RESEARCH ARTICLE

Expression of latent membrane protein 1 of Epstein Barr Virus in oral squamous cell carcinoma: A baseline clinicopathologic study

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Background: In addition to tobacco and alcohol, viruses with oncogenic potential may play a significant role. Epstein Barr virus being latent in oral cavity association of Epstein Barr Virus encoded latent membrane protein 1 expression may provide a link in development of oral cancer.

Aim: To find the association of Epstein Barr Virus in oral squamous cell carcinoma and to find the clinical outcomes of overexpression and non-expression of Latent Membrane Protein in oral squamous cell carcinoma patients

Materials and methods: This observational study comprised of 51 patients with oral squamous cell carcinoma who had never consumed tobacco or alcohol. The resected tissues were subjected to immunohistochemistry to evaluate Epstein Barr virus encoded latent membrane protein 1 overexpression. Overexpression of Epstein Barr virus latent membrane protein1 was compared with various clinical and histological parameters using Chi square tests, Fisher's test, and Wilcoxon rank sum test with P value less than 0.05 being considered significant.

Results: Out of the 51 patients, 6 of them overexpressed Epstein Barr virus latent membrane 1 antigen out of which 2 patients had sharp tooth. Histologically 4 cases were grade 1 and 2 were grade 2. Disease free survival and overall survival was more in Epstein Barr virus latent membrane 1 overexpressed patients (28.6, 33.3 vs 19.8, 22.13)

Conclusion: Epstein Barr virus may be associated in the development of oral squamous cell carcinoma and may influence survival rates in such patients.

Keywords: Epstein Barr Virus, latent membrane protein, oral squamous cell carcinoma, prognosis, survival, sharp tooth

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Introduction

Oncogenic viruses play a significant role in the development of cancers in the body and approximately 64% of cancers in 2018 were attributable to viruses namely Human Papilloma Virus (HPV), Hepatitis B virus (HBV), Hepatitis C viruses (HCV) and Epstein Barr Virus (EBV) [1].

Of these, association of HPV have been strongly implicated in the development of head and neck cancers especially oropharyngeal carcinoma, laryngeal carcinoma, and oral squamous cell carcinoma. On the other hand, EBV, the first identified human oncogenic virus plays a definitive role in nasopharyngeal carcinoma (NPC), some types of lymphomas and gastric carcinomas. Transmitted through saliva, the virus establishes a lifelong latent infection while manipulating host epigenetic mechanisms initiating the different oncogenic pathways. However, role of EBV in oral squamous cell carcinomas is poorly understood [2]. This study is an attempt to evaluate the association of EBV in Oral Squamous Cell Carcinoma (OSCC) patients who have never consumed tobacco or alcohol in their lifetime with the primary objective of finding the prevalence of expression of Latent Membrane Protein 1 of EBV, a surrogate marker for oncogenic role of EBV in OSCC., to compare the clinicopathologic characteristics of the EBV LMP1 positive and negative tumours. The secondary objective is to compare the survival rates of the EBV LMP1 positive and negative tumours.

Methods

60 patients with OSCC who visited a tertiary oncological centre in Madurai, South India were recruited in the study. These patients have never consumed tobacco or alcohol in their lifetime. Informed consent was obtained from the patients or attenders of the patients and the ethical approval was obtained from the appropriate authorities. (NO.303/2023/IEC/TMDCH Dt. 31.1.24). The demographic details, clinical details such as site of the tumour, laterality, presence of sharp teeth and clinically discernible nodes were recorded. The tumour was graded using Tumour, Node, Metastasis TNM staging according to 8th edition of the American Joint Committee on Cancer (AJCC).

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The patients underwent surgery ± chemoradiation depending upon the stage and site of the tumour. The surgically resected specimens were observed for perineural invasion (PNI), lympho-vascular invasion (LVI), Depth of invasion (DOI) and worst pattern of invasion (WPOI). Five-micron thickness of the paraffin embedded tissues were placed in positively charged slides and were incubated at 600 degrees Celsius. After washing with water, antigen is retrieved by placing the slides in Tris Ethylene Diamine tetra acetic acid (EDTA) buffer in pressure cooker for two whistles. Then the slides are placed in distilled water and wash buffer followed by adding peroxidase blocker to block endogenous peroxidase for fifteen minutes. After washed with wash buffer, the Primary antibody EBV LMP-1(3H2104,a,b,c) which was a pre diluted mouse monoclonal antibody (Pathnsitu – Secunderabab, India) was added to the slides marked as positive followed by the secondary antibody , a mouse monoclonal antibody CS1-4 (Dako Envision Flex kit).

The slides were washed serially with wash buffer followed by Diamino Benzidine (DAB) buffer and then again by wash buffer. Then the slides were counter stained with haematoxylin and mount with Distyrene, Plasticizer and Xylene (DPX). Tissue sections positive for nasopharyngeal carcinoma and normal tissues showing no pathology were used as positive and negative control. Block staining method was employed to assess the EBV encoded LMP1 overexpression or positive staining. Strong nuclear and cytoplasmic staining in more than 10% of cells was considered positive staining (Figure 1).

The patients were followed for around 24 to 36 months. Disease free survival, overall survival, and recurrence were all noted.

Results

Though sixty patients with histologically confirmed OSCC were recruited, 9 of them defaulted, and hence 51 patients formed the study sample who were in the age group of 31 to 80 years (mean of 58.68 years). 35 of them were males, and the rest were females. Lateral border of the tongue (19/51) and buccal mucosa (18/51) were the commonest sites of occurrence, with almost equal occurrence in other sites. 6 patients showed EBV LMP 1 overexpression, and their age group was slightly younger (mean age 53.6±11.41). Five of them were males and one was female. EBV LMP 1 overexpression cases were seen on the lips(n=1), buccal mucosa(n=2), and the lateral border of the tongue(n=3). Two patients with EBV LMP-1 overexpression had sharp teeth. Among the EBV-LMP1-positive patients, 4 had grade 1 tumours and 2 had grade 2 tumours. Also, one patient had lymphovascular and perineural invasion. Among the 6 positive patients, 4 had a depth of invasion of less than 5 mm, and 2 had a depth of invasion of 6-10 mm. None of them had recurrence. Disease-free survival and overall survival were higher in EBV LMP-1 overexpressing patients than in the with negative expression. (28.6 ±6.86 months vs 19.8 ±11.74 months and 33.3 ±8.75 months vs 22.13 ±11.27 months)(Figure 2).

These findings were statistically significant. (p value = 0.03 and 0.024).

The descriptive statistics along with the p value are given in detail in Table 1.

Discussion

Oral cancer is the sixth most common cancer in the world [3]. Though the primary etiologic agent of oral cancer is attributed to tobacco and or alcohol, a small subset of

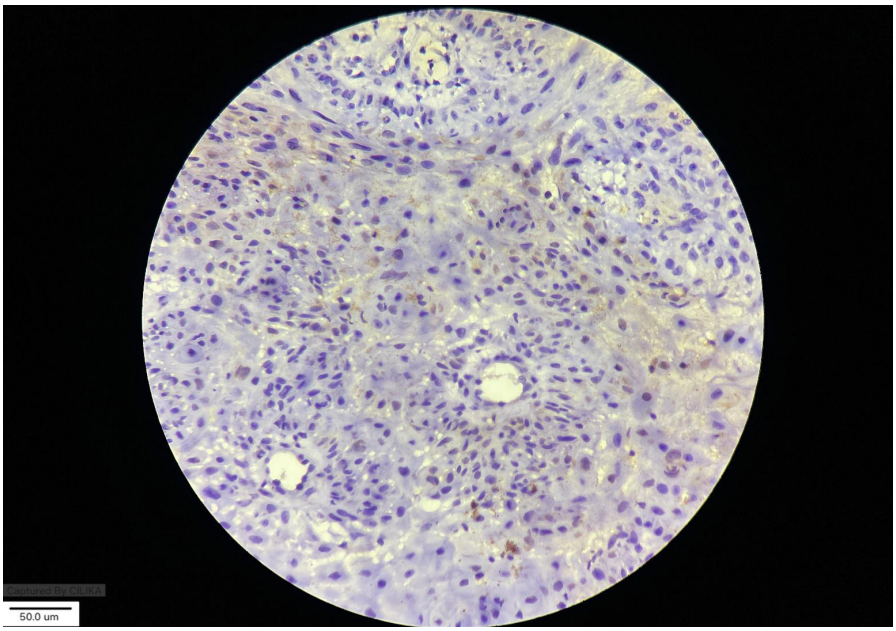

Fig. 1. Photomicrograph in 400 magnification shows the Epstein Barr Virus Latent Membrane Protein 1 stain in brown colour in cytoplasm and nucleus of cells

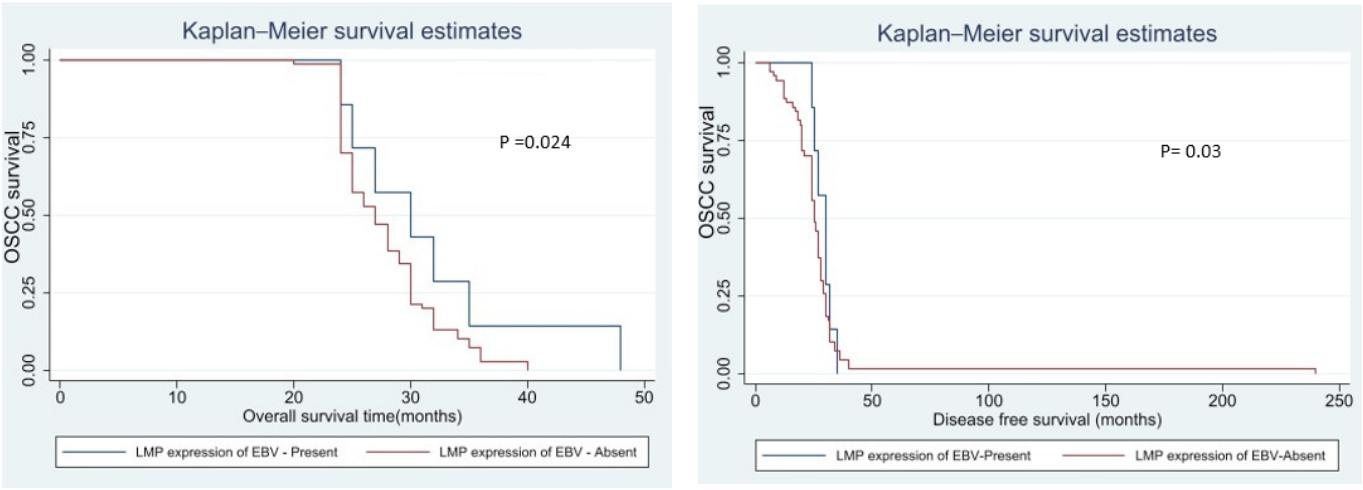

Fig. 2. Kaplan Meier Graphs depicting the overall survival and disease-free survival of patients with EBV LMP 1 expression and non-expression

Table 1. Descriptive statistics

Variable		EBV positivity present: N(%); N=6	Absent: N (%); N=45	P value
Age (years):				0.19T
	Mean (SD)	53.6±11.41	59.3±9.8	
	Range	35-66	42-81	
Gender:				0.41C
	Male	5(83.3)	30(66.7)	
	Female	1(16.7)	15(33.3)	
Site:				0.224F
	Lip	0	1(2.2)	
	Buccal Mucosa	1(16.7)	17(37.8)	
	Retromolar trigone	0	2(4.4)	
	Tongue	0	1(2.2)	
	Palate	1(16.7)	1(2.2)	
	Gingiva	0	1(2.2)	
	Oropharynx	0	1(2.2)	
	Lip & buccal mucosa	1(16.7)	0	
	Tongue lateral border	2(33.3)	17(37.8)	
	Tongue lateral border & dorsum	0	2(4.4)	
	Tongue lateral border & retromolar trigone	0	1(2.22)	
	Buccal mucosa & lateral border tongue	1(16.7)	1(2.22)	
Laterality:				0.241F
	Right	3(50)	22(48.9)	
	Left	2(33.3)	22(48.9)	
	Midline	0	1(2.2)	
	Bilateral	1(16.7)	0	
Sharp tooth:				0.99F
	Yes	2(33.3)	14(31.1)	
	No	4(66.7)	31(68.9)	
Histological grading:				0.21F
	Grade 1	2(33.3)	29(64.4)	
	Grade 2	4(66.7)	12(26.7)	
	Grade 3	0	4(8.9)	
Lymph vascular invasion:				0.548F
	Present	1(16.7)	5(11.1)	
	Absent	5(83.3)	40(88.9)	
Perineural invasion:				0.99F
	Present	1(16.7)	8(17.8)	
	Absent	5(83.3)	37(82.2)	
Depth of invasion:				0.710F
	0-5 mm	4(66.7)	33(73.3)	
	6-10 mm	2(33.3)	10(22.2)	
	11-15 mm	0	2(4.4)	
WPOI:				0.298F
	Type 1-4	6(100)	38(84.4)	
	Type 5	0	7(15.6)	
Recurrence:				0.57F
	Yes	0	9(20)	
	No	6(100)	36(80)	
Neck nodes:				0.284F
	Yes	4(66.7)	38(84.4)	
	No	2(33.3)	7(15.6)	
Disease free survival (months):				0.03T
	Mean ± SD	28.6±6.56	19.8±11.74	
	Range	20-36	0 - 48	
Overall survival (months):				0.024W
	Mean (SD)	33.3±8.75	22.13±11.27	
	Range	24 - 48	6-50	

F-Fisher's Exact test; C-Chi Square test; T-Two sample T test; W-Wilcoxon Rank sum test; Bolded P value is significant (p<0.05); %-Column percentages

them exists without a definable aetiology. Many authors have implicated the presence of sharp teeth in the region of tumour, but a systematic review by Singhvi HR et al has shown no definitive association between broken or sharp teeth with oral carcinogenesis [4].

In this scenario, oncogenic viruses have emerged as important factors in conjunction with environmental factors such as tobacco, alcohol, and inherited genetic mutations [2].

Epstein-Barr virus, one such oncogenic virus, is a primary etiologic factor in nasopharyngeal carcinomas, gastric carcinomas, and Hodgkin's lymphomas. Prevalence of EBV infection ranges from 90 -100% and it establishes a lifelong latency in B cells and /or epithelial cells [5,6].

EBV can cause heritable changes in gene expression and cause cancer progression even in the absence of active viral infection. EBV exerts its oncogenic effects through latent viral proteins, including EBV nuclear antigens (EBNAs) and latent membrane proteins (LMPs), which interfere with normal cellular functions, stimulate cell proliferation, and prevent apoptosis [7,8].

The expression of LMP-1 appears to serve as a potential surrogate marker for EBV in OSCC [9].

LMP1 triggers multiple signalling pathways leading to epithelial cell transformation, cell adhesion and invasion and increased cell growth [10,11].

The prevalence of LMP1 protein in EBV-associated OSSCs ranged from 10.7 to 59.6% [9].

In our study, the overexpression of LMP-1 protein in OSCC was 11.76%. Gonzalez et al [12]. found LMP-1 expression in 85% of OSCC with EBV DNA in OSCC, many of which derived from the lateral border of the tongue. In an Indian study prevalence of EBV LMP 1 expression was 82.65% [13].

In a study in Thailand, 100% of cases showed positive expression of LMP1 protein and about 40% 0f cases were from the lateral border of the tongue [9] Another Indian study also showed a prevalence of 8% of EBV LMP 1 expression, which was seen in 2 OSCC patients 2 with OPMDs, and 2 in healthy controls [14]. Though our study showed comparatively less prevalence, out of 6 positive cases, 3 were seen in the lateral border of the tongue. It is important to note that the lateral border of the tongue is considered a high-risk site for the development of OSCC. EBV latency at this site has been reported in cases of oral hairy leukoplakia [15].

Two patients with EBV LMP1 overexpression had a history of a sharp tooth at the tumour site, while 4 other patients did not. Gupta et al. reported a significant association between chronic mechanical trauma and oral cancer; however, they could not establish it as an independent risk factor [16]. Thus, chronic irritation due to sharp tooth alone may not be implicated in the causation of tumour. Presence of a mechanical irritation along with a viral aetiology may be possible particularly in the absence of tobacco-related insults.

LMP1 positive tumours are found to be more aggressive than LMP1 negative tumours and prone to invade lymph nodes in nasopharyngeal tumours. LMP1 expression has also been found to significantly correlate with the expression of proteins involved in invasion, angiogenesis, metastasis, and reduced overall survival [17].

EBV's role in OSCC disease progression has not been studied in detail. In our study out of the positive 6 cases with LMP1 overexpression, only one case had lymphovascular and perineural invasion. Two cases showed grade 2 depth of invasion while none of the case exhibited worst invasive front and recurrence. Though it is too preliminary to draw conclusions based on this it may be speculated that EBV may provide a favourable prognostic clue in the progression of OSCC.

As LMP1 acts as a potent oncogenic driver, contributing to various stages of NPC progression, LMP1 is recognized as a strong predictor of poor prognosis in NPC [18].

Rajesh et al in their study showed in 20 subjects showing recurrence, 35 % (n = 7) were EBV positive suggesting poor prognosis for EBV positive OSCC subjects. However, the EBV positivity was assessed by nested PCR and no oncogenic proteins were evaluated [19].

In our study, disease free survival and overall survival was increased in EBV LMP1 overexpressed tumours than negatively expressed tumours. In a Finland study have linked the presence of EBV-encoded small RNAs (EBERs) in OSCC tumour cells to a worse prognosis and shorter survival [20].

The limitations of the study include the heterogenous nature of the site of tumour, limited sample size, and assessment of only one oncogenic protein of EBV. Also, co-infection with other viruses especially with HPV was not evaluated. Future prospects include studies being done with larger more site-specific homogenous sample size and detection of all latent proteins of EBV involved in oral carcinogenesis.

Conclusion

This study though preliminary in nature indicate a viral aetiology in oral cancer subjects with no alcohol or tobacco habits. EBV LMP-1 is present in OSCC and can have prognostic impact on the survival of patients.

Conflict of interest

None to declare.

Institutional review board statement

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Author's Contribution details

WC - Conceptualization; Methodology; Validation; Investigation; Resources; Data curation; Writing – original

draft;
US- Conceptualization; Methodology; Validation; Revising final draft Data
SRR – Acquisition of data, analysis of data, drafting the manuscript
ST- analysis, critical evaluation of data, final approval of version to be published
DP- Conceptualization; Validation; Editing; Supervision
AN – Data curation: Methodology; Validation: Final approval of version to be published

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