Iranian Journal of Microbiology
Volume 16 Number 5 (October 2024) 689-697

ORIGINAL ARTICLE

Molecular detection of Epstein-Barr virus in paraffin-embedded tissue samples of patients suffering gastric cancer in Ahvaz, Iran: a case-control study

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Received: November 2023 **Accepted:** September 2024

ABSTRACT

Background and Objectives: Gastric cancer (GC) is the third most common cause of cancer-related mortality. Epstein-Barr virus (EBV) is associated with several human tumors. The present research was performed to investigate the prevalence of EBV-associated gastric cancer (EBVaGC) among Iranian patients.

Materials and Methods: Seventy cases of gastric cancer and 30 cases of gastric ulcer, all preserved in formalin-fixed paraffin-embedded (FFPE), were examined in a case-control study conducted between 2011 and 2018. The specimens underwent analysis to detect the presence of the EBV genome using a Nested-PCR method targeting EBNA1. Subsequently, samples testing positive for the EBNA1 underwent further testing for the presence of the EBER gene using PCR. Finally, Positive samples were subjected to sequencing.

Results: Five out of 70 cases (7%) were found to be positive for EBV based on EBNA1 testing, while all EBNA1 positive samples were negative for EBER. Notably, EBV was not detected in patients with gastric ulcer. The mean age of EBV-positive gastric carcinomas pateints was 64.5 years. Within this group, 60% were male and 40% were female. A higher prevalence of EBV association was observed in diffuse-type cases, with 60% (3 out of 24) testing positive, compared to intestinal-type cases where 40% (2 out of 46) were EBV-positive. Most cases of EBVaGC belonged to grade I.

Conclusion: This research demonstrates a low prevalence of EBVaGC in Iran. Discrepancies in EBVaGC occurrence among countries could be attributed to epidemiological variables and dietary practices. A comprehensive studies will provide significant contributions to understanding of its etiology.

Keywords: Gastric cancer; Gastric ulcer; Epstein-barr virus infection; Iran

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INTRODUCTION

Gastric cancer(GC) is the third most common cause of cancer-related mortality, accountingfor 8.2% of all cancer deaths and is one of the most common tumors of the gastrointestinal tract worldwide (1, 2). The highest rates are reported in Eastern Asia, followed by Central and Eastern Europe, and the lowest rates betong to North America and Western Africa (3). Gastric cancer is a multifactorial disease. The common risk factors for GC include infectious agents such as *Helicobacter pylori* (HP), smoking, high salty-diets, and susceptibility to hereditary gastric cancer syndrome (4). At the beginning of the 1990s, the association between Epstein-Barr virus (EBV) and gastric carcinomas was found (2). Epstein-Barr virus associated gastric cancer (EBVaGC) comprises approximately 10% of gastric carcinomas (5).

EBV is a double stranded DNA virus, also known as human herpes virus 4. It is a gamma-herpes virus and as a member of the Herpesviridae family with oncogenic activityis responsible for approximately 1.8% of all human cancers, including malignancies of both lymphoid and epithelial cell origin such as Hodgkin lymphoma, Burkitt lymphoma, NK/T cell lymphoma, nasopharyngeal carcinoma, B cell-lymphoma in immunosuppressed patients and gastric carcinoma (2).

The oral route is the primary route of the EBV transmission. However, it has been reported that organ transplantation and blood transfusion can also lead to EBV spread (6). Primary infection in early adulthood leads to the establishment of life-long latency in memory B cells (7). During a latent EBV infection, the viral genome persists for a lifetime in multiple circular episomes inside the infected cell nucleus (8). EBV can exhibit one of the four latency programs that differ in the expression of particular EBV-encoded genes. EBVaGC belongs to latency type I or II, in which EBNA-1, LMP-2A, EBERs, BARTs, and BART miRNAs are expressed. EBNA-1 is crucial for the maintenance of the episomal viral genome in infected cells during cell division. In gastric carcinoma cells, EBV is not integrated into the host genome (9).

Several published meta-analyses are addressing the prevalence and association of EBV among gastric cancer patients; however, some important variables such as gender, type of samples, and tumor anatomical location were not included in their meta-analysis. The advanced stage, cardiac tumor localization, older age, and less differentiated histology are adverse prognostic indicators in patients with GC (2).

To date, the mechanisms of EBV-associated gastric cancer are still not comprehensively clarified. Despite these findings, the importance of EBV in gastric carcinogenesis has long been underestimated. In this study, the presence of EBV in GC tissue samples was studied from paraffin-embedded malignant tissues in patients in southern Iran with GC to find the relationship between these viruses and gastric cancer.

MATERIALS AND METHODS

Ethical issues. The ethics committee approved the present research of the Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran, under reference number: IR.AJUMS.MEDICINE.REC.1397.001.

Study population. This was a retrospective case-control study of 100 participants aged between 19-89 years, referred to Imam Khomeini Hospital, Ahvaz-Iran from 2011-2018. This research was designed so that two groups of patients were randomly selected based on the presence or absence of GC and assigned into case and control groups. Formalin-fixed paraffin-embedded (FFPE) tissues are the most common specimen stored in pathology laboratories. All samples were collected from the archives and the diagnostic accuracy of gastric cancer and ulcer was confirmed after endoscopy and biopsy, and after gastrectomy by a pathologist. Of these, 70 FFPE tissue blocks of GC patients as a test group and 30 formalin-fixed paraffin-embedded tissues of patients with gastric ulcer as a control group were selected from the archive pathology.

The following inclusion criteria were used for the case group (i): Patients with gastric carcinoma confirmed by a pathologist; (ii) age >18 years; (iii) living in Khuzestan province. The exclusion criteria (i): patients with severe gastritis atrophic; (ii) treated patient. The inclusion and exclusion criteria for the control group study were gastric ulcer and low/moderate/high grade of metaplasia or dysplasia respectively.

The sampling method was performed in 35 patients (79%) using biopsy by endoscopy and 15 cases (21%) by gastrectomy. In the control group, all samples were prepared by endoscopy. It should be noted that all samples in the control group were negative in terms of dysplasia and metaplasia. Frequency different types and grades of Gastric Cancer Samples are

shown in Figs. 1 and 2 respectively.

Deparaffination samples. One hundred paraffin blocks were sectioned 10 µm-thick about 10 times. Deparaffination was done by xylene and ethanol (Germany, Merk). Initially, all the specimens were placed in microtubes then xylene was added and kept at 45°C for 15 min followed by centrifuge at 14000rpm. This stage was repeated. The supernatant was discarded and 1ml absolute ethanol was added to the precipitate and stored at room temperature for 10 min and centrifuged again at 14000rpm for 1 minute. The supernatant was discarded. This process was repeated by adding 70% ethanol, followed by the same condition and again this process was repeated by adding 50% ethanol, followed by the same condition. Finally, the supernatant was discarded and all microtubes were placed at 65°C for 5 min to vaporize the ethanol residue and the pellet was used in DNA extraction (10).

DNA extraction and quality/quantity assessment. DNA was extracted by the standard proteinase K-sodium dodecyl sulfate (SDS) method, followed by phenol-chloroform purification, and the purity and concentration of viral DNA was measured by a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) (11, 12). PCR method was used to detect β-globin gene in all samples. The extracted DNA was stored at -20°C until PCR amplification.

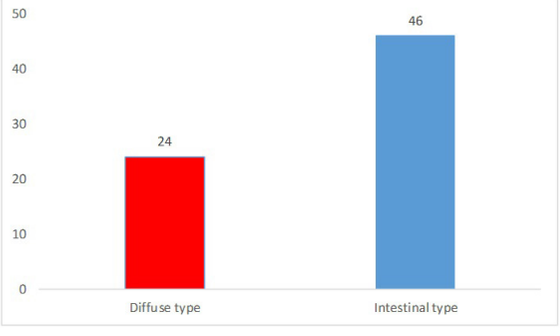

Fig. 1. Frequency of different types of gastric cancer

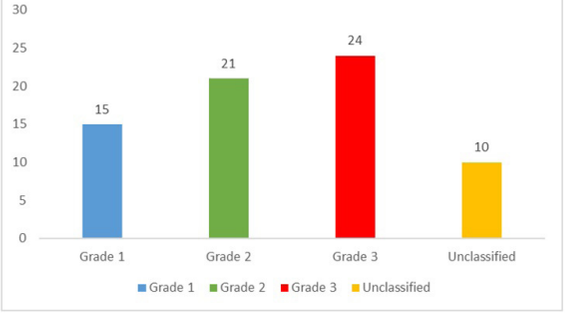

Fig. 2. Frequency of different grades of gastric cancer

Detection of EBV by nested PCR for EBNA 1 region. Nested-PCR test was performed in two steps of primary PCR and Nested for the detection of EBNA 1 (Epstein-Barr virus nuclear antigen-1). The primers for the first round of PCR were a 609 bp region of EBNA-1 and primers for the second round of PCR were a 309 bp region of EBNA-1 described in Table 1. The first round of PCR was performed in a 25 µl mixture, containing 10 µl of extracted DNA, 12 µl of PCR master mix 2x (AMPLIQON), 1 µl (10nM) of each primer sequence, and 1 µl distilled water. Cycling conditions are as follows: denaturation at 95°C for 10 min, followed by an amplification cycle at 95°C for 30 sec, 53°C for 30 sec, 72°C for 30 sec for 30 cycles, and a final extension at 72°C for 10 min. The second round was carried out with 5µl of the first-round product, 1 µl (10nM) of each primer sequence, 12 µl of PCR master mix (2x), and 6 µl distilled water. Cycling conditions are as follows: denaturation at 95°C for 10 min, followed by an amplification cycle at 95°C for 30 sec, 62°C for 30 sec, 72°C for 30 sec for 30 cycles, and a final extension at 72°C for 10 min. PCR product was subjected to electrophoresis on a 2% (0.5 µg/ml) agarose gel, stained with DNA safe stain, and observed under ultraviolet light. The expected PCR product for the second round was 309 bp. We used the B95-8 cell line (EBV-transformed leukocytes) as a positive control, and a mixture of samples without DNA served as a negative control (13).

PCR for the EBER gene. The positive samples for the EBNA1 region were again tested for the EBER gene of the EBV genome by PCR method. PCR was performed in a 25 µl mixture, containing 5 µl of extracted DNA, 12 µl PCR master mix (2x), 1 µl (10nM) of each primer sequence, and 6 µl distilled water. Cycling conditions are as follows: denaturation at 95°C for 10 min, followed by an amplification cycle at 95°C for 30 sec, 48°C for 30 sec, 72°C for 30 sec for 30 cycles and, a final extension at 72°C for 10 min. The expected PCR product for this test was 153 bp. PCR product was subjected to electrophoresis on a 2% agarose gel, stained with DNA safe stain, and observed under ultraviolet light. Primers used for the two steps of PCR are reported in Table 1. PCR for EBER Gene samples was performed in duplicate.

Table 1. EBV gene-specific primers used for Nested-PCR and PCR amplification

Primers name	Sequences (5´-3´)	Product size	Location	References
EBNA-1-F1	GTA GAA GGC CAT TTT TCC AC	609 bp	95502 to 95521	(14)
EBNA-1-R1	CTC CAT CGT CAA AGC TGC		96093 to 96110	
EBNA-1-F2	AGA TGA CCC AGG AGA AGG CCC AAG G	309 bp	95705 to 95729	(14)
EBNA-1-R2	CAA AGG GGA GAC GAC TCA ATG GTG		95989 to 96012	
EBER-F	GAT CCA AAC TTT AGT TTT AG	153 bp	6796 to 6815	(15)
EBER-R	CGC AAC CGT AAC TCT ATA C		6927 to 6945	

Nucleotide sequencing and phylogenetic analysis. The purified products from the second round of PCR amplification for EBNA1 in both the forward and reverse directions were sequenced using an ABI 3730 XL DNA sequencer (BIONEER's Custom Services - Sequencing Service). Analysis and alignment of forward and reverse sequences were performed by SnapGene sequence alignment editor version 3.2.1. The sequences were deposited in the GenBank database under the accession numbers: MT360999, MT361000, MT361001, MT361002, and MT361003. For phylogenetic analysis, MEGA 7 software was utilized. The Maximum Likelihood method with the Tamura Nei-model was used for the construction of the phylogenetic tree. 1000 bootstrap replicates were performed to determine the confidence level of evolutionary distances.

Statistical analysis. SPSS software version 26 (SPSS Inc, Chicago, IL, USA) was used for data analysis. Analysis of data was performed using the K_2 test. Furthermore, Odds Ratio and Confidence Intervals were used for comparing differences between groups. Multiple logistic regression was used for data analysis. A P-value smaller than 0.05 was considered as statistically significant.

RESULTS

Patients characteristics and clinical pathology. The research amided to evaluate the frequency of Epstein-Barr virus in tissue samples of patients suffering from gastric cancer in Ahvaz, Iran. The study enrolled 70 patients with gastric cancer as the test group and 30 patients who suffered from gastric ulcers as the control group. The patient group included 26 women (34%) and 44 men (66%) with a mean age of 64.5 ± 17 years old, while the control group included 15 (50%) women and 15 (50%) men with a mean age of 58.1 ± 19.4 years old. The minimum and maximum age for males were 19 and 89 years old, and for females were 20 and 82 years old.

Prevalence of EBV positive cases. EBV (EBNA 1) was detected in five out of 70 cases (7%) which were considered as EBVaGC (Fig. 3). These positive cases were negative for the EBER gene. All samples of the control group were negative for EBNA 1. Table 2 shows the distribution of EBV-positive and EBV-negative cases according to age and sex in gastric cancer. The results of the frequency, type, and grade of carcinoma, and PCR results are presented in Tables 3 and 4, respectively. Clinical characteristics of EBV associated gastric cancer are shown in Table 5.

Factors associated with EBV positive cases: Gender and age. The mean age for EBV-positive patients was 65.8 ± 15.2 years. EBV positive cases included 2 (40%) females and 3 (60%) males (Table 2).

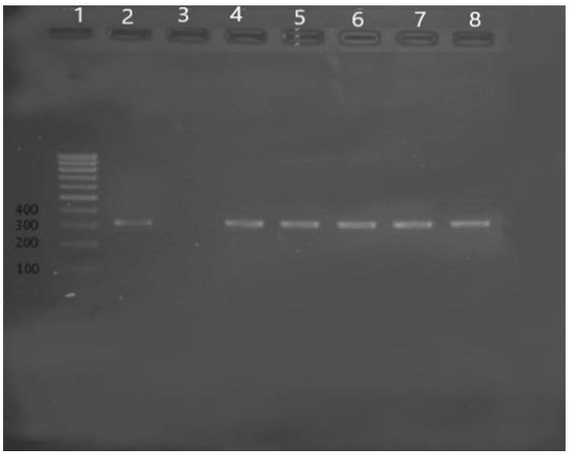

Fig. 3. Gel electrophoresis image of EBNA 1 gene. The length of the fragment is 309 bp. Lane 1 100 bp size ladder, Lane 2 positive control (B95.8 cell line), Lane 3 negative control, Lane 4-8: EBV positive samples

Table 2. The distribution of EBV-Positive and EBV-negative according to age, sex in Gastric Cancer

	EBV-Negative	EBV-Positive	P-value
Age	64.5 ± 17	65.8 ± 15.2	0.282
	Sex, N (%)		
Male	41 (63)	3 (60)	>0.990
Female	24 (37)	2 (40)	

Table 3. The frequency of EBV positivity according to adenocarcinoma type

Type of Adenocarcinoma	Num (%)	EBV-Positive	P-value
Intestinal	46 (65)	2 (40)	>0.990
Diffuse	24 (35)	3 (60)	

Table 4. The frequency of EBV positivity according to adenocarcinoma grade

Grade of Adenocarcinoma	Num (%)	EBV-Positive	P-value
I	15 (21)	2	
II	21 (30)	1	0.794
III	24 (34)	1	
Unclassified	10 (15)	1	

Table 5. Clinical characteristics of EBV associated gastric cancer

Patient No.	Gender	Age	Type of adenocarcinoma	Grade of adenocarcinoma
1	M	55	Diffuse	Unclassified
2	M	77	Intestinal	I
3	F	78	Intestinal	I
4	F	41	Diffuse	III
5	M	78	Diffuse	II

Histological type. Based on Lauren's classification, 3 out of 24 (60%) diffuse-type cases were EBV associated, and 2 out of 46 (40%) intestinal-type were EBV positive. Most cases of EBVaGC belonged to grade I.

Sequencing result. The sequence of PCR products that were amplified by EBNA 1 primers was compared to the EBNA 1 reference gene sequence registered in GenBank. PCR amplicon sequences of the positive samples were identical to the EBNA 1 reference gene with accession number NC_007605.1 (Figs. 4 and 5).

For phylogenetic analysis, the evolutionary history was inferred using the Maximum Likelihood method based on the Tamura-Nei model. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analysis was conducted in MEGA7. The sequences obtained in this project had a 100% similarity to strain MK164621.1 from Canada (Fig. 4).

DISCUSSION

Epstein-Barr virus is the cause of infectious mononucleosis. Many documents show that EBV is associated with a variety of malignant neoplasms. Although the global prevalence of EBV has been consistently reported in 95% of adults, the proportion of tumors associated with this virus has been reported differently in different regions (16). The present work assessed the prevalence of Epstein-Barr virus in tissue samples of patients with gastric carcinoma in Ahvaz, Iran.

To date, several studies have attempted to discover the role of EBV infection in the progression of gastric cancer. EBV enters B lymphocytes in oropharyngeal lymphoid tissues. The virus then enters the gastric epithelial cells, either by the cell-to-cell contact between B lymphocytes and gastric epithelial cells or through direct entry into the gastric epithelial cells (2).

According to the results of the study, the frequency of EBV in patients with gastric cancer in Ahwaz is 7% but not in the benign ulcer cases. The diagnosis of EBV-associated gastric cancer was confirmed by the presence of the EBNA1 gene within the gastric cancer cells and its absence in the control group cases.

The association of EBV and gastric cancer was first reported in 1990. Now, various studies from different countries show that EBV is present in about 10% of gastric cancer patients (17). EBV-associated GC differs in various geographical regions, e.g, 6.4% in China, 8.1% in Mexico, 19.5% in German, 5.6% in Korea, 12% in the United States, 8.5% in France, 13% in Colombia, and 11.3% in Brazil (16). The results of this study were compared with previous studies in Iran. The frequency of EBV in patients with

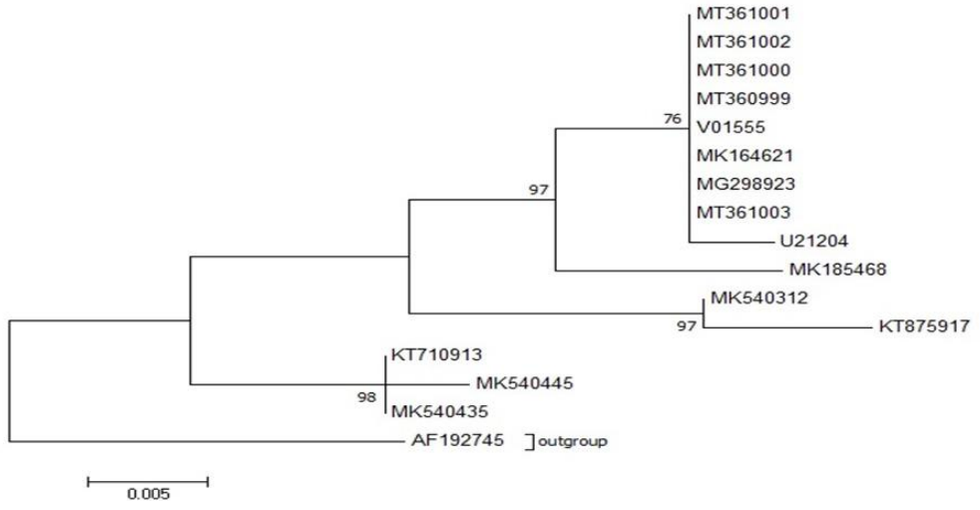

Fig. 4. Phylogenetic analysis of EBV strains and reference sequences of EBV. The phylogenetic tree was constructed with maximum-likelihood and the Tamura-Nei model using MEGA 7 software. Bootstrap values > 70% are displayed at the branch nodes.

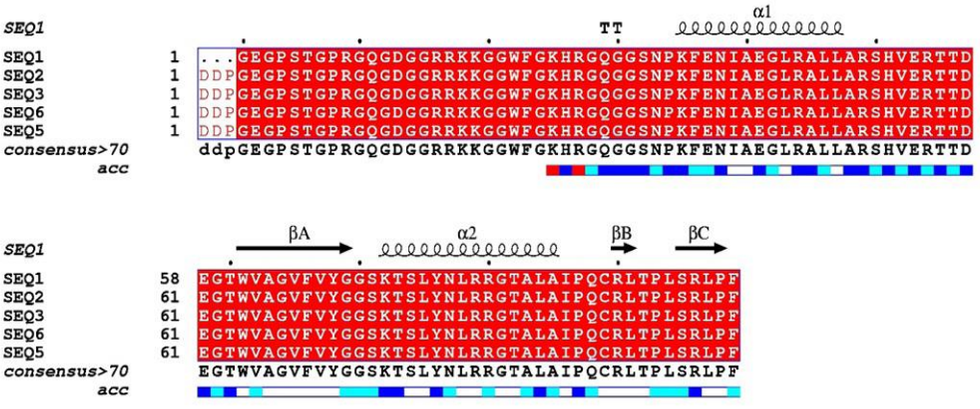

Fig. 5. Nucleotide alignment of the EBNA 1 gene. EBV positive sequences alignment of the EBNA1 region compared with of EBNA 1 reference gene (NC_007605.1)

gastric cancer in Kerman and Tehran was reported as 11% and 7%, respectively (18, 19).

In 2018, Amoueian reported a very high prevalence (62%) of EBV associated GC in the Northeast of Iran compared with other areas of the world and showed an important correlation between EBV infection and the incidence of gastric cancer. EBV in GCs has been examined by some researchers, and they suggest that there are cultural and ethnic differences in tumor pathogenesis and EBV (20).

The incidences of Epstein-Barr virus (EBV)-associated gastric cancer exhibit variability across countries, displaying diverse prevalence rates in developed and developing regions. Such disparities in the prevalence of EBV-associated gastric cancer may be caused by factors including epidemiological patterns, clinicopathological characteristics, dietary practices, and genetic predispositions. It is pertinent to highlight that the limited occurrence of EBV in gastric carcinoma within our investigation might elucidate the lack of statistical significance in the findings (P-value = 0.318).

EBNA-1, LMP1, LMP2, EBERs, and, miRNAs are reported in the majority of tumor cells and EBNA2, 3A, 3B, 3C, LP, but they are not reported in most tumor cells of GC. EBNA-1 targets a single copy of a highly conserved gene that is important for the long-term maintenance of the virus in dividing cells. The possible contribution of EBV to GC pathogenesis is largely unknown. It has been suggested that EBV

may be associated with the majority of the cases, including those diagnosed as EBV negative (EBNA1) or EBER negative by a hit-and-run mechanism. Early during oncogenesis, viral genes are necessary for the initiation of the disease. Gradually, the viral genome is lost to avoid the immune response and host mutations accumulate in the proto-oncogenic cell (15). Based on this hypothesis, the absence of the EBER gene in our study can be justified. Similarly, Lee and co-workers in a study from Korea showed that EBV was indicated in 3 samples (3.30%) by EBER-ISH, 26 samples (28.57%) by Nested PCR, and 3 samples (3.30%) by EBER PCR(15). Furthermore, Lee and colleagues found that EBV was detected in 4 of 40 cases (10%) of gastric carcinoma, whereas LMP1 was negative (21).

While sex differences tend to favor men in most studies, some studies did not find a significant correlation between sex and EBVaGC (22). In this study, EBV-associated gastric cancer was more frequently seen in men, but statistical analysis of the results showed no significant relationship between sex and EBV gastric cancer (P-value>0.99). This finding can be explained by different genetic backgrounds, lifestyles, or hormonal conditions between the two genders (2) (Table 2).

The mean age for patients with EBV-positive gastric carcinomas was 65.8 years. However, in the present study, the highest incidence of EBV-positive gastric carcinomas was in the age group of 70-79 years (4.2%) but there was no significant statistical association between age and EBV-positive gastric carcinomas (P-value =0.282) (Table 2). These findings were in accordance with results reported by other studies from Portugal (2), Russia (23), Mexico, and Several cities in Iran (19, 24). However, several studies have shown a tendency for involvement at a lower age (Kazakhstan and Colombia) (25, 26) or at a higher age (Mexico and Malaysian) (2, 27).

Conventional classification systems like the World Health Organization (WHO) and the Lauren classification have limited utility in clinical management of gastric cancer because of its molecular diversity. Molecular-based classifications are gaining significance. Recently, molecular techniques such as next-generation sequencing (NGS), which encompass DNA and RNA sequencing, whole-exome sequencing, analysis of copy number variations, and DNA methylation arrays, have been employed to categorize gastric cancer into molecular subtypes. These methods offer more comprehensive insights about the tumor compared to traditional histopathological characteristic. A molecular classification categorized gastric cancers into four subtypes according to: Epstein–Barr virus positive status, microsatellite instability, chromosomal instability and genomic stability (28).

Laurén divided the histology of gastric cancer into two groups, i.e., the intestinal-type and the diffuse-type; later, the indeterminate type was included to describe uncommon histology. Most studies showed the intestinal type to be the most common, followed by the diffuse and then the indeterminate type (29). Several constant clinical-pathological features were seen in EBV-associated gastric cancer, such as moderately to poorly differentiated types of gastric cancer and a predisposition to the upper stomach (16). As shown in Table 3, in the present study all patients had adenocarcinoma (65% intestinal and 35% diffuse type). The prevalence of EBVaGC in the diffuse-type gastric carcinoma was higher in our study, a fact which was seen in most studies of Latin America (28, 30) and some countries in Asia such as Korea (21) and Japan (31). Abdirad and co-workers in a study from Iran showed that the proportion of EBV-GC cases in the diffuse-type was higher than in the intestinal-type ($P > 0.05$) (24).

Some studies have demonstrated a better prognosis for EBVaGC than for Gastric carcinoma with EBV negative. Studies have reported that there are also many prognostic factors in GC that affect survival, such as grade, lymphovascular invasion, resection type, and performance status (32). In this research, most cases of EBV were observed in grade 1 cancers. The present study indicated that the prevalence of EBV-associated gastric carcinoma in Khouzestan is low. Differences in the occurrence of EBV-associated gastric carcinoma in different countries can represent epidemiological factors and ethnic differences. Even the EBV genotype should be considered as an important factor for the variation of EBV-associated gastric carcinoma prevalence in different countries. Zanella and collaborators, though combined analysis of EBV genetic structural recombination with that of EBV phylogenetic mutations, and proposed 12 distinct EBV phylopopulations (EBV-p) based on geographic location and tumor type (29). Considering the significant mortality rate of GC, EBV should be more considered in this group in different geographic areas to receive appropriate specific treatment for EBV.

The present study was the first study that showed that the prevalence of EBVaGC in the Southwest of Iran. We used the best materials and methods available to conduct this study. We used PCR for two different genes to investigate more closely and understand possible pathways of virus carcinogenesis We used the Nested PCR instead of regular PCR to be able to detect small copies of DNA.

In situ hybridization is the gold standard for EBV detection in tissue (30), but we used PCR to detect EBV because low copies of EBV DNA are hard to detect with *in situ* hybridization, but can be detected by PCR (21). However, PCR-based methods are more sensitive but less specific than the gold standard ISH method to detect EBV (2). Many studies have reported the higher prevalence of EBV among gastric cancer patients by PCR assay than the EBER-ISH technique. However, PCR is unable to discriminate between cancer cells and lymphocytes infiltrating in tumor stromal, and thus it is impossible to know from where the EBV genome is amplified. It should be noted that the vast majority of people (nearly 95%) are EBV carriers, and their lymphocytes probably contain EBV genomes (2). Therefore, it is better to use both methods to get the most accurate answer. The lack of ISH method was one of the limitations of the present study.

CONCLUSION

Our study showed a prevalence rate of 7% and 0% EBV among gastric cancer patients and control group respectively. Considering this finding, we concluded that EBV could be associated with gastric cancer, and is not accidentally found in gastric cancer. Further studies with larger samples are needed to obtain a better estimate of EBV and to reveal the significance and repercussions of EBV in patients with gastric carcinoma.

ACKNOWLEDGEMENTS

This paper is presents the thesis from MSc by Mehdi Gharibzadeh with registration number OG-9801 and was financially supported by Infectious and Tropical Diseases Research Center, Health Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.

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