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Molecular Quantification of Epstein-Barr Virus Nuclear Antigen Gene and DNA Methylation Patterns of Human Tumor Suppressor Genes p16 and E-cadherin in Relation to Infection with Helicobacter pylori as Early Prognostic Biomarker for Gastric Tumorgenesis in Patients from Baghdad

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ABSTRACT

Objectives: *To investigate the association between EBV viral infectious dose, infection with EBV and/or H. pylori and histologically different gastric diseases and cancer. Also, to study the association between EBV and/or H. pylori infection with DNA methylation patterns of human tumor suppressor genes p16 and CDH1 in progression of primary gastric diseases to neoplasia.* ***Methods:*** *The current prospective cross-section study included a total of 94 GTPs taken from patients suffering from gastro-duodenal manifestations recruited to Gastro-Endoscopy Department at Gastroenterology and Hepatology Teaching Hospital, Baghdad, Iraq, from November 2017 to October 2018. Two gastric tissue biopsies (GTBs) were collected from those patients. Genomic DNA was extracted from fresh GTPs. Direct molecular identification of H. pylori in extracted DNA was performed by amplification of species-specific urea. Identification of Epstein-Barr nuclear antigen 1(EBNA1) in extracted DNA was performed using nested PCR. DNA samples positive to EBNA1 were submitted for viral load estimation using quantitative real time PCR. Methylation patterns of p16 and CDH1 promoters were detected in modified DNA samples by sodium sulfate using MS-PCR.* ***Results:*** *Of total samples, 39 (41.5%) of DNA samples were positive for H. pylori and 18 (19.15%) DNA samples were positive for EBNA-1. Studying EBV load, 8/23DNA samples were showed infectious dose of EBV. Studying methylation patterns of p16 and CDH-1 promoters, 21/42 and 19/42 DNA samples were provide results for MSP-PCR, respectively.* ***Conclusion:*** *Epstein-Barr virus and H. pylori infection may have a synergistic effect in developing different gastric diseases and that enable the clinician to choose the suitable treatment regime.*

Keywords: Epstein-Barr virus, Helicobacter pylori, Gastric cancer, p16 DNA methylation, E-cadherin DNA methylation

INTRODUCTION

Epstein-Barr Virus Nuclear Antigen-1 (EBNA1) is the only EBV protein involved in viral DNA replication and episome maintenance during the proliferation of latently infected cell. Its lack any enzymatic activities such as DNA helicase, therefore, EBV depends heavily on host cellular proteins to replicate its episomes [1]. Epstein-Barr virus strains have been classified into type 1 and type 2 based primarily on the sequence of their EBV Nuclear Antigen-2 gene (EBNA2). Type 1 EBV strains are prevalent worldwide [2].

Gastric cancer (GC) considers as the third most common cause of cancer associated mortality worldwide [3]. Almost, 95% of stomach neoplasms are adenocarcinomas [4]. The percentage of EBV associated GC (EBVaGC) was about 10% of GC cases. Despite the geographical proximity, GC rate varies from very low in Iraq and Egypt to intermediate

in Turkey to high in Iran [5-7]. Epstein-Barr virus associated GC had specific clinic-pathological features, includes male preference and commonly occurs in the upper and middle portion of the stomach [8]. Reactive oxygen species (ROS) induced by, or secreted from, activated inflammatory cells may enhance DNA damage and genomic instability in adjacent epithelial cells, which may generate clones of genetically altered precursors susceptible to latent EBV infection [9].

DNA methylation is the most extensively studied epigenetic modification in which a methyl group added to the fifth carbon position of cytosine residue in a cytosine followed by guanine at the same DNA strand (CpG) in human DNA, some viral DNA and some bacterial DNA [10]. Mainly, increased methylation in the promoter region of a gene leads to reduced gene expression, whereas methylation in the transcribed region has a variable effect on gene expression [11]. In EBV infection, restriction to latent infection is induced by the methylation of the episomal DNA of virus [12].

During latency, EBVaGC possess the most extensive methylation of CpG island motifs on both human and viral genomes [13]. The viral genome is silenced by host-driven methylation of CpG island motifs. Based on the subgroup of viral genes which are expressed, tumors caused by EBV classified into four types; latency Ia, Ib, II and III [14,15].

Helicobacter pylori is a group-1 carcinogen. In developed countries, *H. pylori* prevalence rate was determined as (20%-50%) as compared with developing countries (80%) [16]. Some studies considered co-infection with EBV and *H. pylori* as a risk factor for EBVaGC [17,18], while others suggested that *H. pylori* and EBV comprise different carcinogenic pathways [19,20]. Inflammatory cell infiltration by *H. pylori* infection might be a more imported factor for the induction of aberrant DNA methylation [21]. In human, CpG island methylation in the promoter region of various cancer-associated genes are consider as one of a major mechanisms of development and progression of gastric carcinoma [22]. In patients with GC whom co-infected with EBV and *H. pylori*, a high frequencies of promoter methylation were observed in various cancer-related genes such as p16 and E-cadherin (CDH1) [23,24].

The current study aimed to investigate the association between EBV viral infectious dose, infection with EBV and/or *H. pylori* and histologically different gastric diseases and cancer. Also, to study the association between infection with EBV and/or *H. pylori* and DNA methylation patterns of human tumor suppressor genes p16 and CDH1 in progression of primary gastric diseases to neoplasia.

MATERIALS AND METHODS

Study Design and Sampling

The current prospective cross-section study included a total of 134 patients (70 male and 64 female) suffering from gastro-duodenal manifestations recruited to Gastro-Endoscopy Department at Gastroenterology and Hepatology Teaching Hospital, Baghdad, Iraq, from November 2017 to October 2018. According to consultant physician instructions, those patients were submitted to clinical examination and endoscopy. Two gastric tissue biopsies (GTBs) were collected from those patients, one was placed in 10% formalin and sent to histopathological laboratory and the second one was placed in 1ml of normal saline and preserved at -20°C for molecular analysis.

Data was collected from each patient includes (name, age, gender, smoking, alcoholic and previous treatment and treatment uptake). Histopathology findings of each patient were obtained from laboratory reports. According to esophagus-gastro-duodeno-scopy (OGD) findings, patients suffered from different gastric diseases were grouped into chronic gastritis (CG), gastric ulcer (GU), gastric cancer (GC) and normal (intact mucosa).

Patients with a history of gastric surgery, active gastrointestinal bleeding, who had received treatment like proton pump inhibitors or bismuth compounds in the last four weeks before endoscopy were excluded. This study was approved by Ethical Committee of College of Medicine-AL-Nahrain University.

Genomic DNA Extraction

Genomic DNA was extracted from fresh GTPs using (QIAamp® DNA Mini kit Cat. No. 51304, Qiagen, USA) following manufacturer instructions. Quality of extracted DNA was checked by amplifying of housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH) using conventional PCR [25]. PCR products were electrophoresd on 2% agarose gel. The appearance of band with 240bp indicates that DNA quality suitable for further molecular study.

Direct Molecular Identification of *H. pylori* in Extracted DNA from GTBs

Conventional PCR was performed using specific primers for identification of species-specific ureA of *H. pylori* [26]. PCR products were electrophoresed in 2% agarose gel and the presence of band with 411 bp indicated positivity to *H. pylori*.

Direct Molecular Identification of EBV in Extracted DNA from GTBs

Identification of Epstein-Barr nuclear antigen 1(EBNA1) in extracted DNA from GTBs was done using nested PCR after optimization of procedure [27]. Master mix of first round of PCR was prepared with a final volume of 50 µl per one reaction as following: 1X of Green Go Taq® reaction buffer (Promega, USA), 200 µM of dNTPs (Promega, USA), 20 pmol of each forward and reverse primers (Alpha DNA, USA), 3 Units of Go Taq® DNA Polymeras (Promega, USA). Nuclease-free water was added until the reaction volume reach to sufficient volume. Of extracted DNA, 100 ng/µl was added to reaction mixture tube. To the no template control reaction tube (NTC), nuclease-free water was added instead of DNA. Then, PCR reaction tubes were transferred to the thermal cycler (Eppendorf, Germany) and programmed as following: 94ºC for 5min as pre-denaturation step, a repeated cycles of 35X of 94ºC for 1min, 55ºC for 1 min, 72ºC for 1min and a final extension step of 72ºC for 7 min.

For the second-round amplification, PCR master mix was prepared with final volume of 50 µL per one reaction containing the same components of first round and 6 µL of amplified product from the first round was added to the reaction tube as a template. Amplified program was as following: 94ºC for 5min as pre-denaturation step, a repeated cycles of 20X of 94ºC for 1min, 55ºC for 1 min, 72ºC for 1 min and a final extension step of 72ºC for 7 min. PCR products from first round and second round was electrophoresed in a 2% agarose gel. The appearance of 490 bp and 336 bp bands, respectively, indicates positive for EBNA-1 of EBV.

Quantification of EBV Infectious Load using Quantitative Real Time PCR (Q-PCR)

DNA samples positive to EBNA1 were submitted for viral load estimation using quantitative real time PCR (Mic q Bioline, Australia) and specific kit for EBV quantification (artus® EBV RG PCR Kit, Cat. No. 4501265, Qiagen, USA) was used following manufacturer instructions, to estimate the infectious dose of the EBV. The cut-off value was determined as ≥ 200 copies/ml [28]. Gastric tissue biopsy was weighted (2 µg) and then the weight unite was converted to µl of sample volume. Q-PCR results were calculated as copies/µl then, results converted to copies/ml. The interpretation of results was calculated using the following equation:

$$\text{Results (copies/ml)} = \frac{\text{Q-PCR calculation (copies/ml)} \times \text{Elution volume (µl)}}{\text{Sample volume (µl)}}$$

*(each 1 µg equal 0.001 µl)

Determination of DNA Methylation Patterns of Human p16 and CDH1 Promoters

Bisulfite DNA conversion: DNA samples positive for EBNA-1 were treated with sodium bisulfite using specific kit (EpiTect® Bisulfite Kit cat. No. 59104, Qiagen, USA), following manufacturer instructions [29,30]. The principle of sodium bisulfite conversion reaction is that un-methylated cytosine in DNA will converted to uracil, leaving the methylated cytosine un-changed. During PCR amplification of bisulfite converted DNA using specific primers, uracil in the DNA template will replace by thymine. The bisulfite conversion therefore introduces specific changes in the DNA sequence that reflects the methylation status of individual cytosine.

Methylation Specific PCR (MS-PCR) for Detection of DNA Methylation Patterns

Methylation patterns of p16 and CDH1 promoters were detected in modified DNA samples using MS-PCR30. Two primer sets, one for detection of methylated state and the other for non-methylated state of p16 and E-cadherin promoters were used. The amplified products were electrophoresed on 2% agarose gel each in separated lane. Presence of band with 150 bp means presence of methylated state of p16 promoter, while the presence of band with 151 bp by using the primer for detection of un-methylation of p16 promoter means un-methylated. If both of the above bands were presented, that means partial methylation of p16 promoter.

Regarding CDH1, the presence of band with 116bp means presence of methylated state, while the presence of band with 97 bp by using the primer to detect un-methylation status of gene promoter means un-methylated. If both of the

above bands were presented, that means partial methylation of gene promoter. Thermal cycler (Ependrowff, USA) was used. Optimization of reaction conditions was done by using different concentration of primers (3-10 pmol), different concentrations of DNA (300 ng/µl-500 ng/µl) and gradient annealing temperatures from (48-55)°C.

Statistical Analysis

Statistical analysis Data were summarized, analyzes and presented using statistical package for social sciences (SPSS) version 23 and Microsoft Office Excel 2010. Quantitative variables were expressed as mean, standard deviation (SD), median and inter-quartile range; whereas, categorical variables were expressed as number and percentage. Mann Whitney U test used to compare numeric variables between two groups, while Chi-square test was used to study association between any two categorical variables; however, Yates correction was used instead when more than 20% of cells have expected count less than 5 and Fisher exact test when a cell or more contain an observed value of zero. The level of significance was set at $p \leq 0.05$.

RESULTS

Patients

From a total of 134GTPs, only 94 GTPs taken from patients (45/94 (47.9%) patients were male and 49/94 (52.1%) patients were female) were included in farther test. The mean age of patients was (47.55 ± 17.12) ranging between (17-80) years old. Regarding patients from whom 6/94 (6.4%) GTPs were obtained and considered as negative control (un-remarkable changes in gastric mucosa), 3 patients were male and 3patients were female with age ranged between (24-71) years old.

Histopathological Findings

According reports of histopathological findings of GTBs, patients were grouped as 53 (56.4%) patients with CG related *H. pylori*, 18 (19.1%) patients with CG, 4 (4.3%) patients with GU,13 (13.8%) patients with GC and 6 (6.4%) patients with un attack mucosa.

Direct Molecular Identification of *H. pylori* in Extracted DNA from GTPs

DNA quality was estimated by amplification of GAPDH using conventional PCR. Only 94/134 samples were included in further molecular tests, while 40/134 GTPs were excluded because of low quality of extracted DNA from these samples. Results of direct identification of *H. pylori* in 94 extracted DNA from GTPs showed that 39/94 (41.5%) of extracted DNA samples were positive for *H. pylori*. The distribution of patients related to these samples according to histopathology findings were as 35 (89.7%) patients with CG, 1(2.6%) patients with CU and 3 (7.69%) patients with GC. The association between *H. pylori* positive samples and chronic gastritis revealed that there was statistically high significant association ($p<0.05$).

Direct Molecular Identification of EBV in Extracted DNA from GTBs

The results shown that 18/94 (19.15%) DNA samples were positive for EBNA-1, while 76/94 (80.9%) DNA samples were negative for EBNA-1 (Figure 1). Association between the presents of EBV and histopathological findings in patients positive to EBV were as 11 (61.1%) patients with CG and 7 (38.9%) patients with GC. Association between EBV positive samples and GC was statistically high significant ($p<0.05$).

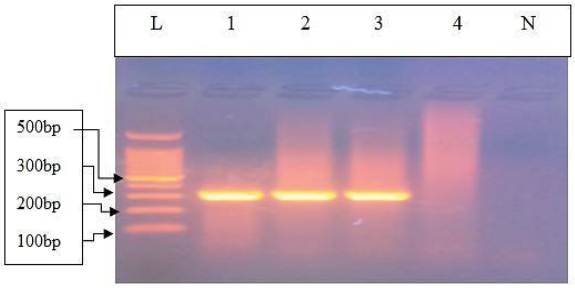

Figure 1 Identification of EBV using specific primers set for amplification of EBNA-1(336 bp), second round. Lane (1,2,3): positive samples for EBNA1; Lane (4): negative samples for EBNA1; Lane N: no template control; Lane L: DNA ladder (100 bp); Electrophoresis was done in 2% agarose gel at (5 V/cm) for 60 min

Quantification of EBV Infectious Dose using Quantitative Real Time PCR

Viral load of EBV was quantified in 37/94 (39.4%) extracted DNA from GTPs to estimate infectious dose of EBV. The selection of these samples was based on positivity to EBNA1 and histopathological findings. Only 23/37 (62.16%) extracted DNA samples were shown a results whether positive or negative for infectious dose of EBV load. Of these, 8/23 (34.8%) samples were showed infectious dose of EBV. Association between the presents of EBV infectious dose and histopathological findings in patients were as 2 (25%) patients with CG, 2 (25%) patients with GU, 3 (37.5%) patients with GC and1 (12.5%) patient have normal mucosa.

Association between Histopathological Findings and Co-infection with *H. pylori* and EBV

Association between histopathological findings and the co-infection with *H. pylori* and EBV shown that 1 patient with CG related *H. pylori* and 2 patients with GC. There was not statistically significant association ($p>0.05$).

DNA Methylation Patterns of p16 and CDH-1 Promoters

A total of 42/94 (44.7%) extracted DNA samples from GTPs were selected based on histopathological findings and positivity to infection with *H. pylori* and/or EBV to detect DNA methylation patterns of p16 and CDH-1 promoters. Only 21/42 and 19/42 DNA samples were provide a results for MSP-PCR of p16 and CDH-1, respectively (Figure 2).

Association between histopathological findings and DNA methylation patterns of p16 promoter in studied patients shown that the most frequent methylated pattern was found in CG related *H. pylori* 3/8 (37.5%) samples and GC 3/8 (37.5%) samples (Table 1).

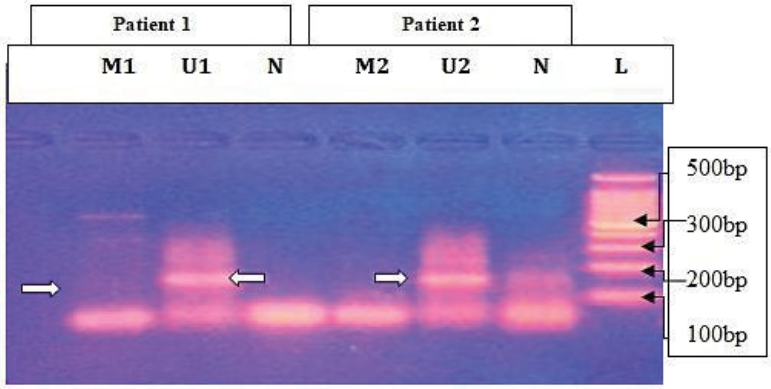

Figure 2 DNA methylation pattern of p16; M: methylated promoter (150 bp), U: unmethylated promoter (151 bp); DNA methylation pattern of patient1 shows partial methylated; N: no template control; L: DNA ladder (100 bp); Electrophoresis was done in 2.5% agarose gel at (5 V/cm) for 60 min.

Table 1 Association between DNA methylation patterns of p16 and histopathological findings

Histopathological Findings	p16 Methylation Patterns			
	U-p16 (N=8)	M-p16 (N=8)	PM-p16 (N=5)	Total (N=21)
Chronic Gastritis	0	1 (12.5%)	0	1 (4.8%)
Chronic Gastritis related *H. pylori*	4 (50%)	3 (37.5%)	4 (80%)	11 (52.4%)
Gastritis Ulcer	2 (25%)	1 (12.5%)	0	3 (14.2%)
Gastric Adenocarcinoma	2 (25%)	3 (37.5%)	1 (20%)	6 (28.5%)
U: Un-methylated; M: Methylated: PM: Partially methylated				

Association between DNA methylated samples of p16 (N=8) and positivity to EBV infection was more frequent in CG related *H. pylori* samples and GC samples.

The results of DNA methylation patterns of CDH-1 in studied patients shown that the most frequent DNA methylated pattern was in CG related *H. pylori* (Table 2).

Table 2 Association between DNA methylation patterns of E-cadherin and histopathological findings

Histopathological Findings	E-cadherin Methylation Patterns			
	U-CDH1 (N=11)	M-CDH1 (N=4)	PM-CDH1 (N=4)	Total (N=19)
Chronic Gastritis	NA	NA	NA	NA
Chronic Gastritis related *H. pylori*	4 (36.4%)	3 (75%)	2 (50%)	10 (52.6%)
Gastritis Ulcer	2 (18.8%)	-	1 (25%)	2 (10.5%)
Gastric Adenocarcinoma	5 (45.5%)	1 (25%)	1 (25%)	7 (36.8%)
U: Un-methylated; M; Methylated; PM: Partially methylated; NA: Not identified				

The association between DNA methylated samples of CDH1 and positivity to EBV infection was ¼ (25%) in each of CG related *H. pylori* samples and GC samples.

Association between DNA methylation patterns of p16 and CDH1 and co-infection with EBV and *H. pylori*

Association between DNA methylated patterns of p16 (21 samples) and CDH1 (19 samples) and co-infection with EBV and *H. pylori* shown in Table 3.

Table 3 Association between DNA methylation patterns of p16 and CDH1 and co-infection with EBV and *H. pylori*

Molecular Identification using PCR	p16 Methylation Patterns (n=21)			CDH1 Methylation Patterns (n=19)		
	U=8 (38.1%)	M=8 (38.1%)	p=5 (23.8%)	U=11 (57.9%)	M=4 (21.1%)	p=4 (21.1%)
EBV	1 (12.5%)	1 (12.5%)	1 (20%)	1 (9%)	1 (25%)	1 (25%)
H. pylori	3 (37.5%)	2 (25%)	3 (60%)	5 (45.5%)	3 (75%)	2 (50%)
EBV and *H. pylori* Positive	0	0	0	1 (9%)	0	0
EBV and *H. pylori* Negative	4 (50%)	4 (50%)	1 (20%)	4 (36.4%)	0	1 (25%)
N: Number of samples; CDH1: E-cadherin gene						

DISCUSSION

Patients

In the present study, 13/94 (13.8%) patients were diagnosed histopathologicaly as have GC. The age range of those patients was (37-80) years, with mean age 43.15 years. Male to female ratio was 1.2:1. Previous Iraqi study revealed that no age group of both gender can be excluded and the ratio of male to female patients with GC was 1.3:l [31]. Other Iraqi study in Baghdad was included 50 patients with GC, their age ranged between 60-70 years and male to female ratio was1.4:1 [32]. Also, Iraqi study was revealed that the incidence of gastric carcinoma was high at ages over 50 years followed by ages between 41-50 years and regarding gender, they found that male was the predominant [33]. A study was done in Duhok city at 2017 revealed that the mean age of patients was 60.27 years ranging between 21-93 years and male to female ratio was 1.38:1. This ratio reflects the results of high exposure to carcinogens in male including smoking, dietary habits, alcohol consumption and probably higher incidence of *H. pylori* [34]. Despite the geographical proximity of Iraq and Iran, the incidence of GC differs hugely between these countries. In Iran high incidence rate of GC (15.3 cases/105) compared to Iraq (3.6 cases/105) [35].

Histopathological Findings

In Iraq, gastric cancer is the ninth common cancer types of all body organs and the second commonest gastrointestinal malignancy after colorectal carcinoma [31,36].

Generally, the prognosis of patients with gastric carcinoma is dependent on its histological type, such as intestinal type (IT) or diffuse type (DT) [37]. In the present study, GTPs from 13/94 (13.9%) patients were diagnosed histopathologically with GC. Of them, 9/13 (69.2%) GTPs samples were intestinal type and 4/13 (30.7%) GTPs samples were diffused type. Iraqi study included 155 patients with GC classified to 84/155 (54%) patients with intestinal type, 40/155 (25.8%) patients with diffuse type and the remaining were mixed type [34].

Direct Molecular Identification of *Helicobacter pylori* from Gastric Tissue Biopsy

Nearly, 50% of the global population is estimated to be infected by *H. pylori* in which, less than 2% develop GC [18]. In the present study, *H. pylori* were identified directly in extracted DNA from GTBs using specific primers for ureA and 39/94 (41.5%) samples were positive to *H. pylori*. Regarding Iraqi studies, using rapid urease test and multiplex PCR to detect cagA, ureC, flagellin A and 16s rRNA of *H. pylori* in extracted DNA from GTBs and they found that 102/210 (48.57%) samples were positive to *H. pylori* by rapid urease test and 97/210 (46.01%) samples were positive to 16SrRNA, flagellin A and ureC, but only 40/97 (19.04%) samples were cagA positive [38,39]. Other study was used PCR specific primers to detect species-specific 23S rRNA in extracted DNA from GTBs and they found that 56/120 (46.6%) samples were positive to *H. pylori*. A study found that 51/69 (73.9%) GTPs were positive to *H. pylori* using specific primers for ureA [40].

A Turkish study was used five different methods (histological examination, bacterial culture, *H. pylori* stool antigen (HpSA), PCR detection of 16s rRNA in stool and Fluorescence in situ hybridization (FISH)) to identified *H. pylori* in 132 GTPs and stool specimens and they found that 56/132 (42.4%) cultured GTPs were positive, 64/132 (48.5%) stool specimens were positive using HpSA test, 85/132 (64.4%) GTPs were positive using histological examination, 98/132 (74.2%) extracted DNA from GTPs was positive using 16s rRNA, 81 (61.4%) GTPs were positive using FISH and 28 (21.2%) stool samples were positive using PCR [41]. Iranian study was included 89 extracted DNA from GTBs using PCR and they found that 49/89 (55%) samples were positive for ureC [42]. In Palestine, a study was revealed that 44/100 (44%) extracted DNA samples from GTPs were positive to *H. pylori* based on direct molecular detection of ureA and the glmM using PCR [43]. In Kingdom of Saudi Arabia, a study was referred to that 20/35 (57.2%) samples from patients with GC and 10/10 (100%) samples from patients with GU were positive to *H. pylori* using 16s rRNA and ureC [44].

Identification of EBV by Detection of EBNA-1 using Nested PCR

In the present study, 18/94 (19.1%) extracted DNA samples were positive to EBV, in which, 7/18 (53.85%) samples from patients with GC. A meta-analysis study carried to estimate the prevalence of EBV-positivity in GC was referred to that the overall prevalence was 8.7% of 15,952 patients with GC using *In situ* hybridization for EBV-EBER. EBV and prevalence was similar in patients from Asia (8.3%), Europe (9.2%), and the Americas (9.9%) [45]. Sudanic study found that 11/50 (22.0%) patients with GC were positive for EBNA1 using conventional PCR [46]. Another Sudanic study found that 7/30 (23.3%) GTPs from patients with GC were positive for EBV by detecting LMP1 using immune-histochemistry [47]. In Portugal, they found that 15/179 (8.4%) FFPE-GTBs from patients with GC were positive to EBV-EBERs using *in situ* hybridization (ISH) [48].

Estimation of EBV Viral Load using Quantitative PCR

Since EBV is a ubiquitous virus that infects the majority of humans without major adverse health consequences, it is important to distinguish between normal background levels of EBV viral load and abnormally increased levels of EBV viral load that could be suggest pathogenicity. In the present study, results of study is associated between infectious dose of EBV and GC by quantification of EBNA-1 using Q-PCR shown no statistically significant ($p>0.05$).

A study was developed Q-PCR assays targeting disparate regions of the EBV genome (BamH1W, EBNA1, LMP1, LMP2, and BZLF1) to measure EBV viral load in tissue and blood samples , and they found that EBV viral load in normal gastric mucosa is extremely low (<1 EBV DNA copy/105 cells), similar to that of whole blood from healthy donors [49]. In India, a study included 200 GTPs from patients (100 non-ulcer dyspepsia, 50 peptic ulcer disease, 50 gastric carcinoma) showed that 113/200 (56.5%) GTBs were positive to EBV using Q-PCR. Data were also analyzed between normal and cancer/ulcer tissue. Out of 50/200 (25%) patients with GC, none of them presented a detectable copy number of EBV DNA in normal mucosa, but they found that 81/200 (40.5%) GTPs from those patients have dual infection with EBV and *H. pylori* [50]. Iranian study showed that 6/90 (6.66 %) samples from patients with GC have DNA viral load more than 2000 copies/105 cells, so they were considered as EBVaGC [51].

Association between Histopathological Findings and Co-infection with *Helicobacter pylori* and EBV

Approximately, 20% of human cancers is driven by infectious agents as the causative inducers [52]. In the present study, a total of 3/8 (37.5%) extracted DNA from GTPs shown positivity to EBV infectious dose were also positive to *H. pylori*. A study referred to that of 48/78 (61.5%) patients whom positive to *H. pylori* using Immunohistochem-

istry (IHC), 18/48 (37.5%) patients were positive to EBNA1 antigens using IHC [53]. Few studies reported that co-infection of patients with both EBV and *H. pylori* enhanced the inflammatory lesions compared to those only infected with *H. pylori* or EBVaGC [18,54]. In Southwest Mexico, a study found that 39/138 (8.3%) extracted DNA from GTPs were identified as positive for *H. pylori* and EBV using PCR amplification of 16s rRNA and EBNA1, respectively [55].

Association between DNA Methylation Patterns of p16 and CDH1, Infection with *H. pylori* and/or EBV in Relation with Histopathological Findings

Prior studies have showed that loss of critical tumor suppressor gene products, such as p16 and CDH1, in EBV-infected gastric cancers more predominant [56]. It was refereed to that methylation of p16 promoter was significantly elevated in the mucosa of *H. pylori* positive compared with *H. pylori* negative healthy volunteers and there was association between promoter methylation of p16 and *H. pylor*i infection in gastric carcinogenesis [57].

In the present study, 8/21 (30.1%) extracted DNA from GTPs were showed methylated promoter of p16. Of these, 3/8 (37.5%) samples from patients with CG and 3/8 (37.5%) samples from patients with GC. Iraqi study found that 53/120 (44.2%) GTPs from patients with different gastric diseases were methylated in p16 promoter and methylation frequency of this gene promoter increased while the disease progressing 39. Turkish study found that abnormal methylation of p16 detected in 6/20 (30%) samples from peripheral blood and GTPs of 20 patients with GC [58]. Other study found that 77/106 (72.6%) of extracted DNA from peripheral blood of patients with GC shown p16 methylated promoter while only 1/18 sample from healthy control, using MS-PCR analysis [59].

In the present study, 4/19 (21.1%) extracted DNA from GTPs were showed methylated promoter of CDH1, of these, 1/4 (25%) sample from patients with GC and 3/4 (75%) samples from patients with CG. Out of 4 samples that have methylated promoter of this gene, 3/4 (75%) samples were positive for *H. pylori* and 1/4 (25%) sample was positive for EBV. It was refereed to that there was a connection between the *H. pylori* infection and CDH1 promoter hypermethylation in pre-cancer lesions and bacterial eradication resulted in cancer reduction [57]. Meta-analysis study included 587 GTPs from cancer sites and 389 normal mucosa tissues of patients with GC from the People's Republic of China, South Korea, Japan, Tunisia, Brazil, and Italy showed that the frequency of E-cadherin hypermethylated promoter ranged from 28.6% to 82.2% (average 61%) in cancer tissues and from 0.00% to 54.5% (average 16%) in normal mucosa, respectively [60]. A study in Egypt detected CDH1 promoter methylation in 36/64 (50.6%) specimens from gastric cancer patients [61].

DECLARATIONS

Ethical Clearance

Ethical clearance was taken by the ethical committee of Al-Nahrain University.

Formatting of Funding Sources

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflicts of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

REFERENCES

[1] Lieberman, Paul M. "Keeping it quiet: Chromatin control of gamma herpes virus latency." *Nature Reviews Microbiology,* Vol. 11, No. 12, 2013, pp. 863-75.

[2] Rickinson Alan Bernard, and Kieff Elliott. "Epstein-Barr virus." In: Fields Virology, 5th ed.; Fields, B. N. edited by Knipe David M, and Howley Peter M; Lippincott-Williams and Wilkins: Philadelphia, PA, USA, 2007; pp. 2655-700. https://www.worldcat.org/title/fields-virology/oclc/71812790

[3] Chan, Bryan A, et al. "Improving outcomes in respectable gastric cancer: A review of current and future strategies." *Oncology Journal,* Vol. 30, No.7, 2016, pp. 635-45.

[4] Bosman, Fred T., et al. "WHO classification of tumours of the digestive system." No. ed. 4. World Health Organization, Lyon, France: IARC Press. 2010, p. 417.

[5] Nouraie, Mehdi, et al. "Ecologic study of serum selenium and upper gastrointestinal cancers in Iran." *World Journal of Gastroenterology,* Vol. 10, No. 17, 2004, pp. 2544-46.

[6] Alireza, S., et al. "Cancer occurrence in Iran in 2002, an international perspective." *Asian Pacific Journal of Cancer Prevention,* Vol. 6, No. 3, 2005, p. 359.

[7] Hussein, Nawfal R. "*Helicobacter pylori* and gastric cancer in the Middle East: A new enigma?" *World journal of gastroenterology: WJG*, Vol. 16, No. 26, 2010, p. 3226.

[8] Yanagi, Ayaka, et al. "Clinicopathologic characteristics of epstein-barr virus-associated gastric cancer over the past decade in Japan." *Microorganisms,* Vol. 7, No. 9, 2019, p. 305.

[9] Tsao, Sai-Wah, et al. "The role of Epstein-Barr virus in epithelial malignancies." *The Journal of Pathology,* Vol. 235, No. 2, 2015, pp. 323-33.

[10] Jones, Peter A., and Daiya Takai. "The role of DNA methylation in mammalian epigenetics." *Science,* Vol. 293, No. 5532, 2001, pp. 1068-70.

[11] Yang, Mihi, and Jong Y. Park. "DNA methylation in promoter region as biomarkers in prostate cancer." *Cancer Epigenetics,* Vol. 863, 2012, pp. 67-109.

[12] Connolly, Sarah A., et al. "Fusing structure and function: a structural view of the herpes virus entry machinery." *Nature Reviews Microbiology,* Vol. 9, No. 5, 2011, pp. 369-81.

[13] Gulley, Margaret L. "Genomic assays for Epstein-Barr virus-positive gastric adenocarcinoma." *Experimental and Molecular Medicine,* Vol. 47, No. 1, 2015, p. e134.

[14] Zhang, Yi-wang, et al. "Epstein-Barr virus latent membrane protein 2A suppresses the expression of HER2 via a pathway involving TWIST and YB-1 in Epstein-Barr virus-associated gastric carcinomas." *Oncotarget,* Vol. 6, No. 1, 2015, p. 207.

[15] He, Baoyu, et al. "Epstein-Barr virus-encoded miR-BART6-3p inhibits cancer cell metastasis and invasion by targeting long non-coding RNA LOC553103." *Cell Death and Disease,* Vol. 7, No. 9, 2016, p. e2353.

[16] International Agency for Research on Cancer, and World Health Organization. "A Review of Human Carcinogens. Part B: Biological Agents/IARC Working Group on the Evaluation of Carcinogenic Risks to Humans." 2009.

[17] Camargo, M. Constanza, et al. "Anti-*helicobacter pylori* antibody profiles in Epstein-Barr virus (EBV)-positive and EBV-negative gastric cancer." *Helicobacter,* Vol. 21, No. 2, 2016, pp. 153-57.

[18] Singh, Shyam, and Hem Chandra Jha. "Status of Epstein-Barr virus coinfection with *Helicobacter pylori* in gastric cancer." *Journal of Oncology,* 2017, pp. 1-17.

[19] de Souza, Carolina Rosal Teixeira, et al. "Occurrence of *Helicobacter pylori* and Epstein-Barr virus infection in endoscopic and gastric cancer patients from Northern Brazil." *BMC Gastroenterology,* Vol. 14, 1, 2014, p. 179.

[20] Shukla, Sanket Kumar, et al. "Transforming growth factor beta 1 (TGF-β1) modulates Epstein-Barr virus reactivation in absence of *Helicobacter pylori* infection in patients with gastric cancer." *Cytokine,* Vol. 77, 2016, pp. 176-79.

[21] Niwa, Tohru, et al. "Inflammatory processes triggered by *Helicobacter pylori* infection cause aberrant DNA methylation in gastric epithelial cells." *Cancer Research,* Vol. 70, No. 4, 2010, pp. 1430-40.

[22] Kawazoe, Akihito, et al. "Clinico pathological features of programmed death ligand 1 expression with tumor-infiltrating lymphocyte, mismatch repair, and Epstein-Barr virus status in a large cohort of gastric cancer patients." *Gastric Cancer,* Vol. 20, No. 3, 2017, pp. 407-15.

[23] Geddert, Helene, et al. "EBV-infection in cardiac and non-cardiac gastric adenocarcinomas is associated with promoter methylation of p16, p14 and APC, but not hMLH1." *Analytical Cellular Pathology,* Vol. 33, No. 3,

2010, pp. 143-49.

[24] Sudo, Makoto, et al. "Promoter hypermethylation of E-cadherin and its abnormal expression in Epstein-Barr virus-associated gastric carcinoma." *International Journal of Cancer,* Vol. 109, No. 2, 2004, pp. 194-99.

[25] Seth, R., et al. "Efficient extraction of DNA from paraffin-embedded tissue using Nucleon HT Kit for clinical molecular pathology research." *Life Science News 1,* 1998.

[26] Smith, S. I., et al. "Comparison of three PCR methods for detection of *Helicobacter pylori* DNA and detection of cagA gene in gastric biopsy specimens." *World Journal of Gastroenterology: WJG,* Vol. 10, No. 13, 2004, p. 1958.

[27] Tsai, Donald E., et al. "Use of EBV PCR for the diagnosis and monitoring of post-transplant lymphoproliferative disorder in adult solid organ transplant patients." *American Journal of Transplantation,* Vol. 2, No. 10, 2002, pp. 946-54.

[28] Ohga, Shouichi, et al. "Epstein-Barr virus (EBV) load and cytokine gene expression in activated T cells of chronic active EBV infection." *The Journal of Infectious Diseases,* Vol. 183, No. 1, 2001, pp. 1-7.

[29] EpiTect® Bisulfite Handbook. For complete bisulfite conversion and cleanup of DNA for methylation analysis, QIAGEN Sample and Assay Technologies, *Epigenomics,* 2014, pp. 1-46.

[30] Herman, James G., et al. "Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands." *Proceedings of the National Academy of Sciences,* Vol. 93, No. 18, 1996, pp. 9821-26.

[31] Al-Bahrani, Zuhair R., and Ahmed Z. Al-Bahrani. "The changes in the incidence of gastric versus colorectal cancer in Iraq during the period between 1965-2006." *TOFIQ journal of Medical Sciences,* Vol. 1, No. 1, 2013, pp. 1-16.

[32] Shahid, Maytham Hatam, Saad Ramadan Jawad, and Asaad Abdullah Abbas. "Experience of gastric cancer in Al-Sadder city in Baghdad." *Iraqi Academic Scientific Journal,* Vol. 16, No. 2, 2017, pp. 129-37.

[33] Lafta, Shurooq A., and Abdul Hussein M. Al Faisal. "Association of *Helicobacter pylori* infection and gastric cancer." *Iraqi Journal of Biotechnology,* Vol. 16, No. 1, 2017, pp. 1-9.

[34] Raziq, Alaa Hani, et al. "Gastric malignancies and the trend of gastric carcinoma in Duhok city-Iraq." *Medical Journal of Babylon,* Vol. 14, No. 1, 2017, pp. 162-68.

[35] Globocan, IARC. Cancer incidence and mortality worldwide, 2008.http://globocan.iarc.fr

[36] Annual Report Iraqi Cancer Registry, 2015.

[37] Lauren, Pekka. "The two histological main types of gastric carcinoma: Diffuse and so-called intestinal-type carcinoma: An attempt at a histo-clinical classification." *Acta Pathologica Microbiologica Scandinavica,* Vol. 64, No. 1, 1965, pp. 31-49.

[38] Kalaf, Elham A. "Multiplex PCR assay for detection of *Helicobacter pylori* isolated from Iraqi dyspeptic patients." *Iraqi Journal of Cancer and Medical Genetics,* Vol. 6, No. 1, 2017, pp. 49-54.

[39] Fawzi, Mohammed, AL-Khafaji, Zahra M, and AL-Thamer Sabah N. "Methylation of P16 gene during gastric carcinogenesis in association Helicobacter infection." *International Journal of Pharma and Bio Science,* Vol. 8, No. 1, 2017, pp.603-9.

[40] Ali, H. S., M. A. R. Dhahi, and J. M. Al-Maliki. "Genotyping of vacA of *Helicobacter pylori* in patients from Baghdad with gastro-duodenal diseases." *Journal of Gastroenterology and Digestive Diseases*, Vol. 2, No. 2, 2017, pp. 25-31.

[41] Aktepe, Orhan Cem, et al. "Five methods for detection of *Helicobacter pylori* in the Turkish population." *World Journal of Gastroenterology: WJG,* Vol. 17, No. 47, 2011, pp. 5172-76.

[42] Pourakbari, Babak, et al. "Diagnosis of *Helicobacter pylori* infection by invasive and non-invasive tests." *Brazilian Journal of Microbiology,* Vol. 44, No. 3, 2013, pp. 795-98.

[43] Essawi, Tamer, et al. "Determination of *Helicobacter pylori* virulence genes in gastric biopsies by PCR." *ISRN Gastroenterology,* 2013, pp.1-4.

[44] Bibi, Fehmida, et al. "Detection and genotyping of *Helicobacter pylori* among gastric ulcer and cancer patients from Saudi Arabia." *Pakistan Journal of Medical Sciences,* Vol. 33, No. 2, 2017, pp. 320-24.

[45] Murphy, Gwen, et al. "Meta-analysis shows that prevalence of Epstein-Barr virus-positive gastric cancer differs based on sex and anatomic location." *Gastroenterology,* Vol. 137, No. 3, 2009, pp. 824-33.

[46] Musa, Hassan Hussein, and Mona Ahmed. "Association of Epstein-barr virus with gastric carcinoma among Sudanese patients." *Journal of Cancer Genetics and Biomarkers,* Vol. 1, No. 1, 2016, p. 46.

[47] Yousif Elemam, Ibrahim Bakhit, and Mahmoud Mohammed Satti. "Association of EBV in Gastric carcinoma among Sudanese patients." *International Journal of Medical Science and Clinical Inventions,* Vol. 3, No. 2, 2016, pp. 1557- 59.

[48] Ribeiro, Joana, et al. "Clinical and pathological characterization of Epstein-Barr virus-associated gastric carcinomas in Portugal." *World Journal of Gastroenterology,* Vol. 23, No. 40, 2017, pp. 7292-302.

[49] Ryan, Julie L., et al. "Epstein-Barr virus infection is common in inflamed gastrointestinal mucosa." *Digestive Diseases and Sciences,* Vol. 57, No. 7, 2012, pp. 1887-98.

[50] Shukla, Sanket Kumar, et al. "Epstein-Barr virus DNA load and its association with *Helicobacter pylori* infection in gastroduodenal diseases." *Brazilian Journal of Infectious Diseases,* Vol. 15, No. 6, 2011, pp. 583-90.

[51] Msc, Ebrahim Faghihloo, and Mohammad Reza Saremi Msc. "Prevalence and characteristics of Epstein-Barr virus-associated gastric cancer in Iran." *Archives of Iranian Medicine,* Vol. 17, No. 11, 2014, pp. 767-70.

[52] De Flora, Silvio, and Paolo Bonanni. "The prevention of infection-associated cancers." *Carcinogenesis,* Vol. 32, No. 6, 2011, pp. 787-95.

[53] Minoura-Etoh, Junko, et al. "Helicobacter pylori-associated oxidant monochloramine induces reactivation of Epstein–Barr virus (EBV) in gastric epithelial cells latently infected with EBV." *Journal of Medical Microbiology,* Vol. 55, No. 7, 2006, pp. 905-11.

[54] Cárdenas-Mondragón, María G., et al. "Case-control study of Epstein-Barr virus and *Helicobacter pylori* serology in Latin American patients with gastric disease." *British Journal of Cancer,* Vol. 112, No. 12, 2015, pp. 1866-73.

[55] Del Moral-Hernández, Oscar, et al. "Multiple infections by EBV, HCMV and *Helicobacter pylori* are highly frequent in patients with chronic gastritis and gastric cancer from Southwest Mexico: An observational study." *Medicine,* Vol. 98, No. 3, 2019, p. e14124.

[56] Ferrasi, Adriana Camargo, et al. "*Helicobacter pylori* and EBV in gastric carcinomas: methylation status and microsatellite instability." *World Journal of Gastroenterology: WJG*, Vol. 16, No. 3, 2010, pp. 312-19.

[57] Perri, Francesco, et al. "Aberrant DNA methylation in non-neoplastic gastric mucosa of *H. pylori* infected patients and effect of eradication." *American Journal of Gastroenterology,* Vol. 102, No. 7, 2007, pp.1361-71.

[58] Erdem, Beril, et al. "Promoter hypermethylation of p16 and APC in gastrointestinal cancer patients." *Turkish Journal of Gastroenterology,* Vol. 25, No. 5, 2014, pp. 512-17.

[59] Guo, Lidong, Huang, Jim C, and, Ji QJ. "Aberrent promoter hypermethylation of p16, surviving and retinoblastoma in gastric cancer." *Bratislava Medical Journal,* Vol. 118, No. 3, 2017, pp. 164-68.

[60] Zeng, Wei, et al. "The clinic-pathological significance of CDH1 in gastric cancer: A meta-analysis and systematic review." *Drug Design, Development and Therapy*, Vol. 9, 2015, pp. 2149-57.

[61] Hamed, Mohamed KF, Gamal Fawzy, and Hanna H. Hanna. "The role of E-cadherin expression and E-cadherin gene promoter hypermethylation in gastric carcinoma." *The Egyptian Journal of Surgery,* Vol. 38, No. 2, 2019, pp. 361-68.