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Is Epstein - Barr virus Infection a Late Event in Gastric Tumour Pathogenesis or a True Aetiological Agent?

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Objectives. To determine the association of Epstein-Barr Virus in gastric tumours in the Kingdom of Bahrain and to explore its role in tumour pathogenesis.
Method. A reterospective study was performed including 58 cases of gastric tumours between January 2001 till December 2005 received in Histopathology department Salmaniya Medical Complex, Kingdom of Bahrain. These cases consist of carcinomas, carcinoids and lymphomas. Formalin fixed tissue sections were used to perform immunohistochemistry and In-Situ Hybridization using Anti-EBV-LMP (Latent Membrane Protein) antibodies and EBER PNA probe respectively.
Results.- The EBV-RNA expression was detected in 44.40% of the intestinal type adenocarcinomas, 25% signet ring cell type adenocarcinomas, 100% carcinoid tumours and 50% lymphomas (p value < 0.036). Tumours showing positive staining pattern for EBER PNA probe were also positive for EBV-LMP except the carcinoid tumours. Of the EBV positive carcinoma cases 75% were poorly differentiated tumours.
Conclusion.-This study is first in the kingdom of Bahrain showing EBV association in gastric tumours. The presence of EBV infection in carcinoma, carcinoid and lymphoma cells, and its absence in normal or metaplastic gastric epithelium suggests that EBV infection is a terminal event rather than an initiating factor in tumour pathogenesis. More prospective studies are required to clarify the epidemiology and aetiology of EBV-associated gastric cancer in the Arabian Gulf region.
Key words. Gastric tumours, Epstein Barr Virus Association.

Introduction

Epstein Barr Virus (EBV) is the first virus associated with neoplasia such as Burkitt's Lymphoma [1] and nasopharyngeal carcinoma [1]. EBV has also been detected in tumour cells of gastric carcinoma [2], Hodgkin's disease [3], T-cell lymphoma [4], parotid carcinoma [5], and thymic carcinoma [6].

EBV sequences have been demonstrated in intestinal and signet ring cell types of gastric carcinomas both in Japan and USA [7,8]. Therefore, a reterospective study was launched to determine the association of Epstein-Barr virus in gastric malignant tumours, based on previous claims in some parts of the world such as Japan and USA. In addition we want to explore the role of EBV in gastric tumour pathogenesis.

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Material and Methods

This is a retrospective study including cases received over a period of five years (between January 2001 and December 2005) at Salmaniya Medical Complex Bahrain, which is the only tertiary care centre providing health services to a population of approximately 650, 000 people including Bahrainis and the expatriates. Total population of Bahrain is 900,000. Only those patients were included who underwent endoscopy with targeted gastric biopsies for upper gastrointestinal symptoms.

Histopathology reports were retrieved from the files of Histopathology Department, Salmaniya Medical Complex. The data was accumulated and all results were merged. Histological slides from cases with diagnosis of gastric tumours were retrieved from the archives and reviewed under the microscope by two pathologists. Overlapping of cases was avoided. A total of 58 gastric tumour cases were identified and divided into two groups: group 1, adenocarcinomas (43 cases) ; and group 2, other tumours including lymphomas (2 cases) and carcinoids (3 cases).

Patients were classified in four age groups <40, 41-60, 61-80 and >80 years. Statistical analysis

were performed using Statistical Package for the Social Sciences (SPSS version 15).

Immunohistochemistry.

Immunohistochemistry was performed on tissue section from 58 cases who underwent endoscopy using monoclonal mouse Anti -Epstein-barr-Virus,LMP, Clone CS.1-4 (Code# M 0897) [DAKO,Glostrup,Denmark]. The sections (3-4 µm) were cut and mounted on saline coated slide, dried, deparaffinized in xylene and rehydrated in alcohol. Endogenous peroxidase was quenched by 3% Hydrogen peroxidase for 10 minutes followed by heat antigen retrieval in 0.01M citrate buffer at pH 6.0 in microwave oven at a medium oven setting. Slides were incubated overnight at 4^0C with primary antibody monoclonal mouse Anti -Epstein- barr-Virus,LMP, Clone CS.1-4 (Code# M 0897) [DAKO] dilution 1:100. Immunoreaction was detected and visualized by using an Envision system (HRP) {Cat# K 406511} [DAKO].

EBER In-Situ hybridization (ISH).

ISH was performed on tissue sections from 58 cases using Epstein Barr Virus (EBER) PNA Probe/Fluorscein (Cat # Y 5200) [DAKO] and PNA ISH Detection Kit (Cat # K-5201) [DAKO]. Paraffin embedded 3-4 µm thick sections cut from each block were deparaffinized, rehydrated and digested with proteinase K. Subsequently sections were hybridized for 2 hours at 37^0C with oligonucleotide probe for EBER. Hybridization products were detected using Anti FITC/AP and substrate. The slides were counterstained with Mayer's haematoxylin. Positive staining was seen as dark blue granules at the site of hybridization under light microscope. Tumour was considered to be associated with the EBV if the cells demonstrated labeling of the tumour cells using probe [9].

Results

Fifty-eight cases were included in this retrospective study. Table 1and figure 1 show cases classified according to histological type of the tumour. Gastric carcinoma was diagnosed according to Lauren's classification ([10]) and classified into intestinal type adenocarcinoma (40 cases) and signet ring cell type adenocarcinoma (13 cases). The gastric tumour site is shown in table 2. Antrum was the commonest site of occurrence. Clinical data including age, gender, nationality according to tumour type was abstracted from histopathological reports as shown in table 3.

Adenocarcinoma was the commonest diagnosis among gastric tumours. Forty of the 58 (69%) cases were intestinal type adenocarcinoma, thirteen cases were signet ring cell adenocarcinomas (Figure 2), two cases were gastric lymphomas (Figure 3) and three were carcinoids.

Intestinal metaplasia was noted in 47 (81%) of the 58 cases as shown in table 4**.**

Partial or complete gastric resections were performed in 41/58 study cases; among these 27 cases were intestinal type and 10 cases were signet ring cell type adenocarcinoma, 2 carcinoids and 2 lymphomas as shown in table 5.

Among the adenocarcinoma cases 65.50% of the intestinal type and 66.70% of signet ring cell type have shown positive staining pattern for Epstein-Barr Virus Latent membrane protein (EBV-LMP) using Immunohistochemistry as shown in Table 6 and figure 4. The tumour cells have displayed brownish cytoplasmic staining pattern as seen in figure 5.

One of the two lymphoma cases have shown positive staining pattern for EBV-LMP. However, the carcinoid tumours were negative for EBV-LMP. In addition, among the intestinal type adenocarcinoma cases showing positive staining pattern for EBV-LMP, 50% were well differentiated, 73% were moderately differentiated and 75% were poorly differentiated tumours as shown in Table 7.

The EBV-RNA expression was detected in 44.40% of the intestinal type adenocarcinomas, 25% signet ring cell type adenocarcinomas, 100% carcinoid tumours and 50% lymphomas as shown in table 8 and figure 7 (p value < 0.036). The tumours showing positive staining pattern for EBER PNA probe were also positive for EBV-LMP but the three carcinoid cases.

Table 1: Frequency of different types of tumours in the study cases.

Tumour Type	Frequency
Adenocarcinoma Intestinal Type	40
Signet Ring Type Adenocarcinoma	13
Large B Cell Lymphoma	2
Carcinoid Tumour	3

Table 2: Distribution of different types of tumours according to topographic location of the gastric tumour.

Type of Cancer	No. of Cases	Antrum	Cardia	Body	Fundus	GE Junction	Location not provided on request form	Total
Adenocarcinoma Intestinal Type	40	17	1	3	1	3	15	40
Signet Ring Cell Type Adenocarcinoma	13	3	1	1	0	0	8	13
Carcinoid	3	1	0	1	0	0	1	3
Lymphoma	2	0	0	0	0	0	2	2
Total	58	21	2	5	1	3	26	58

Table 3: Age groups, gender, and nationality of the cases according to the different types of tumours.

Type of Cancer	No. Cases	Age Group			Gender Ratio		Nationality			
		<45	45-65	>65	Male	Female	Bahraini	%	Non-Bahraini	%
Adenocarcinoma intestinal type	40	4	9	27	31	9	33	82.50%	7	17.50%
Signet Ring cell type adenocarcinoma	13	5	4	4	4	9	8	61.50%	5	38.50%
Carcinoid	3	0	3	0	0	3	3	100%	0	0%
Lymphoma	2	0	0	2	2	0	2	100%	0	0%
Total	**58**		**58**		**37**	**21**	**46**		**12**	

Table 4: Presence of intestinal metaplasia in association with different types of tumours.

Type of tumour	metaplasia present	metaplasia not present
Adenocarcinoma Intestinal Type	31	9
Carcinoid Tumor	1	2
Signet Ring Cell Type Adenocarcinoma	13	0
Lymphoma	2	0
Total	47 (81%)	11 (19%)

Table 5: Resection procedure in different types of tumours

Type of Tumour	Resection done	Resection not done
Adenocarcinoma Intestinal type	27	13
Carcinoid Tumor	2	1
Signet Ring Cell	10	3

Type Adenocarcinoma		
Lymphoma	2	0
Total	41	17

Table 6: Epstein-Barr Virus LMP expression among different types of tumours using Immunohistochemistry.

Type of Cancer	Immunohistochemistry	
	Present %	Not Present %
Adenocarcinoma Intestinal type	65.50%	34.50%
Signet Ring Cell Type Adenocarcinoma	66.70%	33.30%
Carcinoid	0%	100%
Lymphoma	50%	50%

Table 7: Positive staining pattern for Epstein-Barr Virus LMP in intestinal type adenocarcinoma according to tumour differentiation.

Adenocarcinoma (intestinal type) Differentiation	Immunohistochemistry	
	Present %	Not Present %
Well	50.00%	50.00%
Moderate	73%	27%
Poor	75%	25%

Table 8: EBV presence in different types of gastric carcinoma by in situ hybridization method.

	In-Situ Hybridization	
Type of Cancer	Present %	Not Present %
Adenocarcinoma Intestinal type	44.40%	55.60%
Signet Ring Cell Type Adenocarcinoma	25.00%	75.00%
Carcinoid	100%	0%
Lymphoma	50%	50%

The E Colour Films this page displayed as nuclear region using EBER – In situ hybridization technique, as shown in figures 8 and 9.

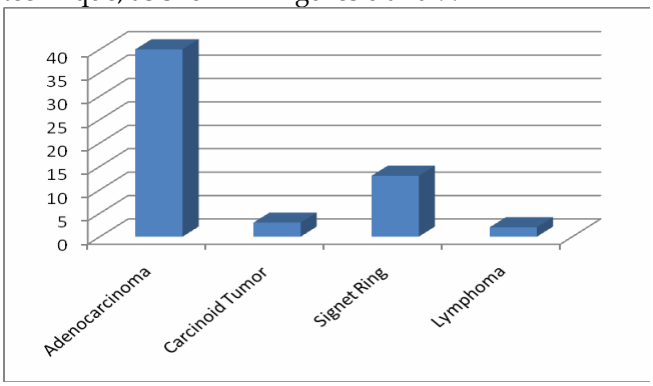

Figure 1: Frequency of different types of cancer among the studied cases.

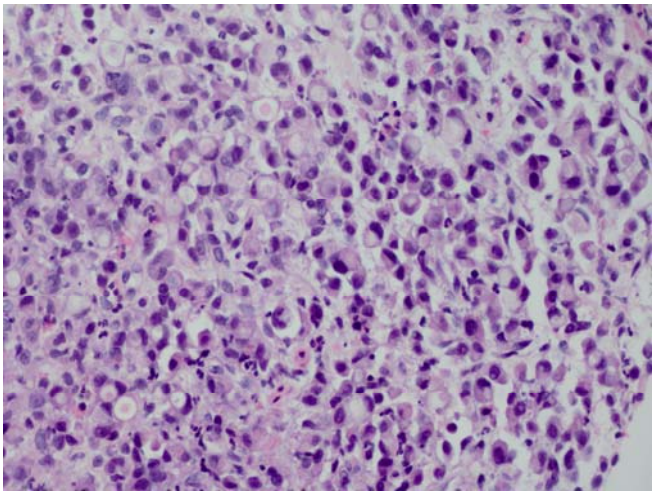

Figure 2: Gastric adenocarcinoma signet ring cell type. (H&E x 400)

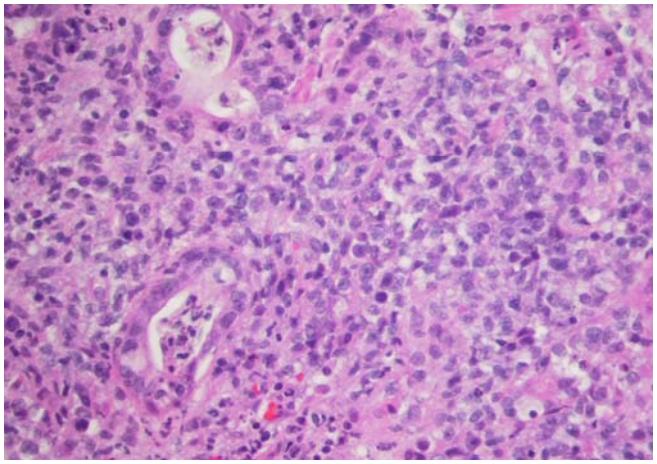

Figure 3: Gastric lymphoma showing lymphoepithelial lesions. (H&E x 400)

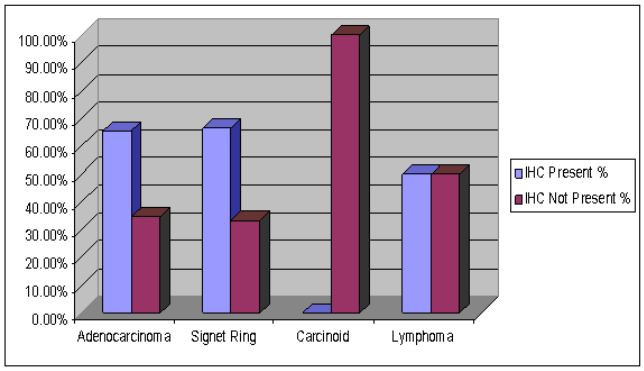

Figure 4: Presence of Epstein Bar Virus in different types of cancer using Immunohistochemistry method.

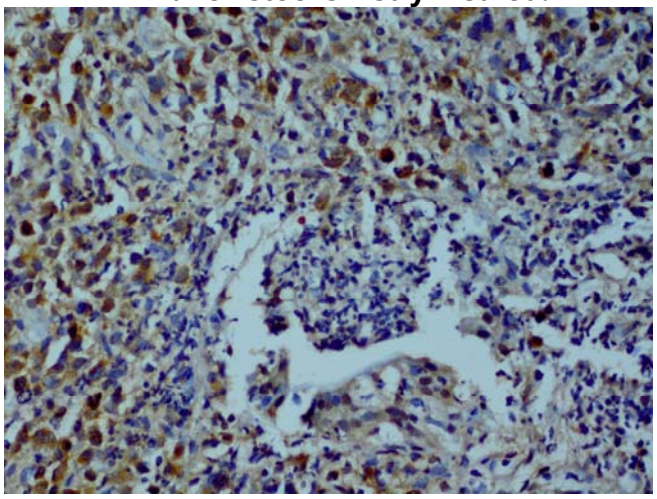

Figure 5: Gastric adenocarcinoma showing malignant signet ring cells exhibiting positive brown cytoplasmic staining. (EBV- LMP Immunostain x 400)

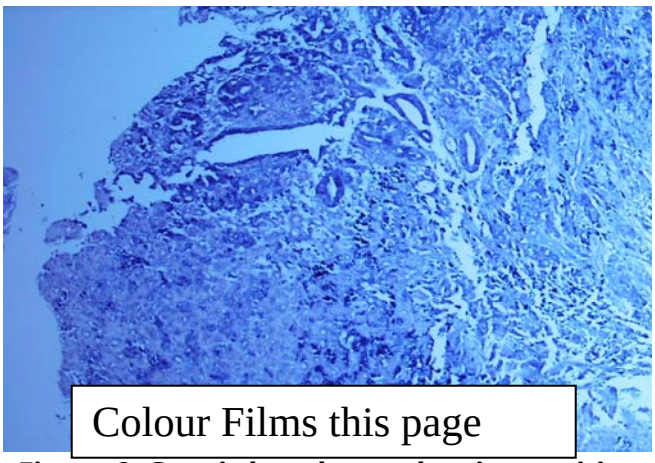

Figure 6: Gastric lymphoma showing positive staining for EBV-LMP (x 40).

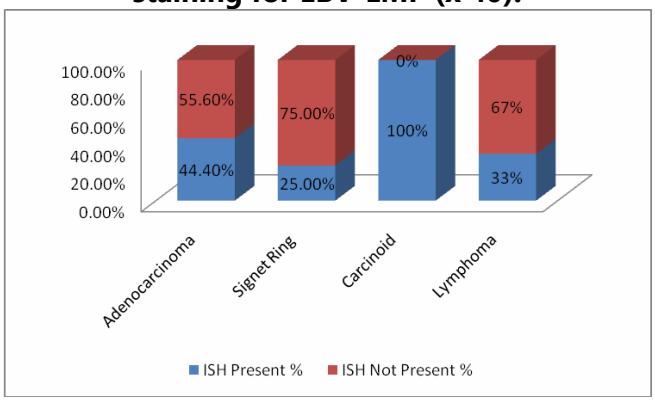

Figure 7: EBV presence in different types of gastric carcinoma by in situ hybridization method.

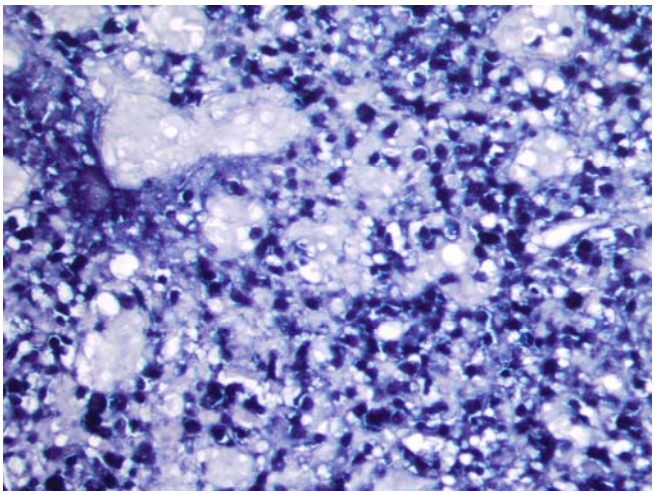

Figure 8. Positive dark blue nuclear staining for EBV RNA in the tumour cell nuclei of signet

ring cell adenocarcinoma, infiltrating the lamina propria singly. Tumour surface is ulcerated.
(In-situ hybridisation x 400)

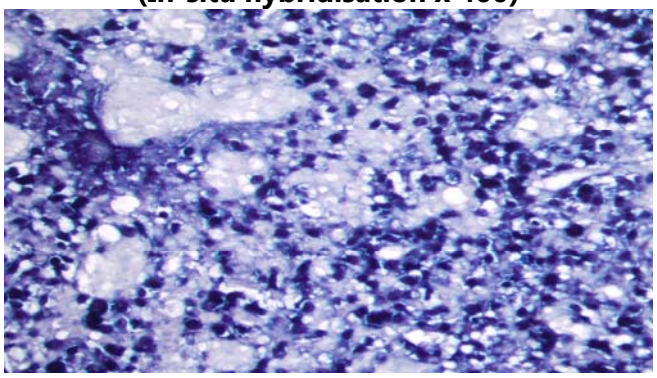

Figure 9. Gastric lymphoma showing dark blue positive nuclear staining in the tumour cells, indicating presence of EBV RNA.
(In-situ hybridisation x 400)

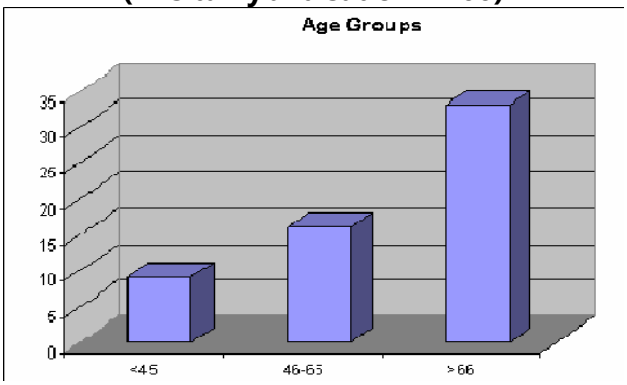

Figure 10. Frequency of gastric tumours according to patient's age groups.

Discussion

Lauren's classification is most commonly used in classifying gastric carcinomas. This recognizes two major histological types of gastric cancer: intestinal type and diffuse signet ring cell type [10]. These two carcinomas are completely different types of tumours with regard to its clinical presentation, epidemiological and morphological features, and genetic alterations. The intestinal type gastric carcinoma is predominant in high risk areas and is usually associated with precursor lesions such as chronic gastritis and intestinal metaplasia, commonly affecting older age group individuals. Whereas diffuse type gastric carcinoma is commonly seen in younger age group associated with locally infiltrative growth pattern at an earlier stage with no precursor lesions. These tumours are often associated with metastasis and peritoneal spread at the time of presentation.

The gastric carcinoid tumour is a generic term applied to any neoplasm composed of neuroendocrine cell, known to be present in stomach. These are small sharply outlined gastric tumours showing trabecular, glandular or insular growth pattern microscopically. Carcinoid tumour cells have cytoplasmic neurosecretory granules under electron microscopy, containing 5-hydroxytryptophan, serotonin, epinephrine[11].

Gastric lymphomas comprise small percentage of malignant tumours of the stomach and nearly all are Non-Hodgkin's type [12].

The common anatomic site of origin for EBV-positive gastric carcinoma is a controversial issue in the literature. Some studies have reported that cardia and middle region of the stomach are the most frequent sites of involvement by EBV positive gastric carcinomas [13]. Whereas other studies have found antrum as the most typical site for EBV positive gastric tumours, which is in agreement with this study [14].

There is growing evidence of demonstrating evidence of EBV infection in gastric tumour cells using molecular biological techniques [11,14,15,16]. The absence of EBER1/2 transcripts in preneoplastic gastric lesions (intestinal metaplasia and dysplasia) and their presence in two distinct types of gastric carcinomas strongly suggest that EBV can only infect neoplastic gastric cells rather than metaplastic or normal gastric epithelium, which supports the fact that EBV infection is a late event in multistep process of gastric cancer pathogenesis [15]. This phenomenon has been supported in this study by the absence of EBV infection in normal or metaplastic gastric epithelium and its presence in gastric carcinoid tumours, gastric lymphomas and gastric carcinomas. In addition EBV is present in 75% of the poorly differentiated carcinomas in contrast to 50% within well differentiated carcinomas as seen in Table 7. This suggests that immunosuppresion may be more commonly present in poorly differentiated tumour cases in contrast to cases with well differentiated tumours.

Contrary to the previous statement, some reports based on southern blot technique and cell culture analysis suggests that carcinoma is initiated and progressed from a single EBV infected cell [16].

The in-situ hybridization technique is a gold standard in detecting EBV, which has been clearly

demonstrated in this study by showing 100% positivity for the viral RNA in carcinoid tumours, where immunohistochemistry failed to detect viral LMP in these tumours. The gastric carcinoma cases and a lymphoma case positive for EBER PNA probe were also positive for EBV-LMP. However, EBV-LMP positivity was observed in more cases than EBER PNA probe in gastric carcinomas. The lymphoma case was positive for EBV by both techniques (Tables 6 and Table 8).

Previously performed studies have demonstrated exclusively moderate to poor histological differentiation of EBV-positive gastric carcinomas [17, 18]. However, this study shows EBV association in a wider range of well to poorly differentiated gastric carcinomas (Table7).

This study has shown no significant correlation between Helicobacter pylori associated gastritis and gastric tumour as shown in previous studies [19]. This may be due to early and prompt treatment of Helicobacter pylori infection. However, a significant association between intestinal metaplasia and gastric carcinoma have been observed as shown in Table 4, which is in agreement with the previous studies [20].

Merit of this study is that all the pathology reports and haematoxylin and eosin stained sections were reviewed by two pathologists, which minimizes the chances of false positive diagnosis, as false increase in gastric carcinoma cases has been reported in some previous studies[14].

The EBV-associated gastric carcinomas are commonly seen in elderly people as shown in the previous studies [17, 18], which has been confirmed in the current study as shown in figure 10.

Conclusion

This study is the first to highlight EBV associated gastric tumours in the Kingdom of Bahrain. The presence of EBV infection in carcinoma, carcinoid and lymphoma cells, while its absence in normal or metaplastic gastric epithelium suggests that EBV infection is a terminal event rather than an initiating factor in tumour pathogenesis. However, more studies are required to clarify the epidemiology and aetiology of EBV-associated gastric cancer in the Arabian Gulf region.

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