Le Infezioni in Medicina, n. 2, 263-271, 2022
doi: 10.53854/liim-3002-12

Higher viral load of Epstein-Barr virus in gastric cancer compared with non-cancerous gastroduodenal tissues

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Article received 14 March 2022, accepted 26 April 2022

SUMMARY

Introduction: Epstein-Barr Virus (EBV)-associated gastric cancer is a distinct molecular subtype of gastrointestinal carcinomas as defined by the Cancer Genome Atlas. Methods: In the present study 237 samples from Iranian patients diagnosed with gastric cancer and gastroduodenal disease were retrospectively examined for EBV infection by quantitative Real-Time PCR. Results: Of the 237 samples tested, EBV DNA was detected in 37 samples (15.6%), in 13 of the 81 gastric cancer cases (16%), and 24 of the 156 non-cancerous samples (15.4%). The EBV infection rate was found higher in patients with gastric ulcer (35%) and duodenal ulcer (21.9%) compared to patients with gastric cancer (16%) and gastritis (19.6%). The EBV-encoded small RNA (EBER) copy number in the gastric cancer group (mean = 2.14×10^{-1} with range of 2.14×10^{-2} to 4.10×10^{-1} copies/cell) was higher than gastroduodenal diseases group (mean = 1.39×10^{-2} with range 1.11×10^{-3} to 2.35×10^{-2} copies/cell), and this difference was statistically significant (P >0.001) Conclusion: The higher number of copies of EBV- EBER in the gastric cancer group compared to the non-cancer group confirmed the possible role of EBV in inducing cancer.

Keywords: Epstein-Barr virus, gastric cancer, gastroduodenal disease, *Helicobacter pylori*, cancer.

INTRODUCTION

Gastric cancer (GC) is the world's fifth most prevalent cancer and the third major cause of cancer mortality [1]. The etiology of GC involves interactions of many risk factors, including environmental, genetic, lifestyle, and infectious factors [2]. *Helicobacter pylori* (Hp) infection has been identified as the most important risk factor in the infectious etiology of GC and other gastroduodenal disorders [3, 4]. Infection with Hp causes an inflammatory response that results in non-neoplastic lesions such as gastritis (GA), gastric ulcer (GU), and duodenal ulcer (DU). In a small group of subjects, atrophy and loss of gastric glands developed, followed by the aforementioned inflammatory lesions, which may eventually develop into neoplastic transformation [5].

Epstein-Barr virus (EBV), a common oncogenic virus, is found in roughly 10% of gastric carcinomas [6]. The mechanism by which EBV may play a part in gastric carcinogenesis is still unknown. However, there is some evidence to support its

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contributing role including the presence of monoclonal EBV episomal DNA in all gastric malignant cells and uniform presence of EBV-encoded small RNA (EBER) in all tumor cells. Epstein-Barr virus persistently infects about 95% of the world population, usually residing in memory B cells in a latent state [7]. Intermittent episodes of viral reactivation are thought to occur in infected memory B cells that circulate in the gastrointestinal tract and produce viral particles that may promote infection of gastric epithelial cells [8, 9]. Although viral reactivation triggers a local inflammatory response similar to the crucial role of Hp infection in the carcinogenic process, the relative role of EBV in early inflammatory gastroduodenal lesions remains unclear.

Quantitation of viral genome copy numbers in blood and other body fluids is an important clinical indicator of viral disease. Viral titer in plasma and peripheral blood has been confirmed as a valuable prognostic criterion for EBV-associated tumors, such as nasopharyngeal carcinoma and hematologic malignancies [10-12]. However, viral load in EBV-associated tumor tissue has not been extensively studied. The study by Ryan et al. found that gastric tissue in which EBV infects epithelial cells has a nearly 3000-fold higher viral load than tissue with only infected B cells [13]. According to the aforementioned study, a cutoff value of 2000 EBV genome copies per 10^5 cells is a valuable threshold to distinguish EBV infection of gastric epithelial cells from gastric tissues with only infected B cells [13]. A recent study provided data supporting a role for EBV in GC and early precursor lesions including non-atrophic GA [9]. However, the exact EBV copy number per cell count has not yet been studied in other gastroduodenal lesions at risk for malignant transformation, including GA, GU, and DU.

Altogether the facts reviewed above encouraged us to investigate whether EBV could have any association with the pathogenesis of gastric cancer and other gastroduodenal diseases either alone or accompanied by Hp. In this study, 237 fresh or formalin-fixed paraffin-embedded (FFPE) samples of Iranian patients diagnosed with gastric cancer and gastroduodenal diseases (GA, GU, DU) as well as gastric congested mucosa (CM) from healthy subjects were tested for the presence of EBV sequences in terms of viral genome copy number per cell.

PATIENTS AND METHODS

Clinical samples

The current retrospective cross-sectional study included 237 gastroduodenal tissue samples from the Ayatollah Rouhani Hospital's Pathology Department, which is associated with Babol University of Medical Sciences. A total of 129 gastroduodenal fresh biopsy specimens, including 52 GA, 20 GU, 31 DU, and 26 GC were obtained by gastroenterologists from individuals who had esophagogastroduodenoscopy (EGD), and 108 FFPE resection specimens including 55 GC and 53 CM were collected from healthy subjects. All registered patients (Fresh biopsy specimens and FFPE samples) were diagnosed between February 2014 and August 2016 and CM and GC samples were collected simultaneously. All patients were living in the Mazandaran province, north of Iran. Study participants had not taken any antimicrobials for two weeks prior to doing endoscopy. Patients with the history of immunosuppressive therapy or chemotherapy were excluded from the study

Demographic characteristics of patients were obtained from the clinical records of the patients in the hospital. Hp infection in all biopsy specimens was determined by a rapid urease test (RUT) as part of a regular procedure in two of the above hospitals. The ethics committee of Babol University of Medical Sciences approved this study (Ethics code: IR.MUBABOL.REC.1396.1), and all subjects gave written informed consent. Also, all methods were performed in accordance with the relevant guidelines and regulations.

DNA extraction

Formalin-fixed paraffin-embedded resection specimens were deparaffinized according to a previously described procedure [14, 15]. The tissue genomic DNA Extraction Mini Kit (Favorgen Biotech Corp., Taiwan) was used to extract DNA from 25 mg (mean weight of starting material as a source of intact DNA) of fresh and FFPE samples according to the manufacturer's instructions. For tissue dissociation, 200 µL of FATG1 tissue lysis buffer and 20 µL of proteinase K (10 mg/mL) were added to each tissue containing a microcentrifuge tube. Samples were subsequently incubated at 60 °C until the tissue was lysed completely. DNA cleanup was done by mini spin column (silica matrix) according to the manufacturer's instructions.

As an extraction negative control, tissue samples were treated concurrently with sterile microcentrifuge tubes containing only reaction mixtures. A NanoDrop spectrophotometer was used to evaluate the quality and amount of isolated DNA (Thermo Scientific, Wilmington, USA). Fresh tissue samples had an isolated DNA yield of 100-400 ng/L, whereas FFPE tissue samples had an isolated DNA yield of 30-70 ng/L. Eluted DNA from fresh and FFPE tissue samples had an A260/A280 ratio of 1.7, indicating that the genomic DNA was of excellent quality.

EBV quantitative real-time polymerase chain reaction

To detect and measure the amount of EBV viral load, using a Rotor-Gene Q Real-Time PCR system (QIAGEN GmbH, Hilden, Germany), quantitative Real-Time PCR was carried out through the primer sets and a TaqMan probe specific for the EBV EBER gene according to a previously described procedure [16]. As previously stated, viral copies were normalized to the amount of cell equivalents by measuring a single copy cellular RNase P gene [17]. Negative results for human RNase P gene amplification were regarded as having insufficient DNA integrity, and samples were re-extracted until RNase P amplification was obtained. Gene synthesis service constructed plasmids with cloned EBV EBER and human RNase P gene target sequences (quantitative standards for Real-Time PCR, Shanghai Generay Biotech Co., Ltd). A total of 100 ng of purified DNA was used in each Real-Time PCR reaction. A standard curve was created utilizing a tenfold dilution series of the EBV EBER plasmid in genomic extracts from EBV negative fresh and FFPE samples to test the sensitivity of quantitative Real-Time PCR. As a negative control, reaction mixtures without a DNA template were used, and DNA extracted from the supernatant of an EBV-producing B-cell line (B95-8) was used as a positive control.

Statistical analysis

The data were analyzed using R 3.4.1, "arm", and "ggplot2" packages. The Shapiro-Wilk test was used to determine if the variables had a normal distribution. Descriptive results were presented as mean ± standard deviation for normal quantitative variables and number (percent) for count data. Median and interquartile range (IQR) was used for the nonparametric variable which did not have a normal distribution. The independent sample t-test or Kruskal-Wallis test was used for comparing the mean or distribution of quantitative variables as appropriate. The Chi-square test was used for assessing the association of qualitative variables. Statistical significance was described as a P-value of less than 0.05.

RESULTS

Patient's characteristics

In this investigation, out of the 237 enrolled subjects (mean age 53.3±16.7 years, range 15-90 years), 81 (34.2%) had GC, 51 (21.5%) had GA, 20 (8.4%) had GU, and 32 (13.5%) had DU; 53 samples (22.4%) were CM of healthy subjects. Out of 237 patients, 130 (54.9%) cases were male and 107 (45.1%) were female. All cancerous samples were primary adenocarcinoma. Cancerous samples were divided into two groups: 69 (85.2%) with gastric adenocarcinoma, intestinal type, and 11 (13.6%) with gastric adenocarcinoma, diffuse type. The type of gastric adenocarcinoma was unknown in one of the samples. The demographic, clinical and pathological characteristics of 237 patients included in this investigation are summarized in Table 1. According to the results, there was a significant relationship between age, gender and pathological diagnosis (P>0.001 and P=0.013, respectively). The majority of GC specimens belonged to men (57/81, 70.4%). Also, the majority of patients with GC (75.3%) and GU (65%) were older than 55 years, while those with GA, DU, and CM, were less than 55 years.

Hp infection was detected in 90.3% of DU, 39.5% of GC, 35.0% of GU, and 22.4% of GA subjects. Hp was not positive in any of the CM specimens from healthy subjects. As shown in Table 1, there was a substantial difference in the prevalence of Hp infection among pathology groups (P >0.001). In contrast, no significant relationship between family history of gastric cancer, smoking, alcohol drinking, or drug use and pathologic diagnosis was observed. In addition, the mean body mass index (BMI) of the participants in the research was 25.5±4.6 (range 16.3-50.1). There was a significant difference between different BMI groups (P = 0.002) and pathology diagnosis and most people with GC were in the range of lean and normal weight (66/7%) (Table 1).

Table 1 - Statistical associations between histopathologic groups and demographic and clinical characteristics of patients.

Variable		*GC*	*GU*	*DU*	*GA*	*CM*	*Total*	*P-value*
Number of patients		81(34.1%)	20(8.4%)	32(13.5%)	51(21.5%)	53(22.4%)	237	–
Age group	≤55	20(24.7%)	7(35%)	25(78.1%)	39(76.5%)	40(76.9%)	131(55.5%)	*P*>0.001
	>55	61(75.3%)	13(65%)	7(21.9%)	12(23.5%)	12(23.1%)	105(44.5%)	
Gender	Male	57(70.4%)	11(55%)	15(46.9%)	24(47.1%)	23(43.4%)	130(54.9%)	*P* =0.013
	Female	24(29.6%)	9(45%)	17(53.1%)	27(52.9%)	30(56.6%)	107(45.1%)	
Smoking status	Smoker	8(10.8%)	2(10%)	3(9.7%)	4(8.3%)	12(24%)	29(13%)	*P* =0.133
	Non-smoker	66(89.2%)	18(90%)	28(90.3%)	44(91.7%)	38(76%)	194(87%)	
Alcohol drinking	Yes	5(6.9%)	3(15%)	2(6.5%)	9(18.8%)	4(8%)	23(10.4%)	*P* =0.215
	No	67(93.1%)	17(85%)	29(93.5%)	39(81.3%)	46(92%)	198(89.6%)	
Family history of GC	Yes	16(21.9%)	5(25%)	4(13.3%)	4(9.8%)	5(10%)	34(15.9%)	*P* =0.213
	No	57(78.1%)	15(75%)	26(86.7%)	37(90.2%)	45(90%)	180(84.1%)	
History of drug use	Yes	8(11%)	2(10%)	3(9.7%)	4(8.3%)	2(4%)	19(8.6%)	*P* =0.741
	No	65(89%)	18(90%)	28(90.3%)	44(91.7%)	48(96%)	203(91.4%)	
BMI	≤24/99	50(66.7%)	4(22.2%)	11(37.9%)	19(43.2%)	24(47.1%)	108(49.8%)	*P* =0.002
	>25	25(33.3%)	14(77.8%)	18(62.1%)	25(56.8%)	27(52.9%)	109(50.2%)	
Hp	Positive	30(39.5%)	7(35%)	28(90.3%)	11(22.4%)	0(0%)	76(33.2%)	*P*>0.001
	Negative	46(60.5%)	13(65%)	3(9.7%)	38(77.6%)	53(100%)	153(66.8%)	
EBV	Positive	13(16%)	7(35%)	7(21.9%)	10(19.6%)	0(0%)	37(15.6%)	*P* =0.002
	Negative	68(84%)	13(65%)	25(78.1%)	41(80.4%)	53(100%)	200(84.4%)	

Notes: GC = Gastric Cancer, GU = Gastric Ulcer, DU = Duodenal Ulcer, GA = Gastritis, CM = Congested Mucosa.

Detection and quantitation of EBV

Of the 237 studied samples, the EBER gene was detected in 37 specimens (15.6%). The EBV infection rate was found higher in patients with GU (35%) and DU (21.9%) than in patients with GC (16%) and GA (19.6%) (Table 1). Epstein-Barr virus EBER gene was not detected in any of the CM samples. The presence of the EBER gene was significantly related to the pathologic diagnosis (P = 0.002). All specimens containing EBV were intestinal-type gastric cancer, and none of the diffuse-type samples were infected with EBV. There was no significant difference between EBER gene frequency in paraffin cancer samples (16.4%) and fresh cancer samples (15.4%) (P=0.911).

To determine the EBV EBER DNA load by Real time PCR method, 37 EBER positive samples were classified into gastric cancer and gastroduodenal disease groups. The EBV EBER DNA load was determined as the viral copy number per cell using a proven single-copy gene, human RNase P. The existence of adequate amplifiable DNA could be indicated by the amplification of this cellular gene. Human RNase P gene was detected in the extracted DNA from all specimens. The EBV EBER DNA copy number in the gastric cancer group (mean $=2.14\times10^{-1}$ with range of 2.14×10^{-2} to 4.10×10^{-1}copies/cell) was higher than gastroduodenal diseases group (mean $=1.39\times10^{-2}$ with range 1.11×10^{-3} to 2.35×10^{-2} copies/cell), and this difference was statistically significant (P>0.001) (Figure 1). Table 2 summarizes the demographic, clinical and virologic parameters of 37 EBER-positive patients with GC and gastroduodenal diseases. In the present study, 13 samples of EBER-positive gastric cancer with DNA viral loads of more than 2,000 copies per 100,000 cells were considered EBV-associated gastric cancers (EBVaGC). As exhibited in Table 2, there is a significant relationship between EBER gene positivity and age, and most people with EBVaGC are over 55 years old (P 0.006). Also, the simultaneous infections of EBV

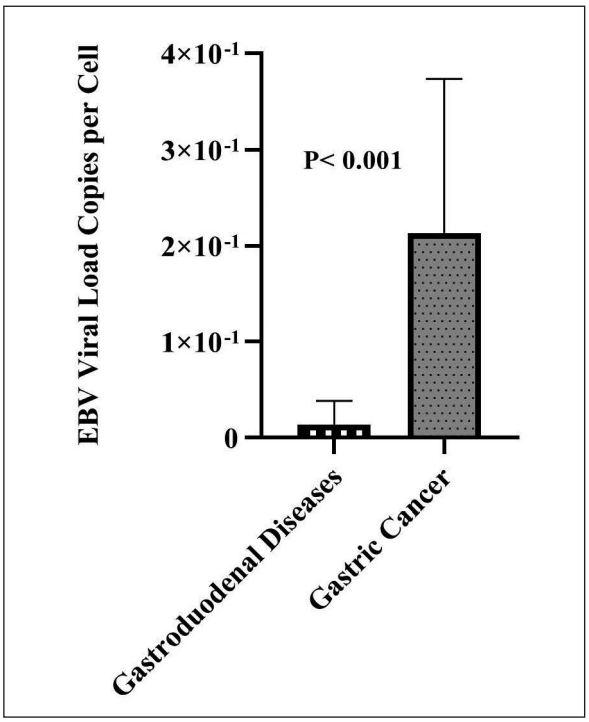

Figure 1 - The mean EBV EBER DNA load in gastric cancer and gastroduodenal diseases groups. The P-value was determined by the Mann-Whitney U test.

and Hp were detected in 17 of 35 EBER positive samples (48.6%), 6 cases (17/1%) in GC group and 11 cases (31/4%) in the gastroduodenal diseases group. The absence of a significant association between dual infection by EBV and Hp indicates the lack of synergistic effect of Hp /EBV coinfection in the induction of gastric cancer (P 0.903).

DISCUSSION

Gastric cancer is recognized as a global health problem with higher than 1,000,000 new cases in 2018 and is the major reason for cancer death in some West Asian countries, including Turkmenistan, Kyrgyzstan, and Iran [1, 18, 19]. The fact that about 50% of gastric tumors are negative for Hp and the presence of the EBV genome in about 10% of gastric cancers worldwide highlights the importance of studying EBVaGC [13, 20-22]. Despite the presence of studies on the interaction of EBV and Hp infection in GC, no definitive results have been obtained [23]. North and Northwest are among the regions of Iran where the risk of gastric cancer is high [24]. The present study aimed to assess the status of EBV infection by Real-Time PCR in 237 gastroduodenal samples, and also to determine the association between EBV and Hp infection with gastric cancer and other gastroduodenal diseases in Babol, Northern Iran. Generally, by observing EBV DNA with more than 2000 copies per 100,000 cells in 13 GC samples (16%), a high incidence of EBVaGC was reported in Babol. Hp as the major cause of gastric cancer, is responsible for more than 60% of GC cases and a different prevalence of this bacteria has been reported in populations studied [22, 23, 25]. There is a higher prevalence of infection by this pathogen in developing countries compared to developed coun-

Table 2 - Demographic, clinical and virologic parameters of 37 EBER positive cases with GC and Gastro duodenal disease.

Variable		*Patients Groups*		*Total*	*P-value*
		Gastric cancer	*Gastro duodenal disease*		
Mean EBV DNA Load copies/cell (range)		2.14×10^{-1} (2.14×10^{-2}-4.10×10^{-1})	1.39×10^{-2} (1.11×10^{-3}-2.35×10^{-2})	8.40×10^{-2} (1.11×10^{-3}--4.10×10^{-1})	*P*>0.001
Age group	≤55	2/37 (5.4%)	15/37 (40.5%)	17/37 (45.9%)	*P* = 0.006
	>55	11/37 (29.7%)	9/37 (24.4%)	20/37 (54.1%)	
Gender	Male	8/37 (21.6%)	12/37 (32.4%)	20/37 (54.1%)	*P* = 0.501
	Female	5/37 (13.5%)	12/37 (32.4%)	17/37 (45.9%)	
Smoking status	Smoker	2/35 (5.7%)	2/35 (5.7%)	4/35 (11.4%)	*P* = 0.594
	Non-Smoker	10/35 (28.6%)	21/35 (60%)	31/35 (88.6%)	
Family history Of GC	Yes	4/31 (12.9%)	3/31 (9.7%)	7/31 (22.6%)	*P* = 0.384
	No	8/31 (25.8%)	16/31 (51.6%)	24/31 (77.4%)	

tries [26]. According to the results, the rate of Hp infection was higher in subjects with DU (90.3%) than that observed in subjects with GC (39.5%), GU (35%), and GA (22.4%). Studies by Moral-Hernández et al, Saxena et al, and Teresa et al had findings similar to those of the current study, *i.e.* a low prevalence of Hp infection was reported in GC specimens, namely, 40.6%, 56.5%, and 50%, respectively [22, 23, 27]. Factors such as Hp concealment within mucosal cells and lack of urease production in the lumen, the bias in obtaining patient information, a history of receiving antimicrobial therapy for Hp, and the use of different diagnostic methods may be reasons for different frequencies of Hp reported in populations [22, 23, 28].

Epstein-Barr virus EBER is one of the transcripts produced in EBV infections. This transcript participates in epithelial cell growth and gastric malignancy by inducing insulin-like growth factor-1 (IGF1). In situ hybridization of EBER (EBER-ISH), defined as the gold standard for EBV status identification in GC samples, and the prevalence of 5-17.9% EBER-positive tumor tissue has been recorded in a systematic review of 34 studies using this method. PCR is another method that has been considered in histological examination of the virus due to its high sensitivity and possible loss of a portion of the EBV infection with the EBER-ISH [13, 29-31]. Using PCR and according to present study results, EBER detection in GU samples was higher than DU, GA, and GC samples (35%, 21.9%, 19.6%, and 16%, respectively) and was significantly associated with pathology diagnosis. It was also higher than the frequency of EBV-positive GC cases reported in three previous studies in Iran in 2007, 2014 and 2016 (3%, 6.66% and 11.1% respectively) [24, 32, 33]. Regarding EBV association with gastric cancer in Iranian population, EBER-ISH technique was only used in one study and other investigators evaluated the presence of EBV by molecular methods [32-34]. In countries with a high risk of GC, like Iran and Japan, a low incidence of EBVaGC has been reported; however, the high frequency EBVaGC (16%) in the current study was inconsistent with the two previous studies in Iran [24]. In addition, our findings were also inconsistent with the evidence of a 2020 study by Aversa et al, on a large population of gastric adenocarcinoma in which a low prevalence of EBVaGC was reported (22 out of 1035 cases) among a high-incidence Chinese population [35]. In studies of different populations to investigate the presence of the EBV in GC and gastrointestinal disease, the detection rate of the EBV genome is very different, and there is conflicting information about the association of this virus with the induction of gastrointestinal malignancies. Factors such as detection methods, population size, geographical and environmental aspects can be the cause of these variations [22-25, 29, 36-38]. For example, in Nogueira et al and Guo et al studies, consistent with many previous investigations, the higher prevalence of EBV among GC subjects was observed by PCR than EBER-ISH, which were 90.2% versus 11%, and 53.7% versus 6.7%, respectively [39].

In many previous studies, EBVaGC was significantly associated with the male gender, and subjects with gastric cancer were predominantly male. In the present study, the lack of EBVaGC association with gender (P=0.501) did not confirm that males are more likely to be diagnosed with EBVaGC than women. Of course, factors such as lifestyle, different genetic backgrounds, or hormonal conditions between the genders can affect the incidence of the disease [25, 29, 39]. Contrary to current investigation findings, a meta-analysis conducted in 2020 interestingly showed a higher incidence of EBVaGC in women [39].

Age is another risk factor for EBV-related gastric carcinoma development, and according to the results of the present study, there is a significant correlation between age with GC and GU; most patients with an average age of over 55 years have GC and GU ($P < 0.001$). Consistent with the current study results, several investigations have reported a high frequency of EBVaGC at older ages [29, 38]. Furthermore, inconsistent with the present study findings some studies showed more frequency of this subtype of cancer at a younger age [35].

In the current study, the determination of EBV DNA quantity in cancer and gastroduodenal disease groups was performed by the Real-Time PCR method. According to the results, more copy numbers of EBV EBER-DNA with a mean of 2.14×10^{-1} copies per cell were observed in the GC group than in the gastroduodenal disease with a mean of 1.39×10^{-2} copies per cell. Also, none of the gastric specimens with normal histology were positive for EBV EBER-DNA. To our knowledge, this is the first study conducted in Iran that reported data on the prevalence and viral load of

EBV DNA in gastric cancer and gastroduodenal diseases. The high EBV EBER DNA quantities in the cancer group may suggest the presence of EBV-related tumors and the possible role of EBV in the development of gastric cancer.

In various studies on the association of the EBV/Hp coinfection with gastric pathogenesis, the main focus on gastric cancer and the inclusion of benign disorders as a control group is considered a major limitation [40]. Some of these studies suggest the existence of a synergic effect between EBV and Hp in the pathogenesis of gastric disorders and carcinogenesis [22, 23, 41-43]. Maintaining the inflammatory state and further damage to the gastric mucosa by increasing IL-17 expression is one of the results of synergy between the two pathogens [22, 25, 36]. In the present study, the absence of a statistically significant difference between dual infections of both pathogens among cancerous and noncancerous groups did not indicate any interaction between EBV and Hp in gastric cancer development (P=0.903). Opposite to many studies, which showed that the frequency of coinfection was higher in gastric cancer samples than other gastric disorders, in the present study, the highest frequency of double infection was observed in DU (70%) in comparison with GC (13%)[22, 23, 27, 36]. Of course, similar to the current study results in some studies such as the study carried by Saxena et al, the prevalence of co-infections in peptic ulcer disease (62.2%) was higher than gastric cancer (46.8%) [23].

In conclusion, this study highlights EBV prevalence in gastric cancer and gastroduodenal diseases. The current investigation showed that EBV viral load was significantly higher in the gastric cancer group as compared to the gastroduodenal disease group. It can be noted that the small sample size, cross sectional design, lack of non-peptic controls and possible bias due to retrospective study were the limitations of the present study, which may be resolved in similar future investigations. Moreover, the etiological contribution of this virus to the development of gastric cancer and other gastroduodenal diseases needs to be further explored in prospective case–control follow-up studies.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

Availability of data and materials

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

Ethics approval

This study was approved by the Ethical Committee of Babol University of Medical Sciences (Ethics code: IR.MUBABOL.REC.1396.1), and for all subjects, written informed consent was obtained.

Funding

This study was financially supported by a grant from Babol University of Medical Sciences (Project code: 9503117).

Acknowledgments

We would like to express our appreciation to the directors and staff of Pathology Department of Ayatollah Rouhani Hospital affiliated to Babol University of Medical Sciences for their collaboration in sample collection. This study was financially supported by a grant from Babol University of Medical Sciences (Project code: 9503117).

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