Original Article

Prevalence and Characteristics of Epstein–barr Virus-associated Gastric Cancer in Iran

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Abstract

Background/Aims: Gastric cancer (GC) is the second leading cause of cancer-related deaths worldwide and is the most frequent cancer in Iran. Epstein-Barr virus (EBV) has been shown to be associated with gastric cancer. The present study was carried out to investigate the prevalence of Epstein-Barr virus (EBV) associated gastric cancer among Iranian patients.

Methods: Ninety formalin fixed paraffin-embedded cases of gastric cancer were studied. The specimens were investigated for the presence of the EBV genome by quantitative real-time polymerase chain reaction.

Results: 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. According to anatomic location, EBV was detected in 4 out of 39 (10.25%) gastric cancer were located in cardia and 2 out of 26 (7.69%) gastric cancer were located in middle/corpus.

Conclusions: 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.

Keywords: Epstein-barr, gastric cancer, Iran

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Introduction

Epstein-Barr virus is a ubiquitous 184 kbp-sized double-stranded DNA virus from herpes virus family and infects 95 % of world's population.[1] In 1964, EBV was the first virus detected from human neoplastic cell.[2] There are many documents that show EBV is associated with a variety of malignant neoplasms including nasopharyngeal carcinoma (NPC), Burkitt lymphoma, Hodgkin lymphoma, nasal T/NK cell lymphom, and B cell-lymphoma in immunosuppressed and gastric cancer patient.[2]

The association of EBV and gastric cancer was first reported in 1990.[3] Now, various studies from different countries show EBV is related with 10% gastric cancer patients. EBV associated gastric cancer varies in different countries, for example 6.4 % in China, 8.1 % in Mexico, 19.5 % in German, 5.6% in Korea, 12% in United States, 8.5% in France, 13% in Colombia and 11.3% in Brazil.[4–11]

Several constant clinical pathological features were seen in EBV associated gastric cancer such as moderately to poorly differentiated type of gastric cancer[12–14] and predisposition to upper stomach.[15–17]

In situ hybridization for EBV-encoded RNA, EBER, is considered as a gold standard assay for EBV detection in paraffin-embedded tissue sections.[18–19] The studies of EBER in situ hybridization has been called into question by showing EBV in EBER-negative cancers based on immunohistochemical or molecular assays.[20–26]

Quantitative PCR for measuring EBV viral load in gastric cancer tissues show that high viral load correlates with EBER localization to malignant cells. According to Ryan, et al. study, a cutoff value of 2000 copies per 100000 cell is a valuable threshold for distinguishing EBV associated cancer that above which insure the EBV is localized to malignant cells in gastric cancer.[27]

There is a high incidence of gastric cancer in Iran. Gastric cancer is the second most common cancer and first leading cause of death for men in Iran.[28] Thus the present study was undertaken to show the EBV associated gastric cancer in Iranian patients by quantitative real-time PCR assay for detection of EBV viral load, and to clarify the clinical pathology features of these cancers.

Materials and Methods

Patient Population

Formalin-fixed-paraffin-embedd blocks from 90 patients with gastric cancer were used for this study from the pathology department of Baqiyatallah Hospital, Tehran, Iran. For each case, information of age, sex, histological classification and cancer anatomic site were collected. These samples were related to patients with confirmed gastric cancer from 2008 – 2010. This study was approved by the research Ethics Committee of Baqiyatallah University of Medical Sciences, Tehran, Iran.

Pathologic Characteristics

Gastric cancers were categorized as intestinal- or diffuse-type according to the Lauren criteria[29] and subclassified as proposed

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by the Japanese Research Society for Gastric Cancer as follows:[30] diffuse types, including por1 (poorly differentiated adenocarcinoma with solid, sheet-like proliferation with an alveolar pattern and indistinct tubular differentiation), por2 (poorly differentiated adenocarcinoma with acinar and trabecular pattern, usually showing diffuse infiltration with abundant fibrous stroma), intestinal types tub1 (well-differentiated adenocarcinoma with distinct glandular pattern and columnar epithelium throughout, moderate or small amount of stroma); tub2 (moderately differentiated adenocarcinoma with small or incomplete tubular structures with cubical or flat epithelium, amount of stroma variable from case to case), as well as sig (signet-ring cell carcinoma) and muc (mucinous carcinoma). The anatomic site of gastric neoplasia was identified as cardia, middle/corpus, or antrum. According to histology, gastric cancer samples were categorized as adenocarcinima.

Extraction of DNA

Formalin-fixed-paraffin-embedd blocks were cut into thin slices. For deparafination, these slices were resuspended three times in xylene for 10 minutes at room temperature and washed two times in 100% ethanol to remove the xylene. Next, samples were dried at room temperature and treated with digestion buffer and Proteinase K overnights at 55˚C and then Proteinase K was inactivated at 95˚C for 10 minutes. After centrifugation at 14000 rpm for 10 minutes, the supernatant was used for DNA extraction using QIAamp DNA minikit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

Quantitative Real-time PCR

Probe-based PCR method was used to measure EBV DNA using FTD kit (Fast-Track Diagnosis, Luxembourg). All PCR assays were performed with the AgPath-IDTM One-Step RT PCR kit (Ambion) as recommended by FTD Company. Quantitative PCR targeting human APOB gene was used to control for quantity and quality of DNA extraction and to normalize for the number of cells amplified per reaction.[31]

PCR was performed and products were detected using ABI Prism 7500 Real-Time PCR instrument and Sequence Detection System software (Applied Biosystems, Foster City, CA). Thermo cycling conditions were as follows: 50°C for 15 minutes; 95°C for 10 minutes; 95°C for 8 seconds and 60°C for 34 seconds for 40 cycles.

Each 25 µl reaction contained: 1X TaqMan Universal Master Mix, forward and reverse primer (15 _mol each), and TagMan probe and 1 µl DNA template. The cycle threshold (Ct) for EBV and the internal control were calculated automatically using system software, and the resulting Ct was used to quantify the EBV copy number by interpolation according to standard curve composed of a series of three clinical pathology features quantitation standards (QS1-QS3: 10000, 100000 and 1000000 copies/µL; FTD).

Statistical Analysis

In the present study, statistical analysis was done using SPSS software (version 12.0, SPSS, Chicago, IL., USA). Relation of EBV status with independent variables including gastric cancer, sex, age and histological types was assessed by Chi Square (X2) and t-test.

Results

Out of 90 patients with confirmed gastric cancer, six cases (6.66 %) had DNA viral load more than 2000 copies per 100000 cells, so they were considered as EBV associated gastric cancer.

Among 90 patients, 64 (71.1%) samples were from men and 26 (28.9%) samples were from women. Four out of 64 (6.25%) male patients and 2 out of 26 (7.69%) female cases were positive for EBV. This study shows that there was not significant association between gender and EBV associated cancer (*P*-value = 0.8).

The mean age for all patients was 65.77 years, for the male patients it was 67.84, and for the female patients it was 60.69. The mean age for patients with EBV associated gastric cancer was 72.16 years: 74.25 years for male patients and 68 years for female cases.

Analysis of histological features showed that 3 and 2 of EBV positive samples were categorized into por1: solid poorly differentiated adenocarcinoma and tub2: moderately differentiated adenocarcinoma, respectively, and 1 of EBV associated gastric cancer was categorized into well differentiated adenocarcinoma.

The distribution of carcinomas according to age, sex, anatomic site and histological features in EBV-negative and EBV-positive gastric cancer are shown in Table 1.

According to anatomic location, 4 out of 39 (10.25 %) cardiac tumors and 2 out of 26 (7.69%) body (corpus) tumors were EBV associated. There was not EBV associated gastric cancer in the antrum. (*P*-value = 0.36)

Clinical pathology and genetic characteristics of EBV associated gastric cancer are shown in Table 2.

Discussion

Gastric cancer is the fourth most common cancer worldwide and it is the second leading cause with cancer associated death.[32] A total of 930000 new patients of gastric cancer are being diagnosed and gastric cancer cause 700000 patients die every year.[32] Gastric cancer is the second most common cancer and first leading cause of death for men in Iran.[28] While there are several geographical areas with low and intermediate incidence of gastric cancers, northern and northwestern areas of Iran are high risk regions for gastric cancer.[33]

Smoking, diet, drinking alcohol and gastric disease including gastritis and Helicobacter pylori infection are risk factors for gastric cancer. The EBV is also associated with gastric cancer.

In this study we performed a quantitative Real-time PCR analysis whereas more studies in this scope were carried out by EBER in situ hybridization. The quantitative Real-time DNA amplification assay is equivalent to EBER in situ hybridization for detection of EBV associated gastric cancer and it is superior to immunohistochemical detection of EBV proteins. The quantitative Real-time PCR is also rapid and less labor intensive than manual EBER in situ hybridization.

According to a prior study, a cutoff value 2000 copies of EBV DNA per 100000 cells is valuable threshold for distinguish of EBV associated gastric cancer and it is a reasonable level beyond which EBV was localized to malignant cells.[27]

In the present study, 90 samples from patients with confirmed gastric cancer were examined. It was shown that 6.66 % of samples have DNA viral load more than 2000 copies per 100000 cells, so they were considered as EBV associated gastric cancer. Viral

Table 1. The distribution of carcinomas according to age, sex, anatomic site and histological in EBV-negative and EBV-positive gastric cancer

	EBV-negative	EBV-positive	*P*-value
Age , mean (SD)	65.3 (10.6)	72.2 (6.6)	0.12
Sex, N (%)			0.80
Men	60 (71.4)	4 (66.7)	
Women	24 (28.6)	2 (33.3)	
Location, N (%)			0.36
Cardia	39 (46.4)	4 (66.7)	
Corpus	24 (28.6)	2 (33.3)	
Antrum	21 (25.0)	0 (0.0)	
Differentiation, N (%)			0.967
Well-diff.	12 (14.3)	1 (16.7)	
Moderately-diff.	27 (32.1)	2 (33.3)	
Poorly-diff.	45 (53.6)	3 (50.0)	
Total	**84 (100)**	**6 (100)**	

Table 2. Clinical pathology and genetic characteristics of EBV associated gastric cancer

	Gender	Age	Anatomic Site	Histological Classification	EBV DNA copies / 100 000 cells
1	Men	67	Cardia,	Tub1; Well Differentiated	9,629
2	Men	73	Cardia	Por1; Poorly Differentiated	14,894
3	Women	62	Corpus	Por1; Poorly Differentiated	490,321
4	Men	80	Cardia	Por1; Poorly Differentiated	18,135
5	Women	74	Cardia	Tub2; Moderately Differentiated	6,396
6	Men	77	Corpus	Tub2; Moderately Differentiated	128,557

loads were considerably higher for EBV positive versus EBV negative cancers (mean111322 versus 235 EBV DNA copies per 100,000 cells).

Some ways to help prevent cross-contamination when designing the project. First, negative control and reagent blank included in each run for the identification of contamination. Second, all PCR reactions were performed in real-time format to prevent risk of contamination by eliminating the post-PCR processing. We have also performed the PCR for each positive and negative specimen on two occasions, and each time we used positive and negative controls in which the results were the same. To check for amplicon contamination, every run contained at least two "no template" controls in which nuclease free H_2O was substituted for template.

Low frequencies of gastric cancer related to EBV in Iran[34] and Japan confirm the hypothesis that high risk countries for gastric cancer show low frequency of EBV associated gastric cancer.[35] EBV associated gastric cancer prevalence was detected in our study was higher than the frequency that was shown in a previous study in Iran (3%).[34] The difference in the prevalence in this study and in an earlier study from Iran might be related to difference in detection approaches. This study was done by quantitative real-time PCR although the earlier study from Iran was conducted by in situ hybridization.

EBV associated gastric cancer varies in different countries,[4–11] it seems that economic conditions are not an important variable for EBV associated gastric cancer because low and high frequencies were found in both developed and developing countries.

The male/female preference in EBV associated gastric cancer was investigated in some studies. Absence of this prevalence was reported by studies in Chile[12] and Mexico.[5,36] According to studies by Galesteky, et al. in Russia[16] and Shibata, et al. in United States,[15] EBV associated gastric cancer was prominent in males.

The male predominance of EBV associated gastric cancer was found in most of studies from different countries, and it shows risk factors of gastric cancer in males are related to the etiology of EBV associated gastric cancer.

Some studies showed that the occurrence of EBV associated gastric cancer are common in elderly persons,[37–39] while other studies showed that there is a tendency of EBV associated gastric cancer to occur in younger age.[10,14,40]

One of the limitations with this study is that the number of EBV positive cancer was low, so it is not possible to get an estimate of male to female ratio or age in EBV associated gastric cancer.

Some reports showed that the cardia and middle/ corpus are the most common anatomic site involved.[41–43] Whereas other studies have reported that the antrum was more frequently involved.[5,40] The relationship between EBV associated gastric cancer and location of cancer was not significant in this study.

Some studies revealed that EBV associated gastric cancer is categorized to solid poorly differentiated adenocarcinoma and moderately differentiated adenocarcinoma.[12–14] In this study we did not observe significant relation between EBV positive cancer and cancer grade.

In conclusion, this study is similar to previous studies,[34] that showed prevalence of EBV associated gastric cancer in Iran is low. However, additional studies are necessary to clarify the etiology and epidemiology factors related to EBV associated gastric cancer in Iran.

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