

Correlation of EBV-LMP Immune Markers Expression with the Clinicopathological Parameters of Gastric Adenocarcinoma by Using Immunohistochemistry

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MBChB, MSc. Histopatholog Correlation of EBV-LMP immune markers expression with the clinicopathological parameters of gastric adenocarcinoma by using immunohistochemistry y/Uruk University-College of dentistry/Baghdad/Iraq

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Abstract: *Background:* Gastric cancer is one of the major causes of cancer-related deaths worldwide. The Cancer Genomic Atlas Consortium (TCGA) has recently proposed a molecular subtyping based on the presence of Epstein-Barr virus, microsatellite instability (MSI), genomic stability (GS), and chromosomal instability (CIN). Meanwhile, the Asian Cancer Research Group (ACRG) proposed a different classification based on tumor P53 (TP53) status, constituted by the subtypes MSI, microsatellite stable/epithelial mesenchymal transition (MSS/EMT), MSS/TP53+, and MSS/TP53 negative. Techniques such as immunohistochemistry and in situ hybridization have been successfully employed to define the molecular subtypes. *Aim of the study:* To evaluate the correlation of EBV-LMP immune markers expression with the clinicopathological characteristics (age, sex, location of the tumor, type of surgery, tumor pattern morphology, TNM staging, tumor grade, lympho-vascular and perineural invasion). *Materials and Methods:* This is a retrospective cohort study that utilizes formalin-fixed paraffin-embedded tissue blocks from 40 patients with gastric adenocarcinoma. The samples were collected retrospectively between January 2020 and January 2023 from archived materials of the Histopathology Department of the Gastroenterology and Hepatology Teaching Hospital, The Teaching Laboratory Institute, and some private laboratories in Baghdad, Iraq. The collection period of data and material spanned from the 1st of October 2022 until 1st of October 2023. One section of 5 micrometers in thickness were taken from each block stained with immunohistochemistry for EBV-LMP (latent membrane protein). The correlation of the EBV-LMP immune markers expression with the clinicopathological parameters were studied by the author. *Results:* EBV-LMP immunological staining was positive in 27/40 patients (67.5%). The EBV-LMP marker was significantly correlated with lymph nodal involvement and tumor stage while the other parameters were not significantly associated with the EBV-LMP expression. *Conclusion:* EBV analysis showed an important relationship to L.N involvement and staging. Therefore, it can be considered as an indicator to determine the prognosis and identify cancer patients who are at greater risk of metastasis.

Keywords: EBV-LMP immune markers; clinicopathological parameters; gastric adenocarcinoma; immunohistochemistry.

INTRODUCTION

Gastric carcinoma with lymphoid stroma or lymphoepithelioma-like carcinoma has been found to be linked with EBV infection and is classified as an EBV-associated gastric malignancy (EBVaGC). Gastric tumors with microsatellite instability [1,2] display a comparable appearance. In general, the prevalence of detecting indications of EBV infection in gastric carcinomas varies between (2-20%) , with an average global rate of approximately (10%) [3-5]. Nevertheless, it is worth mentioning that the involvement of EBV in the development of gastric cancer, either through direct mechanisms or as an indirect consequence, remains a topic of ongoing discussion and analysis. EBVaGCs exhibit distinct macroscopic and histologic features [7]. The major type, gastric cancer with lymphoid stroma, appears in the proximal stomach [8,9] and in the gastric stump of partial gastrectomy patients. It affects younger men and is more common than traditional carcinoma [6,9]. Tumor margins are usually pushing, not infiltrating. Uneven sheets, trabeculae, ill-defined

tubules of polygonal cells, a strong lymphocytic infiltrate of CD8-positive cytotoxic T and B lymphocytes, plasma cells, neutrophils, and eosinophils make up their structure. EBV is linked to most gastric malignancies with lymphoid stroma [6]. Tubular carcinomas with limited desmoplasia, fewer lymphocytes, and prominent lymphoid follicles with active germinal centers (designated "carcinoma with Crohn disease-like lymphoid reaction") and conventional-type adenocarcinomas with scant lymphocytic infiltrate are other EBVaGC variants [8]. The prognosis is better than for typical gastric malignancies [10-13]. Due to the overexpression or amplification of programmed death ligand 1 (PD-L1), gastric malignancies linked to EBV are suitable candidates for immune checkpoint inhibitors [14,15].

The Epstein-Barr directly transforms host gene expression and cell cycle pathways [16]. Oncogenesis involves EBV gene expression and host genome control. At latency I, EBVaGC only expresses EBV-encoded

small RNA (EBER), EBNA1, and BamHI-A rightward transcripts (BARTs) and their microRNA [17]. Nevertheless, new research indicates that the viral latent profile of EBVaGC aligns with either latency I or II. In the latter, latent membrane protein (LMP)-1, 2A, and 2B, as well as BamHI-A rightward transcript 1 (BARF1), are expressed. EBER abundance is proportional to EBV DNA genomes, making it the gold standard for EBVaGC detection [18]. LMPs 1, 2A, and 2B are in the host cell membrane. The LMP1 gene produces a viral protein expressed in Hodgkin lymphomas, nasopharyngeal carcinoma, and GC. Researchers agree that EBVaGC expresses low LMP1 [18-21].

Only 10% of EBVaGC patients expressed LMP1, according to a systematic review. This may be due to LMP1 promoter methylation. By methylation-specific PCR and bisulfite sequencing, Li et al. examined the promoter methylation profiles of LMP1, LMP2A, and LMP2B in 41 EBVaGC samples and 5 EBV-positive cell lines. They found that all LMP promoters were methylated to varying degrees and that demethylation could restore LMP1 expression [21]. According to prevailing viewpoints, LMP1 facilitates the development of cancer by stimulating survival and growth pathways in Hodgkin lymphomas and nasopharyngeal carcinoma [22].

In contrast, it has been observed that LMP1 induces apoptosis in gastric cancer cells, in contrast to its role in promoting proliferation in NPC cells [23]. Recombinant EBV-infected primary gastric epithelial cells had a disrupted cell cycle pattern and restricted expression of viral latent genes, similar to EBV-positive gastric cancer biopsy specimens that lacked EBNA2 and LMP1 [24]. These tumors account for 9% of stomach malignancies and mostly affect men. These fundus-located lesions have a decreased mortality rate and lymph node metastasis rate. EBV-positive cancers are severe CpG (Cytosine-Phosphate-Guanine) island methylators. In 80% of these tumors, CDKN2A promoter hypermethylation and the PIK3CA non-silent mutation are present. Another frequently altered gene is ARID1A, which encodes an anti-apoptotic protein [54]. In about 10% of cases, this subgroup demonstrates recurrent 9p24.1 amplification at the locus encoding JAK2, CD274 which encode PD-L1 and PD-L2. These two proteins bind to cytotoxic T cells' PD-1, allowing neoplastic cells to escape the body's antitumoral immune response. These compounds are important immune checkpoint inhibitor therapeutic targets. EBV-related subtypes have a favorable prognosis, while genomically stable subtypes have the worst [25].

MATERIALS AND METHODS

This is a retrospective cohort study that utilizes formalin-fixed paraffin-embedded tissue blocks from 40 individuals who were diagnosed with gastric adenocarcinoma. The samples were collected retrospectively between January 2020 and January 2023 from archived materials of the histopathology department of the Gastroenterology and Hepatology Teaching Hospital, Teaching Laboratory Institute and some private laboratories in Baghdad, Iraq. The data and material gathering period spanned from October 2022 until October 2023.

All the patients underwent a potentially curative gastrectomy (total, proximal, and distal) with lymphadenectomy, and these cases were histologically classified into 19 intestinal, 14 diffuse, and 7 mixed subtypes. The ages of the patients ranged from 24 to 75 years, and their sexes were 23 males and 17 females. The clinicopathological parameters that were studied include age, sex, location of the tumor, type of surgery, tumor pattern morphology, TNM staging, tumor grade, lympho-vascular invasion, and perineural invasion. One section of 5 micrometers in thickness were taken from each block, the sections were stained by EBV-LMP immunohistochemistry for immune marker expression. These procedures were done in a private laboratory.

Inclusion criteria are: Cases with primary gastric adenocarcinoma. Cases with available clinicopathological data and enough tissue for paraffin blocks. Cases with surgical specimens (total, proximal, proximal and distal gastrectomy).

Exclusion criteria are: other gastric tumors (gastrointestinal stromal tumors, lymphomas, and neuroendocrine tumors). Secondary gastric adenocarcinoma. Endoscopic biopsies and Gastric cancers with pre-operative neoadjuvant therapy.

Table 1: Materials used in the study

Material	Type
Xylene	Analar (England)
Ethanol (absolute)	Merck (Germany)
Distilled water	
Rinse buffer	TBS (DakoCytomation)
Target retrieval solution (heat-induced epitope retrieval (HIER) DAKO PT LINK (code PT100/PT101)	Tris EDTA pH 9.0 (Dakocytomation) EnVision FLEX Target retrieval solution HIGH pH 50x code (K8000 /K8004)

Primary antibody	DAKO FLEX monoclonal mouse Anti-Epstein-Barr Virus, LMP, (Clone CS.1-4). Isotype: IgG1, kappa. Ready-to-use (Link) Code IR753
Hematoxylin	Counter stain EnVision FLEX Hematoxylin (link) (code K80008)
Mounting media	Dakocytomation
Secondary detection system	HRP/DAB detection (Dakocytomation)
Visualization system	EnVision FLEX High pH (Link) (code K8000) for EBV.

METHODS

The immunohistochemistry process involved sectioning tissue blocks, incubating them in a water bath, deparaffinization, applying Xylene, rehydration in alcohol solutions, and being rinsed with tap water. The antigen retrieval phase involved using a tris ethylenediamine tetra-acetic acid (TRIS EDTA) solution heated to 80°C and maintained for 20 minutes before being lowered back to 65°C for each cycle. A PAP pen was used as a reagent blocker, and a wash buffer solution was used. Two drops of peroxidase blocker were applied to stop the endogenous antigen activity. Primary antibody was applied to the samples, targeting EBV-LMP immune marker and each was incubated for 30 minutes. Anti-rabbit antibodies labeled with horseradish peroxidase were applied and washed. (3,3-diaminobenzidine) DAB was prepared by adding poly detector DAB chromogen per milliliter of poly detector DAB buffer. The samples were washed with wash buffer, and hematoxylin counterstain was applied to the background for one minute. EBV-LMP Marker: Neoplastic cells show a moderate to strong predominantly membranous, but also cytoplasmic staining reaction. It was evaluated using IHC, which will be based on the LMP-1 membrane protein encoded by EBV, which cannot detect the location or transcriptional quantity of the virus. However, compared to ISH, IHC methods have the advantages of simple steps, convenient operation, high sensitivity, and low price, making them a reliable primary screening method for EBV[146 thesis]. The interpretation of the slides and the correlation of the immune markers' expression and the clinicopathological parameters: Age, sex, location of the tumor, type of surgery, morphological tumor pattern, TNM staging, tumor grade, lympho-vascular invasion, and peri-neural invasion were done by the authors.

Statistical analysis

The study used Statistical Package for Social Sciences (SPSS) version 26 to describe variables, with serial numbers being the only reference for participant details. Data were managed daily and expressed using frequency/ percentage. The Chi-square and Fisher's exact tests were used to assess the association between categorical variables, with a 95% confidence level and a P-value of 0.05 or less being considered significant.

RESULTS

The study examined 40 cases of gastric adenocarcinoma, with 57.5% being males and 42.5% being females. The age distribution of the patients showed that one case (2.5%) was between 20-29 years of age, 6 cases (15%) were between 30-39 years, 10 cases (25%) were between 40-49 years, 10 cases (25%) were between 50-59 years, and 13 cases (32.5%) were 60 years or over.

The samples were from the proximal stomach gastroesophageal junction and cardia (10%), (2.5%) in the fundus, (50%) in the body and antrum, and (37.5%) in the distal stomach. The cases were treated with total gastrectomy (62.5%), proximal gastrectomy (5%), and distal gastrectomy (32.5%). There were 19 cases (47.5%) of intestinal type adenocarcinoma, 14 cases (35%) of diffuse type adenocarcinoma, and 7 cases (17.5%) of mixed type adenocarcinoma, (Table 2). Four stages were identified: 1A and 1B, 2A and 2B, 3A and 3B, and 4, (Table 3). The cases were graded into G1 well-differentiated adenocarcinoma, G2 moderately differentiated adenocarcinoma, G2/G3 moderately to poorly differentiated adenocarcinoma, and G3 poorly differentiated adenocarcinoma. Only (17) 42.5% of the cases showed lympho-vascular invasion, and (18) 45% showed perineural invasion.

Table 2: Distribution of the samples by site, specimen and diagnosis

Variable	Category	Number	%
Site	Proximal stomach gastroesophageal junction and cardia	4	10.0
	Fundus	1	2.5
	Body and antrum	20	50.0
	Distal stomach	15	37.5
Specimen	Total gastrectomy	25	62.5
	Proximal gastrectomy	2	5.0
	Distal gastrectomy	13	32.5

Diagnosis	Intestinal type adenocarcinoma	19	47.5
	Diffuse type adenocarcinoma	14	35.0
	Mixed type adenocarcinoma	7	17.5

Table 3: Distribution of the samples by stage and grade

Variable	Category	Number	%
Stage	1A	1	2.5
	1B	5	12.5
	2A	5	12.5
	2B	11	27.5
	3A	6	15.0
	3B	10	25.0
	4	2	5.0
T	1	2	5.0
	2	7	17.5
	3	26	65.0
	4A	4	10.0
	4B	1	2.5
N	0	11	27.5
	1	9	22.5
	2	8	20.0
	3	4	10.0
	3A	4	10.0
	3B	2	5.0
	X	2	5.0
M	0	1	2.5
	1	2	5.0
	X	37	92.5
Grade	G1 well differentiated	1	2.5
	G2 moderately differentiated	24	60.0
	G2/G3 moderately to poorly differentiated	2	5.0
	G3 poorly differentiated	13	32.5

Table 4: EBV-LMP marker Immunohistochemical expression in Gastric Adenocarcinoma patients.

Variable	Frequency N	Percent %
EBV-LMP		
Positive	27	67.5
Negative	13	32.5

EBV-LMP immunohistochemical expression and the clinicopathological correlation.

- For EBV-LMP 27/40 (67.5%) +ve patients (Table 4) : patients' Age > 60 Yrs.10/27 (37%) , males = females 13/26 (50%), Site of the tumor in the Body/Antrum 13/27(48%), intestinal type 13/27 (48%), Stage 3B 8/27(29%) and grade 2 14/27 (51%).
- There was a significant association between EBV-LMP score and L.N. involvement and stage), there was no significant association between EBV-LMP score and the other variables as mentioned in (Table 5).

Table 5: Relationship of EBV-LMP immunohistochemical marker expression with the clinicopathological characteristics of the patients.

Variable	EBV-LMP				P value
	Positive		Negative		
	Freq. N=27	Pct. %	Freq. N=13	Pct. %	
Age					
20-29 y	0	0.0	1	7.7	0.132 ¹
30-39 y	5	18.5	1	7.7	
40-49 y	8	29.6	2	15.4	
50-59 y	4	14.8	6	46.2	
≥60 y	10	37.0	3	23.1	
Sex					
Male	16	59.3	7	53.8	0.746 ²
Female	11	40.7	6	46.2	
Site					
Proximal stomach GEJ. and cardia	3	11.1	1	7.7	0.613 ¹
Fundus	0	0	1	7.7	
Body and antrum	13	48.1	7	53.8	
Distal stomach	11	40.7	4	30.8	
Type of surgical Specimen					
Total gastrectomy	17	63	8	61.5	1.000 ¹
Proximal gastrectomy	1	3.7	1	7.7	
Distal gastrectomy	9	33.3	4	30.8	
Histopathological type					
Intestinal type.	13	48.1	6	46.2	1.000 ¹
Diffuse type.	9	33.3	5	38.5	
Mixed type.	5	18.5	2	15.4	
Stage					
1A	1	3.7	0	0.0	0.043* ¹
1B	4	14.8	1	7.7	
2A	5	18.5	0	0.0	
2B	6	22.2	5	38.5	
3A	1	3.7	5	38.5	
3B	8	29.6	2	15.4	
4	2	7.4	0	0.0	
T					
1	2	7.4	0	0.0	0.710 ¹
2	6	22.2	1	7.7	
3	15	55.6	11	84.6	
4A	3	11.1	1	7.7	
4B	1	3.7	0	0.0	
N					
0	11	40.7	0	0.0	0.010* ¹
1	4	14.8	5	38.5	
2	3	11.1	5	38.5	
3	4	14.8	0	0.0	
3A	3	11.1	1	7.7	

3B	1	3.7	1	7.7	
X	1	3.7	1	7.7	
M					
0	1	3.7	0	0.0	0.692 ¹
1	2	7.4	0	0.0	
X	24	88.9	13	100.0	
Grade					
G1 well differentiated	1	3.7	0	0.0	0.571 ¹
G2 moderately differentiated	14	51.9	10	76.9	
G2/G3 moderately to poorly differentiated	2	7.4	0	0.0	
G3 poorly differentiated	10	37.0	3	23.1	
Lymphovascular invasion					
Present	12	44.4	5	38.5	0.720 ²
Absent	15	55.6	8	61.5	
Perineural invasion					
Present	12	44.4	6	46.2	0.919 ²
Absent	15	55.6	7	53.8	

*Significant result ¹Fisher's exact test ²Chi-square test

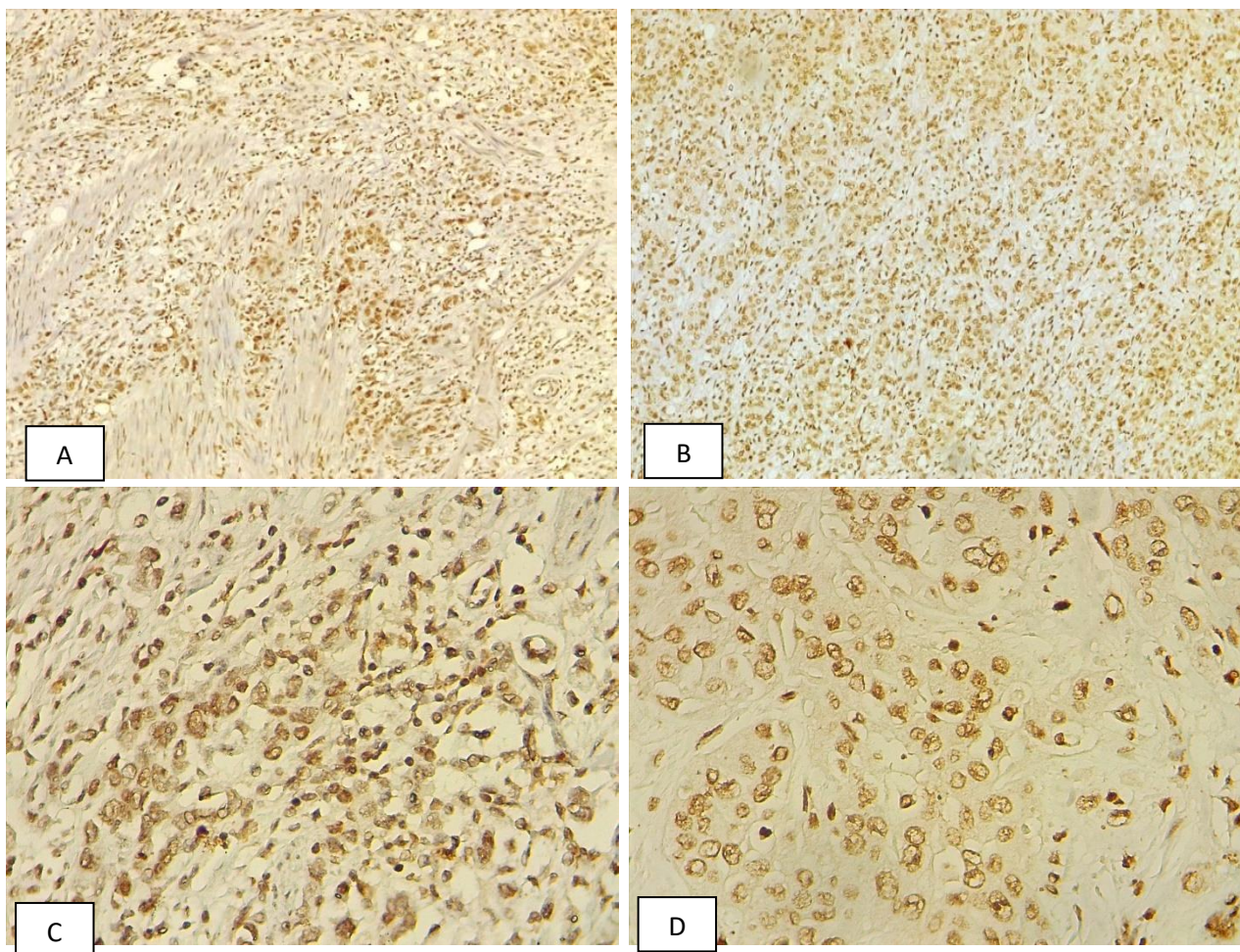


Figure (1) : Microphotograph showing cytoplasmic and membranous brown staining of poorly differentiated gastric adenocarcinoma malignant cells- diffuse type (EBV-LMP), IHC.

A: EBV-LMP positive cytoplasmic expression; 100X. B: EBV-LMP positive membranous expression; 100X.

C: EBV-LMP positive cytoplasmic expression; 400X. D: EBV-LMP positive membranous expression; 400X

DISCUSSION

EBV-LMP immunohistochemical expression and the clinicopathological correlations

AL Noor conducted research in Baghdad in 2016 [157] on 46 adenocarcinoma patients who provided biopsies after gastrectomy. The in situ hybridization found EBV in 39% of individuals. The P53 oncogene was linked to stomach cancer in 65.2% of individuals. The positive findings showed that EBV was highly correlated with age, sex, grade, stage (partial agreement with the study results), and tumor suppressor gene p53 among all patient features

There was a study conducted by **Samir S. et al. in Egypt 2022** [158] which examined a total of one hundred and four surgically removed cases of gastric cancer (GC). Two techniques, namely quantitative real-time polymerase chain reaction (qPCR), were utilized to detect the presence of EBV-derived latent membrane protein-1 (LMP-1) and Epstein-Barr nuclear antigen-1 (EBNA-1) genes. Additionally, immunohistochemistry (IHC) was employed to detect the LMP-1 protein and p16 protein on paraffinized tissue blocks (tumor-suppressor protein p16 as a marker for diagnosis in GC in relation to EBV infection). The results revealed that p16 protein was discovered in (86.5%) of the samples. Additionally, the presence of EBV LMP-1 was seen in (3.84%) of the total cases. The (qPCR) method successfully identified (13.46%) positive for EBV. In situations where EBV positivity was identified using (qPCR), there was an absence of p16 expression. This prevalence was found to be higher when compared to the control group, where none out of the ten individuals (0.0%) tested positive for EBV-LMP1. It was statistically non-significant. The combination of the two methods i.e. IHC and qPCR in addition to p16 is a recommended for improving the accuracy of identification of the infected cancer. Results in this study of p16 protein showed that loss of its expression was associated with EBV infection. Leukemia, brain tumors, malignant melanoma, esophageal carcinoma, and lung carcinoma have all been linked to a loss of p16 expression

In this study, the results for EBV status goes in disagreement with the mentioned studies above due to different sample size, different methods of EBV detection like PCR, FISH and ISH and the usage of different clones of IHC markers.

CONCLUSION:

EBV-LMP immunohistochemical analysis showed an important relationship to lymph nodal involvement and the staging of gastric adenocarcinoma. The evaluation of EBV-LMP expression in gastric cancer tissue can serve as an indicator to determine the prognosis and identify cancer patients who are at greater risk of metastasis.

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