Full paper

Epstein–Barr Virus and *Helicobacter pylori* co-infection in patients with gastric cancer and duodenale ulcer

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Running Title: EBV and *H. pylori* co-infection in gastric cancer

SUMMARY

We aimed to detect EBV/Hp (Epstein–Barr Virus/*Helicobacter pylori*) co-infection by determining the number of copies of EBV/EBER-1 in the gastric biopsy samples of the Hp (+) GC, peptic ulcer (PU), and non-ulcer dyspepsia (NUD) cases.

The patient group (PG), with 34 patients (34 GC and 30 PU patients) and a control group with 40 NUD cases were included. All patients and controls were Hp positive. EBV/EBNA-1 IgG were measured by the Anti-EBNA-1 ELISA IgG kit. Determination and quantification of EBV/EBER-1 gene region was performed by qPCR.

EBV/EBER-1 positivity was 35.29% (12/34), 6.6% (2/30) and 2.5% (1/40) in GC, PU and 40 NUD cases, respectively. A significant difference was found between the GC and NUD cases (p = 0.001). A significant difference was found between the groups for mean EBV/EBER-1 copy numbers (p = 0.019). No significant difference was found between GC and the NUD cases (p = 0.1455) for

EBV/EBNA-1 IgG antibody positivity. EBV/EBER-1 positivity (OR = 3.319), and age ≥ 55 years old (OR = 2.331) were found to be a significant in multivariate logistic regression.

In conclusion, our data suggest that the GC risk by EBVand Hp co-infection increased 3.3 times.

Key Words: Helicobacter pylori; Epstein-Barr virus; EBV-coded small RNAs (EBERs); Gastric cancer; EBER-1 gene; EBV/EBNA-1 IgG antibody

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INTRODUCTION

Helicobacter pylori (Hp) is associated with gastroduodenal pathologies, such as acute gastritis, gastric cancer (GC), intestinal metaplasia (IM), peptic ulcers (PU), non-ulcer dyspepsia (NUD), and mucosa-associated lymphoid tissue (MALT) lymphoma, and has been identified as a group 1 cancer agent by the World Health Organization/International Agency for Research on Cancer (WHO, 2018). Hp colonization is thought to increase the risk of GC up to ten times (Normark *et al.,* 2003). Epstein-Barr virus (EBV) is a member of the Herpes virus family; EBV is known to play a role in the pathogenesis of infectious mononucleosis and many oncological diseases, such as Burkitt's lymphoma and nasopharyngeal carcinoma (Kutok and Wang, 2006). EBV was identified as the first human virus associated with carcinogenesis and is also classified as a group 1 carcinogen (Griffin, 2000). However, in recent years, it has been suggested that EBV and Hp co-infection synergistically cause an increase in the formation of GC and its early onset. It has been suggested that EBV co-infection with Hp may play a role in the association of EBV with Hp as an early participant in GC carcinogenesis processes (Cárdenas-Mondragón *et al*., 2013; Cho *et al*., 2003; Marshall and Warren, 1984).

EBV is known to account for more than 10% of gastric adenocarcinomas in the epithelial cancer group (Chen *et al*., 2012). EBV-coded small RNAs (EBERs) are small, non-coding RNAs that are embedded in the nucleus of human cells infected with EBV. EBERs, first discovered in 1981, are the most common RNAs that settle into infected cells (Nanbo and Takada, 2002). EBER-1 and -2 are short, non-coded RNAs composed of 167 and 172 nucleotides, respectively, that accumulate in the nucleus, and EBERs interact with various host proteins to form ribonucleoprotein (RNP) complexes. Although a definite function of EBERs is still not known, their role in transformation and oncogenesis has been postulated. These two RNAs are transcribed by the host RNA polymerase III during latent infection with EBV (Conrad *et al*., 2006; Moss *et al*., 2014). Saju *et al*. (2016) describe a polymicrobial paradigm consisting of bacterial and viral cooperation that potentially exacerbates a disease, such as GC, a disease that each may cause independent of the other.

In our 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/EBER-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 (+) GC, PU, and NUD cases.

MATERIALS AND METHODS

Study design and population

This case-control study was conducted between January 23, 2019 and January 16, 2021. The two patient groups (PG) consisted of a total of 64 patients (34 GC and 30 DU patients and a control group

consisting of a total of 40 NUD patients) were enrolled in this study. All PG and NUD members had *H. pylori*. The control group was matched with the PG according to the age and gender distribution of the PG (p > 0.05). The antrum and corpus biopsy specimens of the PG and control group members were used for molecular studies.

We excluded patients younger than 18 years old, those who had undergone previous gastric surgery or *H. pylori* eradication treatment, and/or had a history of therapy with antibiotics, antisecretory drugs, bismuth salts, or sucralfate in the month prior to sampling.

Two biopsies from the antrum and the corpus were collected in Brucella broth and transferred immediately to the laboratory. Serum samples with EDTA were obtained from venous blood of the participants and stored in a deep freezer at -80°C. They were brought to room temperature on the day of the study and the serum samples were studied within 24 hours. As Christie *et al.,*(1997) reported, GC is rarely seen in people under 55 years of age. However, GC peaks after 55 years of age, and 55 years is recommended as the screening age in noncomplicated dyspepsia cases. The diagnosis of gastroduodenal diseases was made according to clinical, endoscopic, and histopathological examinations and Sydney and World Health Organization (WHO) classifications (Hu *et al*., 2012). GCs are classified as intestinal and diffuse types according to the classification published by WHO in 2010. EBV/VCA IgM was found to be negative and EBV/VCA IgG was found to be 98% (98/100) positive in all of the study and control group cases. The possible time of exposure to the EBV is likely to have occurred in childhood. The patient and control group members had no clinical signs of lymphomonocytosis. The study was approved by the Clinical Research Ethics Board of Istanbul University, Cerrahpasa Faculty of Medicine (Ethical approval; Ethical approval No: 10/05/2018-171779) and upheld the standards of the Declaration of Helsinki. All patients gave informed consent to participate in the study.

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 manufacturer's scale: (1) < 16 RU/ml negative, (2) ≥ 16 to < 22 RU/ml borderline, and (3) ≥ 22 RU/ml positive.

Detection of Helicobacter pylori

The presence of Hp was histopathologically determined and antrum and corpus biopsy specimens were used in order to diagnose Hp. They were obtained from the greater curvature of the antrum and the greater curvature of the corpus. In our study, the histochemical analysis (Modified Giemsa)

method, which is accepted as a reference test in many studies, was used for Hp detection. 5 mm sections were taken from the formaldehyde-fixed paraffin-embedded blocks to be stained histochemically with Giemsa, and staining was performed according to the specified standard procedure as follows: the section was first placed into distilled water and stained with diluted Giemsa. It was washed again with distilled water and dipped in 0.5% acetic acid; excess dye was removed. Then it was rapidly dehydrated. The presence of Hp on the mucosal surfaces in Giemsa-stained sections was investigated and the Hp concentration in the samples was evaluated according to the Sydney System with the degree of inflammation, neutrophil activity, and atrophy. The presence of Hp was classified as mild (covering less than 1/3 of the surface and scattered microorganisms), moderate (mild to severe density) and marked (large clusters of microorganisms that are not scattered, covering more than 2/3 of the surface) (Hu et al., 2012). Histological evaluations were performed by two experienced pathologists from Cerrahpasa Medical Faculty University Hospital. Biopsy samples were 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.

Detection of EBV

Viral RNA Isolation Steps from Biopsy Sample. 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 Extraction Kit (Canvax Biotect, Cordoba, Spain) according to the manufacturer's recommendations. RNA concentration and purity were checked spectrophotometrically (Nanodrop, ThermoScientific, Wilmington, USA). All RNAs were stored at -80°C until the cDNA step.

EBER-1 and GAPDH Real-Time PCR

Determination of EBER-1 and GAPDH by Quantitative Real-Time PCR*:* a First Strand cDNA synthesis kit (Canvax Biotect, Cordoba, Spain) was used to perform cDNA synthesis according to the manufacturer's instructions from 50 ng isolated RNAs. Combined oligo dT and random hexamer protocols were used for cDNA synthesis and cDNAs were stored at -20 °C until qPCR steps. From the samples whose cDNA processing was complete, the qPCR step was then started. The qMAXsen Green qPCR Master Mix-Low Rox-E0531 master mix (Canvax Biotect, Cordoba, Spain) was used to screen the EBER-1 and GAPDH housekeeping control gene region in the samples. Both primer sets and sequences used in the EBER-1 and GAPDH qPCR study are shown in Table 1.

PCR content in the first well was used in the EBER qPCR assay and the second well was used in glyceraldehyde 3-phosphate dehydrogenase (GAPDH) qPCR to detect the presence of the EBER-1 mRNA molecule transcribed by the pathogen and GAPDH, respectively. The reaction mixture had a final volume of 20 μL and contained 4.4 μl dH$_2$O, 0.3μl of each oligonucleotide (EBER-R and EBER-F or GAPDH-F and GAPDH-R), 10 μl Green qPCR Master Mix, and 5 μl of cDNA added to both tubes.

The qPCR conditions were as follows: (1) denaturation (one cycle at 95°C for 2 min), (2) Amplification (45 cycles at 95°C for 5s, 60°C for 30s (with single acquisition mode)), (3) Melting Curve (95°C for 1 min, 55°C for 1 min and 95°C (with continuous acquisition mode)) and (4) Cooling (40°C for 1 min). All reactions were performed in a LightCycler 480 II (Roche Diagnostics, Mannheim, Germany) real-time PCR instrument.

Quantification of EBER-1 geme expression

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 using the formula below:

$$\frac{2x \text{ number of EBER copies}}{\text{number of human GAPDH copies}} x \ 100,000$$

Statistical analyses

The SPSS 25.0 (IBM Corporation, Armonk, New York, USA) program was used for statistical analysis. Demographic data and EBV/EBER-1 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 EBER-1 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/EBER-1 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/EBER-1 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 (multivariate 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, PU, and NUD cases were 67.71 ± 8.916 (43–79), 60.07 ± 14.76 (26–90), and 60.28 ± 12.30 (35–89), respectively. Four out of 17 (23.52%) of GC cases were the diffuse type, 13/17 (76.48%) 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/EBER-1 positivity rates, and EBV/EBNA-1 IgG qualitative positivity rates of all cases included in the study are shown in Table 2. EBV/EBER-1 copy numbers of cases with EBV/EBER-1 positivity in GC, PU, and NUD groups are shown quantitatively in Table 3.

In our study, categorical investigations were made in terms of the presence of the EBV/EBER-1 gene in the biopsy samples of the study and control groups. In the biopsy samples of the GC cases, the number of cases with EBV/EBER-1 positivity was 12 (35.29%), and EBV-RNA was found in 6.6% (2/30) of cases with PU. EBV/EBER-1 was found to be positive in one (2.5%) out of 40 NUD 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 (35.29%) and the control cases NUD (2.5%) in terms of EBV/EBER-1 positivity. However, no significant difference (p = 0.573) was found between the PU group (6.6%) and the NUD (2.55%) (Table 4).

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/EBER-1 copy numbers were 48,800 ± 10462, 876.14 ± 159.88, and 276.97 ± 43.79 for GC, PU, and NUD, respectively. According to the Kruskal-Wallis analysis performed with the GC, PU, and NUD groups in terms of the mean EBV/EBER-1 copy number, a significant difference was found between the groups (H = 19.450, p = 0.019). 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, a significant difference between the GC and NUD cases and the other case groups (p = 0.019) was found as shown in Table 5.

Categorical investigations were performed to monitor the presence of EBV/EBNA-1 IgG antibody positivity in biopsy samples of study and NUD cases. While the number of cases with EBV/EBNA-1 IgG antibody positivity in serum samples of GC cases was 26 (76.4%), it was also found in 25 (83%) of cases with PU and 35 (87.5%) of NUD cases. Positivity was detected. 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 group (p = 0.1455). Similarly, no significant difference was found when comparing PU cases with NUD cases (Table 6). EBV/EBNA-1 IgG level was found to be positive in the blood samples of 11 out of 12 GC cases in which the presence of EBV/EBER-1 gene was found to be positive, while it was found to be negative in one case (GC 07 numbered case). EBV/EBNA-1 IgG level was found to be negative in one of two PU cases (PU33) with EBV/EBER-1 gene positivity, and both EBER-1 gene and EBV/EBNA-1 Ig G were positive in the one CG/NUD case. No significant difference was found in the comparison of the cases with EBV/EBNA-1 IgG and EBV/EBER-1 gene positivity within GC, PU, and NUD group cases (p = 0.681, 0.310, and 1.00, respectively). No statistical data was calculated for EBV/EBER-1 positivity according to gender (separately in male and females).

Since all EBV/EBER-1 positivity was detected in cases with GC above ≥ 55 years of age, the comparison of only ≥ 55-year-old GC cases with the NUD group was found to be significant (p = 0.0001), as shown in Table 7.

In GC group cases, EBV/EBER-1 positivity, age group variables (< 55 and > 55 years old), and gender (female/male) parameters were taken as independent variables to multiple variate logistic regression. While EBV/EBER-1 positivity was found to be significant (odds ration [OR] = 3.319; p = 0.001, 95% Cl 1.639–6.720), age ≥ 55 years old was found to be significant (OR = 2.331; p = 0.042, 95% Cl 1.030–5.277), as shown in Table 8.

DISCUSSION

GC is a multifactorial and complex malignant disease although its prevalence varies across countries worldwide, and is one of the few malignant diseases known to play a role in the etiology of infectious agents (such as Hp and EBV) (Piazuelo *et al*., 2010). In this study, EBV/EBER-1 copy numbers were determined as 48800 ± 10462, 876.14 ± 159.88, and 276.97 ± 43.79 for GC, PU, and NUD, respectively. According to the Kruskal–Wallis analysis performed in the GC, PU, and NUD groups in terms of the mean EBV/EBER-1 copy number, a significant difference between the GC and NUD cases was found (p = 0.019). The frequency and copy levels of EBV/EBER-1 related to the presence of Hp and EBV co-infection showed a significant difference in GC cases with either the univariate or nonparametric analysis. In addition, when the presence of EBV/EBER-1 and other independent variables (age and gender) in GC cases were evaluated by multivariate analysis, the presence of EBER-1 gene positivity was significant (p = 0.001) with an OR of 3.319. When we examined the frequency of EBV/EBNA IgG for the association of Hp + GC cases with EBV, the frequency of EBNA IgG was determined with a rate of 26 (76.4%) and in NUD cases as 87.5%. As a result of the comparison among the groups, no statistically significant difference was found (p = 0.1455).

In 2008, Lima *et al*. (2008) reported that the EBV/EBER-1 gene region was targeted using the RNA *in-situ* hybridization (ICH) method, and they found the presence of Hp and EBV co-infection in 8.45% of cases with GC. Again, using a qPCR method based on the EBV/EBER-1 region, de Souza *et al*. (2014) found the rate of Hp and EBV co-infection to be 6.9% in the general study and control groups. Hp and EBV coinfection was detected in 1.85% (1/54) of juvenile gastritis patients, 5.4% (2/37) of adult gastritis patients, and 9.6% (12/125) of cases with GC. They found that Hp alone was responsible for 78.4% of these cases, and no statistically significant difference was found between them (p > 0.05); as a result, they claimed that Hp and EBV co-infection could not have had a synergistic effect on the formation of GC. Although they used the ICH method for EBER-1 detection (unlike the qPCR that we used), the rate of Hp + EBV co-infection in GC cases was lower in their findings than in our findings. In India in 2008, Saxena *et al*. (2008)[18] evaluated the presence of EBNA-1 gene in gastric biopsy samples of 62 GC and 241 NUD cases. The presence of EBV-DNA was 50.3% in their whole study population, 46.8% in the GC group, and 29.5% in the NUD. While a significant difference was determined between GC and NUD in terms of the presence of EBV-DNA, the researchers strongly associated EBV-DNA with GC alone or in combination with Hp, similar to our results. In the study of Shukla *et al*. (2011), Hp and EBV co-infection was detected in 40% of the population with general gastroduodenal pathology, and in 54% of the GC group. On the other hand, they found the control group to be 23% in NUD Hp + cases and reported that there was a strong statistical difference (p < 0.001) between them. Their results were in agreement with our results. In 2012, EBV-DNA was detected in 40 (80%) GC cases by Shukla *et al*. (2012) while EBV-DNA was detected in the biopsy samples of 36 (30%) cases in the control group NUD, and they suggested that a highly significant difference (p < 0.001) existed. Shukla *et al*. (2012) suggested that a possible alternative explanation for the higher EBV loads in GC tissues may be due to a simple increase in the number of EBV positive cells, rather than lytic replication. Different studies have suggested that Hp and EBV coinfection may be synergistically more effective in gastric carcinogenesis by playing an active role in modulating the transformation of EBV from the latent to the lytic phase in gastric tissues (de Souza *et al.,* 2014; Saxena *et al*., 2008; Shukla *et al*., 2011). In 2019, del Moral-Hernández *et al*. (2019) conducted a study involving 32 GC and 106 chronic gastritis cases, and found 37.5% (12/32) of the patient group with GC. Their results were similar to the results of our study (35.3%). Hp/EBV coinfection was detected in 25.5% (27/106) of cases with chronic gastritis, and no significant difference between the two groups (p > 0.05) was found. In conclusion, the researchers suggested that the different rates in the presence of EBV in GC cases may be due to differences in the sensitivity and specificity of the molecular methods used in the studies, the diagnostic processes for the studied cases, and the geographical region.

Castenada *et al*. (2019) reported EBV and Hp detection in 40 (10.7%) of 375 patients with GC and in two (1.2%) of 165 patients with chronic gastritis; the difference between them was statistically significant (p < 0.001), and the estimated risk coefficient OR value was significantly (p < 0.011) similar but less so than shown by results. The researchers concluded that Hp and EBV coinfection caused a synergistic increase in Th17-induced proinflammatory interleukin IL-17 cytokine expression and could also have induced a chronic inflammatory process that could damage the gastric mucosa. At the same time, inducing the synthesis of monochloramine would enable Hp to transform EBV from the latent to the lytic stage and cause gastric pathologies. In 2008, in a study conducted by Lima *et al*. (2008) for detecting Hp and EBV co-infection (targeting gene EBER) in gastric pathologies, 71 GC cases were examined and, among the cases, 50 were men and 21 were women. The average age was 56.5 years. Among the 71 patients selected, males dominated with 70.4% (50/71) and 49.3% (35/71) over 65 years old. This result is consistent with our result, which found significant Hp/EBV co-infection in patients over 55 years of age with GC. In our study, age over 55 was also identified as an independent factor that increases the risk of GC in multivariate analysis (OR: 2.331, p = 0.042). Although the mechanisms controlling this synergistic interaction are not fully known, various ideas on this issue can be found. For example, in the presence of an Hp infection, monochloramine, an oxidant produced in the stomach, may induce the transformation of EBV from the latent to the lytic phase. Taken together, all of these data may indicate that the progression of EBV to GC occurs via direct or indirect mechanisms: (1) after infection of epithelial cells by expressing viral oncogenes in latent phase and (2) local viral loads due to tissue damage by increasing chronic inflammatory responses (Martínez-López *et al*., 2014).

In hypotheses based on some mechanisms regarding the role of co-infection in gastric carcinogenesis, it has been suggested that an increase in inflammatory responses in Hp and EBV co-infection exist (Castaneda *et al*., 2019). Another hypothesis is based on gene products interaction (phospholipase C [PLCγ] signaling pathway) (Singh *et al*., 2017), and small heterodimer proteins (SHP1 and 2) (Saju *et al*., 2016). A third hypothesis is based on Hp-associated oxidant monochloramine (NH_2Cl) in gastric epithelial cells (Minoura-Etoh *et al*., 2006). On the other hand, it has been suggested that pro-inflammatory cytokines that become active during the development of Hp-induced gastric inflammation may cause an increase in EBV proliferation. For example, Hp-induced interferon γ (IFN-) provides an inflammatory environment that increases disease severity (Allison *et al*., 2013). Including IL-1β, tumor necrosis factor α (TNF-α), and IL-8 together with IFN-, IL-6, and IL-13 cause an increase in EBV proliferation and development of severe gastritis associated with EBV and Hp co-infection. This process also induces the production of high levels of proinflammatory cytokines (Cárdenas-Mondragón *et al*., 2013). Based on these observations, IFN-γ levels in the plasma of

patients with GC correlate positively with the level of EBV reactivation (Cárdenas-Mondragón *et al*., 2017).

It has been suggested that Hp causes further enhancement of the inflammatory response in chronic gastritis with proinflammatory cytokines and induces the gastric Th17 response via HP0175 protein, a peptidyl prolyl cis-trans-isomerase. In Hp and EBV co-infections, Hp and EBV can act synergistically and cause an increase in IL-17 expression, leading to an inflammatory process that severely damages the gastric mucosa (Amedei *et al.,* 2014).

It is also known that Hp can cause reactivation of EBV in the latent phase into the lytic phase after formation of monochloramine (NH_2Cl). The reactivation of EBV causes more infiltration of B cells loaded with viral particles, which increases the likelihood of epithelial cell infection. Interestingly, tissue samples with EBV and GC show SHP1 hypermethylation with reduced SHP1 expression, and infection of gastric epithelial cells with EBV induces SHP1 promoter hypermethylation, which enhances the phosphorylation-dependent CagA effects (Saju *et al*., 2016). In an *in-vitro* study, it was reported that EBV reactivation occurs via PLCγ signaling and Hp CagA strongly activates PLCγ. As a result of activation and PLCγ signaling pathway induction, EBV reactivation occurs in infected B cells, and both infectious agents activate the-catenin/T cell lymphoid enhance factor (catenin/TCF-4) pathway, which are transforming factors in stomach cells (Singh *et al*., 2017). Saju *et al*. (2016) demonstrated that the SHP1 protein, a host phosphatase, dephosphorylates Cag A, thus preventing its oncogenic activity. The SHP1 protein is a negative regulator of Cag A phosphorylation. However, Hp and EBV co-infection causes methylation of SHP1 and prevents it from dephosphorylating Cag A, thus leading to an increase in the oncogenic potential of Cag A. Phosphorylated CagA with tyrosine interacts with SHP2 (protein with oncogenic properties), after which SHP2 displays oncogenic properties. On the other hand, infection of gastric epithelial cells with EBV disrupts CagA tyrosine dephosphorylation with SHP1 and induces epigenetic silencing of SHP1; consequently, CagA interacts more with SHP2 and causes the pathway to shift toward more SHP2. This process then causes an increase in oncogenic activity.

Some limitations of this study can be noted. We can state that the current number of cases in our study was insufficient to reach a statistically relevant result. In addition, the lytic or latent stage of EBV could not be analyzed in the presence of Hp infection, and due to ethical concerns biopsies could not be obtained from people with a normal gastrointestinal system. The fact that Hp and EBV co-infection in healthy people cannot be compared with co-infection in patients with gastric pathologies is the most important limitation of this and other similar studies. The PCR method cannot distinguish between EBV in epithelial cells and EBV infiltrating into tissue samples from EBV-infected

lymphocytes. In addition, although we found EBV/EBER-1 positivity in tissues with GC, we could not verify it by *in-situ* hybridization due to insufficient funds.

Although we had a small number of GC cases in our study, it can be claimed that the estimated risk created by the synergistic effect based on the addition of EBV increased 3.3 times in the presence of Hp in GC. However, it may not be correct to state this very assertively and precisely with these few fact-control data, and it is still early to reach a plausible conclusion with this minimal amount of data. 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 think that the clear definition of the synergy between Hp and EBV with all its aspects has important implications for managing the diagnosis, prognosis, and treatment of GC and gastroduodenal patient populations in different geographic regions and for identifying individuals with a high risk of possible polymicrobial-based GC. EBV seropositivity and the risk of contracting EBV in Turkey and other countries are extremely high. Defining the combination of Hp and EBV in GC pathogenesis may have particularly strong predictive potential that can lead to the development of non-invasive tests for the vast majority of individuals infected with both Hp and EBV. We suggest that new data that may emerge as a result of this study and similar studies investigating Hp and EBV coinfection on this dual etiopathogenesis proposed in the development of GC and DU may change views in prediagnosis and treatment approaches toward possible polymicrobial pathogenesis of gastroduodenal diseases. However, Hp and EBV coinfection may not be very common in different populations. In addition, we suggest that our preliminary research data will encourage the development of large-series case-control study models based on new or developed methods which will be aimed at demonstrating the molecular-based physiological possible synergistic (cross-talk) relationship of Hp and EBV inside and outside the gastric epithelium and their mutual interactions with host immune responses.

Conflicts of interest: The authors declare no conflicts of interest

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Tables

Table 1. Primer sets and primer sequences used in the EBER and GAPDH Real-time PCR study

Primer or Probe Name	Sequence	Tm (°C)	GC (%)
EBER-Forward	GAG GTT TTG CTA GGG AGG AGA	58	52
EBER-Reverse	CGG ACC ACC AGC TGG TA	58	65
GAPDH-Forward	ACA CTC ACT CTT CTA CCT TTG	60	52
GAPDH-Reverse	CAA ATT CAT TGT CGT ACC AG	60	58

Table 2. Demographic data and laboratory findings (endoscopic, histopathological and microbiological findings) of the study and control groups

Demographic Data and Laboratory Findings	Study Groups		Control Group
	Gastric Cancer (GC)	Peptic Ulcer Disease (PUD)	Non-Ulcer Dyspepsia (NUD)
Subject (n)	34	30	40
Mean Age (min-max)	67.71 ± 8.91 (43-79)	60.07 ± 14.76 (26-90)	60.28 ± 12.30 (35-89)
Gender Male (%) Female (%)	22 (64.7%) 12 (35.3%)	12 (40%) 18 (60%)	18 (45%) 22 (55%)
Site of Involvement n(%)	Diffuse Type 8/34 (23.5%) Intestinal Type 26/34 (76.4%)	-	-
Peptic Ulcer Site	-	Gastric Ulcer 30/30 (100%) Duodenal Ulcer 0/30	-
Hp (+) (n;%)	34 (100%)	30 (100%)	40 (100%)
***EBV/EBER-1 GENE REGION* (+) (n%)**	12 (35.2%)	2 (6.6%)	1 (2.5%)
***EBV/EBER-1 IgG*(+) (n%)**	26 (76.4%)	25 (83%)	35 (87.5%)

Table 3. EBV/EBER-1 gene copy numbers of study and control group cases.

Study and Control Group cases	EBV/EBER-1 Copy Number Log_{10} (copy/100000 cell)	GAPDH Copy Number Log_{10}
GC 01	4.69	3.60
GC02	ND	2.68
GC03	ND	2.72
GC04	ND	3.00
GC 05	3.67	2,98
GC06	ND	2.76
GC 07	3.61	2.92
GC08	ND	2.83
GC09	ND	2.75
GC10	ND	2,89
GC 11	3.53	2.94
GC 12	4.59	2.87
GC13	ND	2.84
GC 14	3.14	2,86
GC 15	3.27	2.88
GC16	ND	2.93
GC17	ND	3.29
GC 18	4.60	3.57
GC19	ND	3,74
GC20	ND	2.83
GC21	4,45	3.39
GC22	ND	3.26
GC23	ND	2.50
GC24	ND	2.60
GC25	ND	3.45
GC26	ND	2.94
GC27	4.58	3.51
GC28	ND	3.59
GC29	4.31	3.37

GC30	ND	2.75
GC31	ND	2.41
GC32	ND	2.53
GC33	4.08	2.91
GC34	ND	2.83
PU01	ND	2.80
PU02	ND	2.68
PU 03	1.62	3.98
PU04	ND	2.57
PU05	ND	2.82
PU06	ND	2.91
PU07	ND	2.89
PU08	ND	3.18
PU09	ND	2.74
PU10	ND	3.39
PU11	ND	2.39
PU12	ND	3.66
PU13	ND	3.92
PU14	ND	2.94
PU15	ND	2.93
PU16	ND	3.08
PU17	ND	2.89
PU18	ND	3.12
PU19	ND	3.50
PU20	ND	2.83
PU21	ND	3.05
PU22	ND	2.72
PU 23	2,94	3.40
PU24	ND	2.77
PU25	ND	2.89
PU26	ND	2.67
PU27	ND	2.75
PU28	ND	2.72

PU29	ND	2.71
PU30	ND	2.91
NUD01	ND	2.30
NUD02	ND	3.81
NUD03	ND	2.48
NUD04	ND	3.20
NUD05	ND	2.74
NUD06	ND	2.80
NUD07	ND	2.63
NUD08	ND	2.77
NUD09	ND	2.81
NUD10	ND	2.60
NUD11	ND	2.84
NUD12	ND	3.21
NUD13	ND	2.80
NUD14	ND	2.73
NUD15	ND	2.44
NUD16	ND	2.67
NUD17	ND	2.80
NUD18	ND	2.51
NUD19	ND	2.52
NUD20	ND	2.30
NUD21	ND	3.84
NUD22	ND	2.91
NUD23	ND	2.39
NUD24	ND	3.14
NUD25	ND	2.65
NUD26	ND	2.48
NUD27	ND	3.34
NUD28	ND	2.74
NUD29	ND	2.45
NUD30	ND	2.53
NUD 31	2,44	3.90

NUD32	ND	2.64
NUD33	ND	2.48
NUD34	ND	2.77
NUD35	ND	2.68
NUD36	ND	2.65
NUD37	ND	2.76
NUD38	ND	2.85
NUD39	ND	2.49
NUD40	ND	2.63

*ND: Not Detected

Table 3. Qualitative comparison of the presence of EBER-1 gene in study and control groups.

EBV-*EBER-1* gene presence	Study Groups		Control Group	*P* value
	GC (n:34) n (%)	PUD (n:30) n (%)	NUD (n:40) n (%)	
EBER-1 gene (+)	12 (35.3%)	2 (6.6%)	1 (2.5%)	GC x NUD **p=0,0001*** PUD x NUD **p= 0.573****
EBER-1 gene (-)	22 (64.7%)	28 (93.4%)	39 (97.5%)	
Total	34	30	40	
*Chi-square Test; χ²; 13.649 ***Fisher's Exact Test*				

*Chi-square Test; χ^2; 13.649

**Fisher's Exact Test

Table 4. Quantitative comparison of the study and control group for EBER-1 gene copy numbers.

Study and Control Groups	n	EBV-RNA copy numbers Log$_{10}$ (Mean ± SE)	EBV-RNA copy numbers Log$_{10}$ (Min.-Max.)	*P* value
Gastric Cancer (GC)	34	3.85 ± 4.15	ND - 4.68	p < 0.001* GC x NUD; p < 0.001**
Peptic Ulcer Disease (PUD)	30	1.48 ± 2.20	ND - 2.94	PUD x NUD; p=1.000
Non-Ulcer Dyspepsia (NUD)	40	0.84 ± 1.64	ND – 2.44	
Total	104	3.37 ± 3.93		
	*Kruskal Wallis non-parametric test; **Post Hoc Dunn Test, ND; not detected, SE; Standard Error			

Table 5. 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=34) (n;%)	Peptic Ulcer Disease (PUD) (n=30) (n;%)	Non-Ulcer Dyspepsia (NUD) (n=40) (n;%)	
EBNA-1 IgG (+)	26 (76.5%)	25 (83.7%)	35 (95%)	X^2= 1.574 $P = 0.455^*$
EBNA-1 IgG (-)	8 (23.5%)	5 (16.3%)	1 (5%)	
Total	34	30	40	
*: Chi-Square Test				

Table 6. Qualitative comparison of GC, PU group 55 years old and NUD ≥55 years old in terms of the presence of EBER-1 gene region positivity.

EBER-1 gene presence	Study Groups		Control Group	*p* value
	Gastric Cancer (GC) (n=32) (n;%)	**Peptic Ulcer Disease (PUD) (n=22) (n;%)**	**Non-Ulcer Dyspepsia (NUD) (n=27) (n;%)**	
EBER-1 gene (+)	12 (37.5%)	2 (9.1%)	-	GC x NUD p=0.0001* PU **x** NÜD p= 0.196**
EBER-1 gene (-)	20 (62.5%)	20 (90.9%)	27 (100%)	
Total	32 (100%)	22 (100%)	27 (100%)	
*Chi-square test, x^2 : 12.710, p = 0.0001 **Fisher exact test				

Table 7. 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)	0.415	0.245	0.091	1.514	0.936- 2.448
Age (≥55 years)	0.846	0.417	0.042	2331	1.030-5.277
EBNA IgG positivity	-0.230	0.304	0.449	0.794	0.437-1.442
EBER-1 gene positivity	1.200	0.360	0.001	3.319	1.639- 6.720
Constant	-0.380	0.540	0.481	0.684	

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