Differential expression of apoptosis related proteins and nitric oxide synthases in Epstein Barr associated gastric carcinomas

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Abstract

AIM: To determine the incidence of Epstein Barr virus associated gastric carcinoma (GC) in Brazil and compare the expressions of apoptosis related proteins and nitric oxide synthases between EBV positive and negative gastric carcinoma.

METHODS: *In situ* hybridization of EBV-encoded small RNA-1 (EBER-1) and PCR was performed to identify the presence of EBV in GCs. Immunohistochemistry was used to identify expressions of bcl-2, bcl-xl, bak, bax, p53, NOS-1, NOS-2, and NOS-3 proteins in 25 EBV positive GCs and in 103 EBV negative GCS.

RESULTS: 12% of the cases of GC (25/208) showed EBER-1 and EBNA-1 expression. The cases were preferentially of diffuse type with intense lymphoid infiltrate in the stroma. EBV associated GCs showed higher expression of bcl-2 protein and lower expression of bak protein than in EBV negative GCs. Indeed, expressions of NOS-1 and NOS-3 were frequently observed in EBV associated GCs.

CONCLUSION: Our data suggest that EBV infection may protect tumor cells from apoptosis, giving them the capacity for permanent cell cycling and proliferation. In addition, EBV positive GCs show high expression of constitutive NOS that could influence tumor progression and aggressiveness.

INTRODUCTION

Gastric carcinoma (GC) is one of the most common malignant tumors in the world, although its incidence has gradually declined in recent years. In Brazil, gastric cancer remains a prevalent neoplasm with high mortality, ranked as the third highest cause of cancer-related deaths[1].

The correlation between Epstein-Barr virus (EBV) infection and GC is well known. EBV infection is found in 2%-16% of GCs[2-4]. However, the pathogenic role of EBV infection in GCs remains uncertain and little is known regarding the molecular characteristics of these tumors.

Apoptosis is a biological phenomenon of critical importance in the regulation of a number of physiological and pathological situations, such as cancer development. In general, it is thought that viral infection and its associated proteins protect against apoptosis, which would normally cause cancer regression[5]. Among the genes that regulate apoptosis are the members of the bcl-2 family. Expression of bcl-2 protein in gastric tumor cells is frequent and may differ according the histological type[6]. There are few studies that compare the relationship between EBV infection in gastric carcinomas with apoptosis related protein expression[7-9].

Nitric oxide (NO) is a short-lived biomolecule with various biological functions. It is an important bioactive agent and signaling molecule that mediates a diverse array of actions such as vasodilatation, neurotransmission, and iron metabolism. NO is also involved in the multi-step process of carcinogenesis by mediating DNA damage, inducing angiogenesis, and suppressing immune responses[10]. It has been demonstrated that low concentrations of NO can protect tumor cells against apoptosis[11]; alternatively, high concentrations of NO

can induce tumor cell death[12]. This small molecule is a product of the conversion of L-arginine to L-citruline by nitric oxide synthases (NOS). There are two groups of NOS, inducible (iNOS or NOS-2) and constitutive (nNOS or NOS-1 and eNOS or NOS-3). Recent studies have examined the expression and activity of NOS in human cancer[13,14]. In gastric carcinomas, many studies addressed the issue, and most of them showed an increased activity and expression of NOS in tumor tissue when compared with normal gastric mucosa[15-18].

To the best of our acknowledgement, the relationship between NOS expression and EBV related gastric cancer has not been investigated. Thus, the aim of the present study is to determine the prevalence of EBV infection in GCs in a Brazilian population and investigate the expression of apoptosis related proteins and nitric oxide synthases in EBV positive and EBV negative gastric carcinomas.

MATERIALS AND METHODS

Materials

Two hundred and eight gastric carcinomas, surgically resected from 1995 to 1998, were analyzed for EBV status using in situ hybridization and PCR. To compare EBV positive and negative carcinomas, we selected 103 EBV negative GC, which were samples that were previously studied for expression of apoptosis related proteins and NOS. The cases were reviewed, and representative formalin-fixed, paraffin-embedded blocks from the tumors were selected. Clinicopathological findings were obtained from surgical records and pathology reports. New paraffin sections were stained with hematoxylin and eosin (H&E), and they were assessed independently by two pathologists (MDB & FAS). Histologically, the tumors were classified according to Lauren's classification system in intestinal or diffuse types[19]. Also we graded the amount of lymphoid infiltrate within or between the tumor cells as minimal/mild or moderate/intense.

In situ Hybridization (ISH)

Sections (5-6 μm thick) on glass slides were prepared for ISH with a fluorescein-conjugated oligonucleotide probe for EBER-1 (Novocastra, Newscastle- Upon- Tyne, U.K.). Briefly, after deparaffinization and dehydration the sections were treated with proteinase K (Sigma, St. Louis, MO) for 15 min at 37℃. After washing and dehydration, the FITC-conjugated probe was applied and hybridized overnight at 37℃. The hybridization was further detected by rabbit anti- FITC antibody conjugated with alkaline phosphatase (Novocastra) for 30 min at room temperature. The slides were counterstained with a light Mayer's haematoxylin. Negative controls had no EBER-1 probe applied.

Immunohistochemistry

Sections (5-6 μm thick) from the same tumor blocks, used for EBER detection, were immunohistochemically analyzed using the standard streptavidin- biotin- peroxidase method. The staining was done using the microwave antigen retrieval technique (95℃ for 30 min in citrate buffer pH 6.0)

for all antibodies. Sections were incubated with antibodies against p53 (DO-7, 1:100, Dako, Copenhagen, Denmark), Bcl-2 (124, 1:50, Dako, Copenhagen, Denmark), Bax (bax, 1:50, Dako, Copenhagen, Denmark), Bcl-xl (bcl-xl, 1:50, Dako, Copenhagen, Denmark), Bak (bak, 1:400, Dako, Copenhagen, Denmark), NOS-1 (nNOS, 1:200, Transduction Laboratories, USA), NOS-2 (iNOS, 1:40, Transduction Laboratories, USA), and NOS-3 (eNOS, 1:50, Transduction Laboratories, USA) at 4℃ overnight. After reagenting them with biotinylated secondary anti-mouse antibodies, the antigen-antibody reactions were visualized using streptavidin-horseradish peroxidase conjugate (Dako LSAB Kit, Los Angeles, CA). The peroxidase activity was localized by 0.05% 3, 3'-diaminobenzidine and 0.03% hydrogen peroxide in Tris- buffered saline. The slides were counterstained with Mayer's hematoxylin. The percentage of positively stained tumor cells in each tumor section was blinded evaluated by counting at least 1000 cells in 10 randomly selected high-power fields. Brown staining for p53 protein was located in nuclei; staining for bcl-2, bcl-xl, bak, bax, NOS-1, NOS-2, and NOS-3 protein was located in cytoplasm. The section was considered as expressing the protein if cellular staining was $\geq 5\%$[5].

PCR studies for EBV

The DNA of cancerous tissues from 25 patients with EBV positive gastric carcinomas was obtained from paraffin-embedded surgical blocks. The DNA was extracted by prolonged proteinase K digestion. Briefly, after deparaffinization with xylene and rehydration in ethanol, the tissue was resuspended in 300 μL of SDS-Proteinase K solution (1% SDS; 9 mmol/L Tris-HCl, pH 9.0; 2.25 mmol/L EDTA; 56.5 mmol/L NaCl; 1 μg/μL Proteinase K (invitrogen) and incubated at 48℃ for 48 h. Each 12 h, 20 μL of 20 μg/μL Proteinase K were added. After extraction with an equal volume of phenol-chloroform, the aqueous layer was transferred to a new tube and the DNA was precipitated by the addition of 2 μL of 20 mg/mL glycogen, 100 μL of ammonium acetate (7.5 mol/L) and 800 μL of 100% ethanol followed by incubation overnight at -20℃. After centrifugation, the DNA was resuspended in 50 μL of water. PCR was performed in a reaction final volume of 25 μL, containing 10 μL of DNA; 2.5 μL of 10 × PCR buffer; 1.5 μL of 25 mmol/L MgCl₂; 2 μL of 2.5 mmol/L dNTP mix; 1 μL of 10 μmol/L EBNA1F and EBNA1R primers; and 0.3 μL of Taq DNA polymerase. The following thermal cycle conditions were used: 94℃ for 5 min, 35 cycles of 94℃ for 30 s, 60℃ for 45 s, and 72℃ for 45 s. After the last cycle, a final extension at 72℃ for 7 min was carried out. The sequences of the primers were: EBNA-1F (5'-GTCATCATCATCCGGGTCTC-3') and EBNA-1R (5'-TTCGGGTTGGAACCTCCTTG-3'). The amplified products were visualized by electrophoresis in 8% poliacrylamide gels.

Statistical analysis

Statistical comparison among groups was performed using χ^2 test and Fisher's exact test when appropriate. P values less than 0.05 were considered statistically significant.

Table 1 Comparison of clinicopathological data between EBV positive and EBV negative gastric carcinomas			
	EBV positive gastric carcinomas (*n* = 25)	EBV negative gastric carcinomas (*n* = 103)	*P* value
Gender			NS
Male	20 (80%)	68 (66%)	
Female	5 (20%)	35 (34%)	
Age: range(mean)[1]	35-78 (60.5)	25-84 (61.2)	NS
Site of tumor			NS
Body and Cardia	8 (32%)	21 (20%)	
Antrum	17 (68%)	82 (80%)	
LN metastasis			NS
Absent	7 (28%)	43 (41%)	
Present	18 (72%)	60 (59%)	
Histological type			< 0.05
Diffuse	18 (72%)	41 (40%)	
Intestinal	7 (28%)	62 (60%)	
Lymphoid stroma			< 0.05
Minimal/mild	6 (24%)	71 (69%)	
Moderate/intense	19 (76%)	32 (31%)	

[1] Age in years; LN: Lymph node; NS: Not significant.

Table 2 Apoptosis related proteins expressions in EBV positive and EBV negative gastric carcinomas *n* (%)			
	EBV positive gastric carcinoma (*n* = 25)	EBV negative gastric carcinoma (*n* = 103)	*P* value
P53			NS
Positive	11 (44)	55 (54)	
Negative	14 (56)	48 (46)	
Bcl-2			< 0.05
Positive	7 (28)	11 (11)	
Negative	18 (72)	92 (89)	
Bcl-x			NS
Positive	22 (88)	96 (93)	
Negative	3 (12)	7 (7)	
Bak			< 0.05
Positive	11 (44)	71 (69)	
Negative	14 (56)	32 (31)	
Bax			NS
Positive	24 (96)	88 (86)	
Negative	1 (4)	15 (14)	

RESULTS

EBER-1 expression and EBNA-1 products were visualized in 25 out of 208 (12%) cases of gastric carcinomas (Figure 1A).

The clinicopathological characteristics of EBV positive and negative GCs are given in Table 1. The male/female ratio in patients with EBV positive GCs and those with EBV negative GCs was 4:1 and 2:1, respectively, and no difference was seen in the average age at 60.5 and 61.2 years, respectively. 68% of EBV positive GCs were in the body and cardia and 32% of them were in the antrum. In the group of EBV negative GCs, 80% were in the body and cardia and only 20% of them were in the antrum, showing predilection for the body and cardia region in both groups. Regarding histological type, 18 of 25 (72%) EBV positive GCs were diffuse type and 7 (28%) were intestinal. 60% of EBV negative GCs were intestinal type and 41 of 103 (40%) were diffuse, resulting in a strong association between histological type and EBV infection in gastric carcinomas (*P* < 0.01). EBV associated GCs also correlated with tumor infiltrating lymphocytes. 19 of 25 (76%) EBV positive GCs showed marked lymphoid stroma evenly distributed with the malignant epithelial cells (Figure 1B). In the EBV negative GCs only 30% of the cases showed marked lymphocytic infiltration (*P* < 0.01). Metastases in lymph nodes were found in 18 of 25 (72%) EBV positive GCs and in 60 of 103 (59%) EBV negative cases. This difference was not statistically significant.

Immunohistochemical results

Table 2 shows the pattern of protein expression in EBV positive and negative GCs. P53 protein expression was observed in the nucleus of the tumor cells with a uniform staining (Figure 1C). 11 of 25 (44%) EBV positive GCs and 55 of 103 (51%) EBV negative GCs were positive for

p53 and no difference was observed between the groups. Expression of bcl-2 protein was observed in 28% of EBV positive GCs and in only 11% of the EBV negative GCs (Figure 1D), showing a statistically significant correlation between EBV status and bcl-2 expression in GCs (*P* < 0.05). The rates of the bcl-xl and bax expressions in EBV positive and negative GCs were 88% and 93%, and 96% and 86%, respectively (Figures 1E and 1F). None of the differences were statistically significant. Bak expression was lower in the EBV positive GCs than in EBV negative tumors (Figure 1G) and it was detected in 11 of 25 (44%) EBV positive GCs, and in 71 of 103 (69%) EBV negative GCs. This difference was statistically significant (*P* < 0.01).

NOS were observed in the cytoplasm of the tumor, epithelial, endothelial, smooth muscle and inflammatory cells. For final analysis, we scored the expression of these enzymes only in the tumor cells (Figures 1H, 1I, and 1J). NOS expressions were significantly different in EBV positive and negative GCs. Overexpression of constitutive forms of NOS (NOS-1 and NOS-3) were more frequently observed in EBV positive than in EBV negative GCs (*P* < 0.01). The majority of GC cases were negative for an inducible form of NOS (NOS-2), independently of EBV status. Comparisons of NOS expressions and EBV status are shown in Table 3.

DISCUSSION

The relationship between Epstein-Barr virus and various epithelioid diseases has already been demonstrated. Involvement of EBV has been described in the etiopathogenesis of not only the nasopharyngeal carcinoma but other carcinomas as well, including gastric carcinomas[20-22].

It is estimated that EBV infection can be found in about 10% of GCs worldwide, especially those with marked lymphocytic infiltration[23-27]. We observed EBV infection in 12% of GCs. Previous studies showed the
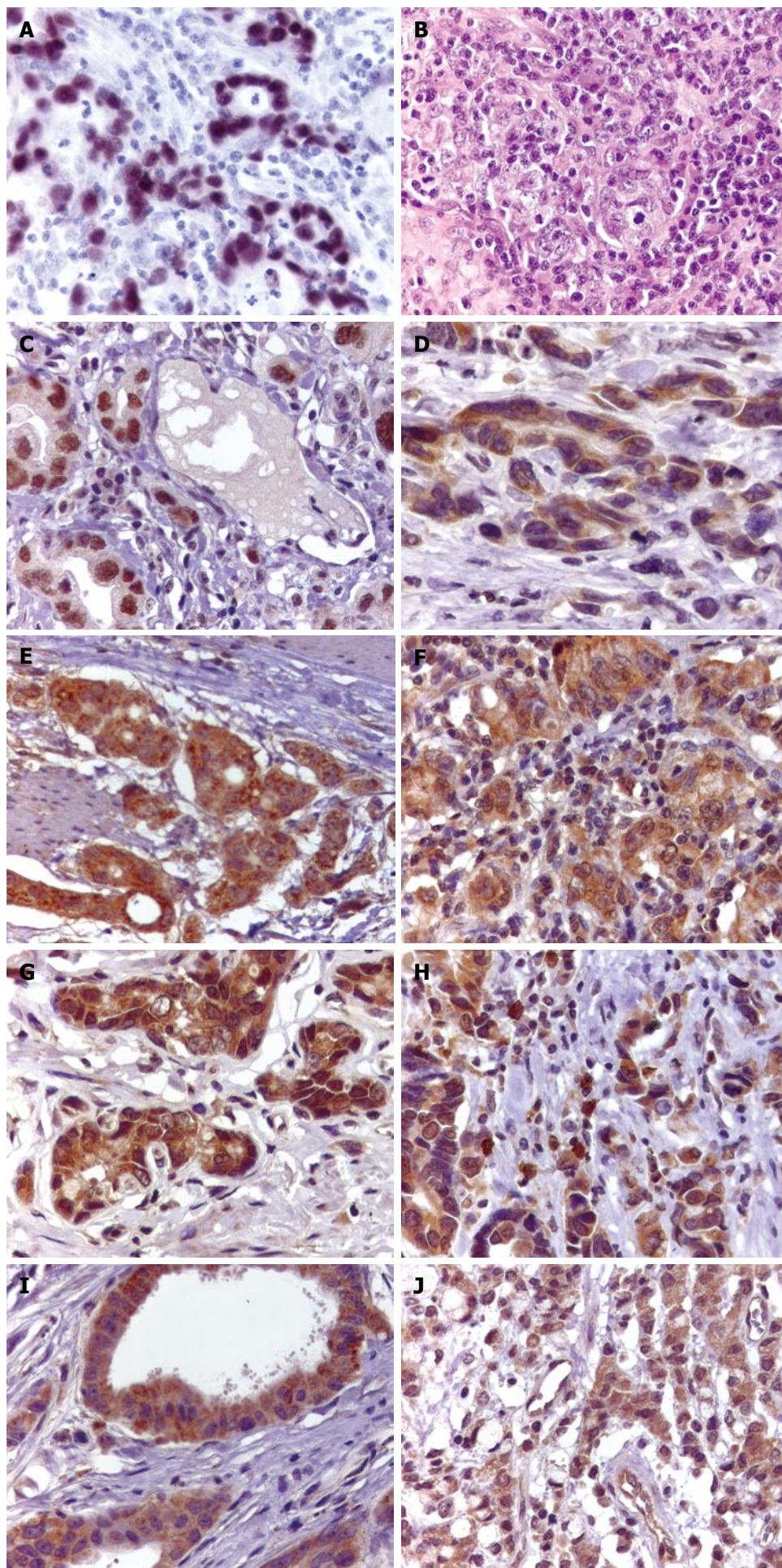
Figure 1 **A**: Gastric carcinoma showing strong positivity of EBER-1 in nuclei of the malignant cells (*in situ* hybridization,400 x); **B**: Overview of gastric carcinoma with clusters of tumor cells separated by prominent lymphoplasmacytic infiltrate (H&E, 400 x); **C**: p53 nuclear immunopositivity in gastric carcinoma cells (400 x); **D**, **E**, **F**, **G**: Immunohistochemical staining for bcl-2, bcl-xl, bax and bak. The cytoplasm of the malignant cells shows irregular brown positivity (400 x); **H**, **I**, **J**: NOS-1, NOS-2 and NOS-3 immunohistochemical staining in tumor epithelial cells, showing a high intensity of staining in the cytoplasm (400 x).

incidence of EBV infection in Brazilian GCs ranging between 5%-11.32%[28-30]. Some reports described the presence of EBV and its products as restricted on the carcinoma cells[31-33], whereas others found this virus in the precursor epithelial lesions as well as in the lymphoid cells[2,7]. In our study the virus was detected in the neoplastic cells but not in the normal, dysplastic gastric epithelium, or in inflammatory cells. This finding indicates that EBV affects the gastric epithelium at a late stage of the multistep process of gastric carcinogenesis.

The role of EBV in neoplastic transformation of gastric epithelial cells is not completely understood. It has been suggested that EBV positive GCs display specific clinicopathological features compared with EBV negative GCs. EBV associated GCs had been characterized by male predominance, preferential location in proximal stomach, and a high prevalence of diffuse types[2,34-36]. In our series, EBV positive GCs were frequently seen in males, in the body and cardia region, and in diffuse carcinomas in agreement with previous reports. Indeed we observed a strong association between EBV infection and lymphoid stroma. The presence of marked lymphoid infiltrate has already been demonstrated and there is growing evidence that extensive lymphocyte infiltration is a consistent characteristic of EBV positive carcinomas and correlates with less aggressive behavior, although no additional information is available regarding follow-up of the patients in the present study.

Molecular analysis of EBV positive carcinoma has been shown to have distinct characteristics including different chromosomal aberrations[37], different and lower frequencies of microsatellite instability[35] and allelic loss[38], and more CpG methylation[39]. However, studies have focused on the relationship between EBV and oncogenes or tumor suppressor genes in EBV associated GCs, but no conclusive results have been reported[5,7,9,40,41]. In this study, expression of apoptosis related proteins and nitric oxide synthases was examined in EBV associated and EBV negative gastric carcinomas. In this report, we observed a correlation between bcl-2 and bak expressions and EBV status. Bcl-2 protein expression was observed more frequently in EBV positive than in EBV negative GCs. On the other hand, positivity for bak protein was more common in EBV negative than in EBV positive GCs. It is already known that the balance between anti-apoptotic and pro-apoptotic members of the bcl-2 family genes determine the outcome of patients with tumors. Bak protein is shown to induce cell death, while bcl-2 can protect cells from apoptosis. Previous reports indicated that EBV infection may protect tumor cells from apoptosis inducing expression of the anti-apoptotic proteins[41]. Our results showed additional information and confirm that EBV infection and apoptosis related proteins interact to negatively influence apoptosis, through high expression of bcl-2 and low expression of bak in gastric tumor cells. Bax and bcl-xl protein expressions were found in both EBV positive and negative GCs and there was no statistical difference between the groups.

There is some indication that EBV modulates and mutates p53 for its own survival[42], however earlier reports showed contradictory results[8,38,43]. In turn, p53

Table 3 NOS expression in EBV positive and EBV negative gastric carcinomas *n* (%)

	EBV positive gastric carcinoma (*n* = 25)	EBV negative gastric carcinoma (*n* = 103)	*P* value
NOS1(nNOS)			< 0.05
Positive	23 (92)	69 (67)	
Negative	2 (8)	34 (33)	
NOS2 (iNOS)			NS
Positive	5 (20)	31(30)	
Negative	20 (80)	72 (70)	
NOS3 (eNOS)			< 0.05
Positive	18 (72)	36 (35)	
Negative	7 (28)	67 (65)	

gene mutations are rarely identified in EBV associated carcinomas[44]. The obtained results in our study showed disturbed function of p53 in almost all cases of EBV positive and EBV negative GCs. Although p53 expression seemed to be slightly lower in EBV positive GCs, that difference was not statistically significant. These results indicated that abnormalities in p53 observed in GCs are independent of EBV infection.

It has been reported that nitric oxide synthases are present in human tumor cell lines and solid tumor tissues[14,16,45-47]. Increased NOS expression has been observed in different tumors tissues when compared with normal tissues[14,48,49].

To the best of our knowledge, this is the first report showing NOS expression is associated with EBV infection gastric cancer. In EBV positive GCs, NOS-1 and NOS-3 proteins were more frequent positive in tumor cells than NOS-2. The results agree with previous data that showed higher activity of constitutive rather than inducible NOS in human gastric tumors[15]. In a recent study, the authors demonstrated significantly high NOS-3 expression in gastric tumors and a directly relationship with tumor angiogenesis and the overall aggressive biology of gastric cancer[50]. Our study showed higher NOS-3 expression in EBV positive GCs than in EBV negative GCs. Increasing evidence suggests that NOS-3 expression, although constitutive, can be regulated by various hormones, cytokines, and growth factors, and by genetic alterations, such as oncogene activation and tumor suppressor inactivation[51-54]. Our data indicate that EBV infection and its products can affect the regulation of this enzyme resulting in elevated NO production. It has been showed that elevated NO production enhances the growth of some tumors through the suppression of anti-tumor immune responses[55-57].

NOS-2 expression was decreased in the whole group of gastric tumors in our study. This feature has been demonstrated by immunohistochemistry in other tumors and there is a possible relationship between loss of NO and carcinogenenesis[15,16]. However, many publications showed elevated NOS expression and increased activity in gastric cancer[58-60]. Although the mechanisms for the antiviral action of NO have not been clarified, the multiplicity of host enzymes it targets makes it possible

that multiple alterations in host cell proteins are involved. An apparent role of NO in inhibiting EBV reactivation from latency was demonstrated in a study using gastric cells lines in culture[61]. This finding led us to propose that EBV latency can be directly and/or indirectly associated with NOS gene expression and also lead us to postulate that NO might also be an important factor in the host EBV relationship. Our data suggest that the increased expression of NOS-1 and NOS-3 in EBV positive GC renders a major contribution to high activity of NOS in this specific group of tumors.

In conclusion, it is clear that EBV is associated with gastric carcinomas. EBV positive GCs have a distinct protein expression profile, as well as distinct clinicopathological features. This present data suggest that EBV infection may protect tumor cells from apoptosis, giving them the capacity for permanent cell cycling and proliferation. Indeed, EBV positive GCs have high expression of constitutive NOS, which might be associated with tumor progression or aggressiveness. A larger number of cases and long-term follow-up are necessary to further investigate this possibility.

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