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ORIGINAL ARTICLE

Epstein-Barr virus is absent in gastric superficial neoplastic lesions

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Abstract

Epstein-Barr virus (EBV) has been associated with about 9% of all gastric carcinomas, but its role in gastric carcinogenesis remains unclear since there is lack of evidence of EBV presence in pre-neoplastic lesions of gastric mucosa. This study intends to determine the prevalence of EBV in gastric dysplasia and superficial neoplasia to clarify whether EBV infection is an early or late event in gastric cancer development. This retrospective study included a total of 242 gastric lesions from 199 consecutive patients who were referred for endoscopic resection. The histological classification of lesions includes 137 low- and high-grade dysplasia and 105 superficial carcinomas. EBV infection was investigated by EBER-ISH. Results showed that EBV was not detected in any epithelial cells of any case with dysplasia or superficial carcinomas, although we observed the presence of a small number of EBV-infected lymphocytes in 2.1% of all lesions. These results showed that EBV is not present in gastric dysplasia neither in superficial carcinomas suggesting that EBV carcinogenesis is a late event in well/moderately differentiated gastric carcinogenesis.

Keywords Gastric dysplasia · Neoplastic lesion · Gastric cancer · Epstein-Barr virus (EBV) · EBVaGC

Introduction

Gastric cancer represents a major public health issue mostly due to the late diagnosis. It ranks as the fifth most common cancer worldwide and it is one of the leading causes of death by cancer [11, 12]. Gastric carcinogenesis has been characterised as a multistep and multifactorial process. The most commonly accepted hypothesis of gastric carcinogenesis has been described as a progressive cascade from normal gastric epithelium through chronic gastritis (CG), chronic atrophic gastritis (CAG), and intestinal metaplasia (IM) ultimately leading to dysplasia and carcinoma. According to the *Vienna Classification of Gastrointestinal Epithelial Neoplasia*, dysplasia can also be graded as high- or low-grade dysplasia [9, 10]. These events are still well accepted for one of the most common type of GC, the intestinal subtype according to the

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Lauren's classification, but for the other types, the identification of precursors lesions still remains a challenge for specialists [8, 17, 29].

The *Cancer Genome Atlas Research Network* (TCGA) proposed four different subtypes of GC based on their genomic and molecular features: microsatellite instability (MSI), genomically stable (GS), chromosomal instability (CIN) and Epstein-Barr virus (EBV)-positive [5]. During the past decade, EBV has been detected in about 10% of all gastric cancer cases worldwide but its role in stomach's carcinogenesis has not been fully understood [4, 35]. Previous studies regarding the detection of EBV in premalignant lesions of gastric cancer are extremely controversial [13, 15, 24, 36]. Some authors have suggested that EBV infection is a late event of gastric carcinogenesis; however, few studies have also detected EBV in patients with gastritis [7, 14]. The knowledge of how and when EBV infects the gastric epithelial cells during the carcinogenesis process would help to determine the role of its infection in gastric cancer development. This study aimed to determine if EBV is an early event of gastric carcinogenesis evaluating its prevalence in patients with gastric dysplasia and superficial neoplasia.

Material and methods

Study description

This study included a retrospective cohort of 199 consecutive patients who were referred for endoscopic resection between March 2003 and December of 2015 at Portuguese Oncology Institute of Porto. Patients were selected accordingly to the following criteria: (1) being submitted to endoscopic resection, (2) with high- or low-grade gastric dysplasia or gastric carcinoma confirmed by histology in the post-resection specimen; and (3) with a representative block for EBV detection.

A total of 242 neoplastic lesions were histologically examined by two pathologists dedicated to gastrointestinal pathology and classified according to the *Vienna classification system* and *WHO classification system*, for tumour and premalignant lesions, respectively [9, 10, 17, 33]. TNM-staging was performed in accordance with the UICC/AJCC system 7th edition [33]. The study was approved by the Ethical Committee of Portuguese Oncology Institute of Porto (CES IPO 80/2014).

EBV detection

EBV infection was detected in all patients using histological sections obtained from formalin-fixed paraffin-embedded (FFPE) tissue blocks. Briefly, EBV was detected by in situ hybridization (ISH) targeting the EBV-encoded small RNAs (EBERs) in FFPE tissue samples as previously described [27]. For quality control, 10% of all samples were retested in independent experiments and positive controls were used in all experiments. An EBV-associated premalignant lesion is defined by the presence of EBV in epithelial gastric cells, whereas the presence of EBV-infected lymphocytes was considered as negative.

Statistical analysis

Statistical analysis was performed using the computer software IBM SPSS statistics for Macintosh, version 24.0.0.1 (IBM Corp, Armonk, NY, USA). Chi-Square (χ^2) or Fisher's exact test was used to compare categorical variables, with a significance level of 5%.

Results

Characterization of the population

A total of 199 patients including 114 males and 85 females with mean age 68.2 ± 10.6 years were submitted to endoscopic resection and included in this study. A total of 242 lesions were identified, being the majority located in the lower third of the stomach ($n = 116$, 48.7%), while the remaining were almost equally distributed between the middle and the upper region of the stomach ($n = 65$, 24.0% and $n = 58$, 26.9%, respectively), and 3 cases (1.2%) were detected in gastric remnant (data not shown).

Post-resection histology revealed a total of 137 gastric lesions ($n = 56.6$) and 105 (43.4) adenocarcinomas (see Table 1). Gastric lesions were classified as low-grade ($n = 37$) and high-grade dysplasia ($n = 100$) and were mainly observed in lower region/gastric remnant ($n = 75$). Intramucosal and submucosal adenocarcinomas were found in 78 (74.2%) and 27 (25.8%), respectively. The location of adenocarcinomas was found to be mainly in the upper/middle region of the stomach ($n = 61$, 58.1%) while the differentiation grade was well/moderate in almost all cases ($n = 98$, 96.1%).

EBV detection

The EBV infection was investigated in lesions by in situ hybridization, and the results revealed that EBV was absent in epithelial cells of all cases. However, the presence of EBV-infected lymphocytes was observed in five different samples, representing 2.1% (5/242) of all lesions. Curiously, we only found EBV-infected lymphocytes in gastric dysplasia and not in the adenocarcinoma group ($p = 0.071$, Table 1). EBV-infected lymphocytes were more frequently found in low-grade dysplasia (80%) comparing to high-grade dysplasia ($p = 0.019$) and 80% were located in the middle and upper third of the stomach (Table 1).

Table 1 Characteristics of the lesions included in this study and detection of EBV

	EBV negative *n* (%)	EBV(+) lymphocytes *n* (%)	*p*
Gender, *n* (%)			
Male, *n* = 137 (56.6)	134 (97.8)	3 (2.2)	1.000
Female, *n* = 105 (43.4)	103 (98.1)	2 (1.9)	
Post-resection histology, *n* (%)			
Dysplasia, *n* = 137 (56.6)	132 (96.4)	5 (3.6)	0.071
Adenocarcinoma, *n* = 105 (43.4)	105 (100)	–	
Dysplasia			
Gender, *n* (%)			
Male, *n* = 74 (54.8)	71 (95.9)	3 (4.1)	1.000
Female, *n* = 63 (45.2)	61 (96.8)	2 (3.2)	
Histological grade, *n* (%)			
Low grade, *n* = 37 (27.0)	33 (89.2)	4 (10.8)	0.019
High grade, 100 (73.0)	99 (99.0)	1 (1.0)	
Location, *n* (%)			
Upper/middle, *n* = 62 (45.2)	58 (93.5)	4 (6.5)	0.176
Lower/gastric remnant, *n* = 75 (54.8)	74 (98.7)	1 (1.3)	
Adenocarcinoma			
Gender, *n* (%)			
Male, *n* = 63 (60.0)	63 (100)	–	
Female, *n* = 42 (40.0)	42 (100)	–	
Histological grade, *n* (%)			
Intramucosal, *n* = 78 (74.2)	78 (100)	–	
Submucosal, *n* = 27 (25.8)	27 (100)	–	
Location, *n* (%)			
Upper/middle, *n* = 61 (58.1)	61 (100)	–	
Lower/gastric remnant, *n* = 44 (41.9)	44 (100)	–	
Differentiation grade, *n* (%)			
Well/moderate, *n* = 98 (96.1)	98 (100)	–	
Poor/signet, *n* = 4 (3.9)	4 (100)	–	

n, number; %, percentage; *EBV*, Epstein-Barr virus

The detailed characteristics of these cases are shown in Table 2. The number of EBV-infected lymphocytes was very low ranging from 1 to 5, except in one of those cases that revealed a higher number (> 30) of EBV-positive lymphocytes surrounding the lesion (Fig. 1). Two of the positive cases (no. 1 and no. 3) had *H. pylori,* which was successful eradicated prior to the endoscopic resection; the other two (no. 2 and no. 4) have never had *H. pylori* infection while case number 5 had no information. Interestingly, the two cases that had a previous *H. pylori* infection have a higher number of EBV-positive lymphocytes.

Discussion

Since its discovery, EBV has been associated with several types of cancer originating from both lymphoid and epithelial cells. Gastric cancer is one of the epithelial malignancies whose development has been linked to EBV infection [1, 28, 30]. In fact, it is described that the EBV-associated gastric carcinomas (EBVaGCs) are a distinct GC subtype, representing almost 10% of all gastric cancer worldwide [16, 18, 26, 28].

EBVaGCs are characterised by presenting EBERs expression in tumour cells and its absence in the normal surrounding tissue [18]. A recent study conducted by our group has characterised the EBVaGC in the North region of Portugal, and it has demonstrated that EBVaGCs represent about 8% of all gastric carcinomas and also that EBV is more often found in intestinal (according to Lauren's classification) and medullary carcinomas [27]. The present study intends to investigate the prevalence of EBV in premalignant lesions and early gastric carcinomas to better understand when EBV infects the gastric mucosa during the carcinogenesis process. We showed no presence of EBV in gastric cancer precursor lesions, which is significantly different from what we describe for GC. Nevertheless, a recent study from the Central Region of Portugal also demonstrated that EBV is present in a very low prevalence in normal gastric tissues in contrast to 11% of gastric tumours [24].

Table 2 Description of patients with EBV-positive lymphocytes

Case no.	Age (y)	Gender	Histology	Location	*H. pylori*	No. EBV-infected lymphocytes
1	52	F	Low-grade dysplasia	Middle third	Eradicated before ER[a]	> 30
2	60	M	Low-grade dysplasia	Upper third	Negative	1
3	71	M	Low-grade dysplasia	Upper third	Eradicated before ER[a]	5
4	72	M	Low-grade dysplasia	Middle third	Negative	2
5	77	F	High-grade dysplasia	Lower third	Unknown[b]	2

[a] *H. pylori* infection was eradicated before the endoscopic resection and was negative at the time of treatment

[b] *H. pylori* infection was not investigated

No., number; *y*, years; *F*, female; *M*, male; *ER*, endoscopic resection

Few studies have reported the presence of EBV in gastric premalignant conditions, mainly in atrophic gastritis adjacent to tumours [6, 7, 14, 15, 19, 20, 23, 34, 36]. The present study was performed selecting consecutive patients submitted to endoscopic resection at Portuguese Oncology Institute of Porto. According to the post-resection histology, patients with low-/high-grade dysplasia and early gastric carcinomas (carcinomas invading no more deeply than the submucosa) compose the study population. The results showed that there was no evidence of EBV infection in both dysplasia and early carcinomas. Interestingly, EBER transcripts were not detected in superficial neoplastic lesions suggesting that EBV infection is a late event in gastric carcinogenesis. The absence of EBV in premalignant lesions could also suggest that the EBVaGCs present an aggressive phenotype, not manifested by premalignant lesions. These findings are in line with previous studies in which EBV has not been detected in adjacent precursor lesions or detected with low frequency [7, 15, 36]. However, some studies showed different results describing the infection of EBV in dysplasia and gastritis cases [13]. These contradictory results could be explained by the use of different methods for EBV detection and interpretation of the results. Some of these studies have used highly sensitive methods, such as quantitative PCR, which could be overestimating the number of EBV-associated cases detecting EBV from the surrounding lymphocytes and ignoring that it might be absent in the tumour epithelial cells. In this study, we showed that 2.1% of the cases show a small number of EBV-positive lymphocytes surrounding the lesion.

EBV-positive gastric cancer is well known for its high rate of poorly differentiated histology [3, 18]. In fact, our results showed that EBV seems not be relevant in the pathogenesis of well-differentiated gastric carcinomas, but the implications for poorly differentiated are limited [23]. Our series includes a limited

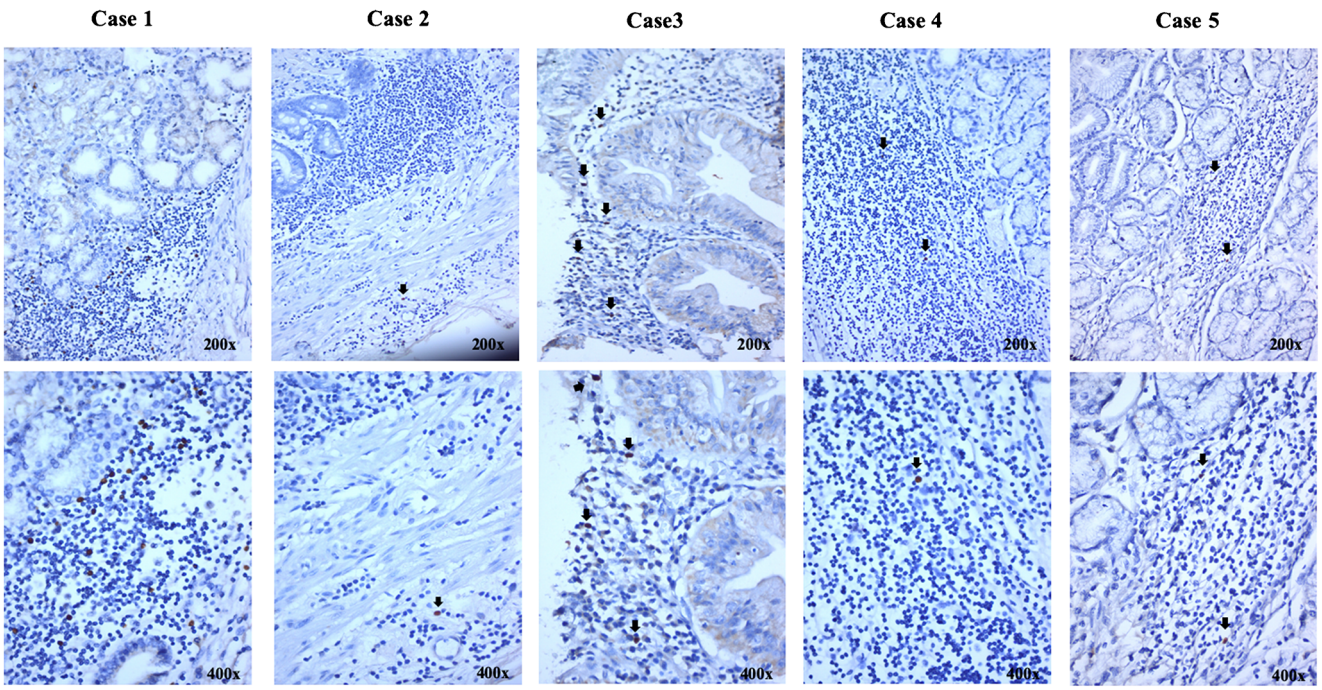

Fig. 1 Representative images of EBER-ISH results of all cases with EBV-infected lymphocytes. A dark brown nuclear staining identified a positive hybridization signal

number of poorly differentiated cases and the conclusion could not be extended to those cases.

How and when EBV gets into gastric epithelial cells during gastric carcinogenesis remains unclear. An overall analysis of literature suggests that EBV participates on gastric carcinogenesis by both direct and indirect mechanisms: infecting epithelial cells and establishing a latent program in which a restrict profile of latent proteins/transcripts are expressed; and/or promoting a chronic inflammatory response contributing to tissue damage and cancer progression [20, 25]. These findings suggest that EBV infects gastric cells at a late stage of stomach carcinogenesis but how EBV is recruited into stomach and how it infects gastric cells needs to be clarified. Corroborating our data, two in vitro studies demonstrated that to be infected by EBV, the undifferentiated nasopharyngeal carcinoma (NPC) cells (other EBV-associated epithelial malignancy) have already had precancerous genetic changes [31, 32]. In fact, pre-existing genetic events, mainly cyclin D1 overexpression and p16 mutations, support the establishment of stable EBV infection in NPC epithelium [31, 32]. Since EBVaGCs are also an EBV-associated epithelial malignancy in which EBV infection seems to be a late event of carcinogenesis, the mechanism of viral carcinogenesis might be similar to the NPC.

It is impossible to discuss gastric carcinogenesis without mention *Helicobacter pylori* (*H. pylori*). The bacterium *H. pylori*, that frequently colonizes the human stomach, has been described as the key factor of several gastrointestinal diseases, ranging from chronic gastritis to gastric adenocarcinoma [29]. Some studies have been suggesting a possible interaction between *H. pylori* and EBV in gastric cancer development. Minoura-Etoh et al. observed a possible antagonism effect between *H. pylori* and EBV where reactive products of *H. pylori* avoid the EBV transformation of gastric cells [22]. In contrast, two other studies have suggested that *H. pylori* may contribute for EBV-associated gastric carcinogenesis by causing gastritis that perhaps might recruit EBV-carrying lymphocytes to the stomach wall, where the virus could be induced to replicate and infect gastric epithelial cells [2, 21]. Moreover, the gastric inflammation may also promote a cytokine-rich microenvironment supporting a clonal growth of EBV-infected epithelial cells [7]. However, it was not possible to investigate the relationship between EBV and *H. pylori* in this study since all lesions were EBV negative.

In conclusion, this study showed that EBV is not infecting the epithelial cells of premalignant lesions, including low- and high-grade of dysplasia and early stages of gastric cancer. These findings suggest that EBV is a late event of well/moderately differentiated gastric carcinogenesis.

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Author contributions JR, HS, PPN, RM, conception and design of the study; JR and MM, EBV detection and data analysis; AG and LPA, histological review of cases and results; DL, MDR and PPN, cases selection and clinical data collection; JR, analysis and interpretation of data, drafting the manuscript, final approval; JR and HS, analysis and interpretation of data, manuscript writing; all authors were able to review the article and give final approval.

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Compliance with ethical standards

All procedures were approved by the Ethical Committee of Portuguese Oncology Institute of Porto (CES IPO 80/2014).

Conflict of interest The authors declare that they have no conflict of interest.

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