RESEARCH ARTICLE

Detection of Epstein-Barr Virus and Cytomegalovirus in Gastric Cancers in Kerman, Iran

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Abstract

Gastric cancer (GC) is a multifactorial disease with different factors having roles in its genesis. *Helicobacter pylori* and Epstein-Barr virus (EBV) are known infectious agents that could contribute. In addition, there is evidence of a relationship with cytomegalovirus (CMV). Since data on CMV prevalence in gastric cancer are limited, we here evaluated the frequency of EBV and CMV in Iranian patients. Ninety paraffin blocks of GC tissues from patients in Kerman were evaluated for the presence of EBV and CMV genomes by real-time polymerase chain reaction. EBV was detected in 10 cases (11.1%) and CMV in seven. One out of 17 female patients (5.88%) and nine out of 73 male patients (12.3%) were positive for EBV, while one out of 17 female patients (5.88%) and six out of 73 male patients (8.22%) were positive for CMV. The mean age for EBV-positive patients was 60.5±14.9 years and the mean age for CMV-positive patients was 67.9±12.3years. This study shows that the frequency of EBV-associated GC is high in Kerman. It also indicates that further studies of associations between GC and CMV are warranted, covering larger samples and populations from different areas of the world.

Keywords: Gastric cancer - tissue samples - cytomegalovirus - Epstein-Barr virus - PCR detection

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Introduction

Gastric cancer is one of the most common tumors of the gastrointestinal tract, it has high morbidity and mortality rates, and it is more than Northeast Asia, including China, Japan and South Korea (Kanda et al., 2016). The pathogenesis of gastric cancer is unclear; however, social-economic environment, lifestyle, nutrition, education, smoking and *Helicobacter pylori* infection are all associated with its occurrence (Park et al., 2015).

Gastric cancer is a multifactorial disease that various factors such as infectious agents involved in its creation, Infectious agents such as H.pylori and EBV are accepted as causative microorganisms for GC (Camargo et al., 2015). In addition the study, which is done in 2014, confirmed the role of latent cytomegalovirus infection in GC (Chen et al., 2015). Human cytomegalovirus (HCMV) is a β-herpes virus that may cause the infection of multiple cell types in human. The virus persists in 30–100% of the population worldwide, particularly in certain areas of Africa and Asia (Reza et al., 2015). Asymptomatic infection, caused by latent state HCMV in healthy individuals, may increase the risk of atherosclerosis and age-related immune senescence (Grim et al., 2010; Mott et al., 2013). Furthermore, severe or acute disease may be induced in immunocompromised cases, including acquired immunodeficiency syndrome patients and transplant recipients, due to the reactivation of latent HCMV (He et al., 2011; Falasca et al., 2014). An increased number of cases of gastrointestinal diseases caused by HCMV infection have been reported, including ulcerative colitis and esophageal ulcers (Jang et al., 2009). In addition, emerging evidence has indicated that HCMV infection may be associated with human malignancies, including colon and prostate cancer (Chow et al., 1997; Lu et al., 2010). However, the association between gastric cancer and HCMV remains unclear.

Epstein-Barr virus (EBV) is a human herpes virus with oncogenic activity. The EBV genome can be detected in malignancies of both lymphoid and epithelial cell origin, such as Burkitt's lymphoma (BL) and nasopharyngeal carcinoma (NPC) (Matsuura et al., 2010). In 1990, EBV genomes were detected in gastric carcinomas using polymerase chain reaction and in situ hybridization (ISH) for EBV-encoded small ribonucleic acid 1 (EBER1) (Saridakis et al., 2005). These findings indicated that EBV-associated gastric carcinomas comprise about 10% of all gastric carcinomas worldwide. Since EBV

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infection may be involved in the early stages of gastric carcinogenesis (Sairenji et al., 2001). EBV spreads by the oral route, after primary infection, EBV establishes the lifelong virus carrier state, called latent infection, which expresses a limited set of viral genes required for viral episome maintenance, thereby conferring a survival advantage to the infected cell (Ryan et al., 2010; Reza et al., 2015). The presence of EBV DNA was linked with severe forms of Gastric cancer. Epstein-Barr virus encoded small RNA (EBER) is non-polyadenylated, noncoding RNA that exists abundantly in EBV-infected cells (Ngan et al., 2001). EBER induces signaling from the Toll-like receptor 3 (TLR3), which is a sensor of viral double-stranded RNA (dsRNA) and induces type I IFN and pro-inflammatory cytokines and the majority of the released EBER existed as a complex with a cellular EBER-binding protein (Cainap et al., 2015).

The most frequently mutated gene in Gastric cancer is the p53 tumor suppressor gene. When the p53 gene is mutated, the cell cycle controlling is lost and this fact is leading to the development of neoplasms (Bargiela-Iparraguirre et al., 2016). The present study was carried out in an attempt to determine whether there is a relationship between mentioned viral infection and p53 expression in Gastric cancer. EBV and CMV belong to the herpesvirals that have linear dsDNA. Gastric cancer is the fourth common cancer in men and the fifth in women and in terms of mortality from cancer is in rank second (Babaei et al., 2010). About 10 percent of all deaths from cancer have been attributed with GC; and around 70 percent of total stomach cancers have been reported from developing countries (Camargo et al., 2015). Mortality from this cancer in developing countries is 10 percent more than in developed countries. In Iran, this cancer is too important, and unlike developed countries its incidence is increasing (Camargo et al., 2014).

This study was carried out evaluation frequency of EBV and CMV in patients with Gastric cancer. We for the first time examined the frequency of CMV in patients with gastric cancer. Molecular techniques are the most reliable assays in viral presence detection in comparison to other tests based on host antibody. Real-Time PCR is the most sensitive methods and have been used frequently to detect viruses. In this study, the presence of EBV, and CMV in GC tissue samples were studied from paraffin-embedded malignant tissues in Iranian patients with gastric cancer by a standard Real Time PCR assay for finding the relationship between those viruses and gastric cancer.

Materials and Methods

Patients

Paraffin embedded blocks samples from patients with Gastric Cancer, referred to the department of Pathology, Afzalipour Pathobiology Lab, Kerman province, Iran, during April 2009 -March 2014 were identified. 90 formalin-fixed paraffin-embedded tumor tissues of patients collected. Types of samples for gastric cancers were Adenocarcinoma (diffuse type and Intestinal type) that in the diffuse type mucinous and in intestinal type signet ring cell were shown. The most type of caricinoma was Intestinal Adenocarcinoma. The present study is based on a retrospective examination of Gastric Cancer diagnostic biopsy or surgery samples from clinical cases, all original hematoxylin and eosin (H&E) slides and Immunohistological stain recut from tissue blocks were reviewed. In total, samples were screened for EBV DNA, CMV DNA , mRNA expression level of EBER, expression level of PP65 antigen and level of expressed p53. This project was approved by the research center ethics committee of the Kerman University of Medical Sciences.

Deparaffination samples

Paraffinated blocks from the 90 tumor samples were cut in 3-μm sections and 5 sections, patients were collected in the same micro-centrifuge tube. Samples were de-waxed in 800 μl xylene, All micro-centrifuge tube located for 10 min in a 60 °C heated block and centrifuged at 10,000 rpm for 1 minute , supernatant was removed. This step was then repeated three times. Add 500 μl absolute ethanol, centrifuge at 10,000 rpm for 1 minute, the samples were then dried in a 70°C heated block with open lids for 5-10 min for remove residual ethanol.

Tissue digestion

According to samples (biopsy or Paraffinated blocks), 200-400 μl of Tissue Lysis Buffer was added to each tube [4 M Urea, 200 mMTris, 20 mMNaCl, 200 mM EDTA; PH=7.4 (25°C)]. To all tubes added 20-40 μl proteinase K, Samples were gently vortexes and located for 10 min in a 60°C heated block, and all samples were subsequently incubated at 37°C overnight.

DNA and RNA Extraction

The next day, 200 μl of Binding Buffer [6 M Guanidine- Hcl, 10mM Urea, 10mM Tris-Hcl, 20% Tritonx-100 (v/v); PH=4.4 (25°C)] was added to each tube with gently vortex. DNA was isolated using a QIAamp DNA Mini kit (Qiagen, Germany) and Total RNA was extracted using RNAeasy mini kit (Qiagen, Germany) according to the manufacturer's instructions. Extracted DNA and RNA pellets were resuspended in 70μl of pre-warmed Elution buffer and stored at -70°C until use.

Virus Detection using Real time PCR

Real time PCR was carried out using EBV, and CMV Real Time PCR kit (Inter Lab Servise, Russia) following instruction manual.

Real Time PCR

For determination expression level of EBER, p53 and PP65 antigen mRNA, reverse transcription real time PCR (rReal Time PCR) was carried out by using the first strand cDNA synthesis kit by RevertAid cDNA synthesis kit (Thermoscientific,USA). Briefly, RNA samples were heated to 65°C for 10 minutes and then chilled on ice. The uniform suspension of bulk first-strand cDNA reaction mix was added according to the manufacturer's protocol.

One μl of DTT solution, and 1 μl of random hexamer (N)6 primer (0.2 μg) were then added to the heat-denatured RNA. Samples were mixed properly by pipetting up and down several times and then incubated for 1 hour at

42°C. For Real time PCR, the Quanti Tect Probe PCR Kit (Qiagen, Germany) used base on instruction kit. Real time PCR primers and probes were design for EBER, p53 and PP65 antigen mRNA after alignment of these regions between all of them in EBML-EBI and as an internal control, β-Actin were purchased from Metabion company (Germany) (Table1).

Statistical analysis

Chi-square test or Fisher's exact test was conducted using SPSS version 17 for the association between the presence of DNA viruses genome and other characterizes (values P=0.05 were considered statistically significant).

Results

Ninety- patients with Gastric Cancer (GC) were selected during April 2009 -March 2014. Paraffinated blocks or biopsy samples from these patients were selected for evaluation frequency of HCMV, EBV virus and evaluation level gene expression of P53, EBER and PP65 using Real Time PCR. From total 90 samples, 17 (18.8%) samples were female and 73 (81.1%) were male. Mean ±SD of age were 60.46±14.88 years old,. Minimum and maximum age for male were 20 and 75 years old and for female were 34 and 86 years old. Of ninety specimens, EBV and CMV were detected in 10 cases (11.1%) and in seven cases (7.77%), respectively. One out of 17 female patients (5.88%) and nine out of 73 male patients (12.32%) were positive for EBV, one out of 17 female patients (5.88%) and six out of 73 male patients (8.22%) were positive for CMV. The mean age for EBV-positive patients was 60.46±14.88 years and the mean age for CMV- positive patients was 67.85±12.25 years (Table 2, 3). Result of PCR in different type of adenocarcinoma was shown in Table 4. Frequency different types of carcinoma were shown in figure 1, Types of samples for gastric cancers were Adenocarcinoma (diffuse type and Intestinal type) that in the diffuse type, mucinous and in intestinal type, signet ring cell were shown. The most type of carcinomas was Intestinal Adenocarcinoma. To catch more information for the role of P53 protein in making tumors, all samples of this study used for P53 detection. Sidelines of benign tumors were collected. Different rate of P53 expression is shown in figure 2 and it is clear that in other groups uninfected for CMV, the expression of p53 is normal and natural but when the PCR test of HCMV or EBV are positive, the rate of P53 dramatically drops which suggests that the reduction in amount of p53 may involve in making tumors. As it can be seen the expression levels of p53 in positive EBV, CMV cases are substantially reduced comparing with negative samples. Probably, other materials that are produced and released in virus life cycle may lead to reduced p53 in these cases.

To find if PP65 is important for GC tumor and the question of its effect on tumor production leaded us to study PP65 in our samples. In figure 3, PP65 mRNA expression in the different groups studied and seen the

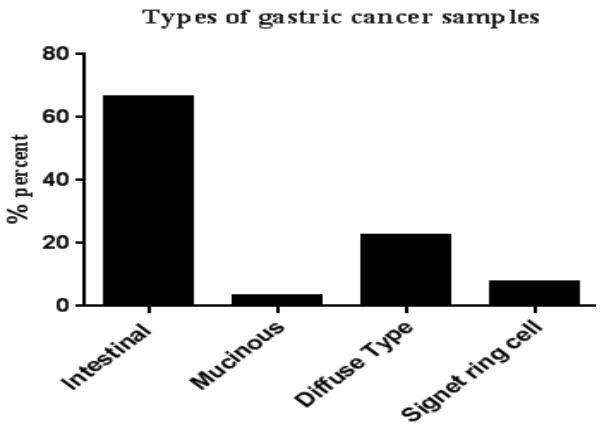

Figure 1. Frequency Types of Gastric Cancer Samples

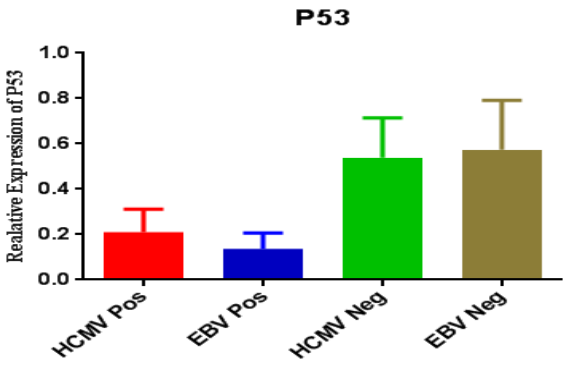

Figure 2. Relative Expression of P53 in Different Samples

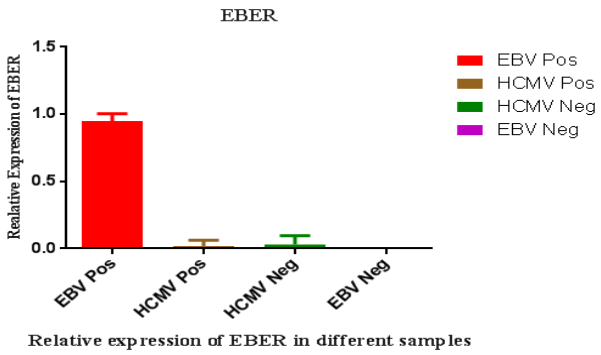

Figure 4. Relative Expression of EBER in Different Samples

Table 1. Sequences of Primers and Probes for Real Time PCR in this Study

Name	Forward	Reverse	Probe	Location
P53	CAGCATCTTATCCGAGTG	GATGGTGGTACAGTCAGA	CCAACCTCAGGCGGCTCATA	147-269
PP65 antigen	GCAGAACCAGTGGAAAGA	GCAGAACCAGTGGAAAGA	CGTACTGGTCACCTATCACCTGC	303-483
EBER	TGAGGAGATGTAGACTTGTA	CTGGTACTTGACCGAAGA	AACCTCAGGACCTACGCTGC	138-321

Table 2. The Distribution of Carcinomas According to age, sex in EBV-negative and EBV-Positive Gastric Cancer

	EBV-negative	EBV-positive	P-value
Age	62.03±12.48	60.45±14.88	0.701
	Sex, N (%)		
Men	16 (20)	1 (10)	0.446
Women	64 (80)	9 (90)	

Table 3. The Distribution of Carcinomas According to age, sex in CMV-negative and CMV-Positive Gastric Cancer

	CMV-negative	CMV-positive	P-value
Age	61.33±12.69	67.85±12.25	0.194
	Sex, N (%)		
Men	16 (19.3)	1 (14.3)	0.746
Women	67 (80.7)	6 (85.7)	

Table 4. Frequency type of carcinoma and PCR result

Type of Adenocarcinoma	Num (%)	CMV Positive	EBV Positive
Intestinal	60(66.6)	6	6
Mucinous	3(3.33)	1*	1*
Diffuse type	20(22.2)	0	1
Signet ring cell	7(7.7)	0	2

*In a sample of mucinous type both CMV and EBV were Positive

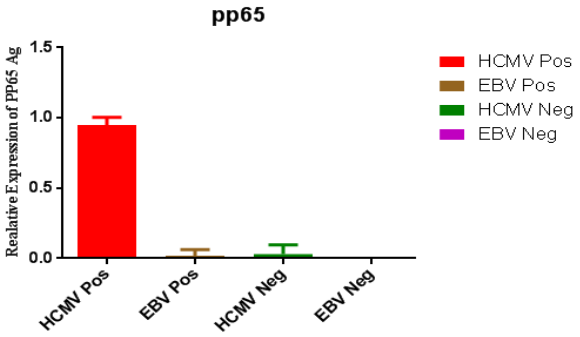

Figure 3. Realtive expression of PP65 in Different Samples

expression rate of PP65 antigen in the positive CMV patients is in the highest level (red column) but as it is shown in one negative CMV DNA sample some levels of expression were observed. In figure 4, EBER mRNA expression in the different groups studied and seen the expression rate of EBER RNA in the positive EBV patients is in the highest level (red column) but as it is shown in one negative CMV DNA sample some levels of expression were observed. Statistical analysis of results showed that there is a significant correlation between p53 and tumor genesis. Our data defined that there is significant relationship between EBER expression and presence of EBV virus. In our study, a relation between the expression levels of pp65 and CMV infection was found in GC tumors production and it cannot be said that CMV, EBV virus is involved in GC tumors genesis.

Discussion

Gastric cancer is the fourth common cancer, and in terms of mortality from cancer is in rank second (Brenner et al., 2009). Every year about 1 million new cases of stomach cancer diagnosed and mortality from this cancer is some 700,000 cases (Parkin et al., 2005).

According to the results of the study, the frequency of EBV in patients with gastric cancer in Kerman is 11.1 percent. The results of this study compared with previous studies in Iran (Tehran) that show a higher incidence of EBV-associated Gastric carcinomas (Abdirad et al., 2007; Faghihloo et al., 2014a). Since prevalence of EBV-associated Gastric cancer is lower in areas with high incidence of Gastric cancer and in areas with low prevalence of Gastric cancer is more and because Kerman is region with low incidence of Gastric cancer (Sadjadi et al., 2007). The results of our study are justifiable. So cannot be said that the prevalence of EBV-associated gastric cancer is low in Iran because EBV-associated gastric cancer have different prevalence in different geographical areas, as in this study, unlike previous studies, the prevalence was higher. Like most studies, not did statistical analysis show a significant association show between age and EBV- associated gastric cancer. However a number of studies were shown tendency to involvement at at lower age (Kazakhstan and Colombia) (Carrascal et al., 2003; Alipov et al., 2005) or higher age (Mexico) (Herrera et al., 1999).

In most studies, sex differences favor of men, but not were some studies shown a significant relationship between sex and EBV-associated Gastric cancer. In this study, The EBV-associated Gastric cancer was seen more in men, but according to the statistical analysis of the results, not was a significant relationship found between sex and EBV-associated Gastric cancer (Camargo et al., 2014). However, the absence of significant correlation can be attributed to the low number of positive samples and the limitation of statistical tests in the difference diagnosis in the low volume. In the present study of 90 cases with gastric cancer, CMV genome in 7 cases (1 female and 6 male) was diagnosed. The frequency of CMV in patients with Gastric cancer in Kerman is 7.1%. In this study show a significant correlation between age and CMV- associated Gastric cancer. In our study according to the statistical analysis of the results, not was a significant relationship found between sex and CMV-associated Gastric cancer. However, the prevalence was in men higher than women. Since no study has been done in this area, there is no possibility of further examination of results. In a study in Iran , of ninety specimens, EBV was detected in six cases (6.66%). The mean age for patients EBV-positive gastric carcinomas was 72.1 years, whereas the mean age for the entire group was 65.7 years. Four out of 64 (6.25%) male patients and 2 out of 26 (7.69%) female cases were positive for EBV. The present study shows that the frequency of EBV-associated gastric carcinoma in Iran is low. Differences of EBV-associated gastric carcinoma incidence in different countries may reflect the epidemiologic factors and dietary habits. Further analysis of clinical pathology features of EBV-associated

gastric carcinoma using a larger number of cases would give invaluable insights into its etiology (Faghihloo et al., 2014b). In a study for Identifying high-risk clusters of gastric cancer incidence in Iran, Gastric cancer was considered as the second most prevalent cancer in Iran. The most likely clusters were found in Ardabil, Gilan, Zanjan, East-Azerbaijan, Qazvin, West-Azerbaijan, Kurdistan, Hamadan, Tehran and Mazandaran between 2007 and 2009. It was statistically significant at the p-value below 0.05. High risk regions included Northern, West-North and central provinces, particularly Ardabil, Kurdistan, Mazandaran and Gilan. More screening tests are suggested to be conducted in high risk regions along with more frequent epidemiological studies to enact gastric cancer prevention programs. (Kavousi et al., 2014).We found negative evidence of an association between HCMV or EBV virus infection and GC in this study. In addition, we showed dramatic differences in the expression of PP65 and EBER mRNA in these tumors which are irrelevant with tumor production. Based on our data, P53 expression is directly linked with CMV and EBV infection and in our positive CMV, EBV samples the rate of P53 dropped and helps to make tumors. In our study, 10 samples out of 90 were positive for EBV virus and EBER mRNA in EBV positive samples and only one sample negative for EBV, were detected. In our survey, P53 was detected in both positive CMV and EBV patients and decreased comparing to negative patients and beside the presence of PP65 in positive CMV, we could report some expression in negative CMV samples. In our study, we found the relation between age and sex in GC patients. We have got more positive GC in elderly cases and also reported that the presence and possibility of GC in men is higher than women. Although researches have a strict causal relationship between HCMV and EBV infection and GC tumors, but future studies need to focus on determining the role of HCMV and EBV as a gastric-initiating event. Moreover, finding different proteins with varied functions and different types of CMV and EBV can be proposed (Corvalan et al., 2005; Fyock et al., 2014; Babacan et al., 2016).

In conclusions, This study shows that the frequency of EBV-associated Gastric cancer is high in Kerman. Our stady is the first study about the frequency of CMV in patients with Gastric cancer. According to the results of this study and other studies, were conducted about the relationship between CMV and other human cancers, CMV can be associated with Gastric cancer that for it aim, we need more studies in worldwide. The results of this study emphasizes on the CMV role in gastric cancer and of hit-and-run theory, which was described by the group Fred Rapp, and the role of cytomegalovirus onco-modulatory support.

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