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Original Article

Association between epstein barr virus and tongue squamous cell carcinoma in iranian patients

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ABSTRACT

Detection of Epstein-Barr virus in oral squamous cell carcinoma suggests its involvement in the carcinogenesis of oral cavity. But, there are few studies on the incidence of EBV genome in squamous cell carcinomas at specific locations in the oral cavity like tongue and with different tumor progression. In this study the presence of EBV genome in tongue Squamous Cell Carcinoma (TSCC) in Iranian patients were investigated. Accordingly, a total of 94 cases with TSCC were firstly analyzed for the presence of viral genome through Nested PCR. Patients were divided into different groups based on their gender and the size, nodal involvement, grade and stage of their tumor. Results showed the presence of EBV genome in 72.3% of TSCCs with no significant difference between two genders, although slightly higher in females. Interestingly, PCR products of EBV genome showed a statistically significant higher distribution in TSCCs at IVa stage ($p = 0.04$), while a considerable low involvement of EBV genome was seen in T1-sized tumors. The result of this study further emphasizes the role of EBV in oral SCCs – mainly at tongue. This is the first investigation to clarify the association between EBV genome and different tumor size and stage in TSCCs; however, more studies in different regions and larger populations should be performed to be able to draw a firmed conclusion.

1. Introduction

Head and neck cancers are the 6th most common cancers around the world, of which about 95% are squamous cell carcinomas (SCC) and can happen in almost any anatomical parts as in nasopharynx, oropharynx and oral cavity [1]. Based on several global surveys, SCC in tongue has been known as the most seen part of intraoral SCCs compared to other sections [2,3]. And unfortunately, not only is its prevalence increasing, but also it has a high morbidity and mortality with a 5-year survival rate of approximately less than 60% – though with a better outcome in elderly patients [4,5].

Based on the suggested risk factors for oral cancers, besides all factors like socioeconomics condition, tobacco and alcohol consumption and genetic predisposition, viral infection plays a very important role [6,7]. Contribution of viruses such as Human papillomavirus (HPV), herpes simplex virus (HSV) and Epstein–Barr virus (EBV) to oral cancers, and specifically SCCs have been shown so far in several studies [1,6,8–10].

EBV, the first tumorigenic virus in humans, was introduced in 1964 as a particle in Burkitt's lymphoma biopsies [11].After this discovery, EBV has been shown to be associated with various malignancies including Hodgkin's lymphoma, mycosis fungoides, testicular germ cell tumor, uterine cervical cancer, gastric carcinoma, and carcinomas of the palatine tonsil, salivary gland, and thymus [11,12].

Association of EBV with nasopharyngeal cancers had long highlighted the possibility of viral – and specifically EBV – causation for oral squamous cell carcinomas (SCC) [1]. And, it was in 1995 that the involvement of EBV in squamous cell carcinomas (SCC) (due to the presence of viral genome in SCC) was firstly reported by Horiuchi et al. [13]. Later on, further studies confirmed such association [14–16]. Most investigations have shown an association between EBV oral SCCs [15], though there are studies reporting not a significant association [17].

Despite the need for firmly clarifying the etiological role of EBV in OSCCs, proposed mechanisms for EBV carcinogenesis strengthen its involvement in oral SCCs.

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EBV belongs to the herpesviridae family and has a 172 kbp linear double-stranded DNA [11,16].This virus primarily infects children during their 3 years of life and has the ability to persist lifelong asymptomatically through latent infection [9]. In vivo, EBV generally has a tissue tropism towards two types of cell during infection: epithelial cells in oro/nasopharynx and/or salivary gland, and the B-lymphocytes [9,11,16].

Interestingly, literatures reveal variation in incidence of EBV in OSCC of different geographical regions, with a high prevalence in south-east Asia and a lower one in North America as well as West and Northern Europe [18]. In spite of the fact that differences in genetic background of each infected individual might play part in existence of such variation, this phenomenon may be due to different behavior of certain EBV strains/subtypes which results in a higher epitheliotropism [19]. Although it has been long believed that Herpesviridae has no subtype, there are possibilities to changes this view by defining EBVs with different pathologic effect [9,19].

Further increase in number of tongue cancers, compared to cancers of other sites in oral cavity, in Iran as in most other Asian countries highlights the importance of studies on tongue carcinomas [6]. To date there has been little finding on the exact correlation between EBV and tongue SCC. So, aim of current study is to describe the presence of EBV DNA in tongue SCC of an Iranian population by Nested PCR.

2. Material and methods

2.1. Study population

Formalin-fixed, paraffin-embedded tissue sections were obtained from the archive of pathology department, Amir-Alam hospital which were examined by an expert pathologist.

The tumors were histologically categorized into three groups of well, moderately, and poorly differentiated, based on the World Health Organization classification (WHO). Staging for tongue SCC was defined according to the American Joint Committee on Cancer (AJCC) and follows TNM staging. The case group consisted of patients with tongue SCC (n = 94). The study was approved by the Ethics committee of Tehran University. Informed consents were obtained from all of the patients in the study.

2.2. DNA extraction & nested PCR

The EBV genomic DNA was extracted from formalin-fixed, paraffin-embedded oral squamous cell carcinomas tissue, using phenol/phenol–chloroform followed by ethanol precipitation. In brief after micro dissection of tissue from paraffin embedded blocks, they were transferred into sterile tubes and xylene was added to dissolve the paraffin. After centrifuge and draining the supernatant, samples were rehydrated with ethanol. In order to lyse other proteins, proteinase K and sodium dodecyl sulfate (SDS) were added and samples were incubated in 37 °C overnight. After addition of phenol, Isoamyl alcohol and chloroform, samples were centrifuged and DNA was extraction from supernatant.

Nested polymerase chain reaction amplification of EBV genome strain B95-8 was performed. The 171 bp and 97 bp DNA fragments in Nest-1 PCR and Nest-2 PCR, were amplified using pairs of primers as Table 1. All reactions were carried out in reaction mixtures of 25 µl containing 2 µ, 10 × PCR buffer(Fermentas), 2.5 mM MgCl2, 0.2 mMdNTPs, 0.5 µM of each primer, and 1 µl of Tag DNA polymerase (Fermentas). In the first reaction, 100–200 ng of EBV genomic DNA was used as template and for the second reaction, 0.5–1 µl of first PCR product was used. DNA from B95-8 strain of Epstein Barr Virus was used as a positive control for each PCR reaction.

Negative controls (ddH2O) were used in all PCRs. Secondary PCR products were resolved by electrophoresis on 3% agarose gels and visualized by staining with safe stain (sinaclon, Iran) Fig. 1.

Table 1
Primers and amplification condition for EBV strain B95-8:.

Nest-1 PCR	Primers & amplification program	PCR product
Outer primer-F	GAGACCGAAGTGAAGGCCCT	171 bp
Outer primer-R	GGTGCCTTCTTAGGAGCTGT	
Amplification program	Simple 3-steps PCR: Step 1: 95 °C, 2 min; Step 2: 95 °C, 30 s.; Step 3: 60.3 °C, 30 s.; Step 4: 72 °C, 30.0 s.; Step 5: Repeat steps 2–4, 30 cycles; Step 6: 72 °C, 5 min.	
Nest-2PCR		
Inner primer-F	GCCAGAGGTAAGTGGACTTTAAT	97 bp
Inner primer-R	GAGGGGACCCTGAGACGGGT	
Amplification program	Touch down PCR: Step 1: 95 °C, 2 min; Step 2: 95 °C, 30 s.; Step 3: 64.5 °C decrease 0.5 °C per cycle, 30 s; Step 4: 72 °C, 10.0 s.; Step 5: Repeat steps 2–4,14 cycles. Step 6: 95 °C, 30 s.; Step 7: 57.5 °C, 30 s.; Step 8: 72 °C, 10 s.; Step 9: Repeat steps 6–8,15 cycles;Step 10: 72 °C, 5 min.	

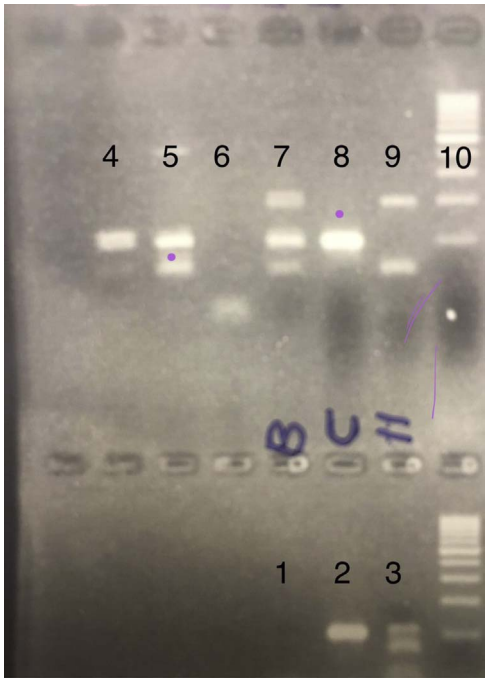

Fig. 1. Gel electrophoresis of nest2 amplification of EBNA-1 gene.
Lane 1: Blank, Lane 2: Positive control; 97 bps (Nest2), Lanes 3,4,5, 8: Nest2 positive (97 bps), Lanes 7: Nest1, Nest2 positive (97 bps, 171 bps), Lane 9: Nest1 positive, Nest2 negative, Lane 6: Negative, Lane 10: DNA ladder

2.3. Statistical analysis

Strength of association between different groups and expression/presence of EBV genes was estimated using odds ratios (ORs) and 95% confidence intervals (CIs). Levels of significance were determined using contingency tables by either Chi-square or Fisher exact analysis.

All analyses were carried out using STATA version 8. $p < 0.05$ was considered a significant statistical difference.

3. Results

Nested PCR analysis of TSCC biopsies revealed presence of EBV DNA (strain B95- 8) in 72.3% of patients. So, most patients were positive for viral genome, while only 26 cases out of 94 patients with tongue SCC had no EBV genome. Of the positive cases for EBV DNA 36 and 32 patients were females and males respectively, which shows a more or

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