International Research Journal of Pharmacy and Medical Sciences
ISSN (Online): 2581-3277

Prevalence and Clinicopathological Correlation of Epstein-Barr Virus-Associated Gastric Cancer in Iraqi Patients

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Abstract—Background*: New horizons are opened for the treatment of Epstein-Barr Virus (EBV)-positive gastric cancers, however, specific clinicopathological characteristics have not been identified in addition to ethnic and geographical incidence variation.* ***Aim****: In this study, we investigated the prevalence of EBV-positive gastric cancer in a sample of Iraqi patients and evaluated the clinicopathological correlation.* ***Materials and Methods****: Thirty formalin fixed paraffin embedded primary adenocarcinoma were retrieved from the archives of the Department of Pathology at Baghdad Teaching Hospital. Demographics and histopathological data were collected from patients' records. EBV-DNA was targeted by an in-situ hybridization technique.* ***Results****: the mean age of the patients was 55.8± 13.39 years, with a range of 30-80 years. The male-to-female ratio is 1.3:1. EBV was detected in 8 (26.6%) cases. EBV-positive gastric carcinoma did not exhibit any demographically significant variations. Although 5 (62.7%) of the EBV-positive tumors were of intestinal type, 6 (75%) were antral/pyloric and 6 (75%) were advanced stage, these characteristics did not differ significantly from those of the EBV-negative tumors.* ***Conclusions****: the prevalence of EBV gastric cancer is relatively high. Association with histopathologic type, anatomical site, and tumor stage did not reach statistically significant warranting larger multicentric study.*

Keywords— *EBV, gastric cancer, ISH, in situ hybridization.*

I. INTRODUCTION

Globally, gastric cancer ranks as the fifth most common cancer and the third leading cause of cancer-related death (1). Although a global decline has been observed since the mid-20th century (1), Iraq witnessed a significant increase rate after 2007 (2).

Gastric adenocarcinoma can be classified into two distinct subtypes: diffuse (undifferentiated), or intestinal (well-differentiated). These subtypes differ in their morphological characteristics, mechanisms of development, and genetic makeup. A comprehensive study of genetic alterations in gastric cancer was documented in the Cancer Genome Atlas which subdivided gastric adenocarcinoma into four subtypes: those that are genetically stable (20%), microsatellite unstable (22%), Epstein Barr Virus (EBV)-positive (9%) or chromosome unstable (50%) (3, 4).

EBV is a member of the Herpesviridae family and the well-known cause of worldwide common infectious mononucleosis (5). Most people become infected with EBV during the first decade of life predominantly in areas with crowded living conditions and poor hygiene (6). As a result of the establishment of equilibrium among EBV persistence, virion generation, and immunological regulation, a significant proportion of the world's populace may remain infected with EBV throughout their lives without encountering adverse health consequences. Serologic studies suggest that more than 90% of the adult population worldwide has been infected by the virus during their lifetime (7). Nevertheless, there exists a correlation between EBV and the development of various malignancies such as nasal NK/T-cell lymphomas, African Burkitt's lymphoma, Hodgkin's lymphoma, and gastric carcinoma; post-transplant lymphoma; lymphoproliferative disorder; nasopharyngeal carcinoma; lymphoepithelioma-like squamous cell malignancies; and leiomyosarcoma (8).

There is significant variation in the viral frequency of the epithelial tumors, ranging from around 100% of nasopharyngeal carcinoma to around 10% of gastric carcinomas (9, 10). Additionally, there are differences in the expression patterns of viral genes, indicating that EBV may impact cell proliferation via many mechanisms (9).

In situ Hybridization is a very useful tool to confirm a potential association between a newly detected pathogen and tissue alterations (10). Directly expressing gene products on tissue sections allows the detection and localization of the virus DNA or RNA inside the target cells providing a more accurate representation (11).

The first identification of EBV DNA in gastric cancer was recorded in 1990 by Burke et al using polymerase chain reaction (PCR) (12). The involvement of EBV in gastric carcinogenesis was then confirmed by Tokunaga et al. (1993) and Fukayama et al. (1999) (13, 14). Subsequently, a substantial body of data has emerged, substantiating the robust etiological correlation between this virus and stomach malignancy.

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The prevalence of EBV in Iraqi patients with gastric cancer has been assessed utilizing various methodologies at varying rates (15, 16). The purpose of this research was to determine the rate of EBV-positive malignancy through the use of in situ hybridization. Furthermore, investigates the correlation between EBV expression and various clinicopathological parameters pertaining to gastric carcinoma, including age, sex, anatomical site, visual appearance, histological subtype, stage, and grade.

II. Materials and Methods

A cross-sectional study was conducted on 30 paraffin blocks of gastric carcinoma specimens from gastrectomy operation which were retrieved from the archive of the Department of Histopathology of Medical City Teaching Laboratories during the period between January 2016- January 2017.

The information regarding the tumor site, age, gender, grade, and stage was extracted from histopathological reports, archival files, and documents about the cases. There were no available data on the patient's prior infection history with infectious mononucleosis.

Two sets of representative FFPE sections of 4µm thicknesses were prepared for each case. One section was stained with hematoxylin and eosin (H&E), while the other sections were prepared for in situ hybridization. Samples were re-examined by two senior pathologists who confirmed that all cases were adenocarcinoma.

In situ hybridization

A ready to use ISH kit was obtained from Maximbio, Rockville. The manufacturer's instructions were followed with minimal adjustments to improve performance. Pre-hybridization was conducted by tissue sections deparaffinize and dehydration. 100 µl of freshly made 1X protease K solution was applied to the whole segment and placed slides in a humid chamber and incubated at 37ºC for 15 minutes. Hybridization was done by applying 10-20 µl of DNA probe/hybridization solution to tissue slices. DNA was denatured in the oven at 95ºC for 10 minutes, then slides were placed in a humid chamber at 37ºC overnight after being removed from the oven. In post- hybridization, 1–2 drops of linker 1 were added to tissue sections and placed in humid chamber, at 37ºC for 1 hour followed by 1–2 drops of linker 2 were and placed in humid chamber at 37ºC for 40 minutes. 1–2 drops of conjugate on tissue sections, slides were maintained in a humid chamber at 37ºC for 40 minutes then the 1–2 drops of substrate at room temperature for 20–40 minutes and monitored under the microscope until a blue precipitate formed at the probe location in the positive cells. All steps are separated by washing. Finally, counterstained with Nuclear Fast Red stain for 15 seconds rehydrated, cleared and mounted.

In situ hybridization signal evaluation: Light microscope examinations assessed EBV expression on slides. Infected cell nuclei show a dark blue positive signal in a diffuse pattern uniformly distributed across most cell nuclei or in a patchy irregular clumped pattern unevenly distributed. Both signal patterns were mixed in most positive situations.

Statistical analysis: Statistical analysis was performed using Statistical Package for Social Sciences (IBM Corp., Armonk, N.Y., USA) software version 25. Descriptive statistics for the clinical features of the patients were done using the range, mean, and standard deviation (SD). Comparison between groups was carried out using the Chi-Square test. Fisher Exact was applied when the expected value in a cell is less than 5.

III. Results

Patient And Tumor Characteristics

The patients had an average age of 55.8± 13.39 years, with a range of 30-80 years. Among them, 73.3% were above the age of 50. The cohort consists of 17 males, accounting for 56.6% of the total, and 13 (43.3%) females. The male-to-female ratio is 1.3:1.

The distal tumors accounted for the bulk of the 22 tumors, representing 73.33%. Out of the total, the intestinal subtype accounted for two-thirds of the cases. More than half of the cases were falling into stages II and III. Additional information may be found in Table 1.

TABLE 1. Patient and tumor characteristics.

Variable		No	Percentage
Age	30-49	8	26.66%
	50-69	16	53.33%
	Over 70	6	20%
Sex	Male	17	56.66%
	Female	13	43.33%
Site	Proximal	3	10%
	Middle	5	16.66%
	Distal	22	73.33%
Microscopy	Intestinal	20	66.70%
	Diffuse	10	33.30%
Grade	I	1	3.33%
	II	18	60%
	III	11	36.66%
Stage	IB	2	6.66%
	II	8	26.66%
	IIIA	7	23.33%
	IIIB	1	3.33%
	IV	2	6.66%

Detection of EBV in Gastric Carcinoma by in Situ Hybridization:

Eight cases (26.6%) out of thirty exhibit EBV DNA expression, as illustrated in Figure 1. The nuclei of the malignant cells exhibited dark blue staining, which manifested in an irregular clumped, diffuse, or mixed pattern, signifying successful hybridization. Lymphocytes and stromal cells in the normal, non-neoplastic gastric mucosa surrounding the adenocarcinoma did not exhibit a positive hybridization signal.

EBV-positive gastric carcinoma did not exhibit any demographically significant variations. As shown in Table 2, although 6 (75%) of the positive tumors were antral/pyloric and 5 (62%) were stage III, these characteristics did not differ significantly from those of the EBV-negative tumors (P=0.231 and 0.338, respectively). There was an even distribution of EBV-positive malignancies across all histological subtypes.

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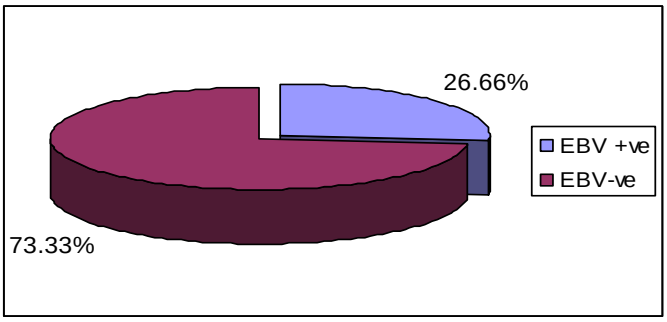

Figure 1: EBV expression in the collected cases.

TABLE 2. Association of EBV expression and demographic and tumor characteristics.

Variables		EBV expression				P value
		Positive n=8		Negative n=22		
		No.	%	No.	%	
age	30-49	3	37.5	5	22.7	0.180
	50-69	2	25	14	63.6	
	Over 70	3	37.5	3	13.6	
sex	Male	4	50	13	59.1	0.657
	Female	4	50	9	40.9	
site	Proximal	1	12.5	2	9.1	0.231
	Middle	1	12.5	4	18.2	
	Distal	6	75	16	72.7	
microscopy	Intestinal	5	62.5	15	68.2	1
	Diffuse	3	37.5	7	31.8	
grade	I	0	0	1	4.5	0.584
	II	4	50	14	63.6	
	III	4	50	7	31.8	
Stage	I	1	12.5	1	4.5	0.338
	II	1	12.5	7	31.8	
	III	5	62.5	13	59.1	
	IV	1	12.5	1	4.5	

IV. DISCUSSION

EBV-positive gastric cancers have the capability to respond to newer immunotherapy drugs (3), however, specific clinical manifestations have not been identified in addition to ethnic and geographical incidence variation (3, 17, 18). In this study, we reported the prevalence of EBV-positive gastric cancer in a sample of Iraqi patients and looked into the clinicopathological correlation.

In the current study, EBV-positive gastric cancer represented 26% of all samples. This was high when compared to Japan, one of the countries with the highest rates of gastric cancer, where EBV gastric cancer is estimated to be 5-to-10% (19). Environmental and cultural variables in various geographic regions (20) and the condition of the patient's immune system (21) likely have a role in the genesis of EBV gastric cancer, as the incidence of EBV-GC in the United States is much greater than in Japan (16%-18%) (22). Pooled analysis of 20,361 gastric cancer revealed that 8.77% (95% CI: 7.73–9.92) of patients with gastric cancer had EBV. The prevalence of EBV was greatest in gastric cancer patients from Poland and lowest in gastric cancer patients from the United Kingdom (25.57, 95%CI: 6.13–64.36% vs. 2.78, 95%CI: 1.51–5.06%) (23). Our findings are in agreement with local studies. A previous local study included 64 gastric cancers, and EBV was detected in 18 (28%) using ISH (24). Another study evaluated EBV presence in gastric cancer using PCR and found that EBV DNA was amplified in 23% of paraffin samples and 33% of fresh samples (16). Al-Abadi et al found that EBV DNA was amplified in 25% of gastric cancer, 80% of them were of intestinal type (15).

In the current study, there was no observed prereplication based on age or gender in EBV-positive gastric cancer. Several studies have shown elevated rates of gastric cancer positive for EBV among males and those under the age of 60 (25). The mean age of individuals with EBV-positive stomach cancer was reported to be 58 years, with 71% of them being male (18, 20, 22). A meta-analysis concluded that EBV was 1.9 times more common in male patients with gastric cancer compared to female patients (P 0.0001). Nevertheless, the odds ratio (OR) for gastric cancer related to Epstein-Barr virus (EBV) was shown to be considerably greater in females compared to men (P = 0.06) (23).

Our findings indicate that EBV-positive gastric cancer was more often seen in cases of intestinal type (62.5%), moderately or poorly differentiated. Additionally, three-quarters of these cases were located in the distal section of the stomach, and 75% were classified as advanced stage. Campos et al reported a higher incidence in distal tumors consistent with our finding (20), whereas other studies found that EBV-positive gastric cancer often occurs in the proximal stomach (cardia and gastric body) (19). The link between proximal tumors and EBV-positive gastric cancer has been confirmed by meta-analytic studies (18, 23). However, it is worth noting that the odds ratio estimate for EBV-associated gastric cancer was much greater in the antrum compared to the cardio and body regions, although this difference did not reach statistical significance (23). The justification for this trait might be attributed to the distinct physiological circumstances shown by the different regions of the stomach (23).

Data regarding histopathological subtypes are conflicting. According to several studies, diffuse gastric cancer predominates EBV-positive cancers in Japan and India (18, 26, 27). Other studies conducted in France and the Netherlands demonstrated a predilection for intestinal form (28, 29). Pooled research has not identified a significant distinction in the prevalence of EBV between the intestinal and diffuse types, consistent with our finding (23).

In terms of tumor grade, Carrascal and colleagues found, in agreement with our finding, that EBV-GC was detected more frequently in poorly differentiated and moderately differentiated adenocarcinomas, with a higher frequency observed in advanced tumors involving the serosa (30). It has been reported that the majority of patients were diagnosed in the advanced stage (52%, stage III, and IV), and 2247 (49%) patients died during the median follow-up period of 3 years (25).

In conclusion, the prevalence of EBV in gastric cancer using ISH was 26.7%. Although a significant correlation was not achieved, EBV gastric cancer was more frequent in distal anatomical sites, intestinal type, and advanced stage.

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