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• *GASTRIC CANCER* •

Relationship between Epstein-Barr virus-encoded proteins with cell proliferation, apoptosis, and apoptosis-related proteins in gastric carcinoma

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Abstract

AIM: To investigate the interrelationship between Epstein-Barr virus (EBV)-encoded proteins and cell proliferation, apoptosis and apoptosis-related proteins in gastric carcinoma, and to explore their role in gastric carcinogenesis.

METHODS: Tissues from 13 cases of EBV-associated gastric carcinoma (EBVaGC) and 45 cases of matched EBV-negative gastric carcinoma (EBVnGC) were collected, and then subjected to analysis for apoptotic index (AI) using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL) assay. Nuclear cell proliferation-associated antigen ki-67 index (KI), bcl-2, and p53 expression were examined by immunohistochemistry. p53 mutation in exons 5-8 of 13 EBVaGC cases was determined by single-strand conformation polymorphism (SSCP) and DNA sequencing. RT-PCR and Southern hybridization were used to detect the expression of nuclear antigens (EBNAs) 1 and 2, latent membrane protein (LMP) 1, immediately early gene BZLF1 and early genes BARF1 and BHRF1 in 13 EBVaGC cases.

RESULTS: The percentage of AI, KI and p53 overexpression was significantly lower in the EBVaGC group than in the EBVnGC group. However, bcl-2 expression did not show significant difference between the two groups. p53 gene mutations were not found in 13 EBVaGCs. Transcripts of EBNA1 were detected in all 13 EBVaGCs, while both EBNA2 and LMP1 mRNA were not detected. Six of the thirteen cases exhibited BZLF1 transcripts and two exhibited BHRF1 transcripts. BARF1 mRNA was detected in six cases.

CONCLUSION: Lower AI and KI may reflect a low biological activity in EBVaGC. EBV infection is associated with p53 abnormal expression but not bcl-2 protein in EBVaGC. BZLF1, BARF1, and BHRF1 may play important roles in inhibiting cell apoptosis and tumorigenesis of EBVaGC through different pathways.

Key words: Epstein-Barr virus; Gastric carcinoma; Apoptosis; Bcl-2; p53; Ki-67

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INTRODUCTION

The correlation between Epstein-Barr virus (EBV) infection and gastric carcinoma is well known. EBV infection is found in 2-16% of ordinary gastric adenocarcinoma cases and 80-100% gastric lymphoepithelioma-like carcinoma cases[1-4]. However, the pathogenic role of EBV in gastric carcinogenesis remains to be elucidated. Recent studies have shown that the form and expression of EBV encoded genes in gastric carcinoma are different from those in Burkitt's lymphoma and nasopharyngeal carcinoma (NPC), suggesting that the oncogenic mechanism of EBV in gastric carcinoma may be unique[5,6]. In this study, we checked the expression of EBV-encoded genes in EBV-associated gastric carcinoma (EBVaGC) and compared cell proliferation and apoptosis and expression of apoptosis-related proteins (bcl-2 and p53) in EBVaGC with those of matched EBV-negative gastric carcinoma (EBVnGC). Our aim was to investigate the relationship between EBV-encoded proteins with cell proliferation and apoptosis and apoptosis-related proteins in gastric carcinoma, which might facilitate the understanding of gastric carcinogenesis.

MATERIALS AND METHODS

Specimens and cases

Between January 2001 and December 2002, 185 surgically resected specimens of gastric carcinoma were collected from the Affiliated Hospital of Qingdao University Medical College, Qingdao Municipal Hospital and Yantai Yuhuangding Hospital. Tumor tissues from each surgical specimen were

separately dissected. DNA was extracted by the standard proteinase K-sodium dodecyl sulfate (SDS) method and purified with phenol-chloroform. Total RNA was extracted with TRIzol reagent (Gibco BRL, Gaithersburg, MD, USA) according to the manufacturer's instructions. The sections were used for histopathological diagnosis, *in situ* hybridization (ISH), immunohistochemical and TUNEL analysis. The cases positive for EBV DNA by PCR-Southern assay were further confirmed by ISH for EBER1 as previously described[4,5,7]. The cases having EBER1 positive signals were classified as EBVaGC group. EBVnGC cases with similar clinicopathological data were chosen as the control group.

RT-PCR and Southern hybridization analysis for EBV genes expression

The sequence and genome coordinate of primers and probes used to detect EBV transcripts are given in Table 1[5,7-9]. The probes were labeled with DIG-ddUTP by DIG oligonucleotide 3'-end labeling kit (Roche Diagnostics, Germany). Approximately 1 μg RNA (treated with DNAase I) of EBV-positive samples was subjected to cDNA synthesis with reverse transcription system (Promega, USA). PCR was performed as described previously[5]. The amplified products were electrophoresed in 2% agarose gel, transferred onto a Hybond N^+ nylon membrane (Amersham Pharmacia Biotec, Ireland) and subjected to hybridization with 3'-end-DIG-labeled oligonucleotide probes. The hybridized signals were detected by alkaline phosphatase (AP) conjugated anti-DIG antibodies. The substrate of AP was CSPD (Roche Diagnostics, Germany). cDNAs from EBV-immortalized lymphoblastoid cell lines (LCL) were used as positive controls, and those from EBV-negative Ramos cells as negative controls. The integrity of RNA was checked by parallel amplification of endogenous control gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

Detection of cell apoptosis by TUNEL assay

In order to observe cell apoptosis, paraffin embedded sections of tissues from EBVaGC cases and EBVnGC cases were checked by TUNEL assay with the Apop-Tag™ peroxidase kit (Zhongshan Biotechnology Company, Beijing). Cell apoptosis was determined using the defined morphological criteria[10,11]. TUNEL signals were detected in nuclei of carcinoma cells with brown staining. Apoptotic index (AI) was determined as the percentage of TUNEL-positive carcinoma cells in comparison with the total number of carcinoma cells in each specimen. Microscopic magnification was 10×40. At least 1 000 tumor cells from 10 randomly selected fields were quantified.

Immunohistochemistry

Paraffin embedded sections of tissues from EBVaGC cases and EBVnGC cases were immunostained by the standard streptavidin-biotin-peroxidase method. The primary antibodies, Do-7, sc-7382 and Ki-S35 (Santa Cruz Biotechnology, Inc.), were anti-human mouse monoclonal antibodies against p53, bcl-2 and ki-67, respectively. Phosphate buffered saline (PBS), instead of the primary antibody, was used for negative control sections. The sections of breast carcinoma tissue with highly expressed p53 served as the positive control for p53 staining while the sections of tonsil tissue expressing both bcl-2 and ki-67 were used as positive control for bcl-2 and ki-67. The percentage of positively stained tumor cells in each tumor section was evaluated by counting at least 1 000 cells in 10 randomly selected high-power fields. Brown staining for p53 and ki-67 was located in nuclei, staining for bcl-2 protein was located in cytoplasm. The section was considered as expressing the protein if cellular staining was $\geqslant 5\%$[11,12]. p53 positivity was divided into two grades: 5-50% positive cells (+) as lower expression, 50-100% positive cells (++) as overexpression. Ki-67 index (KI) was obtained as the percentage of ki-67-positive carcinoma cells in comparison with the total number of carcinoma cells in each specimen[10].

p53 mutation analysis by SSCP and DNA sequencing

p53 mutations were determined by single-strand conformation polymorphism (SSCP) analysis with primers

Table 1 Sequence and co-ordinate of primers and probes for RT-PCR analysis

Transcript		Oligonucleotide sequence (5′-3′)	Product size (bp)	Genome coordinate
EBNA1	5′ primer	GATGAGCGTTTGGGAGAGCTGATTCTGCA	273	67 510-67 539
	3′ primer	TCCTCGTCCATGGTTATCAC		108 075-108 056
	probe	AGACCTGGGAGCAGATTCAC		67 608-67 627
EBNA2	5′ primer	GCTGCTACGCATTAGAGACC	339	47 892-47 911
	3′ primer	TCCTGGTAGGGATTCGAGGG		48 616-48 597
	probe	CAGCACTGGCGTGTGACGTGGTGTAAGTT		48 391-48 420
LMP1	5′ primer	TCCTCCTCTTGGCGCTACTG	490	169 383-169 364
	3′ primer	TCATCACTGTGTCGTTGTCC		168 740-168 759
	probe	GAACAGCACAATTCCAAGGAACAATGCCTG		169 061-169 090
BZLF1	5′ primer	ATTGCACCTTGCCGCCACCTTTG	608	103 194-103 180
	3′ primer	CGGCATTTTCTGGAAGCCACCCGA		102 486-102 463
	probe	CACTGCTGCTGCTGTTTGAACAGT		102 772-102 795
BARF1	5′ primer	GGCTGTCACCGCTTTCTTGG	203	165 560-165 579
	3′ primer	AGGTGTTGGCACTTCTGTGG		165 762-165 743
	probe	CTGGTTTAAACTGGGCCCAGGAGAGGAGCA		165 644-165 673
BHRF1	5′ primer	GTCAAGGTTTCGTCTGTGTG	211	53 830-53 849
	3′ primer	TTCTCTTGCTGCTAGCTCCA		54 480-54 461
	probe	ATGCACACGACTGTCCCGTATACAC		54 435-54 411

of exons 5-8[13]. Samples with putative mutations were confirmed by DNA sequencing.

Statistical analysis

Qualitative data were analyzed by χ^2 test or the Fisher's exact test (two-tail). Quantificative data were expressed as mean±SD and compared between the two groups by Student's *t*-test or *t'*-test. $P < 0.05$ was considered statistically significant. Software SAS 6.12 was employed to process the data.

RESULTS

Clinicopathological features of EBVaGC and EBVnGC

There were 13 cases of EBVaGC among 185 cases of gastric carcinoma (7.03%), 45 cases of EBVnGC with similar clinicopathological data were chosen as the control group. No statistical difference was found in age, sex, tumor location, histological subtype, stage, or lymph node metastasis between the two groups (Table 2).

Table 2 Comparison of clinicopathological data between EBVaGC and EBVnGC patients

	EBVaGC (n = 13)	EBVnGC (n = 45)	P
Age (yr) (mean±SD)[1]	59.76±10.17	57.31±14.05	0.5613 (t = 0.5844)
Sex			
Male	13	35	0.1466 (χ^2 = 2.1070)
Female	0	10	
Tumor location			
Cardia/body	9	21	0.1516 (χ^2 = 2.0566)
Antrum	4	24	
Histological subtype			
Adenocarcinoma	12	41	0.6704 (χ^2 = 0.1811)
Signet ring carcinoma	1	4	
Tumor differentiation			
Moderate	1	7	0.7890 (χ^2 = 0.0716)
Poor	12	38	
Lymph node metastasis			
Present	10	33	0.9999 (χ^2 = 0.0098)
Absent	3	12	
Tumor stage[2]			
Early	0	1	1.0000
Advanced	13	44	

[1]Age was compared using Student's *t* test; [2]Tumor stage was compared using two-tailed Fisher's exact test; the remainders, using χ^2-test.

Expression of EBV-associated genes in EBVaGC

We investigated the expression of EBV-associated genes in 13 EBVaGC cases by RT-PCR and Southern hybridization analysis (Figure 1). The transcripts of EBNA1 were detected in all 13 cases, while both EBNA2 and LMP1 mRNA were not detected. Six of the thirteen cases exhibited BZLF1 transcripts and two exhibited BHRF1 transcripts. BARF1 mRNA was detected in six cases. GAPDH mRNA was amplified to check pertinent RNA extraction. The result showed that the RNA was integrity.

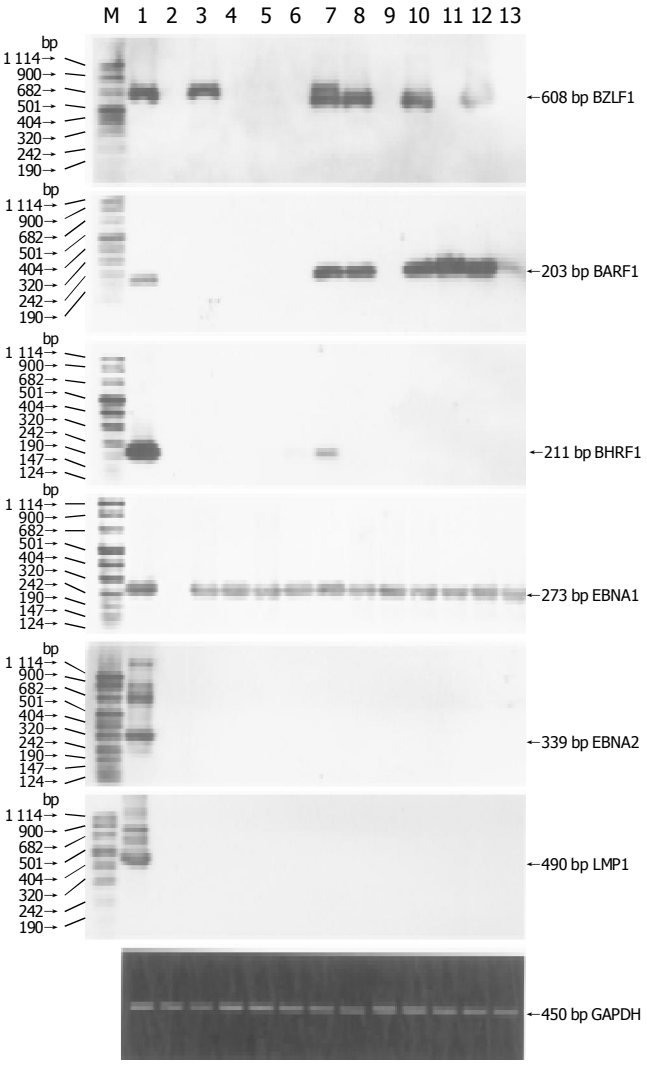

Figure 1 Detection of EBV-associated gene expression by RT-PCR and Southern hybridization in EBVaGC. M: DIG-labeled DNA molecular weight marker VIII (Roche); lane 1: EBV-positive LCL (positive control); lane 2: EBV-negative Ramos cells (negative control); lanes 3-13: EBV-positive gastric carcinoma samples.

TUNEL for apoptosis and immunohistochemisty of ki-67, p53, and bcl-2

Cell apoptosis by TUNEL and immunostaining of ki-67, p53, and bcl-2 are shown in Figure 2. Both the mean values of AI and KI were significantly lower in the EBVaGC group than in the EBVnGC group (Table 3). The bcl-2 and p53 expression was 53.9% (7/13) and 84.6% (11/13), and p53 overexpression was15.4% (2/13) in EBVaGC group, while they were 48.9 (22/45), 86.7% (39/45), and 57.8% (26/45) respectively in EBVnGC group. The difference in p53 overexpression between EBVaGC and EBVnGC was significant. However the difference in bcl-2 and p53 expression between the two groups was not significant (Table 4).

p53 gene mutations

No mutation of p53 gene from exons 5-8 in tissues of 13 EBVaGC cases was detected by PCR-SSCP and DNA sequencing analysis.

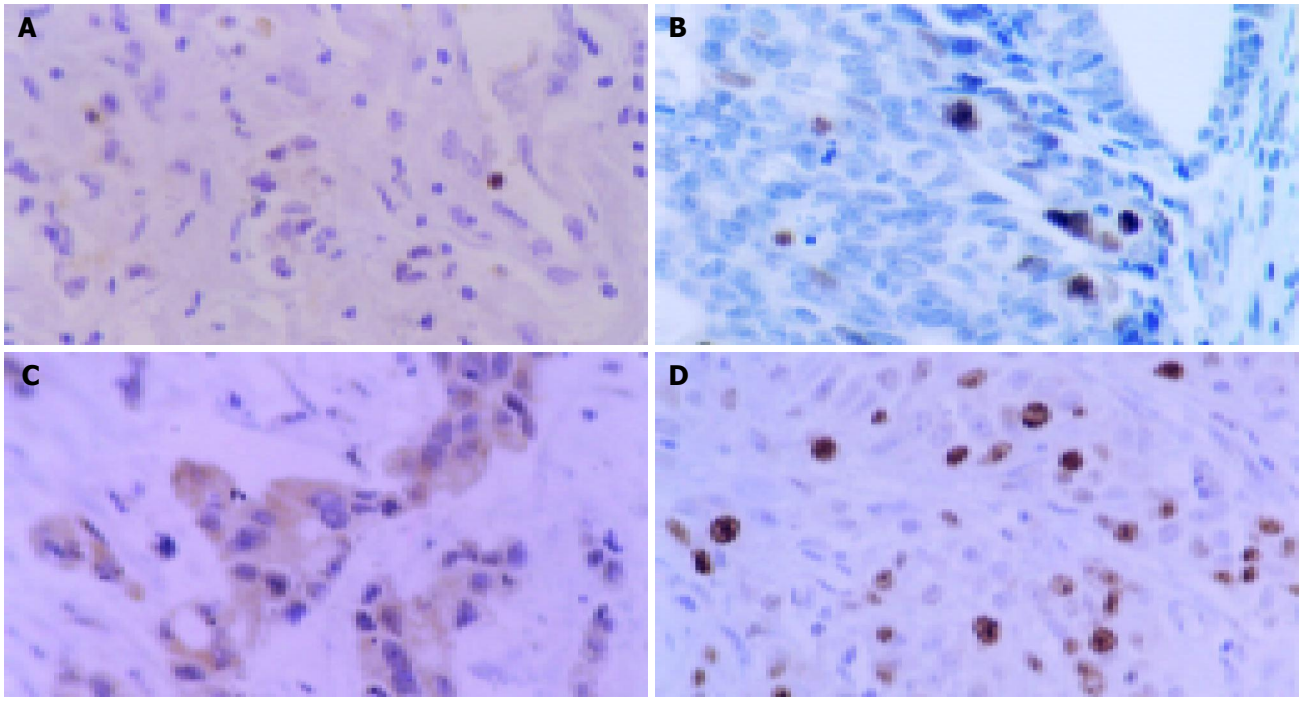

Figure 2 TUNEL for Cell apoptosis and immunohistochemistry of p53, bcl-2, and ki-67. **A**: Apoptotic cells; **B**: Expression of p53; **C**: Expression of bcl-2; **D**: Expression of ki-67. (Original magnification ×400).

Table 3 Apoptotic index and ki-67 index in EBVaGC and EBVnGC (mean±SD)

	AI	KI
EBVaGCs (n = 13)	0.97±0.41[1]	28.25±6.28[2]
EBVnGCs (n = 45)	2.03±0.60	37.86±14.52

[1]t = 5.9795, P = 0.00001<0.01; [2]t' = 3.4579, P = 0.0012<0.01.

Table 4 bcl-2 and p53 expression in EBVaGC and EBVnGC

	n	bcl-2		p53 expression		p53 overexpression	
		+	–	+	–	+	–
EBVaGC	13	7	6	11	2	2	11
EBVnGC	45	22	23	39	6	26	19
χ^2		0.0991		0.0716		7.2593	
P value		0.7529		0.7912		0.0085	

DISCUSSION

EBV is encountered in a subset of tumors but its role in gastric carcinogenesis is not quite understood. Several *in vivo* studies explored the mechanisms by which EBV might contribute to gastric carcinogenesis[10,11,14-17]. Methylation is currently regarded as an alternative mechanism for silencing tumor suppressor genes. In a recent publication more frequent CpG islands methylation were found in EBV-positive gastric carcinomas than EBV-negative carcinomas, suggesting that aberrant methylation might be an important mechanism of EBV-related gastric carcinogenesis[14]. Other studies focused on the relationship between EBV and oncogenes or tumor suppressor genes in EBVaGC, but no conclusive results have been reported[10-12,16,17]. In this study, EBVaGC had a lower rate of p53 overexpression than EBVnGC, indicating that abnormal p53 expression is associated with EBV infection. However, bcl-2 expression did not correlate with the presence of EBV in EBVaGC. In addition, the low rate of cell apoptosis and proliferation may reflect a low biological activity in EBVaGC. The results are inconsistent with previous studies[11,16,17]. For example, Kume *et al*[16], found no correlation between p53 and EBV infection, but found a lower rate of cell apoptosis and higher bcl-2 expression in EBVaGC compared to EBVnGC, suggesting that bcl-2 protein is the main inhibitor of apoptosis in EBVaGC. Ishii *et al*[11], studied cell apoptosis, bcl-2, and p53 expression in early and advanced stage of EBVaGC and EBVnGC, and found p53 overexpression in EBVaGC is significantly lower than in EBVnGC at early stage. Cell apoptosis and bcl-2 expression between two groups have no significant difference at advanced and early stages. No correlation between bcl-2 and p53 expression and presence of EBV is found in other study[17].

Some *in vitro* studies have shown that EBV-encoded proteins during different replication phase play different roles in regulating cell cycle and inhibiting cell apoptosis, which includes LMP1, EBNA, BZLF1, BHRF1, and BARF1[18-22]. EBV-encoded proteins can interact with various kinds of cell regulation factors, making regulation mechanism very complicated. EBNA and LMP1 are the essential genes for cell transformation. LMP1 can induce expression of bcl-2 and block p53-mediated apoptosis through the induction of A20 gene *in vitro*[18,23]. EBV nuclear antigen leader proteins (EBNA-LP) and EBNA2 are the earliest expressed proteins in EBV-immortalized lymphoblastoid cells, and activate cell cycle by inducing transcription of cyclin D2 and promoting the induction of bcl-2 expression by LMP1[19,24]. Our study and other studies failed to detect LMP1 and EBNA2 mRNA

in EBVaGC[5-7], suggesting that LMP1 and EBNA2 may not be essential for tumor formation and not related with cell proliferation and apoptosis, bcl-2, and p53 expression in EBVaGC.

p53 protein is known as a tumor suppressor. The wild-type p53 controls cell proliferation and survival by inducing G1/G2 cell cycle arrest and apoptosis. p53 abnormalities play a critical role in oncogenesis. Several studies showed that EBV-encoding proteins EBNA5 and BZLF1 can bind to p53, possibly resulting in increased p53 half-life and interference with p53 function[25,26]. BZLF1 regulates p53 function through multiple mechanisms *in vitro*. For example, BZLF1 increases the level of cellular p53 by activating p53 transcription. However, BZLF1 can inhibit the function of p53 by preventing induction of p53-dependent cellular target genes, such as p21 and MDM2[27,28]. In the present study, p53 overexpression was lower in EBVaGC than in EBVnGC. Most EBVaGC cases showed a weak p53 expression, but most EBVnGC cases showed strong expression. Some previous studies also found lower level expression of p53 in EBVaGC than in EBVnGC[12,29], suggesting that the mechanism of p53 abnormal expression in EBVaGC is different from that in EBVnGC. No p53 mutation was detected in 13 EBVaGC cases. However, six cases exhibited BZLF1 mRNA, indicating the p53 accumulation in EBVaGC might not be a consequence of mutation but a manifestation of upregulation on p53 by EBV protein such as BZLF1. This viewpoint has been proposed as an explanation for the overexpression of p53 in nasopharyngeal carcinomas in which p53 gene mutation is uncommon[30,31]. Very few papers reported that p53 is not inactivated by EBV[29,32]. van Rees *et al*[32], found an inverse correlation between EBV positivity and loss of heterozygosity (LOH) at chromosomal arm 17p, and also none of the EBV-positive carcinomas showing p53 immunopositivity in contrast to 39% of EBV-negative carcinomas ($P<0.01$), indicating that p53 is not or differently inactivated in EBV-related gastric carcinoma. It is conceivable that binding with EBV proteins, instead of genetic alteration of p53 gene, lead to an accelerated degradation of p53 protein. Further analysis of EBVnGC is needed to identify whether overexpression of p53 is mutated or is derived from other pathways.

bcl-2 is a protein best known for its suppression of apoptosis. *In vitro* studies have shown that LMP1 and EBNA2 can induce bcl-2 expression, but clinical studies have failed to confirm this mechanism *in vivo*[11,17,30,33]. Our study of gastric carcinoma showed that bcl-2 expression did not appear to correlate with the presence of EBV or viral LMP1 and EBNA2 expression. The possible explanations are as follows. bcl-2 expression may not be necessary for cancer cells *in vivo* as indicated by *in vitro* experiments. bcl-2 might localize at different cellular organelles in cancer development, which makes it undetectable by light microscopy. It is the balance between anti-apoptic and pro-apoptic members of the bcl-2 family that determine the outcome. Therefore, it may be necessary to carry out further studies to detect the expression of bcl-2-related proteins[11]. Early gene BHRF1 shows partial sequence homologous to the human bcl-2 proto-oncogene, which is involved in inhibiting cell apoptosis. BHRF1 protein can inhibit apoptosis of B lymphocytes and epithelial cells and promote cell growth and transformation[21,34]. BARF1 shares homology with the cellular proto-oncogene c-fms and is able to immortalize epithelial cells and fibroblast cells and B lymphocyte *in vitro*[6,35,36]. Furthermore, it can activate the expression of bcl-2[22]. We demonstrated that six of 13 EBVaGC cases exhibited BARF1 mRNA and two exhibited BHRF1 mRNA. Zur Hausen *et al*[6], also detected nine BARF1-positive cases and two BHRF1-positive cases in 10 EBV-related gastric adenocarcinomas. Because EBVaGC lacks the expression of LMP1[5-7], BARF1, and BHRF1 might provide an alternative way for the pathogenesis of EBVaGC independent of LMP1.

In summary, this study showed that the mechanism by which EBV inhibits cell apoptosis is not through the induction of bcl-2 expression by EBNA2 or LMP1. BZLF1, BARF1 and BHRF1 may play important roles in inhibiting cell apoptosis and tumorigenesis of EBVaGC through different pathways.

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