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GASTRIC CANCER

Correlation of Epstein-Barr virus and its encoded proteins with *Helicobacter pylori* and expression of c-met and c-myc in gastric carcinoma

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Abstract

AIM: To investigate the interrelationship of Epstein-Barr virus (EBV) and EBV- encoded proteins with *Helicobacter pylori* (*H pylori*) infection and the expression of c-met and c-myc oncogene proteins in gastric carcinoma, and to explore their role in gastric carcinogenesis.

METHODS: One hundred and eighty-five gastric carcinoma tissues were detected by polymerase chain reaction (PCR)-Southern blot for EBV genome and in situ hybridization (ISH) for EBV-encoded small RNA 1 (EBER1). Gastric carcinoma with positive EBER1 signals was confirmed EBV-associated gastric carcinoma (EBVaGC). The status of *H pylori* infection in 185 gastric carcinomas was assessed by rapid urease test and PCR. The samples with positive PCR and urease test were defined as *H pylori* infection. The expression of c-met and c-myc oncogene proteins in tissues of EBVaGC and matched EBV-negative gastric carcinoma (EBVnGC) were examined by immunohistochemistry. RT-PCR and Southern hybridization were used to detect the expression of nuclear antigens (EBNAs) 1 and 2, latent membrane protein (LMP) 1, early genes BARF1 and BHRF1 in EBVaGC cases.

RESULTS: The positive rate of *H pylori* and EBV in 185 gastric carcinomas was 59.45% (110/185) and 7.03% (13/185) respectively. No difference was found in sex, age, pathological differentiation, clinical stages and lymph node metastasis between *H pylori*-positive and *H pylori*-negative gastric carcinomas. However, the positive rate of *H pylori* infection in the antrum gastric carcinomas was higher than that of cardia and body gastric carcinomas. In our series, age, pathological differentiation, clinical stages, lymph node metastasis and location of cancer were not different between EBVnGC and EBVaGC, while the positive rate of EBV in male patients was significantly higher than that of female patients. The positivity of *H pylori* in EBV-associated and EBV-negative gastric carcinomas was 46.15% (6/13) and 81.40%(104/172) respectively. There was no significant correlation between EBV and *H pylori* infection. The c-met overexpression was significantly higher in the EBVaGC group than in the EBVnGC group. However, c-met and c-myc expression did not show significant difference between the two groups. Transcripts of EBNA1 were detected in all 13 EBVaGCs, while both EBNA2 and LMP1 mRNA were not detected. Six of the 13 cases exhibited BARF1 transcripts and 2 exhibited BHRF1 transcripts.

CONCLUSION: The positivity of *H pylori* in EBVnGCs is higher than that of EBVaGCs, but no significant correlation is found between EBV infection and *H pylori* infection. *H pylori*-positive gastric carcinoma is predominant in antrum location, while EBVaGC has a tendency of predominance in cardia/body location. EBV infection is associated with c-met abnormal expression but not with c-myc protein in EBVaGC. c-met overexpression is not induced by LMP1. BARF1 and BHRF1 may play important roles in the tumorigenesis of EBVaGC through different pathways.

Key words: Epstein-Barr virus; *Helicobacter pylori*; Gastric carcinoma; c-met; c-myc

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INTRODUCTION

Helicobacter pylori (*H pylori*) infection is one of the important environmental risks for gastric carcinoma. In 1994, the

Working Group Meeting of the International Agency for Research on Cancer (IARC) concluded that *H pylori* is a definite carcinogen to gastric carcinoma(GC). Chronic gastritis caused by *H pylori* infection may progress to intestinal metaplasia and even GC[1,2]. 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% of gastric lymphoepithelioma-like carcinoma cases[2-5]. However, the pathogenic role of EBV in gastric carcinogenesis remains to be elucidated. Recent studies have shown that the expressions 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[6-8]. The development of gastric carcinoma is a multistep event proceeding from normal to preneoplastic lesions to highly malignant tumors, accompanied by participation of multiple factors and multiple genes. To shed further light on gastric carcinogenesis, we determined the clinicopathologic characteristics and EBV status in 185 patients with gastric carcinoma and correlated with the status of *H pylori* infection, genetic alterations in proto-oncogenes c-myc and c-met.

MATERIALS AND METHODS

Specimens and cases

One hundred and eighty-five 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. Partial tissue was used to detect *H pylori* by urease test. 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 tissue sections were used for histopathological diagnosis, *in situ* hybridization (ISH) and immunohistochemical analysis.

Detection of EBV infection

The cases positive for EBV DNA by PCR-Southern blot assay were further confirmed by ISH for EBER1 as previously described[6]. The cases with EBER1 positive signals were classified as EBVaGC group.

Detection of H pylori infection

The resected tissues were used to detect *H pylori* infection by urease test kit. Simultaneously, PCR was used to detect 16 sRNA of *H pylori*. The specific primers were designed as previously described[9]. The sequence of sense primer is 5'-CTGGAGAGACTAAGCCCTCC-3', and that of antisense primer is 5'-ATTACTGACGCTGATTGTGC-3 '. The PCR products were 109 bp. Three microliter DNA was added into a solution containing 200 μmol/L dNTPs, 0.5 μmol/L each primer, 1.5 mmol/L $MgCl_2$ and 1 U Taq DNA polymerase (Promega, USA) in a total volume of 25 μL. PCR was carried out under the following conditions: first denaturation at 94℃ for 5 min; then denaturation for 30 s at 94℃, annealling for 30 s at 55℃, extension for 45 s at 72 ℃ in 35 amplification cycles; and finally extension for 5 min at 72℃. The amplified products were electrophoresed in 2% agarose gel and visualized by ethidium bromide staining and ultraviolet illumination. DNA from the culture of *H pylori* was used as positive control, and that from human leukocyte as negative control.

Immunohistochemistry

Paraffin-embedded sections of tissues from EBVaGC cases and 45 cases of EBVnGC with similar clinicopathological data were immunostained by the standard streptavidin-biotin-peroxidase method. Anti-human mouse polyclonal antibodies against c-met and anti-human mouse monoclonal antibodies 9E10 against c-myc (Santa Cruz Biotechnology Inc) were used as primary antibodies. Phosphate buffered saline (PBS), instead of the primary antibody, was used for negative control sections. The sections of breast carcinoma tissue with highly expressed c-met and c-myc served as the positive controls. 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 c-myc was located in nuclei, staining for c-met protein was located in both membrane and cytoplasm. The section was considered as expressing the protein if cellular staining ⩾5%, following the methods described previously [10,11]. c-met positivity was divided into three grades: 5%-30%, 30%-50% and >50%. Positive cells >30% (++) was regarded as overexpression.

RT-PCR and Southern hybridization analysis for EBV genes expression

According to the methods previously described[12], RT-PCR and Southern hybridization were used to detect the expression of nuclear antigens (EBNAs) 1 and 2, latent membrane protein (LMP) 1, early genes BARF1 and BHRF1 in EBVaGC cases. 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.

Statistical analysis

Qualitative data were analyzed by χ^2 test or the Fisher's exact test (two-tail). Quantitative data were expressed as mean±SD and compared between the 2 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

Detection of H pylori infection

H pylori infection in 185 gastric carcinomas was assessed by rapid urease test and PCR. The samples

Table 1 *H pylori* status in relation to clinicopathologic characteristics

	n	*H pylori* (+)	*H pylori* (-)	*P*
Sex				
Male	134	78	56	0.574 (χ^2=0.315)
Female	51	32	19	
Age (yr)				
30-	11	6	5	
40-	29	15	14	
50-	41	23	18	0.779 (χ^2=1.764)
60-	59	38	21	
≥70	45	28	17	
Histological subtype				
LDAC[1]	125	75	50	
MDAC[2]	49	29	20	0.986
WDAC[3]	7	4	3	
Signet ring carcinoma	4	2	2	
Tumor stage				
Ⅰ	27	16	11	
Ⅱ	98	57	41	0.975 (χ^2=0.216)
Ⅲ	41	25	16	
Ⅳ	19	12	7	
Lymph node metastasis				
Present	130	79	51	0.577 (χ^2=0.311)
Absent	55	31	24	
Tumor location				
Cardia[5]	23	8	15	
Body[6]	47	23	24	
Antrum	95	68	27	0.003 (χ^2=13.817)
Multiple	14	8	6	
Remanent carcinoma[4]	6	3	3	

[1]LDAC: Lowly-differentiated adenocarcinoma; [2]MDAC: Moderately-differentiated adenocarcinoma; WDAC: Well-differentiated adenocarcinoma; [4]6 cases of remanent gastric carcinoma were not statistically analyzed; [5]Compared with antrum, q=4.528, P=0.005; [6]Compared with antrum, q=3.681, P=0.010.

Table 2 EBV status in relation to clinicopathologic characteristics

	n	EBV (+)	EBV (-)	*P*
Sex				
Male	134	13	121	0.047 (χ^2=3.940)
Female	51	0	51	
Age (yr)				
30-	11	0	11	
40-	29	3	26	
50-	41	2	39	0.669
60-	59	6	53	
≥70	45	2	43	
Histological subtype				
LDAC[1]	125	11	114	
MDAC[2]	49	1	48	0.141
WDAC[3]	7	0	7	
Signet ring carcinoma	4	1	3	
Tumor stage				
Ⅰ	27	1	26	
Ⅱ	98	5	93	0.259
Ⅲ	41	4	37	
Ⅳ	19	3	16	
Lymph node metastasis				
Present	130	10	120	0.818 (χ^2=0.053)
Absent	55	3	52	
Tumor location				
Cardia[5]	23	1	22	
Body[6]	47	7	40	
Antrum	95	3	92	0.064
Multiple	14	1	13	
remanent carcinoma[4]	6	1	5	

[1]LDAC: Lowly-differentiated adenocarcinoma; [2]MDAC: Moderately-differentiated adenocarcinoma; WDAC: Well-differentiated adenocarcinoma; [4]6 cases of remanent gastric carcinoma were not statistically analyzed.

with positive PCR and urease test were defined as *H pylori*- positive. No difference was found in sex, age, pathological differentiation, clinical stages or lymph node metastasis between *H pylori*-positive and *H pylori*-negative gastric carcinomas. However, the location of tumor was significantly different between the two groups (χ^2=13.817, P=0.003). The positive rate of *H pylori* infection in the antrum gastric carcinomas was higher than that of cardia and body gastric carcinomas (q=4.528, P=0.005; q=3.681, P=0.010) (Table 1).

Detection of EBV infection

The positive rate of EBV in 185 gastic carcinomas was 7.03% (13/185). Age, pathological differentiation, clinical stages, lymph node metastasis and location of cancer were not different between EBV-negative gastric carcinomas (EBVnGC) and EBVaGC (P=0.669, 0.141, 0.259, 0.818, 0.064, respectively), while sex was significantly different between the two groups (χ^2=3.940, P=0.047) (Table 2).

Relationship of EBV and H pylori

The positivity of *H pylori* in EBV-positive and EBV-negative gastric carcinomas was 46.15% (6/13) and 81.40%(104/172) respectively. There was no significant correlation between EBV and *H pylori* infection (χ^2=1.027, P=0.317, r=-0.075) (Table 3).

Immunohistochemistry of c-met and c-myc

Forty-five 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 lymphnode metastasis between the two groups [12]. Immunostaining results of c-met and c-myc are shown in Figure 1. The c-myc and c-met expression was 61.5% (8/13) and 76.9% (10/13), and c-met overexpression was 69.2% (9/13) in EBVaGC group, while they were 55.6 (25/45), 64.4% (29/45) and 37.8% (17/45) respectively in EBVnGC group. The difference in c-met overexpression between EBVaGC and EBVnGC was significant. However, the difference in c-myc and c-met expression between the two groups was not significant (Table 4).

Expression of EBV-associated genes in EBVaGC

We investigated the expression of EBV-associated genes in 13 EBVaGC cases by RT-PCR and

Table 3 Relationship between EBV and *H pylori* in gastric carcinoma

	EBV(+)	EBV(-)	Total
H pylori (+)	6	104	110
H pylori (-)	7	68	75
Total	13	172	185

χ^2 =1.02, P=0.317, r=-0.075

Table 4 c-myc and c-met expression in EBVaGC and EBVnGC

	n	c-myc expression		c-met expression		c-met overexpression	
		+	-	+	-	+	-
EBVaGC	13	8	5	10	3	9	4
EBVnGC	45	25	20	29	16	17	28
χ^2		0.147		0.259		4.035	
P value		0.701		0.611		0.045	

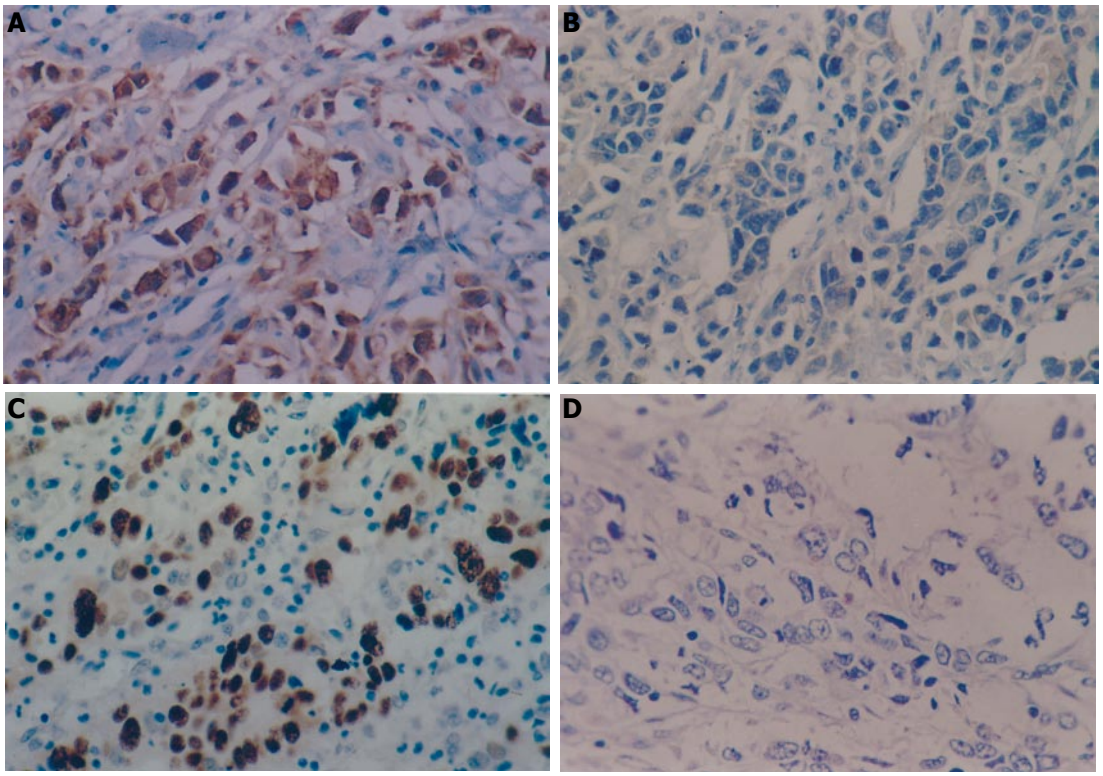

Figure 1 Immunohistochemistry of c-met and c-myc. **A**: Positive expression of c-met; **B**: Negative expression of c-met; **C**: Positive expression of c-myc; **D**: Negative expression of c-myc. (Original magnification × 400).

Southern hybridization analysis (Figure 2). The transcripts of EBNA1 were detected in all 13 cases, while neither EBNA2 nor LMP1 mRNA was detected. Six of the 13 cases exhibited BARF1 transcripts and 2 exhibited BHRF1 transcripts. GAPDH mRNA was amplified to check pertinent RNA extraction. The result showed that the RNA was in integrity.

DISCUSSION

H pylori is believed to be a carcinogen of gastric carcinoma. Recently, it was found that EBV is also linked with the development of partial gastric carcinomas. In this study, we simultaneously detected the status of EBV and *H pylori* infection in gastric carcinomas. No statistical relationship was found between *H pylori* infection rate and sex, age, pathological differentiation, clinical stages and lymph node metastasis. However, the positive rate of *H pylori* infection in the antrum gastric carcinomas was higher than that of cardia and body gastric carcinomas. These results are consistent with those of previous studies[1,13]. In our series, age, pathological differentiation, clinical stages, lymph node metastasis and location of cancer were not different between EBVnGC and EBVaGC, while the positive rate of EBV in male patients was significantly higher than that of female patients. Eleven cases of EBVaGC were low differentiated adenocarcinoma, and 8 cases of EBVaGC were body or cardia cancer. Although no statistical difference was found, it revealed a tendency that included predominance of cardia/body location and low differentiated adenocarcinoma. It remains controversial whether there is a significant clinicopathologic difference between EBVaGC and EBVnGC. Several reports showed that EBVaGC was characterized by male predominance, preferential location in proximal stomach, and a high prevalence of low differentiated adenocarcinoma[14,15]. Wu *et al*[2] found EBV-positive lymphoepithelioma-like carcinoma (LELC) showed less node metastasis and higher survival rate, and tended to be at cardia/body location in contrast to EBV-negative gastric carcinomas, while no significant clinicopathologic difference was found between EBV-positive ordinary gastric carcinoma and EBV-negative gastric carcinoma.

In the present study, no significant correlation between EBV and *H pylori* infection was found in gastric carcinomas. Currently, only a few studies have investigated the effect and interaction of EBV and *H pylori* infection in gastric carcinomas, but no conclusive results have been

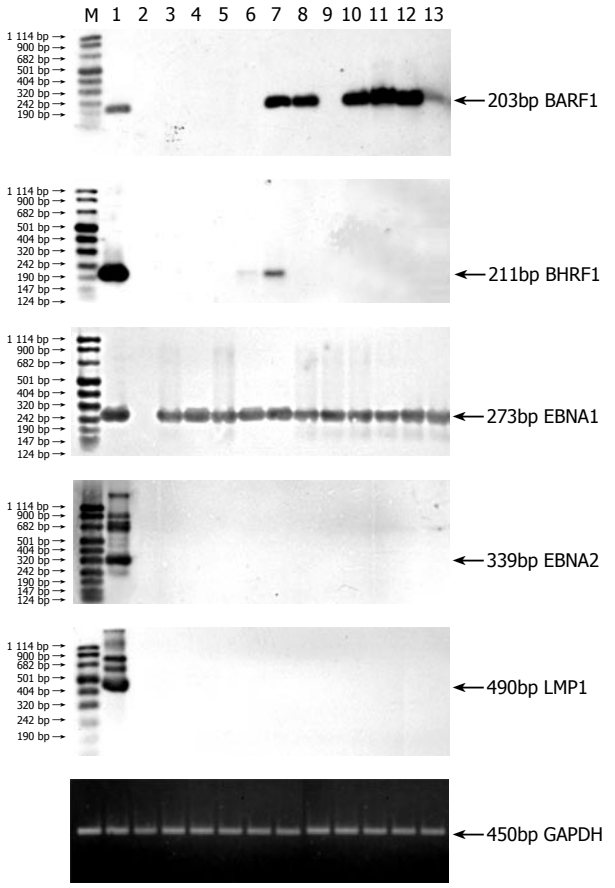

Figure 2 Detection of EBV-associated gene expression by RT-PCR and Southern hybridization in EBVaGC. M: DIG-labeled DNA molecular weight marker VIII (Roche); lane 1: EBV-positive LCL (positive control); lane 2: EBV-negative Ramos cells (negative control); lanes 3-13: EBV-positive gastric carcinoma samples.

reported[2,16-18]. Levine *et al*[16] documented that specific IgG antibody levels against *H pylori* in 39 EBV-negative gastric carcinomas were significantly higher than those of 5 EBV-positive gastric carcinomas. It was thus inferred that an inverse relationship between specific IgG titer against *H pylori* and EBV status existed. In the present study, the positivity of *H pylori* in EBVnGCs was higher than that of EBVaGCs, but no reverse correlation is found between EBV infection and *H pylori* infection. Wu *et al*[2] found EBV was detected in 11 (100%) gastric LELC and 19 (13.7%) of 139 non-LELC. Compared with the EBV-negative gastric carcinomas (68.4%) and EBV-positive non-LELC (68.3%), EBV-positive gastric LELC had a significant lower positive rate of *H pylori* IgG (36.4%). This finding implicates that there is a tendency for gastric LELC, with the high frequency of EBV and predominant proximal location, to have less association with *H pylori* infection than the other two groups, and also suggests that EBV may play a role in *H pylori*-seronegative gastric carcinomas, especially those located at the proximal stomach. Same results were found in other two reports[19,20]. Our result showed that in ordinary non-LELC gastric carcinomas, *H pylori*-positive gastric carcinoma is predominant in antrum location, while EBVaGC has a tendency of predominance in cardia/body location. Several studies found equal *H pylori* infection and distribution of intestinal metaplasia and atrophic gastritis among EBV-positive and -negative gastric carcinomas, indicating that EBV and *H pylori* may play roles together in the pathogenesis of gastric carcinomas. Chronic atrophic gastritis and subsequent intestinal metaplasia caused by *H pylori* infection enhances the susceptibility of EBV to gastric mucosal epithelia, and then EBV facilitated the carcinogenesis of gastric carcinoma[17,18].

Many studies have been focused on the relationship between EBV and the oncogenes or tumor suppressor genes in the carcinogenic mechanism of EBV. c-met protein is a receptor for hepatocyte growth factor (HGF), also known as scatter factor (SF), a tyrosine kinase encoded by the proto-oncogene c-met. HGF/SF-met signaling has been shown to affect a wide range of biological activities in mammalian cells, including cellular proliferation, migration, invasion, morphogenesis, and angiogenesis[21,22]. The oncogene c-myc also has numerous biological activities, such as transformation, immortalization, blockage of cell differentiation and induction of apoptosis[23]. Amplification and abnormal expression of oncogenes of the c-myc and /or c-met are involved in the development of tumor. In this study, EBVaGC had a higher rate of c-met overexpression than EBVnGC, however the difference in c-myc and c-met expression between the two groups was not significant, indicating that EBV infection induces c-met over-expression. The results are inconsistent with previous studies[11,24-30]. The study about the amplification and expression of c-met oncogene and EBV infection in tumor tissues has been little reported, majoring in lymphoproliferative disorder such as Hodgkin's disease (HD). Kijima *et al*[24] found the amplification and overexpression of c-met in EBV-positive and -negative gastric carcinomas did not differ significantly. Teofili *et al*[25] also reported c-met expression was independent of the presence of EBV in HD patients. However, Weimar *et al*[26] found a strong correlation between the expression of the c-met proto-oncogene and EBV infection in patients with HD. Six out of eight EBV-positive samples from HD patients expressed c-met protein, while none of the 10 EBV-negative samples from HD expressed c-met. Furthermore, circulating B lymphocytes,c-met-negative, express c-met after infection by EBV. In the same way, no consistent results were found in the study of c-myc expression in EBV-associated tumor. Several studies showed that EBV facilitates the development of tumor by inducing c-myc and inhibiting p53 expression[27-29]. However, Park *et al*[30] found that significant increase of c-myc gene copy numbers was only found in 12 out of 31 non-Hodgkin's malignant lymphomas (MLs) (38.7%), in which 6 cases were EBV positive and 6 cases were EBV negative, indicating c-myc gene amplification did not correlate with EBV infection. Ishii *et al*[11] found c-myc expression in early stage of EBVaGC was higher than that of EBVnGC, while the c-myc expression in advanced stage of EBVaGC was lower than that of EBVnGC. It was inferred that EBV might cause the host cell to induce c-myc expression and inhibit p53 overexpression in the initial development of the cancers (early stage), but then influence c-myc expression negatively in advanced

stage cancers, making them less likely to have a natural regression via apoptosis. In the present study, most of the samples were advanced stage gastric carcinomas. Apparently, it could not infer whether EBV induces c-myc expression in early stage EBVaGC, but it was certain that EBV does not inhibit c-myc expression in advanced stage EBVaGC.

Some *in vitro* and *in vivo* studies have shown that EBV-encoded genes, such as LMP1, EBNA, BHRF1 and BARF1, can interact with oncogenes and tumor suppressor genes in the carcinogenesis of tumor[28,31,32]. LMP1 are the essential genes for cell transformation. LMP1 can induce c-met expression through the activation of Ets-1 transcription factor *in vitro*, which may contribute in part to the highly metastatic potential of NPC[31]. The expression of c-myc can be induced by LMP1 and EBNA2 *in vitro* and *in vivo*[28, 29, 32, 33]. Our study and other studies failed to detect LMP1 and EBNA2 mRNA in EBVaGC[6-8], suggesting that LMP1 and EBNA2 may not be essential for EBVaGC formation and may not be related with c-met and c-myc expression in EBVaGC. EBV 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[34,35]. 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*[8,36,37]. Furthermore, it can activate the expression of bcl-2[38]. We demonstrated that 6 of 13 EBVaGC cases exhibited BARF1 mRNA and 2 exhibited BHRF1 mRNA. Zur Hausen *et al*[8] also detected 9 BARF1-positive cases and 2 BHRF1-positive cases in 10 EBV-related gastric adenocarcinomas. Because EBVaGC lacks the expression of LMP1[6-8], BARF1 and BHRF1 might provide an alternative way for the pathogenesis of EBVaGC independent of LMP1. *In vitro* studies showed that BARF1 can activate the expression of c-myc in BARF1 transformed cells, and EBNA1 and c-myc cooperate in lymphomagenesis[38,39]. In our study no correlation was found between c-met and c-myc expression with the expression of BARF1 or BHRF1 in 13 EBVaGCs. So further study is needed to elucidate the relationship of EBV infection and EBV-encoded proteins with expression of c-met and c-myc.

In summary, this study shows that *H pylori* infection is closely linked to the distal location gastric carcinoma, but EBVaGC is predominant proximal location. No correlation exists between EBV infection and *H pylori* infection in the development of gastric carcinomas. EBV infection is associated with c-met abnormal expression but not with c-myc protein in EBVaGC. c-met overexpression is not induced by LMP1. BARF1 and BHRF1 may play important roles in tumorigenesis of EBVaGC through different pathways.

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