

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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Abstract

Background

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

Materials and Methods

Seventy formalin-fixed paraffin-embedded (FFPE) cases of gastric cancer and 30 FFPE cases of gastric ulcer were studied from 2011–2018 in the case-control study. The specimens were investigated for the presence of the EBV genome by Nested-PCR for EBNA1. The positive samples for the EBNA1 region were again tested for the EBER gene by the PCR method. Positive samples were sequenced and aligned to construct the EBV phylogenetic tree.

Results

Five out of 70 cases (7%) indicated positive results for EBV by EBNA1, while all EBNA1 positive samples were negative for EBER. Additionally, EBV was detected in none of the gastric ulcer patients. The mean age for patients with EBV-positive gastric carcinomas was 64.5 years. Three (60%) male patients and 2 (40%) female cases were positive for EBV. Three 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 were seen in grade I.

Conclusion

The present study shows that the frequency of EBVaGC in Iran is low. Differences in EBVaGC incidence in different countries may represent epidemiologic factors and dietary habits. Further analysis of clinical pathology characteristics of EBVaGC using a greater number of cases will offer useful insights into its etiology.

Introduction

Gastric cancer(GC) is the third most common cause of cancer-related mortality accounted for 8.2% of all cancer deaths and one of the most common tumors of the gastrointestinal tract worldwide(1, 2). There are the highest rates in Eastern Asia, followed by Central and Eastern Europe, and the lowest in North America and Western Africa (3). Gastric cancer is a combination of multifactorial disease. The common risk factors of GC include infectious agents such as Helicobacter pylori (HP), smoking, high salty-diets, 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 doublestranded DNA virus, also known as human herpes virus 4.It is a gamma-herpes as a member of the Herpesviridae family with oncogenic activity and 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, and nasopharyngeal carcinoma 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 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 life-long in multiple circular episomes inside the infected cell nucleus (8). EBV can exhibit one of 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. (9)EBNA-1 is crucial for the maintenance of the episomal viral genome in infected cells during cell division (9). 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 did not include in their meta-analysis. Advanced stage, cardiac tumor localization, older age, and less differentiated histology are adverse prognostic indicators in the 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 Iranian patients with GC for finding the relationship between those 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 case-control study of 100 participants with gastric cancer and gastric ulcer aged between 19–89 years, referred to Imam Khomeini Hospital, Ahvaz-Iran from 2011–2018. This research was designed in 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

diagnostic accuracy of gastric cancer and ulcer were confirmed after endoscopy and biopsy, and after gastrectomy by a pathologist.

We examined 70 paraffin-embedded tissue blocks from gastric cancer patients as a test group and 30 paraffin-embedded tissues block from gastric ulcer patients as a control group. The inclusion criteria for the case group: all of the gastric carcinoma untreated patients with age > 18 years. The exclusion criteria: The patients with severe gastritis or atrophic and treated patient. The inclusion and exclusion criteria for the control group study were a gastric ulcer and low/moderate/high grade of metaplasia or dysplasia 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 14000 rpm. This stage was repeated. The supernatant was discarded and 1 ml absolute ethanol was added to the precipitate and stored at room temperature for 10 min and centrifuged again at 14000 rpm for 1 minute. The supernatant was discarded. This process was repeated by adding 70% ethanol, followed the same condition and again this process was repeated by adding 50% ethanol, followed 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

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 were measured by a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) (11, 12). The extracted DNA was stored at -20°C until PCR amplification.

Detection of EBV by Nested PCR for EBNA 1 region

Nested-PCR test was performed in two steps of primary PCR and Nested for detection of EBNA 1 (Epstein-Barr virus nuclear antigen-1). The primers for primary PCR was a 609 bp region of EBNA-1 and primers for Nested PCR was a 308 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 PCR master mix (2x), 1 µl (10 nM) of each primer sequence, and 1 µl distilled water. Cycling conditions are as follows: denaturation at 95 °C for 10 min, followed by amplification cycle at 95 °C for 30 sec, 53 °C 30 sec, 72 °C 30 sec for 30 cycles, and a final extension at 72 °C 10 min. The second round was carried out with 5 µl of the first-round product, 1 µl (10 nM) of each primer sequence, 12 µl PCR master mix (2x), and 6 µl distilled water. Cycling conditions are as follows: denaturation at 95 °C for 10 min, followed by amplification cycle at 95 °C for 30 sec, 62 °C 30 sec, 72 °C 30 sec for 30 cycles, and a final extension at 72 °C 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 308 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. The PCR was performed in a 25 µl mixture, containing 5 µl of extracted DNA, 12 µl PCR master mix (2x), 1 µl (10 nM) of each primer sequence, and 6 µl distilled water. Cycling conditions are as follows: denaturation at 95 °C for 10 min, followed by amplification cycle at 95 °C for 30 sec, 48 °C 30 sec, 72 °C 30 sec for 30 cycles and, a final extension at 72 °C 10 min. The expected PCR product for this test was 150 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 two steps of PCR are reported in Table 1.

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 deposit in the GenBank database under the accession numbers: MT360999, MT361000, MT361000, MT361002, and MT361003. For phylogenetic analysis, MEGA 7 software was utilized. The Maximum likelihood method with Tamura Nei-model was used for the construction phylogenetic tree. 1000 bootstrap replicates performed the confidence level of evolutionary distances.

Statistical analysis

SPSS software version 26 (SPSS Inc, Chicago, IL, USA) was used for data analysis. Analyses of data were performed using the K_2 test. Furthermore, Odds Ratio and Confidence Intervals were used for

comparing differences between groups. The P-value smaller than 0.05 was considered statistically significant.

Results

Patients characteristics and clinical pathology

The research was aimed to evaluate the frequency of Epstein-Barr virus in tissue samples of patients suffering gastric cancer in Ahvaz, Iran that enrolled 70 patients with gastric cancer as a test group and 30 patients who suffered from gastric ulcer as a control group. The patient group included 26 women (34%) and 44 men (66%) with a mean age of 64.5 ± 17 years old and the control group included 15 (50%) women and 15 (50%) men with a mean age 58.1 ± 19.4 of years old. Minimum and maximum age for male were 19 and 89 years old and for female were 20 and 82 years old.

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 different grades of Gastric Cancer Samples are shown in Figs. 1 and 2 respectively.

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 1 shows the distribution of EBV-Positive and EBV negative according to age, sex in Gastric Cancer. The results of the Frequency type and grade of carcinoma and PCR result are presented in Tables 2 and 3 respectively.

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 include 2 (40%) female and 3 (60%) male. (Table 1)

Table 1
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)	

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 were seen in grade I.

Table 2
The Frequency type of carcinoma and PCR result

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

Table 3
The Frequency grades of carcinoma and PCR result

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

Sequencing result

The sequence of PCR products that were amplified by EBNA 1 primers was compared to the EBNA 1 reference gene sequence registered to GenBank. PCR amplicon sequences of the positive samples were identical to the EBNA 1 reference gene and molecular Phylogenetic analysis was done for these sequences (Fig. 3, 4).

For phylogenetic analysis, the evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood (-555.19) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial

tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 16 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 284 positions in the final dataset. Evolutionary analyses were conducted in MEGA7. The sequences obtained in this project had a 100% similarity to strain MK164621.1 from Canada (Fig. 3).

Table 4
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	□
3	F	78	Intestinal	□
4	F	41	Diffuse	□
5	M	78	Diffuse	□

Discussion

Epstein-Barr virus is the cause of infectious mononucleosis. Many documents show EBV is associated with a variety of malignant neoplasms including nasopharyngeal carcinoma (NPC), Burkitt lymphoma, Hodgkin lymphoma, nasal T/NK cell lymphoma, and B cell-lymphoma in the immunosuppressed and gastric cancer patient. 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) .

Gastric cancer is the third most common cause of cancer-related mortality and one of the most common tumors of the gastrointestinal tract worldwide(1, 2). 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 Ahvaz is 7% but not in the benign ulcer cases. The diagnosis of EBV-associated gastric cancer is 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 EBV is related to 10% of gastric cancer patients. 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 compared with previous studies in Iran. The frequency of EBV in patients with gastric cancer in Tehran, Sari, and Kerman was reported 7%, 3%, 11%, respectively. (16–18)

Since the prevalence of EBV-associated gastric cancer is lower in areas with a high incidence of gastric cancer and in areas with a low prevalence of gastric cancer is more and because Ahvaz is the region with a high incidence of Gastric cancer. (19, 20) In contrast, 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 incidence of Gastric cancer. Some investigators checked EBV in GCs and suggested that there are ethnic differences in tumor virology and pathogenesis. (21)

EBV-associated gastric cancer varies in different countries, 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 (16). Therefore, these variations in EBV-associated occurrence of GC in different areas may represent epidemiological and clinicalopathological factors; dietary habits and genetic.; dietary habits and genetic. (21) We should consider that the low number of positive cases in our study might be the reason for the insignificant result in the analysis (P-value = 0.318).

EBV encoded genes in EBV associated gastric carcinoma as follows: EBNA-1, LMP1, LMP2, EBERs and, miRNAs reported in the majority of tumor cells and EBNA2, 3A, 3B, 3C, LP and not reported in most tumor cells of GC. EBNA-1 targets a single copy highly conserved gene and 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 mechanism of *hit-and-run*. Early during oncogenesis, viral genes are necessary for the initiation of 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 negation of the EBER gene in our study can be justified. In the same way, Lee and co-workers in the study from Korea showed that the presence of 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) In this way, Lee and colleagues found that EBV was detected in 4 of 40 cases (10%) of gastric carcinoma whereas LMP1, was negative. (22)

In most studies, sex differences favor men, but not were some studies have shown a significant relationship between sex and EBVaGC. (23) In this study, 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 EBVaGC-. (P-value > 0.99) (Table 1).

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 is no significant statistical was seen between age and EBV-positive gastric carcinomas (P-value = 0.282) (Table 1). These findings are in accord with results reported by other studies investigating from Portugal (24), Russian(25), Mexico, and Several cities in Iran. (17, 18, 20) However, several studies were shown a tendency to involvement at lower age (Kazakhstan and Colombia). (26, 27) or higher age (Mexico and Malaysian). (28, 29)

The division of gastric cancer is as follows: 1- Adenocarcinoma 2- Lymphoma 3- Stromal sarcoma 4- Liposarcoma 5- Squamous cell carcinoma. More than 95% of gastric cancers are adenocarcinomas (17, 30). 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 indeterminate type. (31) Several constant clinical-pathological features were seen in EBV associated gastric cancer such as moderately to the poorly differentiated type of gastric cancer and predisposition to upper stomach (16). As shown in Table 2 in the present study all patients had adenocarcinoma (65% intestinal and 35% diffuse type). The prevalence of EBVaGC in diffuse type gastric carcinoma was more in our study, a fact which was seen in most studies of Latin America (28, 32) and some countries in Asia such as Korea (33), China, (34) and India (35). Abdirad and co-workers in a study from Iran showed the proportion of EBV-GC cases in diffuse- type was higher than intestinal- type ($P > 0.05$). (20)

Some studies have demonstrated a better prognosis of EBVaGC than for Gastric carcinoma with EBV negative. Studies reported that there are also many prognostic factors in GC that affect survival, such as grade, lymphovascular invasion, resection type, and performance status. In this research, most cases of EBV have been observed in grade 1 cancers(36).

The present study indicates that the prevalence of EBV-associated gastric carcinoma in Iran is low. Differences in the occurrence of EBV-associated gastric carcinoma in different countries can represent epidemiological factors and dietary trends. Regarding the significant mortality rate of GC, EBV should be more considered in this group in the different geographic areas to receive appropriate specific treatment for EBV.

Conclusion

Our study showed the prevalence rate of 7% EBV gastric cancer patients. 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.

Declarations

Availability of data and materials

The datasets during and/or analysed during the current study available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Azarakhsh Azaran: Analyzed and interpreted the patient data and writing the manuscript.

Mehdi gharibzadeh: Data collection and performed the virological examination.

Shahram Jalilian: Interpreted the patient data.

Seyed Saeid Seyedian: Contributor in writing the manuscript.

Manoochehr Makvandi: Revised the manuscript.

All authors read and approved the final manuscript.

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Figures

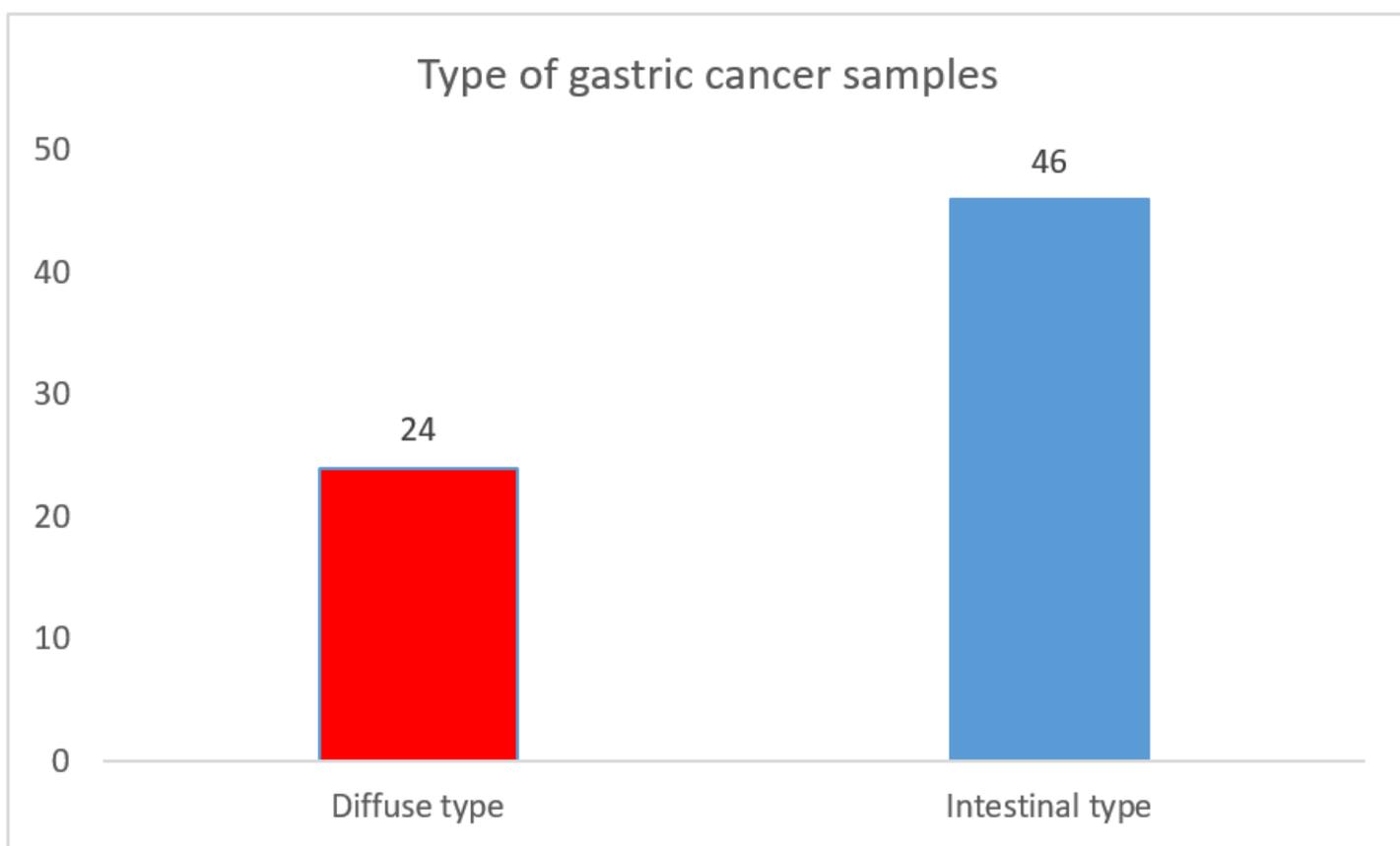


Figure 1

Frequency Types of Gastric Cancer Samples

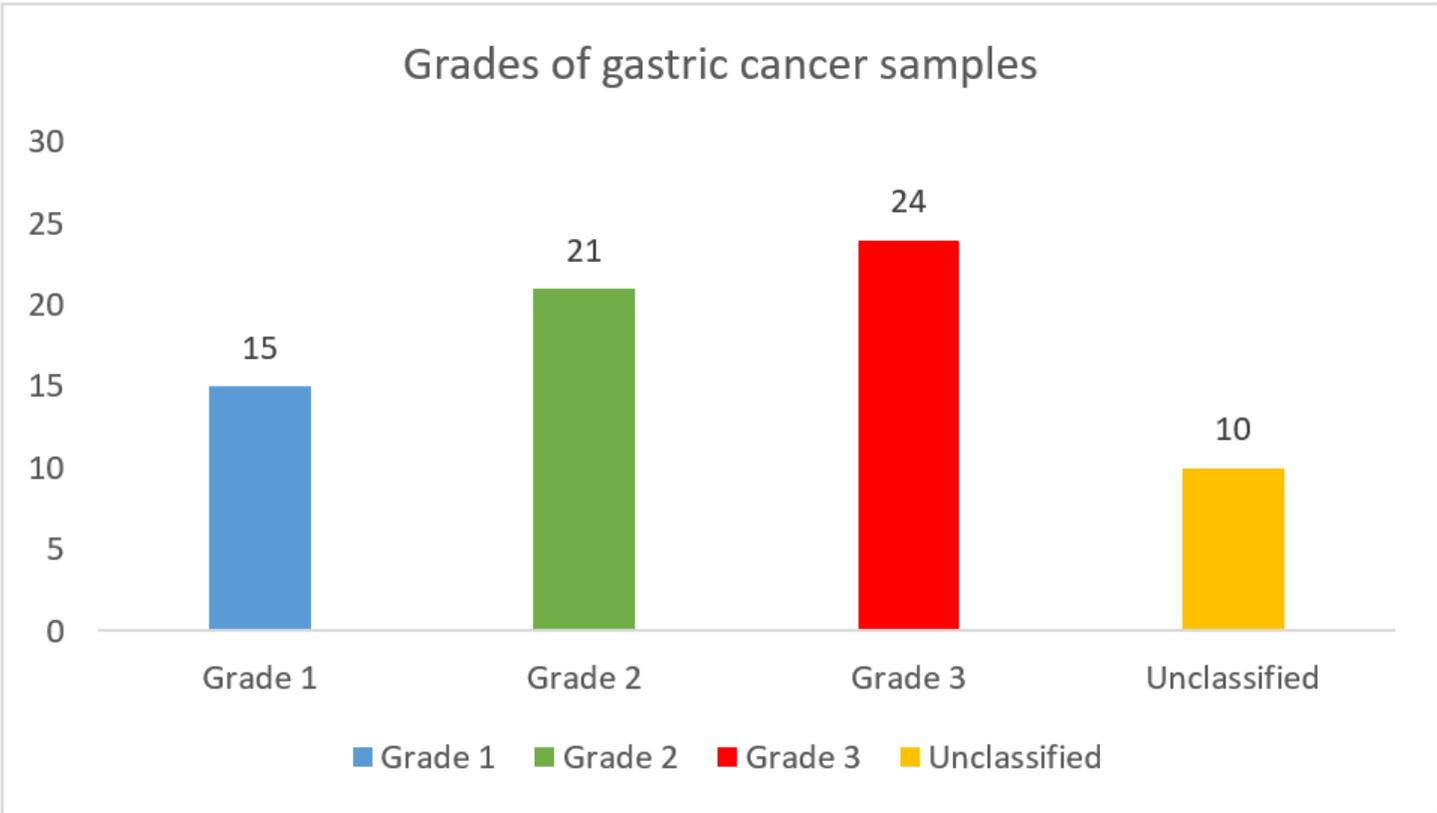


Figure 2

Frequency grades of Gastric Cancer Samples

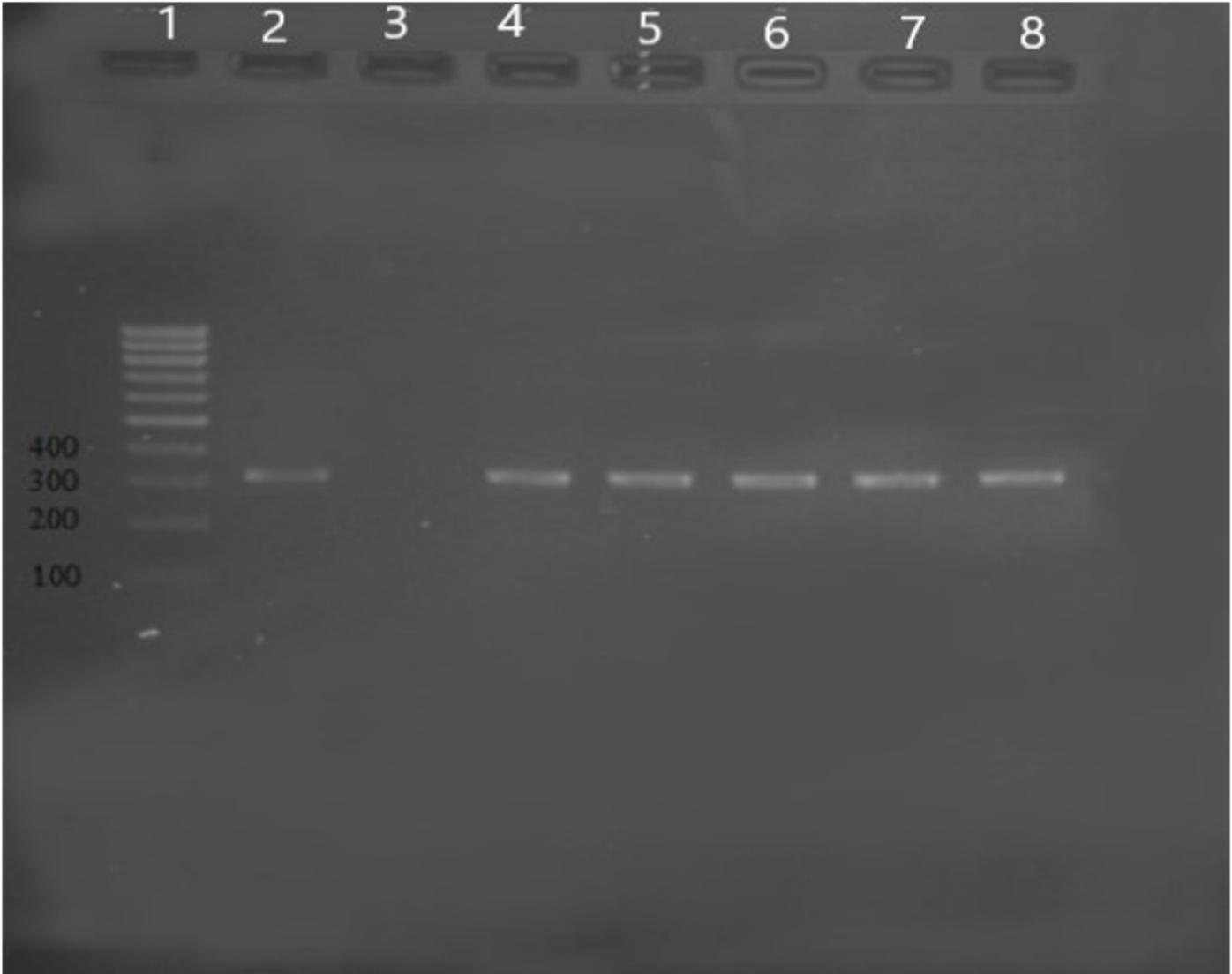


Figure 3

Analysis of EBV using the EBNA1 gene. Lane 1 100 bp size marker, Lane 2 positive control (B95.8 cell line), Lane 3 negative control, Lane 4-8: EBV positive patients

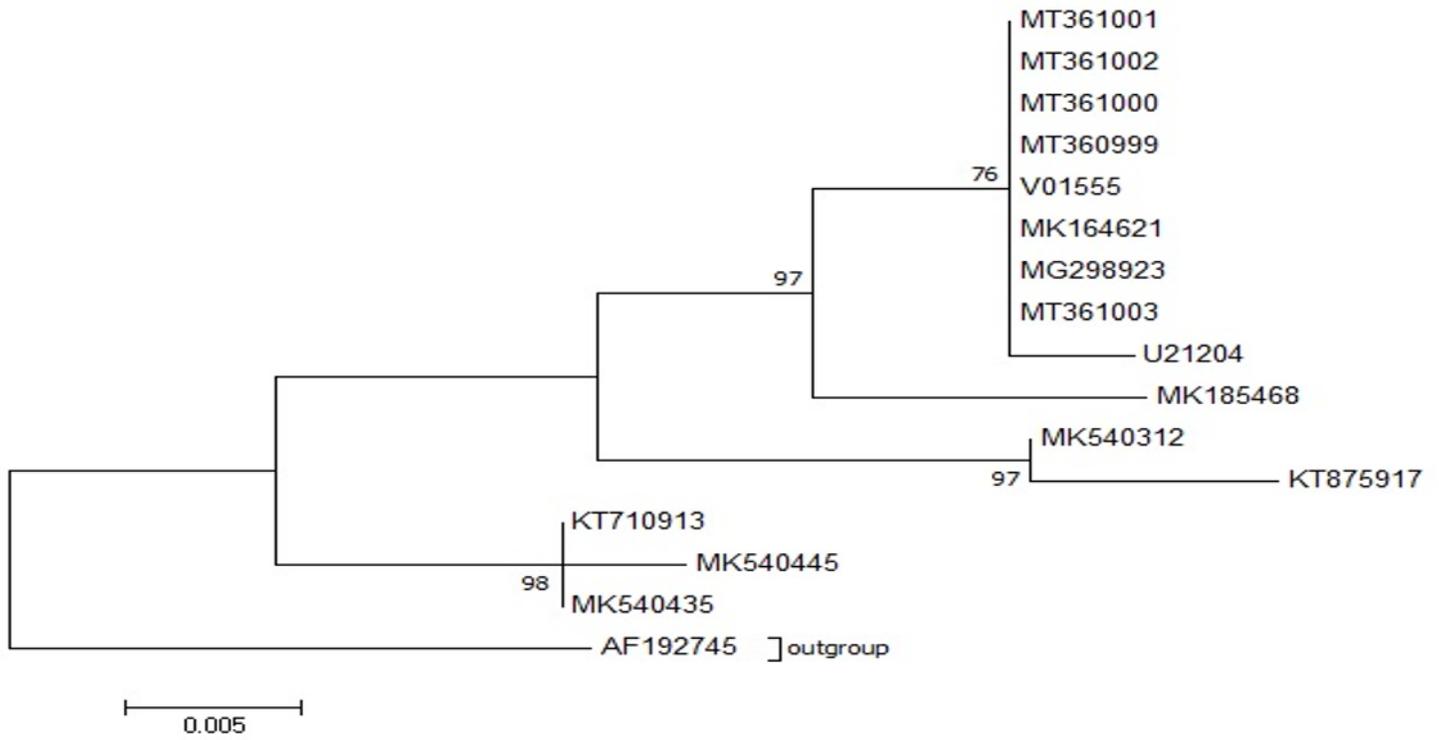


Figure 4

Molecular Phylogenetic analysis by Maximum Likelihood method

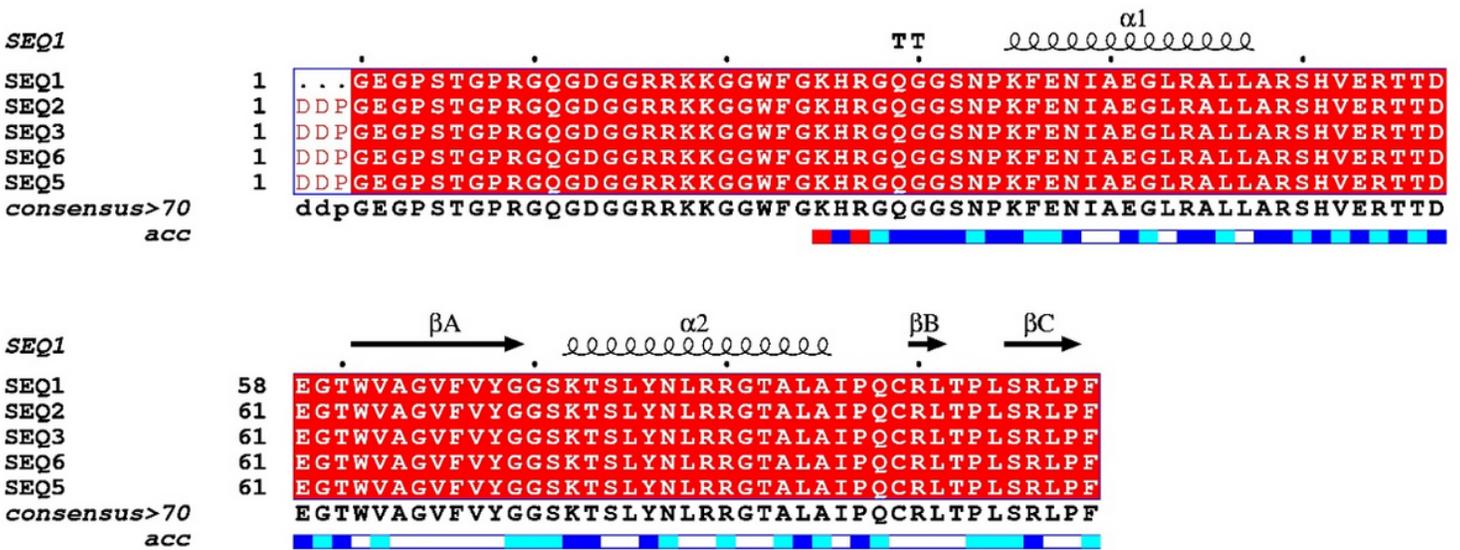


Figure 5

Comparison of the DNA sequence between EBNA 1 reference gene sequence and PCR amplicon of EBV positive samples in gastric cancer patients. DNA sequences of each sample and EBNA 1 reference gene sequence were the same.