Original Article

Prevalence of *Epstein–Barr virus, Human Papillomavirus* and *Porphyromonas Gingivalis* in Oral Cancer

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Abstract

Background: Multiple risk factors are supposed to progress oral cavity carcinoma and among them, the role of neither bacterial nor viral infections should be underestimated. Despite relentless efforts, the accelerating effects of human papillomavirus (HPV), Epstein–Barr virus (EBV), and *Porphyromonas gingivalis* (*P. gingivalis*) on oral cancer has not yet been recognized successfully. Taking advantage of these facts, in this study we evaluated the prevalence of HPV, EBV, and *P. gingivalis* in oral cavity carcinoma.

Materials and Methods: A total of 43 oral cavity cancerous tissues and 29 healthy oral ones were collected from Loghman Hospital, Tehran, Iran, between 2016 and 2018. After DNA extraction, the prevalence of HPV, EBV, and *P. gingivalis* was evaluated by PCR.

Results: There were 53.5 well-differentiated (15 male, 9 female), 41.8% moderate (10 male, 5 female), and 4.7% poor (1 male, 3 female) adenocarcinoma paraffin-embedded tissue samples. PCR analysis has shown that there were 1 HPV (age: 46; moderate adenocarcinoma) and 1 EBV (age: 62; moderate adenocarcinoma) positive in different samples. No *P. gingivalis* was found and there was not any infected tissue with both EBV and HPV. In 31% of control tissues, blisters were observed and in 51.7% there was no mucus. We did not find any association between age, sex, and HPV, EBV positive samples.

Conclusion: As sample size can affect the results of epidemiological and clinical study, and due to the low number of positive samples in this study, we concluded that HPV, EBV, and *P. gingivalis* may not have a detrimental effect on the progression of oral cancer, but further studies are needed.

Keywords: EBV, HPV, *P. gingivalis*, Oral cancer

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Introduction

Nowadays, cancers are considered as one of the serious health problems worldwide which have a detrimental

effect on health care systems. Among many different kinds of cancers, it is estimated that the global incidence of oral cavity carcinoma has more than 60% up-regulation[1]. Moreover, 1400 new cases per 100,000 individuals suffer from oral carcinoma in Iran annually[2]. Oral carcinoma is a life-threatening disease, with ranging stages from benign to invasive malignant tumor which can intervene in the patients' lives. Actually, different symptoms, such as pre-malignant dysplastic lesions with leukoplakia, erythroplakia, or a combination of two can be associated with oral cavity carcinoma[3]. Although there are a lot of progressions in detecting this disease in the early stages, oral cavity carcinoma accounts for a high rate of mortality. As chemo-resistance in oral cancer is observed and the carcinoma cells are able to spread in all parts of the body, this health problem can be classified as an incurable disease[4,5]. Previous studies suggest that some risk factors can play a crucial role in this disease. The consumption of tobaco and alcohol, low intake of fruit and vegetable and poor oral hygiene, family history of this cancer, body mass index (BMI), personal medical history, and even tea consumption has an effect on the progression of oral carcinoma[6-10]. Moreover, the role of neither viral nor bacterial risk factors should be underestimated. These factors can express different oncoproteins and lead to cell division[11].

Human papillomavirus (HPV) is one of the viral risk factors which has a double-stranded DNA genome[12]. This virus with some oncoproteins, such as E6 and E7 can stimulate the division of cells and deteriorate the situation of patients, suffering from different kinds of cancers. Some cancers have a direct relationship with this virus, for example, the relationship between cervical cancer and HPV has been confirmed. E6 is able to destroy the structure of P53 with ubiquitin, and E7 can separate the Rb from E2F; hence, the protein overexpression may be observed in cells[13-15].

Furthermore, herpesviridea, especially Epstein–Barr virus (EBV), is supposed as one of the infectious risk factors to progress cancer[16]. This family possesses some special strategies to escape from the immune system. Recurrence and latency are observed in some of the virus members of this family; therefore, in most of patients who suffer from immune deficiency, *herpesviridea*, especially EBV, may cause serious health problems[17]. In previous studies, the association between EBV and gastric cancer by using some oncoprotein, such as LMP1 and EBNA-1 was reported[18,19].

Apart from HPV and EBV, there is an argument that *Porphyromonas gingivalis* (*P. gingivalis*) can stimulate cell division. This bacterium belongs to gram-negative bacteria, and is able to express serine-glycine dipeptide lipid classes which can engage human Toll-like receptor 2 (TLR2), and induce tumor necrosis factor alpha (TNF-α) expression; therefore, inflammation and cancer may be observed[20].

As the prevalence of EBV, HPV, and *P. gingivalis* in oral cavity carcinoma is not yet clear, in this study we evaluated the HPV, EBV, and *P. gingivalis* prevalence in this type of cancer.

Methods

Samples

This study has been submitted and approved by the ethics committee of Shahid Beheshti University of Medical Sciences, School of Medicine (SBMU. IR.SBMU.RETECH.REC.1397.456). In this case-control study, 43 oral cavity cancer and 29 healthy oral tissues were collected from Loghman Hospital, Tehran, Iran, between 2016 and 2018.

DNA extraction

After removing paraffin digesting tissues by 1ml xylene for 3 hours, and digesting buffer containing proteinase K, the DNA of all samples was extracted by Exgene (GeneAll, Seoul, Korea), and eluded in 50 µl of elution, according to manufacturer's protocol[21].

β-globin gene PCR

As an internal control, the *β-globin* gene was employed to evaluate and confirm the quality of samples DNA. In this study, we used 12.5 µl master mix, 1 µl forward and 1 µl reverse primer (10 pmol), 1 µl DNA and 8.5 µl sterile water in final 25 µl in following PCR procedure: 5 min in 95°C as the first denaturation, 30 cycles of 95°C for 30s, 55°C for 30s, 72°C for 30s and 72°C for 7 min. Every negative *β-globin* gene was double-checked.

PCR

We investigated HPV, EBV, and *P. gingivalis* prevalence in three different PCR schedules. In final 25 µl, we combined 12 µl PCR master mix 5X (Cinaclone,

Table 1: The primers sequence for PCR procedure.

GAPDH forward	**GGTGCTAAGCAGTTGGTGGT**
GAPDH reverse	**GGTACACCTGCAGACACCATTGAT**
HPV forward	**CGGACAGAGCCCATTACAATATT**
HPV reverse	**CGCACAACCGAAGCGTAGA**
EBV forward	**GATTCAGGCGTGGTCCTTGG**
EBV reverse	**CCGAAGAGGTTGAAAACAAA**
P. gingivalis forward	**GCGTATGCA ACTTGCCTTAC**
P. gingivalis reverse	**GTTTCAACGGCAGGCTGAAC**

Iran), including 8 µl sterile distilled water, 2 µl DNA template, 1.5 µl forward and 1.5 µl reverses primers, and incubated in following PCR temperature. PCR temperature for HPV: 5 min in 94°C as first denaturation, 29 cycles of 94°C for 30s, 48°C for 30s, 72°C for 30s and 72°C for 7 min. PCR schedule for EBV: 5 min in 94°C as first denaturation, 29 cycles of 94°C for 30s, 56°C for 30s, 72°C for 45s and 72°C for 10 min. And PCR for *P. gingivalis*: 5 min in 94°C as first denaturation, 29 cycles of 94°C for 30s, 54°C for 30s, 72°C for 30s and 72°C for 10 min. (The primers were presented in Table.1). The PCR products were run in 2% in gel electrophoresis[21].

Statistical analysis

All data were assessed by IBM SPSS Statistics software version 22, and Chi-square test to find the relationship between patient's sex, age, and the prevalence of EBV, HPV, and *P. gingivalis*. P-value less than 0.05 were considered meaningful.

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Results

To evaluate the prevalence of HPV, EBV, and *P. gingivalis*, 43 cancerous embedded paraffin tissues [26 males (60.5%) and 17 females (39.5%)] were collected. The mean age was 59.72 (max: 97 and min: 19). 29 matched control samples were also included. There were 53.5% well-differentiated (15 males, 9 female), 41.8% moderate (10 males, 5 female), and 4.7% poor (1 male, 3 female) adenocarcinoma. PCR analysis has shown that there were 1 HPV (age: 46; moderate adenocarcinoma) and 1 EBV (age: 62; moderate adenocarcinoma) positive in different samples. No *P. gingivalis* was found and there was not any infected tissue with both EBV and HPV. In 31% of control tissues, blisters were observed and in 51.7% there was no mucus. All control tissues were negative. Positive samples were EBNA-1, HeLa cell line, and positive sample collected from hospital for EBV, HPV and *P. gingivalis*, respectively. We did not find any association between age, sex, and HPV, EBV positive samples.

Discussion

Oral cavity carcinoma can be supposed as one of the major reasons of mortality. Studies reported that about 60% of patients suffering from oral cavity carcinoma, have stage III or IV of this cancer[21]. Alcohol, smoking, age, sex, and high fat diet have a detrimental effect on this kind of cancer. For example, there is a higher chance (about 10 to 15 times) in the smoker to get oral cavity carcinoma[23,24]. Many studies made an effort to identify the novel biomarkers which can induce cells to divide uncontrollably. Investigations showed that infectious risk factors can be considered as one of the main causes of oral cavity carcinoma. As some different infections are able to express oncogenes, these risk factors can progress cancer diseases, especially oral cavity carcinoma. Recent studies have shown that HPV oncogenes (E6 and E7) are able to knock down P53 and deregulate the protein expression[25].

There were 1 HPV (age: 46; moderate adenocarcinoma) and 1 EBV (age: 62; moderate adenocarcinoma) positive in different samples. No *P. gingivalis* was found and there was not any infected tissue with both EBV and HPV. Other studies showed different rates of HPV prevalence in different parts of the world. For example, it was reported that there was not any relationship between HPV and oral squamous cell carcinoma (SCC) of the oral tongue, in non-smoking and non-drinking patients between 2003 and 2006[26].

In another study executed by Shih-Wei Yang, HPV was not introduced as a prognostic indicator of malignant transformation in oral leukoplakia[27]. In 2020, among 50 patients, there were no HPV positive tissues and it was reported that p16INK4a expression was not dependent on the presence of HPV in patients suffering from oral cancer[28]. However, some other studies detected HPV in oral cancer tissues. For example, Janecka-Widła showed that more than 20% of patients were infected with HPV. [81.25% HPV-16, 9.38% HPV-35, and double infections with HPV-16 and 35 (6.25%) or HPV-35 and 18 (3.12%)][29]. In 2020, 94 out of 158 were HPV positive, resulted that HPV has a strong impact on oral cavity carcinoma[30].

In Qatar, one of the Iran's neighbor countries, HPV types 59 (54.8%), 31 (53.7%), 52 (49.1%), 51 (48.6%), 58 (47%), and 35 (45.5%) had higher prevalence[31]. Among 96 patients (23 female/73 male), HPV was detected in 112 patients (19 female/93 male) in 2020[32]. About 61% of patients, HPV-16 was positive[33].

Another virus evaluated in this study was EBV. Our results indicated one positive EBV tissue; therefore, EBV may not involve in progressing the oral cancer. Some studies reported similar results, for example, in the study of Torben Wilms, there was no evidence of EBV presence in squamous cell carcinoma of the mobile tongue[34]. In India, there was a higher prevalence of EBV in control samples than cancerous tissues[35]. In some other investigations in 2018, the role of EBV and HPV DNA in accelerating the oral cavity cancer progression was emphasized [36]. In Thailand, a tight relationship between EBV and oral cancer was reported[37] and 27.3% of all samples were EBV positive, and 7.8% of samples were infected with both HPV and EBV[38].

In this study, we did not detect any *P. gingivalis*. It was reported that *P. gingivalis* has a profound impact on oral cancer by upregulating IL-8 and MMPs[39]. By blocking apoptotic pathways in gingival epithelial cells, *P. gingivalis* can prevent cells to become cancerous[40]. However, in some other studies, *P. gingivalis* was reported with a higher level (more than 33%, $P<0.05$) in cancerous tissues[41]. By changing in epithelial-mesenchymal transition-like, *P. gingivalis* can increase the chance of oral cancer diseases42. This bacterium may be able to interact with TLRs and stimulate the division of oral cells[43].

Conclusion

As sample size can affect the results of epidemiological and clinical study, and due to the low number of positive samples in this study, we concluded that HPV, EBV, and *P. gingivalis* may not have a detrimental effect on the progression of oral cancer, but further studies are needed.

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