Original Article

Prevalence of Epstein-barr Virus (EBV) among Patients with Oral Squamous Cell Carcinoma from Ahvaz, Iran: A Case-Control Study

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ABSTRACT

Epstein-Barr virus (EBV), one of the most significant causes of lymphoid and epithelial cancers, has been linked to oral carcinogenesis; however, this etiological association remains controversial. To investigate this association, the present study aimed to determine the prevalence of EBV in cancerous and non-cancerous oral tissues from Ahvaz, Iran. In total, 164 blocks of formalin-fixed paraffin-embedded tissues from oral squamous cell carcinoma (OSCC), including 76 tongue squamous cell carcinomas and 88 non-cancerous tongue tissues, were collected from Ahvaz Imam Khomeini Hospital, Ahvaz, Iran, from December 2014 to March 2019, for this case-control study. The tissues were cut into 15-µm-thick sections, and DNA was extracted using a solution of Phenol, Chloroform, and Isoamyl Alcohol. The EBV detection and typing were performed using nested polymerase chain reaction. The EBV was detected in 9 (5.48%) out of the 164 samples studied, including 4 (5.26%) of the 76 SCC cases and 5 (5.68%) of the 88 samples in the control group (P>0.05). The EBV was positive in 2.40% of the 83 male and 8.6% of the 81 female samples (P>0.05). In terms of the histological grades of the case group, 3 (3/57) and 1 (1/13) of the EBV-positive samples were well and moderately differentiated, respectively (P>0.05). For EBV typing, the 9 EBV-positive samples were tested, and it was found that 2 and 7 of the cases were EBV type I and II, respectively. Results of the current study demonstrated the low frequency of EBV in Iranian patients with OSCC, with EBV type II predominating. Further studies are required to clarify the association between EBV and OSCC.

Keywords: Epstein-barr virus, Oral squamous cell carcinoma, Nested Polymerase Chain Reaction, Prevalence

1. Introduction

Oral squamous cell carcinoma (OSCC), as a major public health problem, is the sixth most prevalent malignancy worldwide, accounting for up to 90% of all head and neck tumors (1, 2). Annually, cancer leads to high morbidity and mortality all over the world; accordingly, about 263,900 new cancer cases and 128,000 cancer-associated deaths are contributed to this malignancy each year (3). The OSCC has a variable incidence rate among the different regions of the world which is the result of various lifestyle factors (e.g., tobacco smoking, alcohol drinking, and betel quid chewing habits) among various geographical regions (4).

The OSCC incidence rate in Asia is high among Southeast countries, in particular, Pakistan and India where this cancer is reported as the first and second most prevalent malignancy in males and females, respectively (5, 6). In Iran, OSCC has a similar incidence rate to Pakistan and India (7, 8), with a prevalence rate of about 20-36.3 cases per 100,000 people (8). Tobacco smoking, alcohol drinking, and betel quid chewing habits are the major risk factors for OSCC development; however, studies have suggested other etiological factors, such as oncogenic viruses, for this cancer (4).

The first evidence of the association of viral pathogens with OSCC was reported about two decades ago (9). Currently, there are several human viruses, such as human papillomavirus, Cytomegalovirus, herpes simplex virus, and Epstein-Barr virus (EBV), which have been suggested to be implicated in the development of OSCC (9). Nonetheless, these data are controversial and need to be more investigated. The EBV is a common viral pathogen from the human herpesvirus family with a worldwide distribution; accordingly, over 90% of human adults are seropositive for this virus (10). This virus is a major cause of infectious mononucleosis and is associated with the development of nasopharyngeal carcinoma, Burkitt's lymphoma, and gastric cancer (11).

Regarding DNA sequence divergence in the EBNA-3C gene, EBV is categorized into types I and II (12). Studies propose a possible oncogenic role for EBV in OSCC; however, these reports are variable and controversial, which required further study (4). Therefore, the current work aimed to assess the prevalence of EBV DNA among cancerous and non-cancerous oral tissues through a case-control investigation of OSCC cases in Ahvaz, Iran, to study the association of this oncogenic virus with OSCC.

2. Materials and Methods

2.1. Study population

The current work was approved by the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (No; IR.AJUMS.REC.1398.522). The clinical samples, including 76 OSCC specimens (tongue SCC) and 88 non-cancerous tongue tissues, were collected from formalin-fixed paraffin-embedded (FFPE) tissues of the patients who visited Imam Khomeini Hospital, Ahvaz, Iran, from December 2014 to March 2019. To perform this study, the clinical history of the patients (such as age and gender) was retrieved and documented for subsequent investigations. Finally, the clinical samples of both case and control groups were enrolled based on their quality for molecular studies and the presence of their clinical history.

2.2. DNA extraction

To perform DNA extraction, the tissues were first cut into 15-µm-thick sections. To avoid DNA cross-contamination between the samples, the microtome blade was thoroughly cleaned with 10% Clorox after cutting each tissue sample. The sections were deparaffinized via 1 h of incubation at 37 °C with 1 mL of xylene and then removal of the xylene by washing for 30 min with a descending concentration of ethanol (100%, 70%, and 50%). Subsequently, the sections were dried at 65° C, lysed via 300 µL of lysis buffer with 30 µl of proteinase K (Bioneer, Korea), and incubated overnight at 37 °C. A solution of Phenol:Chloroform:Isoamyl Alcohol

(Sigma-Alrdich, USA) was used to wash and remove proteins from the suspension. The DNA was precipitated by the addition of 1 ml of absolute ethanol and was assessed for purity via NanoDrop (Thermo Fisher, USA).

Finally, a 110 bp fragment of the β-globin gene was amplified with PCO3/PCO4 primers (Table 1) to check for the DNA extraction process. This was performed based on the following condition using a 25 µL PCR reaction with 12.5 µL of mastermix (Ampliqon, Denmark), 9.5 µL of sterile water, 1 µL of each primer (10 pmol), and 1 µL of DNA sample; first denaturation for 4 min at 95 °C as followed by 35 cycles for 30 s at 95 °C as denaturation, 30 s at 55 °C for annealing, 30 s at 72 °C as extension, and finally one cycle as extension at 72 °C for 4 min. The positive β-globin gene samples were enrolled for subsequent investigations.

Table 1. The Primers used for PCRs

Primer Name	Sequences (5'–3')	Amplicon Size (bp)
PCO3	ACACAACTGTGTTCACTAGC	110
PCO4	CAACTTCATCCACGTTCACC	
EBNA1F1	GTAGAAGGCCATTTTTCCAC	609
EBNA1R1	CTCCATCGTCAAAGCTGC	
EBNA1F2	AGATGACCCAGGAGAAGGCCCAAGG	309
EBNA1R2	CAAAGGGGAGACGACTCAATGGTG	
EBNA3CF	CGG AAG AGG TGG AAA ACA AA	153 or 246
EBNA3CR	GTG GGG GTC GTC ATC ATC TC	

2.3. Epstein-Barr virus detection and genotyping

The EBV was detected by a nested PCR to amplify the EBV-EBNA-1 gene by two primer pairs (Table 1). The first amplification was carried out based on the following condition to amplify a 609 bp amplicon from the EBNA-1 gene: 4 min of pre-denaturation at 94 °C followed by 38 cycles at 94 °C for 45 s, at 54 °C for 45 s, at 72 °C for 45 s, and at 72 °C for 5 min as the final extension. The second PCR run to amplify a 309 bp fragment was performed as follows: 4 min of pre-denaturation at 94 °C, and then 35 cycles at 94 °C for 45 s, at 58 °C for 45 s, and at 72 °C for 45 s, and finally one cycle at 72 °C for 5 min.

The amplifications were performed by using a 25 µL PCR reaction with 12.5 µL of mastermix (Ampliqon, Denmark), 9.5-10 µL of sterile water, 1 µL of each primer (10 pmol), and 1 µL of DNA sample (0.5 µL for the second amplification). The PCR products were assessed and visualized by performing agarose gel electrophoresis and using a UV transilluminator (GENE FLASH, UK), respectively.

The EBV typing was performed using EBNA3CF/EBNA3CR primers (Table 1) to amplify a type-specific region of the EBV EBNA-3C gene (12). These primers amplify two fragments from the EBNA-3C gene with two different amplicon sizes (a 246 bp fragment for EBV type II and a 153 bp amplicon for EBV type I), which can be assessed by agarose gel electrophoresis. The reaction was carried out according to the following conditions: 4 min of pre-denaturation at 94 °C, 35 cycles at 94 °C for 45 s, at 53 °C for 45 s, at 72 °C for 45 s, and finally one cycle at 72 °C for 5 min. This was carried out using a 25 µL PCR reaction with 12.5 µL of mastermix (Ampliqon, Denmark), 9.5 µL of sterile water, 1 µL of each primer (10 pmol), and 1.5 µL of DNA sample.

2.4. Statistical analysis

Statistical analysis was performed using the Fisher's exact test and independent samples t-test in IBM SPSS software (Version 22). Accordingly, a *P* value of less than 0.05 was considered statistically significant.

3. Results

This work studied 76 OSCC samples and 88 non-cancerous oral tissues, including 83 (50.6%) males and 81 (49.4%) females (Table 2). The SCC group included 35 (46%) males and 41 (54%) females with a mean age of 53.51±13, while the control group included 48 (54.5%) males and 40 (55.5%) females with a mean age of 49.61±17. Furthermore, 102 (62.19%) cases were below 56 years old, and 62 (37.8%) cases were 56 years old and above. Besides, 40 (52.63%) cases (40/76) of the SCCs and 62 (70.45%) cases (62/88) of the controls were below 56 years old.

In the SCC group, all the samples were anatomically tongue SCC, 57 (75%), 13 (17.1%), and 6 (7.89%) of which were histologically well, moderately, and poorly differentiated, respectively. Regarding PCR findings, 9 (5.48%) out of the 164 studied samples were EBV positive, including 4 (5.26%) out of the 76 SCC cases and 5 (5.68%) out of the 88 samples in the control group (P>0.5) (Table 3) (Fig. 1).

Table 2. Characteristics of the studied groups and EBV-status

Study Group (n)	Variable	Group (%)	EBV+	EBV-	P-value
SCC Group (76)	**Mean age (SD)**	53.51 ± 13	49.9 ± 17	51.2 ± 13	P>0.5
	Gender				
	Male	35 (46%)	1 (2.85%)	34 (97.14%)	P>0.5
	Female	41 (54%)	3 (7.31%)	38 (92.68%)	
	Age				
	<56 years	40 (52.63%)	3 (7.5%)	37 (92.5%)	P>0.5
	≥56 years	36 (47.36%)	1 (2.77%)	35 (97.22%)	
	Histological grade				
	Well	57 (75%)	3 (5.26%)	54 (94.73%)	P>0.5
	Moderately	13 (17.1%)	1 (7.69%)	12 (92.3%)	
	poorly	6 (7.89%)	0	6 (100%)	
Control Group (88)	**Mean age (SD)**	49.61 ± 17	51.4 ± 13	52.3 ± 13	P>0.5
	Gender				
	Male	48 (54.5%)	1 (2.08%)	47 (97.91%)	P>0.5
	Female	40 (55.5%)	4 (10%)	36 (90%)	
	Age				
	<56 years	62 (70.45%) 26	3 (4.83%)	59 (95.16%)	P>0.5
	≥56 years	(29.45%)	2 (7.69%)	24 (92.3%)	

Table 3. The frequency of EBV-Positive and –Negative according to groups

Group	EBV		Total	P-value
	Positive	Negative		
SCC	4	72	76	
Control	5	83	88	P>0.05
Total	9	155	164	

regarding the genders, EBV was positive in 2 (2.40%) out of the 83 males and 7 (8.6%) out of the 81 females (P=0.08). In the SCC group, 1 (2.85%) male (1/35) and 3 (7.31%) females (3/41) were EBV positive (P>0.5), while in the control group, 1 (2.08%) male (1/48) and 4 (10%) females (4/40) tested positive for EBV (P>0.5). Moreover, 6 (5.88%) out of the 102 cases with < 56 years of age and 3 (4.83%) out of the 62 cases with ≥ 56 years of age were EBV positive (*P*>0.5). Among the EBV-positive SCC cases, three cases had < 56 years of age and one case had ≥ 56 years of age (*P*>0.5). Regarding the histological grades and EBV positivity of the SCC cases, 3 (3/57) and 1 (1/13) of the EBV-positive samples were well

and moderately differentiated, respectively (P>0.05). In terms of EBV typing, the 9 EBV-positive samples were tested by a type-specific primer pair, and the results showed that 2 and 7 cases were EBV types I and II, respectively (Fig. 2).

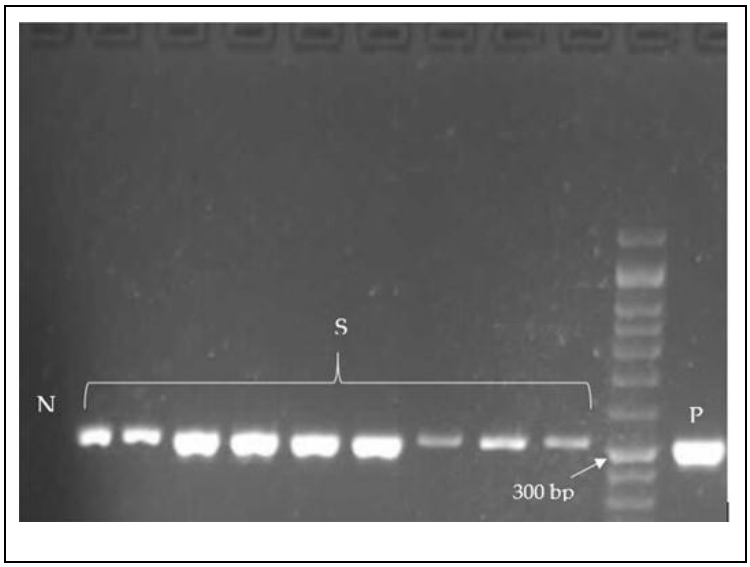

Figure. 1. The EBV positive samples. N; negative control, S; 309 bp positive samples, p; positive control

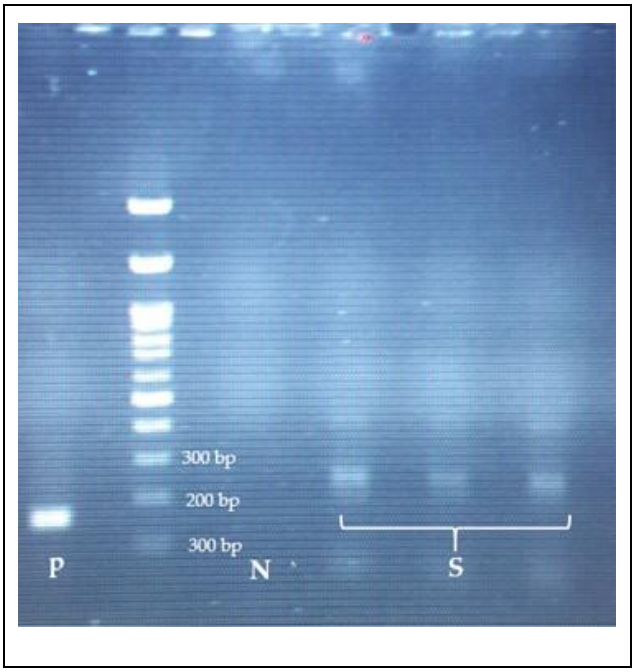

Figure. 2. Some results of EBV typing. N; negative control, S; three 246 bp positive samples, p; positive control (type I; 153 bp)

4. Discussion

Oral squamous cell carcinoma is globally known as the sixth most prevalent malignancy, which can be mediated via several risk factors, including tobacco smoking, alcohol drinking, betel quid chewing, and

particularly oncogenic viruses (13, 14). The EBV has shown various prevalence rates among OSCC studies all over the world (15-19); accordingly, researchers have reported different ideas about the role of this virus in OSCC. Therefore, more investigations are needed to clarify this inconsistency.

The present work evaluated the prevalence of EBV among cancerous and non-cancerous oral tissues in a case-control investigation in Ahvaz, Iran, to study the association of this oncogenic virus with OSCC. The results showed that 5.48% of the 164 studied samples were EBV-positive, including 5.26% of the 76 SCC cases and 5.68% of the 88 control subjects (P>0.5). These findings indicated that EBV had a low prevalence rate among the study population. Moreover, it was found that it was more prevalent among controls, compared to SCC cases.

Results of this study are in line with those of several studies in which EBV has shown a higher prevalence rate in control samples, compared to SCC cases. For instance, in a study performed in India by Prathyusha et al., 20% and 50% of the OSCC cases and controls were positive for EBV, respectively (20). Rensburg et al. by using FFPE tissues found that EBV was present in 25% of OSCCs and 42% of controls (21). In another study on FFPE tissues, Heerden et al. showed that 24% of OSCCs and 37% of controls were positive for EBV (22). As mentioned earlier, until now, studies conducted in different regions of the world have shown an inconsistent prevalence rate of EBV in OSCC cases, including investigations reporting a high prevalence rate of this virus and those without any association between EBV and OSCC.

In a study performed by Horiuchi et al., EBV was reported in 52.8% and 27.5% of OSSC cases via PCR and in-situ hybridization, respectively (23). In another investigation performed by Cruz et al., the authors detected EBV in 100% of OSCC cases, 77.8% of cases with pre-cancerous lesions, and 8.3% of cases with normal mucosa, demonstrating a high prevalence rate of EBV in OSCC cases (24). Likewise, Tsang et al. showed a high rate of EBV positivity (82.5%) in biopsy samples of OSCCs by using an EBV genomic microarray (25). Similarly, other studies assessing FFPE samples by PCR technique have also reported a higher rate of EBV positivity in OSCC cases, compared to non-cancerous oral tissues (20, 26). In this regard, Kis et al. found that the EBV prevalence rate was significantly higher among OSSC cases, compared to the control non-cancerous oral tissues (26).

However, there are other investigations whose results have not indicated an association between EBV and OSCC. For instance, in a study conducted by Lamaroon et al. in Thailand, EBV was not detected among 24 OSCC cases; therefore, the authors did not regard this virus as a risk factor for OSCC (27). Similarly, in a study performed by Wilms et al., EBV was not present in OSCC and they could not indicate any association between EBV and this cancer (28). These differences in EBV prevalence in OSCC may be the consequence of using different methods for detection, various anatomical sub-site and sample sizes, different geographic locations, and also the effects of other OSCC risk factors.

Regarding gender, although EBV was more positive among females (8.6%) than males (2.40%) (P=0.08), there was no statistically significant difference between EBV detection and gender and the mean age of patients in the present study. Consistent with the findings of this study, there was also no association between EBV positivity and the gender and age of patients in a study conducted by Zebardast et al. (29).

Regarding the histological grades and EBV positivity, the findings of the present research did not show any statistically significant difference between the different histological grades and EBV detection. The EBV has two sub-types, known as types I and II, with different transforming capacities. Evaluation of the EBV sub-types has indicated that type I can transform human B cells better than type II (30). In this study, the findings demonstrated that the majority of positive cases were EBV type II. However, limited data are available regarding the EBV sub-types among

the Iranian population with malignancy. In a study performed by Tabibzadeh et al., it was shown that 8.8% of patients with hematologic malignancies were positive for EBV type II (12). Nonetheless, the findings of this study are in contrast to those reported by Shahani et al. in their study which indicated that EBV type I was the predominant sub-type among patients with nasopharyngeal carcinoma (31).

In conclusion, the present study indicated that the prevalence of EBV among the Iranian patients with OSCC was low and there was no statistically significant association between EBV positivity and either of the two studied groups. This observation suggests that EBV might not have a significant effect on OSCC in the study population, highlighting the role that other risk factors play in this cancer, such as tobacco smoking, alcohol drinking, and genetic factors. However, in several investigations without detection of EBV in OSCC cases, the "hit and run" theory has been suggested, according to which, viral DNA is only required for induction of tumorigenesis and is disappeared afterward during the subsequent uncontrolled cell cycles. Furthermore, EBV-encoded RNAs were shown to be overexpressed in OSSC samples which may show the possible role of EBV infection in oral carcinogenesis (29). Accordingly, more investigations are needed to determine the possible role of EBV in OSCC development.

Authors' Contribution

Study concept and design: SH.J, A.A
Acquisition of data: H.M
Analysis and interpretation of data: SH.M
Drafting of the manuscript: S.SH
Statistical analysis: A.R
Revision of the manuscript: SH.J, SH.B

Ethics

This work was approved by the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences (AJUMS), Ahvaz, Iran (Ethical code; IR.AJUMS.REC.1398.522).

Conflict of Interest

The authors have no relevant conflicts of interest to declare.

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