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ORIGINAL ARTICLE

Epstein-barr virus/*Helicobacter pylori* coinfection and gastric cancer: the possible role of viral gene expression and shp1 methylation

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ABSTRACT

Background and Objectives: Among the various factors involved in the development of gastric cancer (GC), infectious agents are one of the most important causative inducers. This study aimed to investigate the possible role of EBV gene expression on SHP1 methylation in co-infection with *Helicobacter pylori* in patients with GC.

Materials and Methods: Formalin-fixed paraffin-embedded samples were obtained from 150 patients with gastrointestinal disorders. The presence of the *H. pylori* and EBV genome were examined by PCR. The expression level of viral gene transcripts and methylation status of the *SHP1* cellular gene was assessed by quantitative real-time PCR and methyl-specific PCR.

Results: EBV and *H. pylori* coinfection were reported in 5.6% of patients. The mean DNA viral load was significant in patients coinfected with *cag*A-positive *H. pylori* (P= 0.02). The expression of BZLF1 and EBER was associated with GC. Also, the expression level of BZLF1in GC tissues was significantly higher in coinfection (P = 0.01). SHP1 methylation frequency was higher in the GC group than in the control group (P = 0.04). The correlation between the methylation rate and the *H. pylori* infection was highly significant (P<0.0001). The strongest positive correlation was observed in GC specimens between SHP1 methylation and *H. pylori cag*A-positive strains (p= 0.003).

Conclusion: Our results suggested that *cag*A might involve in the elevation of EBV lytic gene expression and SHP1 methylation, and the development of gastric cancer. Understanding the mechanism of EBV *H. pylori* - *cag*A + coinfection, as well as host epigenetic changes, can play an important role in diagnosing and preventing gastric cancer.

Keywords: Epstein-barr virus; *Helicobacter pylori* coinfection; Viral gene expression; SHP1 methylation; Gastric cancer

INTRODUCTION

Gastric cancer (GC) is one of the most prevalent types of cancer and the third leading cause of cancer-related mortality worldwide (1). The Agency for Research on Cancer (GLOBOCAN, 2018) estimated that the prevalence of GC in Iran is 25.37 cases per 100,000 people (2). *Helicobacter pylori (H. pylori)*

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and Epstein-Barr virus (EBV) are known as group I carcinogens that have enhanced the risk of GC (3). *H. pylori* is infecting more than 50% of the world's population, although only 5% of infected individuals develop gastric cancer (4). Among *H. pylori* virulence factors, CagA and VacA can alter cell function and promotes cell autonomy to malignant transformation (5). EBV is involved in several types of human epithelial and lymphoid cancers. The prevalence of EBV-associated GC (EBVaGC) is between 2 and 20% (with a global average of 10%). EBVaGC belongs to latency type I or II, in which EBERs, EBNA-1, BARTs, LMP-2A, and BART miRNAs gene expression in epithelial cells can alter the homeostasis of host gene expression (3). *In vitro* studies in an established coinfection model system using human gastric epithelial cells have shown that *H. pylori*-EBV coinfection is more severe in comparison to alone infection by *H. pylori* or EBV (6).

The viral protein also affects cellular processes by dysregulating cell cycle regulation, inflammation, angiogenesis, and hypermethylation (7, 8). It has been shown that coinfection of *H. pylori* and EBV can affect the epigenetic modification of host cells (9). Accordingly, SHP1, a host cell phosphatase, is present in the gastrointestinal linings. SHP1 forms a complex with CagA and counteracts phosphorylation-dependent *H. pylori* CagA actions (10). *In vitro* infection of gastric epithelial cells with EBV induces epigenetic modification of SHP1 by promoter hypermethylation of the SHP1-encoding gene, which enhances phosphorylation-dependent CagA action (11). However, there is a paucity of studies that describe the relationship between SHP1 and EBV/ *H. pylori* coinfection in clinical specimens. Therefore, we evaluated the expression levels of viral latent gene (EBNA-1), lytic gene (BZLF-1), and EBV-encoded RNA 1 (EBER1) in GC clinical specimens using Real-time RT-PCR. In addition, we determined the correlation of clinicopathological findings and *H. pylori* infection on the expression of several EBV transcripts. Furthermore, we examined the correlation between EBV and *H. pylori* pathogenic factors with SHP1 methylation.

MATERIALS AND METHODS

Patients and samples. This study was performed on 150 formalin-fixed paraffin-embedded (FFPE) clinical samples from patients attending endoscopy service at the referral Al-Zahra Hospital (Isfahan, Iran) and referral Pathobiology Center (Mashhad, Iran). Clinical diagnosis was conducted based on histology, endoscopy, and clinical presentation. A representative formalin-fixed tissue specimen embedded in paraffin blocks was selected, and histological sections were examined by an experienced pathologist and graded based on Lauren's classification (9). Demographic and clinical data were obtained from the medical records. Exclusion criteria were as follows: the previous history of cancer, any kind of *H. pylori* treatment, including the use of antibiotics, non-steroid anti-inflammatory drugs, or proton pump inhibitors during a month before endoscopy. None of the patients received chemotherapy or radiation therapy.

Ethics approval and consent to participate. Ethics approval was obtained from the Ethics Committee of Isfahan University of Medical Sciences (IR. MUI. Rec. 1396.3765). Informed consent was obtained from all subjects involved in the study. All methods in this study were carried out in accordance with international relevant guidelines and regulations of ethics in human research.

DNA extraction. FFPE tissues were dewaxed/deparaffinized by the procedure including 3 washes in xylene for 3 minutes followed by 3 washes in 99.8% ethanol for 3 minutes. Genomic DNA was extracted from paraffin tissue using the QIAmp DNA FFPE Tissue Kit (Qiagen GmBH, Hilden, Germany) according to the manufacturer's protocol. DNA extraction from the B-95.8 cell line (ATCC: CRL-1612, Pasteur Institute, Iran) was performed by the phenol-chloroform method and used as a positive control (12).

Detection of *H. pylori* and determination of *cag*A positive strains. PCR was performed on the extracted DNA using primers specific for the *H. pylori ure*A gene. All primers used in this study are listed in Table 1. Each 25 µl PCR reaction mixture contained PCR master mix (2×PCR master mix red (Ampliqon, Denmark), 25 pM of each specific primer, and 200 ng of the template DNA. The reaction conditions were as follows: 95°C for 5 minutes, followed by 30 cycles of 95°C for 30 s, 59°C for 45 s, 72°C for 1 min, and a final extension at 72°C for 5 minutes. The *cag*A gene in *H. pylori*-positive samples was detected by PCR using primers and the amplification program as de-

scribed by Estaji et al. (13). For each PCR reaction, *H. pylori* (ATCC 26695) was used as a positive control.

Identification and quantification of EBV genome. For detection of EBV, SYBR Green Real-Time PCR was performed by targeting nonpolymorphic EBNA-1 using specific primer sets (14). Each 25 µl PCR reaction contained 1× RealQ Plus Master Mix (Green High ROX, Amplicon, Denmark), 200 nM of each specific primer (Table 1), and 200 ng of the extracted DNA. The Real-Time PCR was carried out on a Corbett Research 6000 Real-Time PCR instrument and Rotor gene 6000 software (Qiagen, AG Hilden, Germany) under the following conditions: 95°C for 3 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 1 min. Melting curve analysis was done on amplified products to verify the specificity of the amplicons. Each PCR run contained two non-template controls (NTC) in which nuclease-free H_2O was used instead of the DNA template to check for amplicon contamination.

EBV DNA quantification was determined by the absolute quantitation method, as described previously (15). A standard curve was generated using 10-fold dilutions of Namalwa DNA (C126, Pasteur Institute, Iran) equivalent to 5×10^4 - 5×10^1 copies of EBV DNA. The standard curve was accepted when the correlation coefficient was at least 0.99 and if a difference of 3.3 ± 0.3 cycles was demonstrated between each of the 10- fold dilutions. For clinical samples, the quantification results were derived from the standard curve. Experimental samples were run in duplicate, and a mean viral load was calculated. A Real-Time PCR of GAPDH (internal control) was run in parallel for each tissue sample to control the efficacy of DNA extraction and to normalize the number of cells amplified in each reaction. EBV viral load in paraffin-embedded tissue was calculated based on the ratio of the number of EBV copies to the GAPDH in a given volume of the extracted DNA. The resulting ratio was then multiplied by 100,000 to provide the number of copies of EBV per 100,000 cells (16).

RNA extraction and cDNA synthesis. RNA was extracted from EBV-positive paraffin-embedded tissues using the RNX-Plus (Cinnagen, Iran, Cat. No. RN7713C) according to the manufacturer's instructions. Subsequently, RNA was treated with RNase-free DNaseI (GeneAll, Seoul, Korea) to eliminate DNA contaminations by the manufacturer's protocol. The extracted RNA was evaluated by a nanodrop 2000 spectrophotometer (NanoDrop 2000, Thermo Scientific, Wilmington, DE).

Quantitative RT-PCR. A quantitative RT-PCR was used for the detection of BZLF1, EBNA1, and EBER1 in EBV-positive samples. The isolated RNA (1 µg) was reverse transcribed to cDNA with oligo-dT primers (Cinnaclone, Iran) using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific / Invitrogen, USA). The cDNA from the B95 cell line (ATCC: CRL-1612, Pasteur Institute, Iran) was used as a positive control.

The Real-Time PCR reaction was performed on a Corbett Research 6000 Real-Time PCR instrument and Rotor gene 6000 software (Qiagen, AG Hilden, Germany) in a final volume of 25 µl reaction containing 1× RealQ Plus master mix (Green High ROX, Amplicon, Denmark), 20 pM of each forward and reverse primers (Table 1), and 200 ng of cDNA template. The

Table 1. Primers used in this study

Gene	Primer (5'-3') forward	Primer (5'-3') reverse	Size	Tm	Ref.
UreA	AGTGGGTATTGAAGCGATG	TGCTTTCGTTGTCTGCTTG	395	59	13
*cag*A	AATACACCAACGCCTCCAAG	TTGTTGCCGCTTTTGCTCTC	400	59	34
EBV	CCGGTGTGTTCGTATATGGAG	GGGAGACGACTCAATGGTGTA	106	60	16
Shp1	M TGTGAACGTTATTATAGTATAGCG	M CCAAATAATACTTCACGCATACG	174	60	35
	U GTGAATGTTATTATAGTATAGTGTTTGG	U TTCACACATACAAACCCAAACAA	162	60	
EBNA-1	CCGGTGTGTTCGTATATGGAG	GGGAGACGACTCAATGGTGTA	106	60	19
EBER-1	AGGACCTACGCTGCCCTAGA	AAAACATGCGGACCACCAGC	160	60	18
BZLF-1	ACGCACACGGAAACCACAA	CTTAAACTTGGCCCGGCATT	112	60	15
GAPDH	CATGAGAAGTATGACAACAGCCT	AGTCCTTCCACGATACCAAAGT	113	58	36

M: methylated, U: unmethylated

thermocycling conditions were 10 min pre-incubation at 95°C, followed by 45 cycles of 95°C for 30 s, 58°C for 1 min. A Non-Template Control (NTC) in which nuclease-free H_2O was substituted for the template was prepared in parallel with the clinical samples to check amplicon contamination. All experiments were performed in triplicate. The GAPDH (reference) was used for normalization (17). The relative quantitation values were calculated by the Pfaffl method and normalized using the reference gene (18). Amplified products were analyzed by melting curve analysis to verify the specificity of the amplicons.

Bisulfite modification and methylation-specific PCR. To find out whether EBV influences DNA methylation patterns of the SHP1 promoter, methylation-specific PCR (MSP) was performed. The extracted DNA from 25 GC tissues and 25 control (non-GC) tissues were treated with sodium bisulfite according to the manufacturer's protocol (EpiTect Bisulfite Kit, Qiagen, Germany). During this process, all non-methylated cytosines are converted to uracil, but methylated cytosines remain intact. To detect methylated or unmethylated status at the SHP1 promoter, each PCR reaction was performed separately in 25 µl reaction volumes, containing PCR master mix (PCR master mix red, Ampliqon, Denmark), 0.5µM of each specific forward and reverse primers (Table 1), and 2ng of treated DNA. The reaction condition was as follows: 94°C for 2 min for initial denaturation, followed by 40 cycles of 94°C for 30 s, 60°C for 1 min, 72°C for 1 min, and final extension for 72°C for 10 min. Fully methylated and non-methylated DNA (Humdiagnostics, Tehran, Iran) were used as positive and negative controls, respectively. PCR products were separated on 2% agarose gel. The samples that provided a band with methylation-specific primers were marked as methylated (M). The tissues that only presented a band with primers of unmethylated DNA were scored as unmethylated (U). Each sample was tested independently three times.

Statistical analysis. Statistical analysis was performed using Graph Pad Prizm (version 6). Fisher's exact and Mann-Whitney test was used to analyze and test whether there was a difference between the 2 independent groups. We used the media and the standard deviation (SD) for all descriptive analyses. Data are expressed as the mean ± SD. $P < 0.05$ was considered statistically significant, adjusted by Bonferroni's correction test.

RESULTS

The profiles of study parameters. Table 2 summarizes the pathological and demographic information of the study patients (n=150), including age, gender, and coinfection with *H. pylori* based on EBV detection. Among 150 collected FFPE samples, 95 (63.3%) were male, and 55 (36.7%) were female. The mean age was 56.2 (± 18.2), ranging between 8-95 years. The relationship between gender, age, and EBV infection was not significant ($P>0.05$), (Table 2). In this study, the lowest and highest cancer stages were I (4%) and IVA (52%), respectively. The EBV and *H. pylori* DNA were found in 6% (9/150) and 59.3% (89/150) of tested samples, respectively. We divided samples into three groups according to histological diagnosis: gastritis, intestinal metaplasia, and gastric cancer (GC). All gastritis specimens were negative for EBV and used as control for comparison between groups. EBV mono-infection was found in 6% (3/50), *H. pylori* mono-infection was detected in 46% (23/50), and EBV/ *H. pylori* coinfection was detected in 6% (3/50) of 50 GC tissue samples. Out of 50 intestinal metaplasia tissue specimens, EBV mono-infection was detected in 2% (1/50), *H. pylori* mono-infection was detected in 52% (26/50), and EBV/ *H. pylori* coinfection was detected in 4% (2/50). The relationship between EBV-positive patients in GC patients as compared to patients with intestinal metaplasia was significant (OR=4.0, 95%CI= 1.9-18.7, P= 0.03). However, there were no significant relationship between the presence of *H. pylori* (OR = 0.6 95% CI = 0.3-1.3, P = 0.14), EBV/ *H. pylori* coinfection (OR = 2.51, 95% CI = 0.4-15.8, P = 0.31) with GC.

Quantification of EBV using Real-Time PCR. DNA viral load was measured in EBV-positive samples. The median copy number of EBV in our study population was 13697.5. The median DNA copy number in GC patients was higher as compared to intestinal metaplasia [18888.1 (IQR=10421.9- 27602.6) vs. 4196.5 (IQR=3679.9- 4784.1); $P<0.05$, Fig. 1A. The overall infection rate of EBV and *H. pylori* (coinfection) in the study population was 5.6%. The rate of coinfection was higher in patients with GC than in patients with intestinal metaplasia. In our study

Table 2. Comparison of the research parameters between EBV positive and negative groups.

Parameters	Total (150)	EBV (+) (9)	EBV (-) (141)	P
Age (±SD)	56.5 (18.2)	65.4 (20.4)	60 (14.8)	>0.05
Sex (%)				
Male	95 (63.3)	7 (7.3)	88 (92.7)	>0.05
Female	55 (36.7)	2 (3.6)	53 (96.4)	
Disease (%)				
Gastritis	50 (30.3)	0	50	
Metaplasia	50 (30.3)	3 (6)	47 (94)	0.05*
Gastric cancer	50 (30.3)	6 (12)	44 (88)	
H. pylori (%)				
Presence	89 (59.3)	5 (5.6)	84 (94.4)	>0.05
H. pylori (%)				
cagA (+)	54 (60.7)	3 (5.5)	51 (92.5)	>0.05
Gastric cancer (%)				
Mono EBV	3/50 (6)			0.03
Mono HP	23/50 (46)			0.14
Coinfection	3/50(6)			0.3
Non EBV/HP	21/50(42)			
Stage of Cancer				
I	1	0	1	
IIA	5	1	4	
IIB	9	0	9	
IIC	2	0	2	
IIIA	8	1	7	0.012
IIIB	7	0	7	
IIIC	13	2	11	
IVA	0	0	0	
IVB	0	0	0	
ND	5	2	3	

*Significant (between gastritis and GC); HP, *H. pylori*; ND Not determined; NA Not applicable

population, the difference between the median copy number of EBV DNA in *H. pylori*-positive samples and *H. pylori*-negative samples was not statistically significant: 15,637 (IQR: 5077.9 -32,347.3), 6919.1 (IQR: 3163.3-15298.3); (P =0.38), respectively (Fig. 1B). Although, the median copy number of EBV DNA in *H. pylori cag*A-positive patients was significantly higher than in *H. pylori cag*A-negative patients [29310.8 (IQR: 22477.9- 41456.7) vs. 6919.1 (3938.2-10422), P= 0.02]. The EBV load was compared between *H. pylori*-infected and non-infected patients in distinct disease groups. The EBV DNA viral load was higher in *H. pylori*-positive GC patients. Although the difference was slightly significant (P=0.046), Fig. 1C.

Expression levels of EBV latent and lytic genes. We investigated the expression level of latent and lytic EBV marker genes (EBNA1 and BZLF1, respectively) by quantitative Real-Time PCR. EBNA1 transcript was detected in all EBV-positive patients. The expression level of EBNA1 was increased in GC patients (20.9 ± 5.1) when compared to intestinal metaplasia patients (2.45 ± 0.05). However, the difference was not significant (P=0.055). The expression level of the BZLF1 gene in gastric cancer and intestinal metaplasia samples specimens was 11.08 ± 6.3, and 3.64 ± 0.05, respectively (P=0.03). The relationship between EBER expression level and GC was statistically significant (P=0.001), Fig. 2A. The lowest expression of BZLF1 and EBER genes were observed in stage IIB

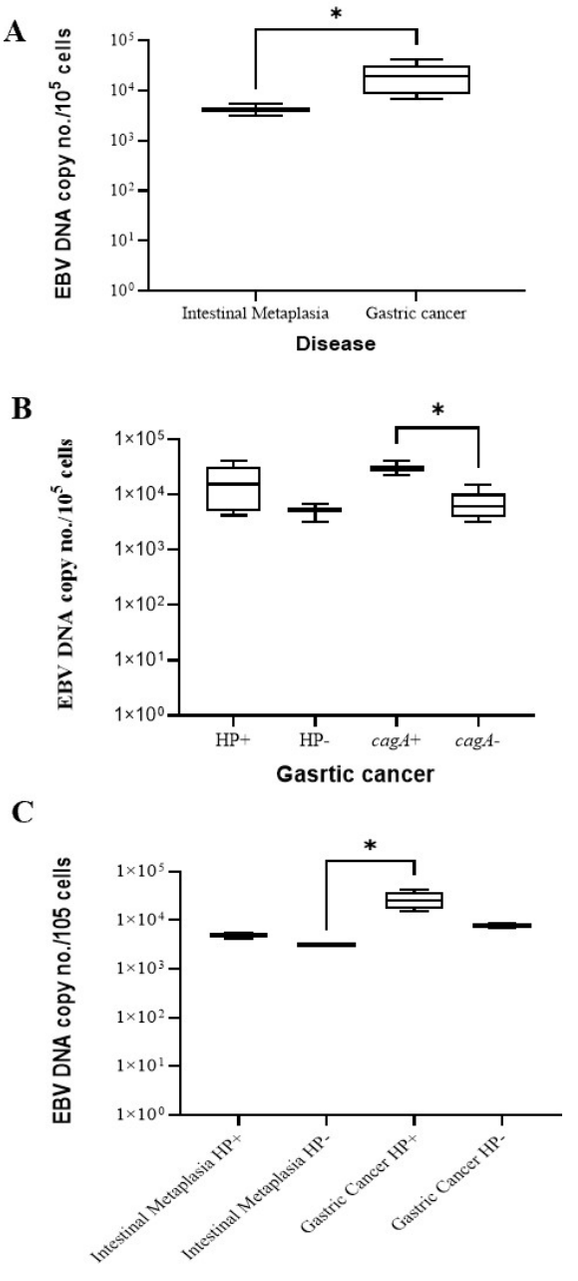

Fig. 1. Comparison of EBV viral load between (A) two groups of patients, (gastric cancer and intestinal metaplasia). (B) DNA viral load comparison in gastric cancer patients with *H. pylori*-positive (Hp+) vs. *H. pylori*-negative (Hp-), and *cag*A positive (*cag*A+) *H. pylori* vs. *cag*A negative (*cag*A-) *H. pylori* strains. (C) Increased EBV viral load in *H. pylori*-positive compared to *H. pylori*-negative patients. The lines inside the boxes denote the medians, the boxes denote the interquartile ranges, and the bars indicate the 10 and 90 percentiles.

and IIA samples, while the highest expression of these genes was seen in stage IIIA and IIIC, respectively (data not shown). The maximum expression level of investigated EBV genes was BZLF1 in stage IIA samples, and the EBER in stage IIA samples showed the lowest expression level of the examined EBV genes. Fig. 2B shows that patients infected with *H. pylori* (coinfection) had a higher expression level of BZLF1 when compared to the patients infected with EBV alone (P=0.032). However, the expression level of EBNA1 and EBER was not associated with *H. pylori* infection (P=0.09, and 0.049, respectively).

The expression level of viral transcripts and EBV DNA load was studied in EBV-positive samples. Particularly, we noticed a marked increase in the level of

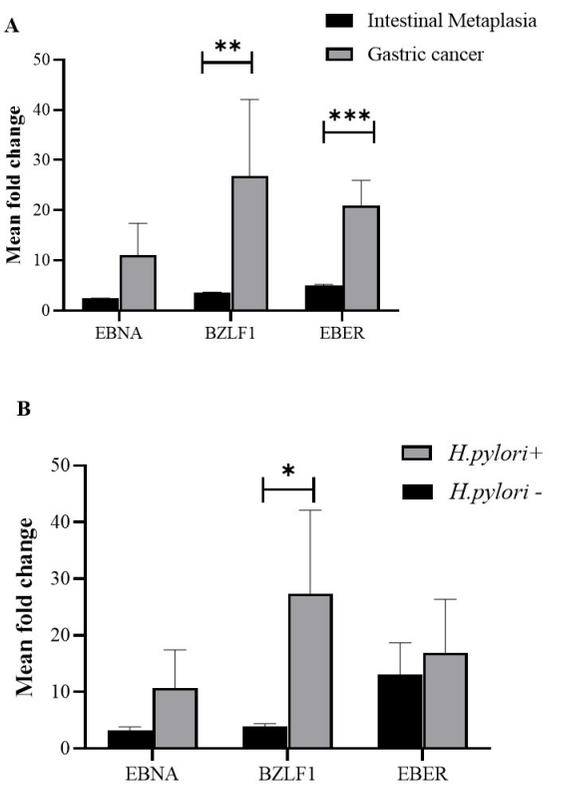

Fig. 2. Epstein–Barr virus gene expression concerning disease, and *H. pylori* infection. (A) The increased expression level of EBNA, BZLF1, and EBER transcripts in gastric cancer patients was significant compared to intestinal metaplasia. The relationship between EBER, BZLF1 expression level, and GC was statistically significant (P=0.001, 0.038). (B) Increased BZLF1 expression in *H. pylori*-positive significantly compared to *H. pylori*-negative patients (P=0.032).

BZLF1 (immediate early transcript) in GC patients with a viral load greater than 10,000 copies per 100,000 cells (Fig. 3A). Interestingly, BZLF1 expression in *H. pylori cag*A negative patients (including GC 1-3 and IM1,2 patients) was significantly lower when compared to *H. pylori cag*A positive patients (P=0.004). Those GC patients with viral loads greater than 10,000 EBV DNA copies per 100,000 cells (three samples) all express higher EBER (21.9-fold), BZLF1 (43.5-fold), and EBNA (17.6-fold), whereas in patients with viral loads less than 5,000 copies per 100,000 cells the expression level of viral transcripts was 17.2, 12.4, and 4.01-fold, respectively. BZLF1 gene expression is considered the most abundant viral transcript and was seen in all GC EBV-positive patients. Consistently, as shown in Fig. 3B, the ratio of lytic gene transcripts to the latent gene transcripts was analyzed. The results showed that EBV samples with higher DNA load had a higher lytic-to-latent ratio. To further study global differences in EBV gene expression patterns in EBV (+) samples, we performed a correlation analysis of the viral gene expression data and viral DNA loads for GC and Intestinal metaplasia samples (Fig. 3C and D). Consistently, BZLF1 and EBNA were found to group together. The BZLF1 and EBNA1 show the highest expression correlation in GC samples. In contrast, EBER shows a poor correlation with EBNA1 and BZLF1 expression in GC samples which suggests that EBER might not coregulate with the BZLF1 and EBNA1.

Methylation pattern of SHP1. Table 3 illustrates the association of SHP1 gene methylation status and patients' characteristics, *H. pylori*, *cag*A, EBV, and pathological features. The association between *cag*A positive strains and methylation was signifi-

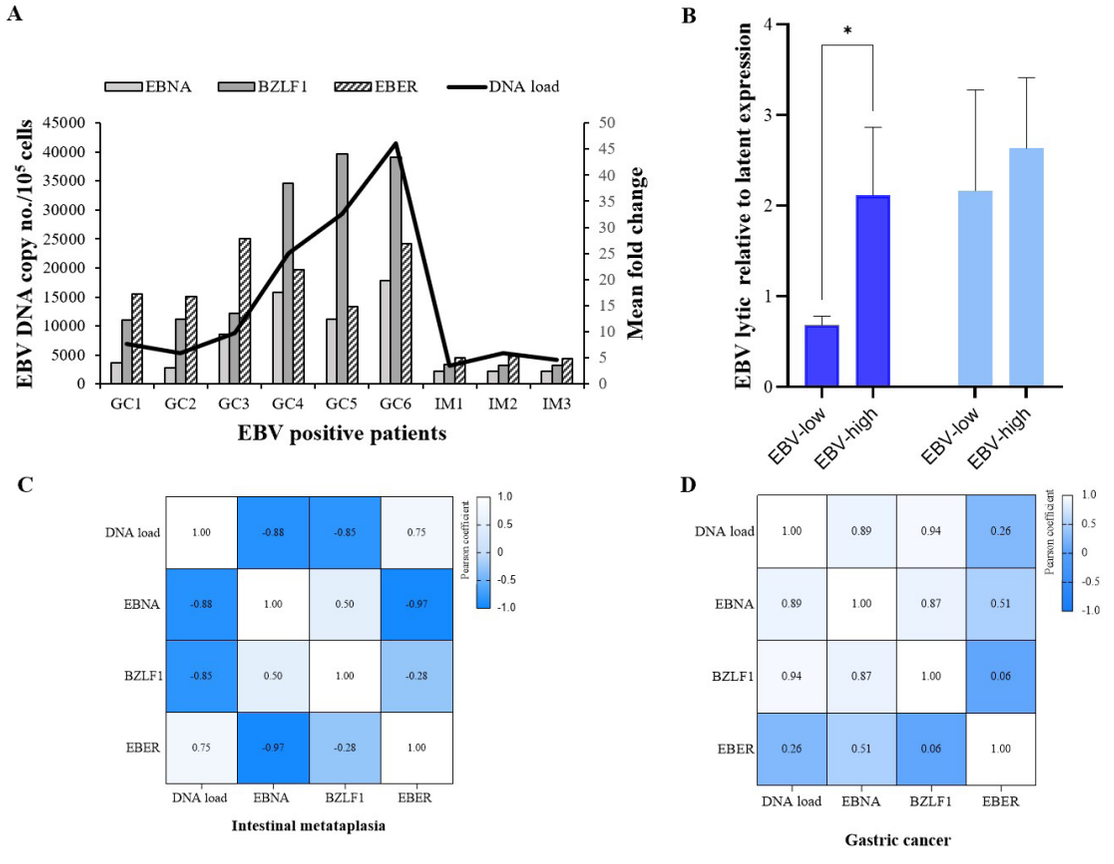

Fig. 3. EBV viral load relation with viral gene expression levels and type of disease. (A) A significant increase in BZLF1 expression was seen in GC patients with a viral load greater than 10,000 copies per 100,000 cells. (B) The ratio of EBV lytic (BZLF1)-to-latent (EBNA, EBER) expression in EBV-high and EBV-Low copy no. (C, D) A plot of the EBV gene expression pattern was determined by correlation analyses of the EBV(+) patients in intestinal metaplasia and gastric cancer patients, respectively.

cant (P=0.0001). We observed that the frequency of methylation in diffuse and intestinal types of GC was the highest with a tendency to the early stage of tumors that was decreased with increasing tumor stages. Moreover, based on Table 3, the association between histopathological types of tumors and SHP1 methylation was not statistically significant (P = 0.22). We compared the SHP1 methylation rate between the GC samples and the control group, and between EBV/ *H. pylori*-positive samples with EBV/ *H. pylori*-negative samples. The result is shown in Tables 4 and 5. SHP1 methylation frequency was higher in the GC group than in the control group (P = 0.04). The results in Table 4 showed that the correlation between the methylation rate and the *H. pylori* infection was highly significant (P<0.0001).

Furthermore, in GC specimens, the strongest positive correlation was observed between SHP1 methylation and *H. pylori cag*A positive strains (P= 0.003). To determine whether SHP1 methylation was the result of the effect of EBV- or *H. pylori* mono-infection, and/or EBV- *H. pylori* coinfection, we analyzed the SHP1 methylation status among the mentioned groups. In Table 5, the methylation pattern was compared between the co-infected and mono-infected samples. Interestingly, we found that the SHP1 methylation rate in *H. pylori* mono-infection, and *H. pylori cag*A positive groups were higher when compared to the coinfection group. We compared EBV gene expression level and SHP1 methylation between the mono-infection and coinfection groups, and second between methylation and unmethylation groups in each

Table 3. Associations between SHP1 methylation status and patient characteristics, *H. pylori*, EBV infection, and clinical features.

Characteristics	SHP1			
	Methylation (+)	Methylation (-)	P Value	OR (95% CI)
Mean age (year)	54.2 ± 21.9	60.7 ± 18.4	0.57	-
Age group (%)				
<50	10 (41.6) [a]	11 (42.3) [c]	0.00001*	0.7
>50	14 (58.3) [b]	15 (57.7) [d]		(0.3-3)
Gender (%)				
Male	13 (54.2)	18 (69.2)	0.38	0.53
Female	11 (45.8)	8 (30.8)		(0.2- 1.7)
H. pylori (%)				
*cag*A+	15 (88.2)	2 (14.3)	0.0001	45
*cag*A-	2 (11.8)	12 (85.7)		(5.5-368.1)
EBV (%)	6 (25)	3 (11.5)	0.28	2.56
Gastric cancer (%)				(0.6-11.7)
Diffuse	7 (46.7)	5 (50)		
Intestinal	6 (40)	4 (40)	0.22	ND
Mixed	2 (13.3)	1 (10)		
Stage of cancer				
I	0	1		
IIA	4	1		
IIB	0	1		
IIIA	3	0	-	ND
IIIB	1	2		
IIIC	4	3		
IVA	0	0		
IVB	0	0		
ND	3	2		

*One-Way ANOVA: comparison between group a versus group b, and comparison between group c versus group d, was meaningful, P=0.00000. HSD for pairwise comparison by Post Hoc Tukey method. ND: not determined.

Table 4. Comparison of SHP1methylation pattern between the EBV, *H. pylori*- positive and negative samples

	SHP1 methylation pattern					
	Control group (%)	**ŧp**	**GC group (%)**	**ŧP**	**Total**	**P**
Methylation						
+	9 (36)	0.05	15 (60)	0.16	24 (48)	<0.05
-	16 (64)		10 (40)		26 (32)	
H. pylori						
+	8 (88.9)	0.001**	12 (80)	0.02*	20 (40)	<0.0001***
-	1 (11.1)		3 (20)		4 (8)	
EBV						
+	2 (66.6)	0.43	6 (100)	0.7	8 (16)	>0.05
-	1 (33.4)		0 (0)		1 (4)	
*cag*A						
+	6 (71.4)	0.17	8 (90)	0.004**	14 (28)	<0.05
-	1 (28.6)		2 (10)		3 (6)	

FDR correction for multiple comparisons by Bonferroni correction method. ŧ test of proportions. *, **, *** significant.

Table 5. Comparison of SHP1 methylation patterns between the co- and mono-infection groups

Variable	**SHP1 methylation**		
	Gastric cancer group (n=15)	**Control group (n=9)**	**P**
Coinfection vs not coinfected	3 (20)	2 (22)	0.8
	12 (80)	7 (78)	
	p (0.01) *	p (0.04)	
Coinfection vs *Hp* mono-infection	3 (23)	2 (22.2)	0.8
	10 (77)	7 (77.8)	
	p (0.01) *	p (0.04)	
Coinfection vs EBV mono- infection	3 (50)	2 (88)	0.2
	3 (50)	1(12)	
	p (1)	p (0.4)	
Coinfection vs. non- *Hp* /non- EBV co-infection	3 (100)	2 (0.67)	0.3
	0	1 (0.33)	
	p (0.6)	p (0.05)	
*Hp cag*A+ vs	6 (66.7)	6 (100)	0.4
*Hp cag*A+ / EBV coinfection	3 (33.3)	0	
	p (0.01) *	p (0.00001) ***	

Hp: H. pylori; *, ***, significant. FDR correction for multiple comparisons by Bonferroni correction post hoc method.

infection type. The findings revealed that there was no significant mean difference between mono-infection and coinfection groups in either of the methylation types (Fig. 4). Likewise, a descriptive analysis of the gene expression level of EBV in the methylated SHP1 group showed no specific difference in the expression level of the assessed transcripts in the EBV mono-infection group as compared to the coinfected group in GC patients (Fig. 4)

DISCUSSION

Until now, most research in clinical findings unequivocally specified the EBV and *H. pylori* coinfection in GC (14). Furthermore, how *H. pylori* interact-

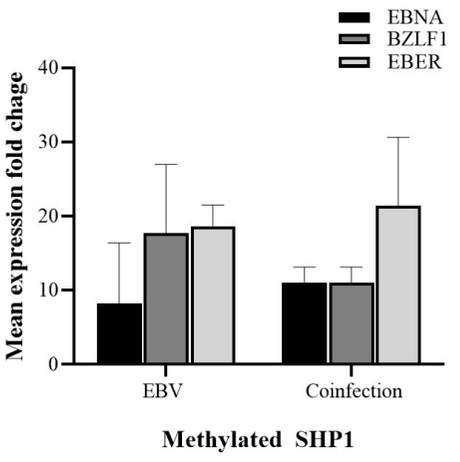

Fig. 4. Mean of EBV gene expression in mono-infection and coinfection groups in SHP1 methylated patients. (The variations in the data are represented through the illustrated SD).

ed with EBV and these pathogens target host factors and downstream pathways into the progression of aggressive GC is still undetermined. In this study, we investigated the EBV frequency of infection in patients with and without *H. pylori* infection in Iran and found the virus-*H. pylori* coinfections are more frequent in gastric cancer than gastritis, and the EBV frequency of infection in GC is lower than that reported in other populations. The pooled estimate of EBVaGC frequencies in North and South America, Asia, and Europe is 9.9%, 8.3%, and 9.2%, respectively (19). Other studies from Iran reported a frequency of 6.7-21% of EBV infections in patients with GC (20-22). A higher rate of coinfection in cancer patients than in the control group in our results supports the role of interaction of these two pathogens in the progression of gastric adenocarcinoma. Additionally, the presence of the EBV genome was positively associated with the detection of *H. pylori* virulence factor (*cag*A) in clinical specimens. de Souza et al. reported an association between the cytotoxic product of the *cag*A gene and EBV in clinical gastric adenocarcinoma samples and concluded that coinfections of *H. pylori-cag*A positive and EBV were correlated with the most advanced tumor stages (23). We showed that the mean DNA load of the virus in the *H. pylori*/ EBV-coinfected GC group was slightly higher than the control group. Notably, the coinfection of *H. pylori cag*A positive/EBV had a stronger association with the median copy number of EBV DNA than *H. pylori cag*A negative/EBV samples. Pandey et al. reported an elevated EBV DNA copy number as well as higher green fluorescent protein (GFP) expression in the presence of *cag*A-positive *H. pylori* in a coinfection model system (14). Our observational study in clinical samples confirmed the *H. pylori*-encoded secretory factor. enhance viral copy numbers and levels of infectivity.

We found that *H. pylori*/ EBV co-infected samples enhanced the expression of the lytic (BZLF1) gene, and the expression level was significantly associated with GC as compared to intestinal metaplasia. Few reports have compared BZLF1 expression levels in mono-EBV and EBV/ *H. pylori* coinfection in GC patients. Shukla et al. demonstrated that BZLF1 expression was significantly associated with GC and peptic ulcers compared to dyspepsia and that BZLF1 expression is higher in *H. pylori*- EBV coinfected patients (24). Particularly, those GC patients with EBV-high viral loads expressed higher BZLF1 as compared with EBV-low DNA loads. High viral load is a risk factor for the progression of EBV to malignancy and the expression of genes. High expression of BZLF1, an immediate early gene, supports the entry of the virus into the lytic phase, and that may increase the number of EBV-infected cells and the risk of development of malignancy. Expression of BZLF1 has been identified in nasopharyngeal carcinoma (NPC) and gastric carcinoma (GC). Most of these identifications analyzed the mRNA profiles of EBV-associated tumor cells (15, 25, 26). Interestingly, we found that BZLF1 expression in *H. pylori cag*A positive patients was significantly higher compared to EBV coinfection with *H. pylori cag*A negative subjects (P=0.004). Some studies detected EBV/ *H. pylori cag*A positive coinfection in cases of GC and concluded that the *H. pylori cag*A infection may have some effect on the development of EBVaGC (14, 27). However, these studies did not analyze the lytic or latent stage of EBV in presence of *H. pylori cag*A positive infection. To the best of our knowledge, this is one of few studies that analyzed the relevance of EBV early and late transcripts and *cag*A in gastroduodenal clinical samples. Previous reports cited that *H. pylori* is dependent on CagA to enhance EBV infection in gastric epithelial cells (10, 14). Moreover, it has been shown that protein kinase C (PKC) chiefly induces the expression of BZLF1 and the EBV lytic cycle *in vitro* (28), and CagA is a known activator of this kinase (29). It might conclude that CagA, could

trigger the EBV lytic cycle. However, further studies are needed to reach a clear-cut conclusion.

Evidence that EBV's lytic phase contributes to EBV oncogenesis is now increasing (30, 31). We investigated the epigenetic changes of SHP1 for the first time in GC patients coinfected with *H. pylori* and EBV. Our data demonstrated that the promoter region of SHP1 was methylated in 60% of cancer cases and that the SHP1 methylation rate in *cag*A positive *H. pylori* patients was significantly higher than in *cag*A negative samples. In EBV-positive and -negative GC groups, the difference in methylation levels was not significant. In the present study, it was observed that methylation in the SHP1 promoter was more frequent in the *H. pylori-cag*A+ group than in those with *H. pylori-cag*A+/EBV coinfection. This result suggests that extended activity of *H. pylori* CagA inhibits SHP1 and may consequently involve in the development of EBVaGC. However, due to the limitation of our study (especially the limited number of EBV-positive GC cases), a definitive association between gene expression data and SHP1 methylation in the EBV mono-infection and coinfection groups cannot be confidently established at this moment. Further study with the inclusion of more EBV (+) patients should be performed to solve this puzzle.

CONCLUSION

The current study demonstrated that the *H. pylori* /EBV co-infection, compared to mono-infection, might lead to more changes in the mean levels of EBV DNA and viral genes expression and progression of malignancies. BZLF1 expression in *H. pylori cag*A positive patients was significantly higher than *cag*A negative GC. It can be concluded that the *H. pylori* CagA oncoprotein may be involved in the methylation process of SHP1 methylation, and /EBV/ *H. pylori* coinfection is likely to affect other pathways involved in tumorigenesis more than mono-EBV or mono-*H. pylori* infection.

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