pathogenesis of OLP, or whether the EBV infection is secondary to the OLP lesions as a result of a possible local immunosuppression, is difficult to say. In this study, PCRmethod have failed to detect any difference in EBV prevalence between OLP and control subjects. Other investigators using PCR have failed to detect any difference in OSCC and control subjects (19-21), too, and the EBV prevalence differs from 0% to 100% in previous studies of OSCC with PCR (16, 19-26). In another study, PCR method was employed to detect members of the human herpes virus (HHV) in 38 OLP specimens and 20 normal control buccal mucosa tissue

samples. EBV was detected in small percentages of tissue samples, therefore, this result does not suggest a causative role for members of HHV family in the pathogenesis of OLP (34). In similar study, PCR was carried out in 10 specimens of oral erosive lichen planus (ELP) and 5 specimens of normal oral mucosa. No EBV was demonstrated in ELP patients' and control subjects' specimens (27). Although the numbers of patients in our research are more than theirs but the results are the same. This similarity in results may be due to common social behaviors in Asian countries, because that research has been also carried out normal flora component of their mouth and higher prevalence of EBV in their country. Three theories for the presence of EBV DNA in oral premalignant and malignant lesions have proposed by Horiuchi et al. (11): [i] EBV infection may be involved in the carcinogenesis of oral squamous cell epithelium; [ii] EBV easily infects squamous cell carcinoma cells [iii] EBV exists in cancer cells as a passenger.

V. Conclusion:

This investigation showed no EBV positivity in OLP and control subjects but this result cannot reject the role of EBV in oral lesions such as OLP, therefore, further studies need to be done for definitive conclusions.

Authors' contributions

KRN, SVH and BBK designed the study. KRN and KB collected the clinical specimens and allied clinical data. BBK carried out the molecular studies, and drafted the manuscript. BBK and SVH analysed the data of the study. KRN and KB approved the final version to be published. All authors read and approved the final manuscript.

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