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Immunohistochemical Expression of Epstein-Bar Virus in Biopsies Bearing Oral Squamous Cell Carcinoma

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Background: Oral Squamous cell carcinoma is the sixth most common cancer across the world. In Pakistan oral squamous cell carcinoma ranks second among all cancer in both men and women. Several risk factors of oral squamous cell carcinoma have been established which includes; smoking, alcohol, betel quid and Human Papilloma Virus infection. Epstein-Bar virus has been recently linked with development of oral squamous cell carcinoma in multiple studies. We therefore, attempt to investigate the expression of Epstein-Bar virus in oral squamous cell carcinoma biopsy samples using immunohistochemistry.
Methods: A total of 150 biopsy proven oral squamous cell carcinoma cases were investigated for expression of Epstein-Bar virus protein using immunohistochemistry. The data were then compared for association with age, gender and biological behavior of oral squamous cell carcinoma.
Results: 34 out of 150 samples were positive for Epstein-Bar virus infection. Most of the positive cases were males and predominantly affected buccal mucosa for neoplastic lesions. Most well known risk factors of the disease was significantly associated with EBV. Uniform distribution of EBV and different morphological patterns of oral squamous cell carcinoma was also observed.

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Conclusion: Epstein-Bar virus may be considered as a risk factor in etiopathogenesis of all forms of oral squamous cell carcinoma. We suggest that oncogenic effect of EBV may be enhanced by other chemical carcinogens; although more investigation is needed on a large series of cohort to confirm these findings.

Keywords: Epstein bar virus (EBV); oral squamous cell carcinoma; immunohistochemistry; buccal mucosa.

1. INTRODUCTION

Oral Squamous cell carcinoma is the sixth most prevalent cancer across all races and sex globally, which accounts for 300,400 cases and 145,400 deaths during the year 2016 [1-2]. Malaysia, South Central Asia, and East and Central Europe has the highest incidence of oral squamous cell carcinoma [3]. In Pakistan, the prevalence of oral squamous cell carcinoma is reported to be 10%, whereas 40% of burden disease is observed in neighboring India [4-5]. The high burden of oral squamous cell carcinoma in the region is largely contributed by risk factors such as age, gender, tobacco chewing and smoking, alcohol, betel quid, poor oral hygiene, low socioeconomic status, diet, genetics and viruses [6-7].

Biopsy of oral lesion remains the gold standard for diagnosis of oral squamous cell carcinoma. Histologically, oral squamous cell carcinoma is characterized by marked cellular and nuclear pleomorphism, atypical mitosis and keratin pearls formation [8]. Oral squamous cell carcinoma is characterized as well, moderate and poorly differentiated criteria, where later represents the worst prognosis [9]. Immunohistochemistry supplements routine H & E evaluation for differentiating the tumor as well as determining the viral etiopathogenesis.

Buccal mucosa is most commonly observed site of lesion for oral squamous cell carcinoma, however the tumor may also involve tongue, palate, floor of the mouth and lips [10-11]. Beside known risk factor, Epstein-Bar virus has been recently linked with development of oral squamous cell carcinoma [12]. Despite the well-established oncogenic role of Epstein Barr virus in development of various cancers, the influence of Epstein-Bar virus in oral squamous cell carcinoma remains unclear. However, it is hypothesized that Epstein-Bar virus binds to CD21 receptor on inactive B-lymphocytes through envelope glycoprotein and enters the cell. The viral capsid dissolves and the viral genome is transported to the cell nucleus .The virus leads to extensive methylation of both the host and viral genome, and these changes inhibit apoptosis, promote viral persistence and propagation, thus inducing malignant trans-formation in the infected B-cell [13].

Multiple western studies have documented the expression of Epstein-Bar virus in oral squamous cell carcinoma cases, however this finding has not been investigated in our population [14]. Therefore, we aimed to evaluate the expression of Epstein-Bar virus in oral squamous cell carcinoma biopsy samples using immunohistochemistry. We also sought to compare the expression of Epstein-Bar virus with age and biological behavior of the tumor upon diagnosis.

2. METHODS

The ethical approval for study was sought from ethical review committee (Ziauddin University) to collect a total of 150 clinically suspected cases of oral squamous cell carcinoma (OSCC) followed by histological examination. All cases were ranged between 18-75 years of age. The cases were received at Ziauddin University hospital & the laboratory Saddar Karachi, between years 2016-2017. The diagnosis of OSCC was confirmed by histological evaluation of multiple biopsies sections by panel of histopathologists. All OSCC confirmed biopsies specimen were graded according to morphological criteria and selected for study after informed consent.

The immunohistochemistry was performed using routine Avitin biotin complex method according to manufactures protocol. Briefly, tissue blocks were cut into 3 µm sections using standard microtome and was deparaffinized in xylene and hydrated through alcohol to normal saline. After 10 minutes of incubation in hydrogen peroxide to block endogenous peroxidase, slides were treated with Ficin and then incubated with the monoclonal mouse EBV antibody at a 1:100 dilution overnight at 2-8°C. The slides were then

incubated with a biotinylated antimouse secondary antibody (1:100 dilution for 30 minutes at room temperature) [15]. The slides were paramount with cover slip and bound antibody was detected for EBV. To authenticate the reaction biopsies blocks harboring Hodgkin lymphoma with known level to EBV expression were used as positive control. The tissue sections considered positive by dark brown cytoplasmic staining in the tumor cells observed by light microscopy.

Statistical evaluation was performed using SPSS version 20. Chi square was used to determine the association of EBV protein expression with independent variable. P-value less than 0.05 was regarded as statistically significant.

3. RESULTS

34 out of 150 oral squamous cell carcinoma cases were positive for EBV protein which account for 22.6% of total cases. This figure reflects that EBV is a significant risk factor (p-value =0.001) for the development of OSCC (Table 1). Fig. 1 shows immunohistochemical expression of EBV protein in oral squamous cell carcinoma. We compared the association of lesion sites, risk factors and biological behavior of tumor with EBV infection in oral squamous cell carcinoma cases. Tables 2 and 3 demonstrate the frequency and statistical estimates of EBV infection with research parameters.

Majority of positive cases were males, whereas only one female case was positive for EBV protein. The mean age of oral squamous cell carcinoma cases with EBV protein expression is 49.6 and 53.6 in males and females respectively (Table 2). Oral squamous cell carcinoma lesions predominately affected buccal mucosa followed by tongue and other sites. All anatomical locations for tumor observed significant statistical difference with EBV infection with exception of maxilla (Table 3). We have determined that smoking is the leading cause of oral squamous cell carcinoma in our region, however other risk factors may also be involved in the development of tumor. EBV infection showed a significant correlation with most risk factors of the disease (Table 4). In present research, majority of cases were reported as moderately differentiated tumors based on the morphological pattern. Overall, all morphological patterns correlates positive with EBV infection (Table 5).

Table 1. Burden of EBV infection in oral squamous cell carcinoma

	Oral squamous cell carcinoma	p value
EBV positive	34	
EBV negative	116	0.001
Total	150	

Table 2. Age distribution of gender of oral squamous cell carcinoma

Gender	Mean age	Total
Male	49.61	115
Female	53.63	35
Total	50.55	150

4. DISCUSSION

The association of oral squamous cell carcinoma with EBV is controversial. Additionally, the link of EBV with oral squamous cell carcinoma from our population in not been investigated yet. The present analysis focused on detection of EBV infection in oral squamous cell carcinoma biopsy specimens. High prevalence of EBV in oral squamous cell carcinoma is reported in different parts of the world [16]. Contrary to this, we have observed EBV infection in relatively low frequency in present investigation. We believe

Table 3. Distribution of EBV across tumor sites

Sites	EBV positive	EBV negative	p value
Oral cavity	07	19	0.001
Tongue	02	18	0.005
Lips	02	11	0.001
Buccal mucosa	21	47	0.001
Hard Palate	01	03	0.046
Mandible	01	06	0.008
Maxilla	00	07	1.00

Table 4. Comparison of known risk factors with EBV infections

Risk factors	EBV positive	EBV negative	p value
Smoking	17	43	0.001
Alcohol	01	17	0.056
Pan	10	58	0.001
Gutka	04	13	0.001
Betel nuts	03	10	0.003
Tobacco	01	07	0.125
Naswar	03	09	0.005
None	06	04	0.005

Table 5. Prevalence of EBV infection across tumoral differentiation

Grades	EBV positive	EBV negative	p value
Well differentiated	13	41	0.001
Moderately differentiated	17	62	0.001
Poorly differentiated	04	13	0.001

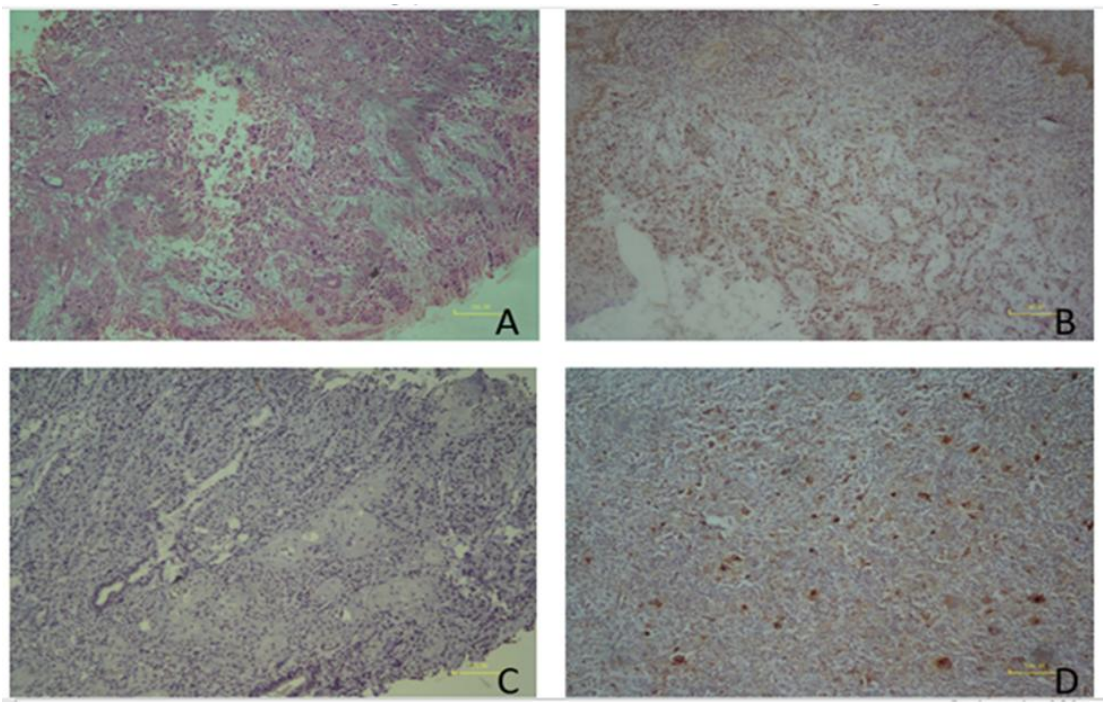

Fig. 1. Photomicrograph of moderately differentiated oral squamous cell carcinoma exhibiting neoplastic squamous epithelial cells arrange in nest, cords and strands along with keratin pearls formation; 10x magnification. (A) Photomicrograph of moderately differentiated oral squamous cell carcinoma displaying EBV immunohistochemical expression in atypical epithelial cells; 10x magnification. (B) Photomicrograph of EBV negative oral squamous cell carcinoma with moderate differentiation; 10x magnification. (C) EBV immunohistochemical expression in Hodgkin lymphoma control; 10x magnification. (D)

that differences in genetic makeup and immune status of the patient would have contributed to these low rates. Alternatively, variations in detection method and technical skills may have been responsible for these discrepancies. Despite low prevalence, strong statistical association of EBV infection with oral squamous cell carcinoma has been determined in our analysis. This finding in line with western and regional series [17]. Therefore,

we assumed that EBV may be a potential risk factor for development of oral squamous cell carcinoma.

In present research, majority of oral squamous cell carcinoma cases were men which is parallel with other studies reported across the world, however, contrary to Acharya et al., which reported female predominance [18]. Additionally, we have determined the mean age of oral squamous cell carcinoma upon diagnosis were 50.55 years which is almost a decade younger than recorded by many regional and international reports [19]. Thus, we believe that differences in mean age may be attributed by exposure of oral carcinogens and risk factors common in our population; particularly by male gender.

In current study, we have determined that oral squamous cell carcinoma lesion predominantly affected buccal mucosa followed by tongue and other sites. Consistent to our findings Khan et al also reported buccal mucosa as the most common site of oral squamous cell carcinoma, whereas Higa et al. and Maeda et al. reported tongue as chief anatomical location for this lesion [10,20-21]. However, unlike present study, both investigations failed to compare EBV across various anatomical sites of tumor. Our study reveals uniform distribution of EBV across all tumor sites. The significant difference was observed with respect to all anatomical sites, except maxilla. We believed that the reported statistical difference between anatomical sites and EBV infection may be related to specific receptor C3D; exclusively express on surface of keratinize squamous epithelium lining oral cavity [22]. The receptor mediated pooling of virus has been previously establish in cancer such as nasopharyngeal carcinoma. Moreover, squamous epithelium may become affected by circulating EBV carrying lymphocytes as suggested for gastric epithelial cells [23].

Previously, Acharya et al. investigated the link of EBV with risk factors of disease and determined strong association with betel nuts [18]. Interestingly, we have observed that all risk factors of oral squamous cell carcinoma, apart from alcohol and tobacco, correlated significantly with EBV infection. However, strong link of EBV infections with smoker, pan and gutka was noted. Thus, it is possible that EBV could have acted in conjunction with other factors to induce full transformation [24]. To best of our knowledge, this study is the first to report association of EBV with well-known risk factors of oral squamous cell carcinoma. We recommend, more research to investigate this link using a more sensitive detection method, on a large series of Cohort method could confirm our finding.

Positive statistical difference across all degree of tumoral differentiation is another aspect of present histopathological analysis. Previously, Raab-Traub et al. and Pearson et al. indicated EBV in histological subtype of nasopharyngeal carcinoma. However both investigation utilize EBV DNA to compare with tumoral differentiation [23]. Although, present study detected EBV protein in oral squamous cell carcinoma, in present study we found a positive link of EBV infection with all patterns of differentiation. These results may be comparable with studies carried out nasopharyngeal carcinoma [25].

There are several limitation of present research. Firstly, a series of large Cohort may have proven more useful in establishing the reported link of EBV with oral squamous cell carcinoma and its parameters. Secondly, more robust technique such as PCR and FISH could have provided us with more sensitive tool in determining our outcomes. Thirdly, the present study was focused particularly core biopsies specimens which limited us in considering other histological parameter for comparative analysis.

5. CONCLUSION

In conclusion, the results of the present work suggested that EBV as the potential risk factor for oral squamous cell carcinoma, this will have to be establish with specific pathogenic mechanisms. Although tobacco and alcohol drinking do not affect the EBV prevalence, however, all other known risk factors seem to enhance the carcinogenic role of EBV in oral squamous cell carcinoma. The presence of EBV across all tumor grades suggested that the infection may not be useful in determining the biological course of the disease, however, more studies are required to a meaningful conclusion for this comment.

CONSENT

As per international standard or university standard, patient's written consent has been collected and preserved by the author(s).

ETHICAL APPROVAL

As per international standard or university standard, written approval of Ethics committee has been collected and preserved by the author(s).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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