Co-infection relationship with Epstein-Barr virus in gastroduodenal diseases with *Helicobacter Pylori*. Quantitative PCR and EBNA-1 gene-based approach

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Abstract

***Objective: Helicobacter pylori* (Hp) and Epstein-Barr virus (EBV) are involved in gastric cancer (GC) etiology. EBV/Hp co-infection was thought synergistically increase gastroduodenal disease occurence. We aimed to determine the presence of EBV/Hp co-infection in gastroduodenal diseases.**

***Methods:* The study group had 68 Hp (+) cases [25 GC, 13 IM (intestinal metaplasia), 30 PU (peptic ulcer)], and the control group had 40 NUD (non-ulcer dyspepsia) cases [20 Hp+, 20 Hp-]. EBV-DNA was detected by non-polymorphic EBNA-1 gene-based qPCR. EBV/EBNA-1 IgG levels were determined by quantitative and qualitative ELISA methods, respectively.**

***Results:* EBV-DNA positivity was 32% (8/25), 6.6% (2/30) and 5% (1/20) in GC, PU and NUD Hp (+) cases, respectively. There was a significant difference (p = 0.001) between GC (32%) and NUD Hp (+) (5%) cases in terms of EBV-DNA positivity. Mean EBV-DNA copy numbers were 6568.54 ± 20351, 30.60 ± 159.88 and 13.85 ± 61.93 for GC, PU, and NUD, respectively. In terms of the mean EBV-DNA copy number, a significant difference was found between the groups (p = 0.005). In terms of EBV/EBNA-1 IgG antibody positivity, no significant difference was found between GC and NUD cases (p = 0.248). EBV DNA positivity was found to be significant (odds ration [OR] = 26.71 (p=0.009, %95CI 2.286-312.041) in multivariate logistic regression.**

***Conclusioun:* Although we had a small number of GC cases, it can be suggested that the estimated risk created by the synergistic effect based on the addition of EBV increased 26 times in the presence of Hp in GC.** (Acta gastroenterol. belg., **2022,** 85, **301-308**).

Keywords: *Helicobacter pylori*, Epstein-Barr virus, Epstein-Barr nuclear antigen 1 (EBNA-1), gastric cancer, EBV/EBNA-1 IgG antibody, peptic ulcer.

Introduction

International Agency for Research on Cancer (IARC), a branch of the World Health Organization, first classified *Helicobacter pylori* (Hp) as Type I carcinogen in 1994 (1). Hp mostly causes asymptomatic gastritis if the bacteria are not eradicated, and it can cause gastric cancer (GC) within 10 years in 2-4% of these patients. GC is the third leading cause of cancer-related deaths worldwide, and thus, Hp infection is an important health issue (2). Epstein-Barr virus (EBV) is a gamma herpes virus, and it was found to be associated with lymphomas such as Burkitt's lymphoma and carcinomas such as GC and nasopharyngeal carcinomas (3). Epstein-Barr virus nuclear antigen 1 (EBNA1) is an EBV-encoded protein, and its presence is required for episome replication and maintenance. EBNA-1 expression is present latently with EBV infection, and it allows localization of EBV DNA to the nuclear chromatin in latent EBV infections (4,5). EBNA-1 is required for the protection and persistence of the viral genome in latent infections, and it has been found to be expressed in all EBV-related cancers (6).

Resent research has revealed a new polymicrobial paradigm in which EBV-Hp co-infection is thought to synergistically increase GC formation compared to a monomicrobial infection. Various studies from different countries have suggested that the association between EBV and Hp may play a role as an early participant in the gastric carcinogenesis processes. Studies in this area also suggest that these two pathogens could potentially cause chronic gastritis and GC, and that they could synergistically play a common role in the pathogenesis of such diseases (7). A few studies from different geographical regions have been conducted internationally on the gastric carcinogenesis process based on this dual factor (EBV-Hp co-infection). The relationship between gastroduodenal pathologies and Hp shows regional differences in some parameters (e.g., virulence, pathogenesis, and resistance), and it would be more beneficial to study GC in different regions of the world (8,9).

In this study, we aimed to detect the presence of EBV and Hp co-infection (dual infection) by determining the presence and number of copies of EBV DNA (EBNA-1 gene in the antrum/corpus gastric biopsy samples) and the frequency of EBV/Epstein-Barr nuclear antigen-1 (EBV/EBNA-1) IgG levels in the serum of the Hp (+)

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GC, intestinal metaplasia (IM), peptic ulcer (PU), and non-ulcer dyspepsia (NUD) cases.

Materials and methods

Study design and patients

In this case-control study, the study group had 68 Hp^+ cases [25 GC, 13 IM (intestinal metaplasia), 30 PU (peptic ulcer)], and the control group had 40 NUD (non-ulcer dyspepsia) cases [20 Hp^+, 20 Hp^-]. The SG (study group) was matched with the CG (control group) according to the age and gender distribution of the SG ($p > 0.05$). The antrum and corpus biopsy specimens of al cases were used for molecular studies

Gastric antrum and corpus biopsy and blood samples were obtained from the patients who applied to the General Surgery Department Endoscopy Unit with dyspeptic complaints and. had an indication for endoscopy. After the biopsy procedure, the tissue samples of 195 cases were sent to the pathology and microbiology laboratories. A total of 75 cases were diagnosed with GC, intestinal metaplasia IM, PU and nonulcer dyspepsia (NUD) were included in the study. Accordingly, out of 195 cases, a total of 60 cases, 17 with GC, 13 with IM, and 30 with PU, whose histopathologically were found to have Hp (+) were included in the study as the SG, and 20 NUD cases with histopathologically Hp (+) and 20 NUD cases with histopathologically Hp (-) included as the CG. We used NUD (non-ulcer dyspepsia) term for funcional dyspepsia which was defined by Rome IV classification (10) . The symptoms of patients with NUD are included bothersome postprandial fullness and early satiationepigastric pain and burning with no evidence of structural disease (including at upper endoscopy) that is likely to explain the symptoms in this study. Patients younger than 18 years old, those who had undergone previous gastric surgery or Hp eradication treatment, and/or had a history of therapy with antibiotics, proton pump inhibitors, bismuth salts, or sucralfate in the month prior to sampling were excluded from the study.

The collection of two biopsy samples from the antrum and the corpus were performed in Brucella broth and transferred to the laboratory. Collected serum samples were also used for serological assays. Clinical, endoscopic, and histopathological examinations and Sydney and World Health Organiation (WHO) classifications were used for the diagnosis of gastroduodenal disorders (11). GCs are classified as diffuse and intestinal types according to WHO. The study was approved by the Clinical Research Ethics Board of Istanbul University, Cerrahpasa Faculty of Medicine (Ethical approval; Ethical approval No: 18.04.2018, A-25 64414572-903.99-146239) and upheld the standards of the Declaration of Helsinki. All patients gave informed consent to participate in the study.

Serological Assays

Epstein-Barr Nuclear Antigen-1 IgG Test

EBV/EBNA-1 IgG serum levels of all patients were measured using an automated and open system Triturus device (Triturus, Grifols, Spain) with the EUROIMMUN ANTI-EBNA-1 enzyme-linked immunosorbent assay (ELISA) IgG kit (Medizinische Labordiagnostika AG, Lubeck Germany). We followed the recommendations of the manufacturer while performing the assays. Results were interpreted in relative unit milliliter (RU/ml) according to the manufacrturer's scale: (1) < 16 RU/ml negative, (2) ≥ 16 to < 22 RU/ml borderline, and (3) ≥ 22 RU/ml positive.

Molecular methods: ureC gene Detection in H. pylori

The presence of Hp was histopathologically determined from patient biopsy samples and then homogenized with the MagNA Lyser Homogenizer (Roche Diagnostics, Basel, Switzerland) device. DNA was isolated from these homogenized samples using the QIAamp DNA Mini Kit (Qiagen GmbH, Germany). In order to verify Hp DNA, the ureC gene region (glmM) of Hp was determined by the real-time polymerase chain reaction (qPCR) method with a Fluorion device (İontek, Turkey) and *H. pylori*-QLS 1.0 kit (İontek, Turkey) device.

Determination of EBV/EBNA-1 Gene Region by qPCR

Viral DNA Isolation Steps from Biopsy Samples

The solution containing the homogenized tissue samples was used as the starting material, and the following isolation protocol was applied using the CANVAX brand HigherPurity™ Viral RNA/DNA Extraction Kit (Canvax Biotect, Cordoba, Spain) according to the manufacturer's recommandations. Viral DNA's stored at -80 °C

EBNA-1 qPCR Study Protocol: Determination of EBNA-1 by Quantitative Real-Time PCR

From the samples whose viral DNAs were isolated, the qPCR step was then started. In order to detect EBV/EBNA-1 gene region primers in the following references were used (12,13). The qMAXsen Green qPCR Master Mix-Low Rox-E0531 master mix of CANVAX. was used to screen the target gene region (EBNA) in the samples. Both primer sets and sequences used in the EBNA-1 qPCR study are shown as supplementary file.

PCR content in the first tube was used in the EBNA qPCR assay to detect the presence of the EBNA-1 molecule transcribed by the pathogen. PCR content in the second tube was used to detect the control gene. The reaction mixture had a final volume of 20 µL and contained 6.25 µl dH_2O, 0.3µl of each oligonucleotide (EBNA-F, EBNA-R and EBNA-P), 10 µl Green qPCR

Master Mix, and 2 µl of DNA. The conditions consisted of several steps: (1) one cycle at 95°C for 10 min, (2) 45 cycles at 95 °C for 15 s, and (3) 60°C for 1min. All reactions were performed in a LightCycler 480 Real-Time PCR instrument.

Evaluation of data obtained from the EBNA-1 Real-Time PCR Study

The data obtained from the qPCR assays were analyzed using LightCycler 480 software. The copy numbers of synthetic genes whose copy numbers were known and serial dilution were made were recorded in the program. The determination of the number of copies over the Ct values of the samples was made automatically throughout the program. The copy numbers calculated by the program based on the Ct values of the samples and the samples whose copy numbers are known, were converted into copies/100.000 cell units using the formula described below.

$$\frac{\text{2x number of EBNA copies}}{\text{number of human GAPDH copies}} \text{ x } 100.000$$

Statistical analyses

The SPSS 25.0 (IBM Corporation, Armonk, New York, USA) program was used for statistical analysis. Demographic data and EBV DNA (EBNA-1 gene region positive) and EBV/EBNA-1 IgG antibody positivity rates of the study and control groups were shown as categorical variables and expressed as frequency and ratio values (n%). Analysis of variance (ANOVA) was used to compare the study and NUD groups. Chi-square and Fisher's exact tests were applied for comparisons of EBV DNA (EBNA-1 gene region) and EBV/EBNA-1 IgG antibody positivity rates among the categorical variables and NUDs, and the results were expressed as Hochberg corrected p-values. The Kruskal-Wallis analysis was applied to compare the mean of EBV-DNA copy numbers of the study and NUD cases. The results of Kruskal-Wallis non-parametric test were shown as H and p values. After the Kruskal-Wallis non-parametric test was applied, a post-hoc Dunn test was performed for paired comparison analysis. In the context of determining the cause and effect relationship with explanatory variables that are suggested to increase the risk of developing GC, EBV-DNA positivity, together with other group variables (age group variables [< 55 and ≥ 55 years] and gender [female/male] parameters) were included as an independent variable for binary logistic regression (multivaryet logistic regression) analysis using the Enter method. Variables were analyzed at 95% confidence level (CI), and $p < 0.05$ was considered significant.

Results

The mean ± standard deviation (SD) and range of ages (min-max) of the GC, IM, PU, NUD Hp (+) ve NUD Hp (-) cases were 67.7±9.053, 58.15±12,29, 48.46±16.65, 49.15±13.88 and 55.15 ± 10.85, respectively. Four out of 25 (16%) of GC cases were the diffuse type, 21/25 (84%) were the intestinal type, and no mixed type (mixed type) cancer cases were detected. All of the cases in the PU study group were gastric ulcers.

Demographic data, endoscopic-histopathological findings, EBV/EBNA-1 gene region qPCR positivity rates, and EBV/EBNA-1 IgG qualitative positivity rates of all cases included in the study are shown in Table 1.

Table 1. — **Demographic data and laboratory findings (endoscopic, histopathological and microbiological findings) of the study and control groups**

Demographic Data and Laboratory Findings	**Study Groups**			**Control Groups**	
	Gastric Canser (GC)	Intestinal Metaplazia (IM)	Peptic Ulser (PU)	Non-Ulcer Dyspepsia [NUD *Hp* (+)]	Non-Ulcer Dyspepsia [NUD *Hp* (-)]
Subject (n)	25	13	30	20	20
Mean Age (min-max))	67.7 ± 9.053 (43-79)	58.15 ± 12.29 (35-79)	60.06 ± 14.76 (26-90)	60.85 ± 13.59 (35-89)	59.7 ± 9.84 (43-77)
Gender **Male (%)** **Female (%)**	 16 (64%) 9 (36%)	 6 (46.15%) 7(53.84%)	 12 (40%) 18 (60%)	 10 (50%) 10 (50%)	 8 (40%) 12 (60%)
Site of Involvement n(%)	Diffuse type 4/25 (%16)				
	Intestinal type 21/25 (%84)				
Peptic Ulcer Site n(%)			Gastric Ulcer 30/30 (%100)		
			Duodenal Ulcer 0/30 (%0)		
Hp (+) (n;%)	25 (%100)	13 (100%)	30 (100%)	20 (100%)	0 (0%)
EBV/EBNA-1 GENE REGION (+) (n%)	8(32%)	0(0%)	2(7%)	1(5%)	0(0%)
EBV/EBNA-1 *IgG* (+) (n%)	13(76.3%)	13(100%)	25(85%)	19(95%)	16(86%)

Table 2. — **Qualitative comparison of the presence of EBV-DNA (EBNA-1 gene region positive) in study and control groups**

EBV-DNA *EBNA-1* gene presence	Study Groups**		Control Group**	*P* value
	GC (n:25) n (%)	PUD (n:30 n (%)	NUD Hp (+) (n: 20) n (%)	
***EBNA-1* gene (+)**	8 (32%)	2 (6.6%)	1 (5%)	GC vs. NUD **p=0.03*** PUD vs. NUD **p= 1.00***
***EBNA-1* gene (-)**	17 (68%)	28 (93.4%)	19 (95%)	
Total	25	30	20	

*Fisher's Exact Test. ** IM and NUD Hp (-) groups are not included in the comparisons.

Table 3. — **Quantitative comparison of the study and control groups in terms of EBV-DNA EBNA-1 gene region positive) copy numbers**

Study and Control Groups***	n	EBV-DNA copy numbers (Mean ± Sd)	*P* value
Gastric Cancer (GC)	25	6568.54 ± 20351	p < 0.005* GC vs. NUD Hp(+); p < 0.05**
Peptic Ulcer Disease (PUD)	30	30.60 ± 159.88	PUD vs. NUD; p=1.000
Non-Ulcer Dyspepsia [NUD Hp (+)]	20	6.92 ± 43.79	
Total	75		

*Kruskal Wallis non-parametric test; **Post Hoc Dunn Test. ***IM and NUD Hp (-) groups are not included in the comparisons.

Table 4. — **Qualitative comparison of EBV / EBNA-1 IgG presence in the study and control groups**

EBV/ EBNA-1 IgG presence	Study Groups**		Control Group**	*p* value
	Gastric Cancer (GC) (n= 25) (n;%)	Peptic Ulcer Disease (PUD) (n= 30) (n;%)	Non-Ulcer Dyspepsia (NUD) (n= 20) (n;%)	
EBNA-1 IgG (+)	21 (84%)	25 (83.7%)	19 (95%)	X^2= 1.331 *P* = 0.248*
EBNA-1 IgG (-)	4 (16%)	5 (16.3%)	1 (5%)	
Total	34	30	40	

*: Chi-Square Test. **IM and NUD Hp (-) groups are not included in the comparisons.

EBV-DNA copy numbers of cases with EBV-DNA positivity in GC, IM, PU, and NUD groups are shown quantitatively in supplemantary file.

In our study, categorical investigations were made in terms of the presence of EBV-DNA (EBNA-1 gene region positive) in the biopsy samples of the study and control groups. In the biopsy samples of the GC cases, the number of cases with EBV-DNA positivity was 8 (32%), EBV-DNA was found in 6.6% (2/30) of cases with PU. EBV-DNA was found to be positive in one (5%) out of 20 NUD Hp (+) cases used as the control group. According to these data, there was a significant difference (p = 0.001) between the cases in the GC cases (32%) and the control cases NUD Hp (+) (5%) in terms of EBV-DNA positivity. However, no significant difference (p = 0.573) was found between the the PU group (6.6%) and the CG/ NUD Hp (+) (5%) (Table 2).

When the data of the study and control group cases in our study were analyzed in terms of noncategorical variables in another way, mean EBV-DNA copy numbers were 6568.54 ± 20351, 30.60 ± 159.88 and 13.85 ± 61.93 for GC, PU, and NUD Hp (+), respectively. According to the Kruskal-Wallis analysis performed with the GC, PU, and NUD Hp (+) groups in terms of the mean EBV-DNA copy number, a significant difference was found between the groups (H = 12.684, p = 0.005). Later, according to the post hoc Dunn analysis that was performed to determine which of the gastroduodenal diseases constituted the study group and the control group, only a significant difference between the GC and NUD Hp (+) cases (p = 0.05) was found as shown in Table 3.

Categorical investigations were performed to monitor the presence of EBV/EBNA-1 IgG antibody positivity in biopsy samples of study and NUD Hp (+) cases. While the number of cases with EBV/EBNA-1 IgG antibody positivity in serum samples of GC cases was 21 (84%), it was also found EBV/EBNA-1 IgG antibody positivity in 25 (83%) of cases with PU and 19 (95%) of NUD Hp (+) cases. According to these data, in terms of EBV/EBNA-1 IgG antibody positivity, no significant difference was found between the cases with GC and the NUD Hp (+) group (p = 0.248). Similarly, no significant difference was found in the comparison of PU cases with NUD Hp (+) cases (Table 4). EBV/EBNA-1 IgG level was found to be positive in the blood samples of 7 out of 8 GC cases in which the presence of EBV-DNA (EB-1 gene region positive) was found to be positive, while in one case (GC 07 numbered case) it was found to be negative. EBV/

Table 5. — **Results of logistic regressions analysis according to the variables in gastric cancer cases**

Independent Variables	B[a]	S.E.[b]	Sig[f]	OR[c]	95% C.I.[d] (Min-Max)
Gender (Male)	0.786	0.714	0.271	2.195	0.541-8.900
Age (≥55 years)	2.329	1.357	0.086	10.270	0.718-146.869
EBNA-1 gene positivity	3.285	1.254	0.009	26.711	2.286-312.041
Constant	-0.523	0.742	0.497	0.593	

[a] B, beta regression coefficient, [b] SE, standard error , [c] OR, odds ratio , [c] CI, confidence interval, [f]Sig, significance.

EBNA-1 IgG level was found to be negative in one of two PU cases (PU33) with EBV-DNA (EBNA-1 gene positive), and in the one CG/NUD case, both EBNA-1 gene and EBV/EBNA-1 Ig G were positive.

In GC group cases, EBV-DNA (EBNA-1 gene region positive) positivity, age group variables (< 55 and > 55 years old), and gender (female/male) parameters were taken as independent variables to multivariate logistic regression analysis. EBV DNA positivity was found to be significant (odds ration [OR] = 26.71 (p=0.009, %95CI 2.286-312.041) in logistic regression analysis as shown in Table 5.

Discussion

GC is a multifactorial and complex malignant disease, and it is one of the few malignant diseases that are known to play a role in the etiology of infectious agents (such as Hp and EBV) (14,15). The frequency and distribution of GC subtypes and the prevalence of Hp differ according to the geographic region (16). However, there are also different opinions regarding the prevalence of EBV (17). The first of these opinions is that EBV-associated GC shows a similar distribution throughout the world regardless of regional and racial differences and without any regional cluster (18). The oncogenic effects of EBV are thought to be mediated by nuclear antigens (EBNAs) and latent membrane proteins (LMPs) that interact with tumor suppressor genes and signaling pathways (7). Some genes that are associated with EBV (i.e., EBNA1, EBER-1, EBER2, BARF1, BARF0, and LMP2A) are expressed in GCs and appear as oncogenic EBV proteins. However, the role of EBV in the pathogenesis of GC is not well understood (19,20). According to a new polymicrobial paradigm that has recently emerged, Hp and EBV infections are common in childhood, and it has been suggested that simultaneous infections with both Hp and EBV can synergistically increase the change in the gastric mucosa to chronic gastritis and GC (21,22,23).

In our study, the presence of Hp and EBV co-infection was detected in 10.2% (11/108) of the Hp (+) or (−) study and control group patients, while it was 12.5% (11/88) for only Hp (+) patients. EBV DNA was detected in 32% (8/25) of biopsy specimens from patients with GC. All of the patients in whom EBV DNA was detected had the intestinal type GC. EBV DNA was not detected in the diffuse type of GC. With or without lesions, all biopsy specimens that were taken from IM cases were negative for EBV DNA. In patients with PU, the presence of Hp and EBV co-infection was detected in 7% (2/30) of patients (PU33 and PU57 patients). Additionally, 2.5% (1/40) of co-infection was detected in NUD Hp (+/−) patients, who were used as the control group, and EBV DNA positivity was found only in the NUD Hp (+) group.

The presence of EBV DNA was detected in eight (32%) patients in the GC group, while it was detected in only one (5%) patient in the control group of NUD Hp (+) patients. If the data were analyzed using a nonparametric test, the EBV DNA copy numbers were 6568.54 ± 20351 for GC and 13.85 ± 61.93 for NUD Hp (+), respectively. In both the univariate and nonparametric analysis, the EBV DNA frequency and copy levels were significantly different between patients with GC or NUD Hp (+) values, respectively, and a this difference was shown using a post-hoc Dunn's test. Additionally, when the presence of EBV DNA and other independent variables (age and gender) in GC patients were evaluated using a multivariate analysis, the presence of the EBNA-1 gene region that showed a positive result for EBV DNA was highly significant, with an OR of 26.7. The frequency of EBV/EBNA IgG was detected in 21 (84%) patients in both GCs and 19 (95%) patients in the NUD Hp (+) group. No statistically significant difference was found in the analysis between groups.

Saxena et al. (24) reported 62 patients with GC and 241 NUD in their study, and the presence of Hp/EBV DNA was 46.8% in the GC group, 62.2% in the PU group, and 29.5% in the NUD control group. They showed a significant difference between the GC and NUD groups. Shukla et al. (25) found the presence of Hp/EBV co-infection to be 54% in the GC group and 23% in the NUD Hp (+) patients, which was significantly different. In the non-parametric analysis of the EBV DNA copy number, they found a significant difference between the Hp (+) GC and NUD Hp (+) groups for EBV DNA median values. The frequency of Hp/EBV coinfection was higher in this study from India, and it was significantly higher in patients with GC compared to the control group; this was consistent with our study results. In the study by Shukla et al. (26) on the expression frequency of latent and lytic EBV transcripts according to the Hp (+/−) presence in malignant and non-malignant gastropathologies, EBV DNA was detected in 40 (80%) patients in the GC group and 36 (30%) patients in the control group NUD, which was a significant difference. They suggested that there is an increasing trend in EBV DNA load in Hp (+)

gastropathologies, especially in GCs, and that Hp and EBV interact and activate synergistic mechanisms in the etiopathogenesis of GC. In our study, the OR of the EBNA-1 gene that indicated a positive result for the presence of EBV DNA was 26 in a multivariate analysis. Thus, the presence of EBV increases the GC risk 26-times when Hp (+) pathologies have developed. In a 2006 study, Hp-associated monochloramine induced the latent infection process in the EBV-infected gastric epithelium towards lytic infection (27). Another study investigated whether Hp infection increased the EBV viral load in patients with GC, and BZLF-1 and BARF-1 expression, which are lytic transcripts of EBV, was high in Hp (+) patients; this supports the hypothesized mechanism that it modulates the transition from the latent phase to the lytic phase. However, it has been suggested in different studies that Hp and EBV co-infection may be synergistically more effective in gastric carcinogenesis by playing an active role in modulating the transformation of EBV from the latent phase to the lytic hase in gastric tissues (25,28,29).

In 2019 in Mexico, Moral-Hernández et al. (30) evaluated the presence of the EBNA-1 gene that targeted EBV DNA in 32 GC and 106 chronic gastritis patients using their gastric biopsy specimens, and they found that it was present 37.5% (12/32) of GC patients and 25.5% (27/106) of patients with chronic gastritis, which was not significantly different. In chronic gastritis, 53% of Hp (+) patients were EBV (+). In conclusion, the researchers suggested that the different rates in the presence of EBV in GC cases may be due to the differences in sensitivity and specificity of the molecular methods that were used in the studies, the diagnostic processes for the patients who were studied, and the geographical region.

In 2019 in Peru, Castenada et al. (31) reported that Hp and EBV co-infection was found in 7.8% of GC patients, and it was also higher in GC in univariate and multivariate analyses. They suggested that EBV/Hp co-infection synergistically increases the Th17-induced proinflammatory cytokine IL-17 expression and can induce a chronic inflammatory process that can damage the gastric mucosa. Additionally, Hp can contribute to the formation of gastric pathologies by inducing the synthesis of monochloramine, which will transform EBV from latent to lytic. In 2006, Minoura-Etoh et al. (27) reported that 48 (61.5%) of 78 NUD patients were Hp (+) and that 18 (37.5%) of them had EBV DNA positivity. None of 30 (38.4%) patients who were Hp (−) had EBV DNA, which was significantly different between the two groups.

In our study, the EBNA-1 IgG frequency results were high (76.4% and 95%) in patients with GC and the NUD Hp (+) control group, respectively, but this difference was not significant .This is an expected result when the common transmission routes of EBV infections are considered.

In the literature, hypotheses based on some mechanisms have been proposed regarding the role of Hp and EBV co-infection in gastric carcinogenesis. The following are the hypothesis that suggests an increased inflammatory response in Hp and EBV co-infection: the hypothesis based on gene product interaction (PLCγ signaling pathway, SHP1 protein, SHP2 protein) and Hp-associated oxidant monochloramine (NH2Cl) in gastric epithelial cells; and hypotheses claiming that latent EBV induces its reactivation. It is also suggested that Hp stimulates the gastric Th17 response via the HP0175 protein (32). In Hp/EBV co-infections, Hp and EBV can act synergistically and increase IL-17 expression, leading to an inflammatory process that severely damages the gastric mucosa (33). The reactivation of EBV causes more infiltration of B cells that are loaded with viral particles, which increases the likelihood of epithelial cells becoming infected (22,34). EBV co-infection enhances the induction of IL-1β, TNF-α, and IL-8 production, and causes more severe chronic inflammation, resulting in more damage to the gastric mucosa (22).

In an *in vitro* study, EBV reactivation occurred via PLCγ signaling and *Hp* CagA strongly activates PLCγ (35). This activation induced the PLCγ signaling pathway, which caused EBV reactivation in infected B cells, and both infectious agents activated the β-catenin/TCF-4 pathway, which are transforming factors in stomach cells (8,36). Saju et al. (21) demonstrated that the SHP1 protein, which is a host phosphatase, dephosphorylates CagA and prevents its oncogenic activity. The SHP1 protein performs this dephosphorylation as a negative regulator of CagA phosphorylation. However, Hp and EBV co-infection cause SHP1 methylation, and they prevent it from dephosphorylating CagA, thereby increasing CagA's oncogenic potential. Thus, in the absence of EBV, when SHP-1 interacts with CagA, CagA becomes dephosphorylated and its oncogenic activity decreases. In EBV/Hp co-infection, the SHP-1 promoter is methylated by EBV, which downregulates its expression and increases the oncogenicity of tyrosine-phosphorylated cagA (21). Hp-associated oxidant monochloramine (NH2Cl) latently induces the reactivation of EBV into the lytic phase in EBV-infected gastric epithelial cells. Thus, Hp-associated gastric inflammation induces the production of oxidant monochloramine, which is formed by infiltrating neutrophils. This induction converts a latent EBV infection to an EBV infection by early gene activation, resulting in induction of a lytic EBV infection (27).

If we briefly summarized the role of Hp/EBV co-infection in gastric carcinosis. Mechanistically, both pathogens increase immune cell recruitment to the gastric mucosa and pro-inflammatory cytokine secretion. Hp-associated monochloramine (MCA) induces a lytic transformation of EBV in the gastric epithelium with a latent EBV infection, and an active EBV infection involves polymorphonuclear cells (PMNCs), natural killer (NK) cells, and dendritic cells (DCs). It also induces an inflammatory response in the gastric mucosa by attracting immune cells, including CD8, Th17, and Th1 cells (37).

Our study has some limitations. In the presence of an Hp infection, the lytic or latent stage of EBV was not analyzed, and due to ethical concerns, biopsies were not taken from people with a normal gastrointestinal tract. In addition, the PCR method cannot distinguish between EBV DNA in epithelial cells and EBV DNA that infiltrates tissue samples from EBV-infected lymphocytes. EBNA-1 that was detected using qPCR can also be an indicator of the severity of the inflammatory response, unlike an EBV infection in epithelial cells. The PCR method that we used is more sensitive but less specific than the gold standard method (ISH). Additionally, the PCR method cannot distinguish between cancer cells and lymphocytes that have infiltrated the cancer stromalin, and therefore, it is not possible to know the origin of the EBV DNA that was replicated by PCR.

Additionally, in the presence of Hp in GC, the estimated risk that is created by the synergistic effect based on adding EBV increases 26-times. However, there are many questions about whether each factor initiates the carcinogenesis process in Hp and EBV co-infection alone or with a synergistic effect. In addition, we suggest that the clear definition of synergy between Hp and EBV with all its aspects has important implications for managing the diagnosis, prognosis, and treatment of GC for populations in different geographic regions and identifying individuals who have a high risk of possible polymicrobial-based GC. Additionally, determining the Hp/EBV relationship will have a strong predictive potential, which requires the development of non-invasive tests for an early GC diagnosis. However, because it has been suggested that the molecular interaction between Hp/CagA and EBV is observed only in a small number of GC (10%) cases, it is thought that this may not be very common in different populations. We suggest that new data, which may emerge as a result of our or other studies on this dual etiopathogenesis, may also change the views on the treatment approaches for possible polymicrobial pathogenesis processes of gastroduodenal diseases. In addition, our research data aimed to demonstrate the molecular-based physiologically possible synergistic (cross-talk) relationship between Hp and EBV inside and outside the gastric epithelium and their mutual interaction with the host immune responses.

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Conflict of interest statement

All authors state that we don't have any potential conflict of interest including any financial activities, additional affiliations, personal or other relationships with other people or organizations that could influence, or be perceived to influence, their work, such as employment, consultancies, stock ownership, honoraria, patent applications/registrations, grants or other funding.

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