Original Article

Investigation of the Potential Presence of *Porphyromonas gingivalis* in Esophageal Squamous Cell Carcinoma (ESCC) Paraffin-Embedded Tissue Samples

Afsoon Taghavi[1, 2], Masoud Dadashi[3, 4], Saeideh Khaleghnejad[5], Somayeh Delfani[5], Parisa Abedi Elkhichi[5, 6], Parto nasri[7], Bahareh Hajikhani[1, 5*]

[1]Men's Health and Reproductive Health Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[2]Department of Pathology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
[3]Department of Microbiology, School of Medicine, Alborz University of Medical Sciences, Karaj, Iran.
[4]Non-Communicable Diseases Research Center, Alborz University of Medical Sciences, Karaj, Iran.
[5]Department of Microbiology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
[6]Medical Microbiology Research Center, Qazvin University of Medical Sciences, Qazvin, Iran.
[7]Department of Pathology, Isfahan University of Medical Sciences, Isfahan, Iran

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Abstract

Background and Aim: Esophageal cancer is the eighth most common cancer and the sixth leading cause of cancer death worldwide. Evidence suggests that there is a link between bacterial infection and malignancy. There are few studies on the prevalence of *Porphyromonas gingivalis* in esophageal squamous cell carcinoma (ESCC), so this study aimed to investigate the possible presence of this bacterium in ESCC tissue samples.

Materials and Methods: In this study, 34 esophageal squamous cell carcinoma samples were collected to evaluate the potential presence of *Porphyromonas gingivalis.* After extracting the DNA, the polymerase chain reaction (PCR) technique was used to detect the presence of the bacterium molecularly.

Results: The age range of the study population was 26 to 90 years, with a mean age of 63 years. Most tissue samples come from stage I cancer (73.5%). Based on the molecular analysis, no *P. gingivalis* was detected in any biopsy specimens

Conclusion: *P. gingivalis* infection and ESCC were not correlated based on the current in this study. Likely, the use of fresh samples, more accurate diagnostic methods, geographic differences, and larger sample sizes all contribute to the differences in results between related research, which can be clarified through large-scale studies.

Keywords: *Porphyromonas gingivalis*, Esophageal squamous cell carcinoma, Polymerase chain reaction

***Corresponding Author:** Bahareh Hajikhani. Department of Microbiology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran. Email: b.hajikhani@gmail.com Orcid ID: 0000-0001-7966-6267

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Introduction

Esophageal cancer is the eighth most common cancer and the sixth most common cause of death due to cancer. This cancer is frequently malignant, accounting for 2% of all malignant tumors[1]. Histologically, there are two forms of esophageal cancer: squamous cell carcinoma and adenocarcinoma.

Squamous cell carcinoma is more common than adenocarcinoma carcinoma[2]. Most esophageal squamous cell carcinomas (ESCC) are found in the middle of the esophagus, while esophageal adenocarcinoma is generally detected at the distal end of this organ[3]. ESCC is more prevalent in developing countries. Eastern and Southeast Asia, in particular, had the highest rates, while adenocarcinoma is more prevalent in developed countries[4]. ESCC is often diagnosed with morphological features and suspicious lesions.

Despite advances in multimodal treatments such as surgery, chemotherapy, radiotherapy, and chemoradiotherapy, ESCC has a poor prognosis[5]. Age, heredity, gene mutations, vitamin deficiency (particularly riboflavin and nicotinic acid), smoking, and alcohol consumption increase the risk of esophageal cancer[6,7]. In addition, a growing body of research suggests that microorganisms are closely related to many human cancers[8,9]. The upper respiratory tract comprises many microbes that may play an important role in esophageal cancer due to cellular and genetic changes[2]. The distal esophagus has detected 166 microbial species from 9 phyla[10,11].

Porphyromonas gingivalis (*P. gingivalis*) is one of the bacterial agents that appears to have a potential role in the establishment or progression of esophageal cancer[12]. *P. gingivalis* is an anaerobic gram-negative bacterium considered a pathogen in periodontal disease that disrupts the homeostasis of a microbial population[13-15].

Some studies have suggested the critical role of this bacterium in facilitating the development and progression of orodigestive and pancreatic cancers[16,17]. The possibility of this bacterium being implicated in ESCC has also been considered[18]. It has been shown that *P. gingivalis* in saliva is associated with developing oral squamous cell cancer, which can increase the risk of esophageal cancer[19].

P. gingivalis uses different possible mechanisms for inducing ESCC, such as immune evasion, inhibition of apoptosis, alteration in carcinogens, induction of MMP-9, and imbalance in oral microbiota[20-22]. Given the importance of ESCC in Asia and especially in our country, the poor prognosis of the disease, poor response to treatment, its rising incidence, and the relationship between the infection and cancer, here, we conducted a study to investigate the possible presence of *P. gingivalis* in ESCC tissue samples.

Materials and Methods

Samples and DNA extraction: This descriptive research was conducted between April 2018 and March 2019. In this survey, 34 esophageal carcinoma paraffin-embedded tissue blocks were examined. These samples were taken in past years at Shohada-e Tajrish Hospital in Tehran, Iran. A pathologist performed a microscopic assessment to determine the malignant tissues. The samples were sent to the Department of Microbiology, Shahid Beheshti University of Medical Sciences, for further evaluation. After cutting the paraffin blocks into thin slices, they were placed in 1.5 mL microtubes, and then DNA was extracted from all paraffin-embedded tissue blocks using G-spin TM Total DNA extraction Kit (GeneAll, Korea) according to the manufacturer's instruction. After confirming the quality and quantity of extracted DNA using the NanoDrop spectrophotometer, the samples were kept at -20 ° C.

Standard PCR: The human beta-actin gene was used as the target of PCR to support successful DNA extraction. The second PCR was performed using the specific primers (Table 1), and the reaction mixture contained 12.5 µL of PCR master mix, 1 µL of each forward and reverse primer, and 5 µL of DNA template. The final volume was brought to 25 µL by adding PCR-grade water. The negative and positive controls were each prepared using 5 µL of PCR-grade water and 5 µL of *P. gingivalis* ATCC 33277 DNA, respectively. The PCR conditions were as follows: one cycle of initial denaturation at 94°C for 5 minutes, 30 cycles of denaturation at 94 C for 45 seconds, annealing at 57°C for 45 seconds, extension at 72°C for 45 seconds, and final extension at 72°C for 10

Table 1: Primer sequences and predicted sizes of PCR products. Gene bank access number: D26470.

Primer	Sequence	Product size
Forward	5' CGAAGTCTTCATCGGTCGTT 3'	498 bp
Reverse	5' GTACCTGTCGGCTTACCATCTT 3'	

minutes. The PCR products were run on a 1.5% agarose gel and stained with ethidium bromide (0.50 mg/mL). The gel was then examined with a UV transilluminator.

Statistical Analysis: All the data in this study were analyzed using Statistical Package for Social Sciences (SPSS) software (version 23).

Ethics approval to perform the study was obtained from the Shahid Beheshti University of Medical Sciences (IR.SBMU.RETECH.REC.1395.1003).

Results

The study population consisted of 34 patients (17 men and 17 women) with a mean age of 63 (ranging from 26 to 90 years). The highest ESCC patients in both genders were in the age group of 51 to 75 years (11 males and nine female). Also, in the age group under 25, there was only one sample related to a female patient with ESCC. More details about the age groups and cancer stages of patients are given in Table 2. A total of 34 paraffin-embedded tissue specimens were prepared and studied. The frequency of different SCC stages was as follows: stage I, 25 (73.5%) specimens, stage II 3 (8.8%), stage III 1(2.9%), and stage IV 5 (14.7%). According to our results, all the analyzed biopsy specimens were negative for *P. gingivalis* using the molecular technique. All samples were examined in the presence of positive and negative controls, and the results showed the accuracy of the procedure.

Table 2: gender * grade of cancer* age Crosstabulation.

Age-gender			Grade of ESCC				Total
			1	2	3	4	
0-25	gender	female	1	--	--	--	1
	Total		1	--	--	--	1
26-50	gender	male	3	0	--	1	4
		female	4	1	--	0	5
	Total		7	1	--	1	9
51-75	gender	male	6	1	1	3	11
		female	7	1	0	1	9
	Total		13	2	1	4	20
76-99	gender	male	2	--	--	--	2
		female	2	--	--	--	2
	Total		4	--	--	--	4
Total	gender	male	11	1	1	4	17
		female	14	2	0	1	17
	Total		**25**	**3**	**1**	**5**	**34**

Discussion

ESCC is a particular form of gastrointestinal tumor that causes a significant public health burden. Despite advancements in diagnostics, this cancer is often diagnosed at an advanced stage, resulting in a low overall survival[23]. Even though esophageal cancer has been identified worldwide, the Asian belt, including Iran, has a very high occurrence rate of this cancer[24].

Due to the importance of ESCC, identifying the factors involved in its occurrence is of particular importance. According to mounting evidence, microorganisms could be closely related to many human cancers[25]. It has been discovered that the oral microbiota defines the esophageal microbiome, which can theoretically lead to esophageal carcinogenesis[26].

Recent research has found a link between inflammatory periodontal disease and the possibility that microorganisms can play a role in developing esophageal cancer, such as ESCC[27,28]. One of the key etiological factors in the pathogenesis of periodontal disease is *P. gingivalis*, which can impair the oral microbial balance, resulting in a dysbiotic host-microbiota relationship. It may particularly play a role in the growth of periodontal disease and upper orodigestive tract cancers[18]. Ahn et al., in their research, showed that periodontitis and serum IgG against *P. gingivalis* are linked to orodigestive cancer mortality[29]. This bacterium has been implicated in the etiology of several unrelated chronic disorders, including diabetes, rheumatoid arthritis, cardiovascular disease, and several forms of orodigestive cancers[18,30].

Lipopolysaccharide (LPS), fimbriae, gingipains, and outer membrane vesicles are the most significant virulence factors in *P. gingivalis*. The ability to build a dysbiotic microbiota and a dysregulated immune response are two major pathogenic mechanisms. It is also worth mentioning that *P. gingivalis* can infect oral epithelial and endothelial cells, causing potent pro-inflammatory cytokine release[17,31,32].

P. gingivalis interactions with epithelial cells could lead to an oncogenic process, activating the TGF β and PI3K/AKT signaling pathways[33,34]. In response to this

bacterium, TGF β stops the cell cycle and reduces apoptosis[35]. In this regard, the results of a study showed that *P. gingivalis* induced IL-6 production during the JAK2 and GSK3β pathways activation, and IL-6, in turn, increases inflammation[36,37]. Furthermore, the bacterium decreases IL-1 β activity by secreting nucleoside diphosphate kinase (NDK), which cleaves ATP and prevents the proapoptotic P2X7 receptor activation[38]. T $CD8^+$ cells are affected by a reduction in IL-1 β, which allows the bacterium to escape from the host immune[15]. On the other hand, Moffatt and colleagues showed that *P. gingivalis* can upregulate microRNAs that inhibit apoptosis in primary gingival epithelial cells, such as miR-203[39]. The metabolism of potentially carcinogenic compounds is another possible mechanism for *P. gingivalis*-induced carcinogenesis. This bacterium, for instance, transforms ethanol into its carcinogenic derivative, acetaldehyde, at levels sufficient to induce DNA destruction, mutagenesis, and epithelial hyperproliferation which can lead to the development of cancerous lesions[40].

Due to the various mechanisms of malignancy stimulated by this bacterium and its potential role in the development of cancers such as ESCC, various studies have investigated the presence of this microorganism in cancerous tissues. The present study aimed to evaluate this bacterium's presence in paraffin-embedded ESCC tissues. In this study, we used 34 paraffin-embedded samples from cancer tissue due to problems obtaining fresh samples from patients. Despite confirming and repeating all laboratory steps in this study, bacterial DNA was not isolated from ESCC biopsy specimens.

Similar to our finding, Dantong Shao and colleagues showed no significant difference in the relative abundance of *P. gingivalis* between ESCC tumors and non-tumor tissues[41]. Failure to isolate the bacterial DNA from the samples does not necessarily mean denying the role of this bacterium in the development and progression of ESCC. Numerous factors, including paraffin-embedded tissues, limited sample sizes, and techniques used to detect the bacterial genome. They can all influence the rate of bacterial detection in the tested tissue specimens.

However, a group of studies have shown the presence of this bacterium or its components in the examined tissues using different diagnostic methods. In 2016, Shegan Gao and colleagues evaluated the presence of *P. gingivalis* in the ESCC and normal tissues by immunohistochemistry using antibodies targeting whole bacteria and its unique secreted protease. This study showed that *P. gingivalis* was detected in 61 % of cancerous tissues, 12 % of adjacent tissues, and none of the normal control tissues[12]. These results showed for the first time that *P. gingivalis* infects the esophageal epithelium of ESCC patients.

2017 Yuan and colleagues 2017 conducted another study on ESCC specimens collected and treated promptly after surgery using qPCR and immunohistochemistry. They discovered that *P. gingivalis* was preferentially and regularly present in ESCC specimens but only infrequently in matched noncancerous parts[42].

The use of fresh samples, more accurate diagnostic methods, differences in a geographic region, and higher sample size may be the reasons for the differences between the results of these studies and our research. A study by Chen et al. reported that the abundance of *P. gingivalis* in the samples was associated with a higher risk of ESCC. As a major pathogen, this bacterium attacks epithelial cells and modulates host immune responses. Hence, *P. gingivalis* can play an important role in esophageal cancer[43].

The study by She-Gan Gao et al. provided evidence for the involvement of *P. gingivalis* in the pathogenesis of ESCC. This study measured serum immunoglobulin G and A (IgG / A) antibody levels against P. gingivalis. Evidence showed that IgG and IgA levels for *P. gingivalis* were significantly higher in ESCC patients than non-ESCC patients[44]. In addition to the differences in the number of samples and diagnostic methods used, most studies in this field have been conducted in China, which, in terms of population type, genetic background, lifestyle, and eating habits, were different from the study population in our study which can partly explain the reason for the differences in results.

Conclusion

Due to the importance of ESCC, identifying the factors involved in its occurrence is of particular importance. Although there is evidence of the prevalence of *Porphyromonas gingivalis* in

esophageal squamous cell carcinoma, our findings show no association between the prevalence of this bacterium and esophageal squamous cells. This may be due to the limited number of clinical specimens. In general, the importance and novelty of this issue, to determine the true role of this microorganism in the incidence of ESCC, more extensive studies are needed.

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Conflict of interest

The authors further declare that, they have no conflict of interest.

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