Short Communication

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Comparing the Frequency of Epstein–Barr Virus in Esophageal Cancer Tissue

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ABSTRACT

BACKGROUND AND OBJECTIVE: Esophageal cancer is one of the most common cancers of the digestive system and infection with cancer-causing viruses is considered as one of the risk factors for this type of cancer. Epstein-Barr virus (EBV) is a human carcinogenic virus that can infect epithelial cells and lead to their malignancy. Some studies indicate the role of EBV in Esophageal Squamous Cell Carcinoma (ESCC). However, studies reveal different results. This study aims to determine the frequency of EBV in samples obtained from patients with ESCC and normal samples or samples without esophageal cancer lesion.

METHODS: In this cross-sectional study, 100 samples of paraffin-embedded tissue in patients with esophageal cancer and 68 samples of normal paraffin-embedded tissue or noncancerous lesion were examined. After deparaffinization of tissue samples, DNA was extracted. All the DNA samples extracted using Real Time PCR method were examined to assess the presence of EBV genome using EBV specific primers and probes.

FINDINGS: EBV EBER gene was detected in 10 cases (10%) of esophageal cancer samples and in 3 (4.4%) of the non-cancerous control samples, indicating the presence of EBV in cancerous and non-cancerous cells.

CONCLUSION: Results of this study demonstrated the Frequency of Epstein–Barr virus in esophageal cancer tissue. However, these results cannot confirm or reject the role of EBV in occurrence of esophageal cancer.

KEY WORDS: *Epstein-Barr Virus, Esophageal Squamous Cell Carcinoma, Real time PCR.*

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Introduction

Esophageal cancer is one of the most common cancers that involves the digestive system and is the sixth lethal human cancer in the world. This type of cancer is highly prevalent in a region of the world called "Asian Esophageal Cancer Belt", which extends from South coast of the Caspian Sea in Iran to North China (1). Smoking, alcohol consumption, nutritional and environmental factors are among the known risk factors for esophageal cancer (2).

Other risk factors are also suggested for esophageal cancer and one of them is infection with cancer-causing viruses (3). Human tumor viruses cause about 10 to 15% of all cases of cancer in the world (4). Epstein-Barr virus (EBV) is a human gamma herpesvirus and human is its only natural host. EBV is also known as a human herpesvirus – 4 (HHV – 4) and is one of the 8 human herpesviruses and has tropism for B lymphocytes and epithelial cells. Around 95% of adults in the world are infected with EBV (5).

The initial infection caused by this virus usually occurs during childhood and the infected individuals usually carry the virus for the rest of their life. This virus is an etiologic agent of infectious mononucleosis and is associated with malignancy of B lymphocytes and epithelial cells. In 1997, the International Agency for Research on Cancer (IARC) confirmed the cancer-causing effects of this virus on human.

We currently have strong evidence regarding the role of EBV as an etiological agent in Burkitt's lymphoma, Nasopharyngeal carcinoma, Hodgkin's disease and lymphoma associated with immunosuppression (6). EBV is also associated with cancers of the pharynx, esophagus and stomach (7–9). Cancers of epithelial tissue and lymph such as cancers of the abdomen, lungs and uterus are also related to this virus (10, 11). The risk of esophageal cancer in northern China, southern Asia and northern Iran is higher than other regions of the world (2).

In recent years, some studies have indicated the role of EBV in the incidence of Esophageal Squamous Cell Carcinoma (ESCC) (12). A limited number of studies are available regarding the relationship between EBV and esophageal cancer; some of them do not show any significant relationship between EBV and esophageal cancer, while some of them show percentages of 1.8–35.5% (13-16). However, no definite relationship between EBV and esophageal cancer has been proved so far. Since EBV genome have been found in the upper digestive tract (oral cavity and esophagus), the continuous exposure of this duct to this cancer-causing virus may play a role in the incidence of malignancy. Studies show that high alcohol and tobacco consumption may be the cause of 90% of all ESCC cases in these regions (18). It is interesting that people in Iran, particularly in the northern region that is located on the Asian Esophageal Cancer Belt, alcohol and tobacco consumption is very low (19).

Thus, we cannot a proper justification for the high prevalence of ESCC in these regions. Therefore, we need to consider other risk factors for the high prevalence of ESCC in these regions. The viral agents such as EBV can be the factors to justify the high prevalence of this type of cancer in these regions. Different studies indicate that this virus can infect the epithelial cells and transform them toward malignant (21, 22).

The high detection rate of EBV may be related to several factors. Factors such as race and geographical location seem to play significant role. In addition, the detection rate of EBV may differ based on the sensitivity and specificity of the detection method. Some detection methods such as Southern blotting, PCR and Nested PCR are known as highly sensitive and specific methods of viral DNA detection (20).

Therefore, further studies are required to focus on the role of EBV in creation of ESCC. Mazandaran Province is located on the Asian Esophageal Cancer Belt and is considered as one of the high-risk regions for this type of cancer and few studies have been conducted in this regard in the north of Iran. Thus, this study was conducted to determine the frequency of EBV in samples obtained from patients with ESCC and normal samples or samples without esophageal cancer lesion using Real Time PCR method to measure the presence of EBV genome.

Methods

Clinical Samples: In this cross-sectional study, 100 samples of paraffin-embedded tissue in patients with esophageal cancer (squamous cell carcinoma) (59 men and 41 women) in the age range of 38 – 91 years and 68 samples of paraffin-embedded tissue collected from patients who underwent endoscopy and were pathologically diagnosed as normal tissue or noncancerous lesion in the age range of 42-90 years (42 men and 26 women) were studied. Samples were examined in terms of Epstein-Barr virus, age, sex and place of residence. This study has the moral permission

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DNA extraction: To extract DNA from paraffin-embedded samples, the samples were deparaffinized using xylene and absolute ethanol according to the instructions provided by previous studies (17). Extraction of DNA from tissue was done using High Pure PCR Template Preparation kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Ultimately, the quality of the extracted DNA was assessed by NanoDrop (Thermo Scientific, Wilmington, USA).

Real Time PCR Assay: Real Time PCR assay was done to determine the presence of EBV genome using YTA Probe qPCR MasterMix (Yekta Tajhiz) and specific primers and probes of EBV (table 1) and based on the combination of components (table 2) and an established program (table 3) using Applied Biosystems 7300 Real-Time PCR System.

The positive control sample for EBV: To achieve EBV genome as positive control to run EBV detection test in the target samples, B-cell type lympho-blastoid cell lines (B-95-8) that inherently secrete EBV in supernatant cell culture was prepared from Cell Bank of Pasteur Institute of Iran. After extraction of viral DNA from cell supernatant, it was used as the positive control in Real Time PCR assay.

Statistical analysis: In this study, the data were analyzed using SPSS 22 and Chi – square test and $p<0.05$ was considered statistically significant.

Table 1. Primers and probes used in Real Time PCR to determine the presence of EBV genome

Target gene	Primers and probes	The sequence of bases (3'-5')
EBV EBER Gene	EBER-F-Primer	5'- TGACGTAGTCTGTCTTGAGGAGATG -3'
	EBER-R-Primer	5'- CGTCTCCTCCCTAGCAAAACC -3'
	EBER-Probe	FAM-TGCAAAACCTCAGGACCTACGCTGC-TAMRA

Table 2. The combination of components used in Real Time PCR to determine the presence of EBV genome

Volume	Combination
12.5 µl	2X qPCR MasterMix for probe
1.2 µl	Combination of primers and probes (100 pmol/µl)
0.3 µl	Passive Reference Dye I (50 X)
6 µl	Nuclease-free water
5 µl	DNA sample
25 µl	Final volume

Table 3. The program used in Real Time PCR to determine the presence of EBV genome

Steps	Number cycles	Time	Temperature (ºC)
Initial Denaturation	1	10 minutes	95
Denaturation	40	30 seconds	95
Annealing/ Extension		60 seconds	60

Results

The mean age of patients with ESCC was 66.8±10.7 and the mean age of patients without cancer was 64.3±12.5 in this study. Based on the results of Real Time PCR, EBV EBER gene was detected in 10 cases (10%) of cancerous samples and 3 cases (4.4%) of non-cancerous control samples (Fig 1). The presence of EBV DNA in men in both case and control groups was more than women (60% and 100%, respectively). The highest relative frequency of EBV DNA presence was observed in patients 65 to 74 years old (50%). On the other hand, the presence of EBV genome in the samples of city dwellers was more than villagers (tables 4, 5). There was no significant relationship between the presence of EBV DNA and variables of age, sex and place of residence in both groups.

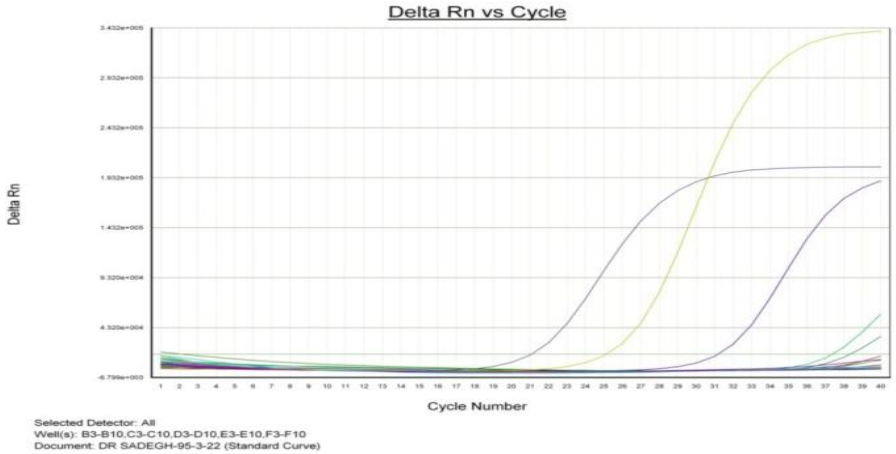

Figure 1. Real Time PCR assay results to determine the presence of EBV genome in clinical samples of ESCC tissue and noncancerous esophagus tissue (case and control samples) as well as positive control (B-95-8)

Table 4. The relative frequency of EBV DNA presence in ESCC tissue samples in Mazandaran province

EBV DNA Variable	Positive N(%)	Negative N(%)	Total N(%)
Sex			
Man	6(60)	53(58.9)	59(59)
Woman	4(40)	37(41.1)	41(41)
Total	10(100)	90(100)	100(100)
Age (years)			
Below 55	1(10)	19(21.1)	20(20)
55 to 64	2(20)	13(14.4)	15(15)
65 to 74	5(50)	37(41.1)	42(42)
75 or above	2(20)	21(23.3)	23(23)
Total	10(10)	90(100)	100(100)
Place of residence			
City	7(70)	41(45.6)	48(48)
Village	3(30)	49(54.4)	52(52)
Total	10(10)	90(90)	100(100)

Table 5. The relative frequency of EBV DNA presence in noncancerous esophagus tissue samples in Mazandaran province

EBV DNA Variable	Positive N(%)	Negative N(%)	Total N(%)
Sex			
Man	3(100)	39(60)	42(61.8)
Woman	0(0.0)	26(40)	26(38.2)
Total	3(100)	65(100)	68(100)
Age (years)			
Below 55	0(0.0)	19(29.2)	19(27.9)
55 to 64	1(33.3)	15(23.1)	16(23.5)
65 to 74	1(33.3)	15(23.1)	16(23.5)
75 or above	1(33.3)	16(24.6)	17(25)
Total	3(100)	65(100)	68(100)
Place of residence			
City	1(33.3)	36(55.4)	37(45.6)
Village	2(66.7)	29(44.6)	31(45.6)
Total	3(100)	65(100)	68(100)

Discussion

Results of this study detected the presence of EBV DNA in 10 of ESCC samples and 4.4% of noncancerous esophagus tissue samples using Real Time PCR method. Esophageal squamous cell carcinoma is the most common type of esophageal malignancy and is highly prevalent in developing countries including Northern provinces of Iran. The risk of esophageal squamous cell carcinoma is generally low in developed countries.

Investigating 164 samples from esophageal squamous cell surgery, Wu et al. detected the EBV EBER proteins in 6.7% of samples and detected LMP-1 in 6.1% of samples using ISH and IHC methods (immunohistochemistry) (14). Jenkins et al. reported the presence of EBV in 8.3% of samples collected from patients in United States (22). Wang et al. in Taiwan detected EBV in 35% of cases (23). Awerkiew et al. also detected EBV in 35% of cancerous samples (8 of 23 samples) in Germany (24).

Sedaghat et al. only examined 28 cancerous samples of Iranian patients and found the presence of EBV in 12 cases (42.8%) (25). Haghshenas et al. also reported the presence of EBV DNA in 10% of cases using PCR method, which was consistent with the results of the present study (26). However, considering the larger sample size in the present study and compared with noncancerous samples, this percentage is much lower than the study of Sedaghat et al. (10% as opposed to 42.8%). Yet, it is similar to the study of Haghshenas et al. on samples in Mazandaran and Golestan, except that the sample size in the present study is two and a half times larger and a more accurate molecular method and a noncancerous control sample are used in the present study.

In a study conducted in Shantou region of Guangdong province in China, Zhang et al. analyzed the presence of HPV-1, HSV-1, CMV and EBV viruses in tissue samples of patients with esophageal cancer using Nested PCR method and investigated the level of EBV in ESCC etiology in this region and reported EBV DNA in 70 mucosa samples to be 30%. Moreover, they found that the presence of EBV in the mucosa of ESCC samples to be considerably more than the mucosa of normal samples (7.4%) and reported the highest level of infection to be among 45 to 58 years old patients (8). The difference in the level of EBV DNA between cancerous samples and noncancerous samples was less than the study of Zhang. Awerkiew et al. analyzed 72 ESCC and 40 adenocarcinoma samples from Germany and 43 ESCC samples from Russia and demonstrated the presence of EBV DNA using PCR and ISH methods. They reported the presence of EBV DNA in 34% of ESCC samples and 26% of adenocarcinoma using Nested PCR method and detected a copy of EBV genome in each 27 – 200000 cells using Real Time PCR method. However, they did not detect any specific copy of EBER RNA of EBV in the nucleus of tumor cells using In – Situ Hybridization method, while in EBER versions in the nucleus, 7 ESCC samples and 1 AC samples of 24 EBV DNA samples were detected to be positive (27).

In addition, some reports such as the studies of Yanai et al. in Japan (28), Sunpaweravong et al. in Thailand (16) and Hong et al. (29) using ISH and PCR methods found negative results for all the studied ESCC samples. In other studies, EBV DNA or some versions of EBER were observed in very rare cases (4–7%) or in no case at all (30–32).

Considering that the relationship between EBV and esophageal cancer was only observed in paraffin-embedded tissue, it seems that studying fresh tissue samples can demonstrate this relationship more accurately. Moreover, it is suggested that the expression of some important proteins of this virus be tested on fresh tissue. According the results of this study, although the prevalence of EBV DNA in noncancerous control samples was lower than cancerous esophagus samples, this does not demonstrate a causal relationship between EBV and its role in the incidence of ESCC. Considering that the level of EBV DNA detected are different in different regions of the world and considering different results achieved from various laboratory detection methods, conducting more extensive studies on different ethnic populations and using more standard detection methods will help us gain a more clear picture of this relationship.

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