Differential expression of EBV proteins LMP1 and BHFR1 in EBV-associated gastric and nasopharyngeal cancer tissues

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Received February 5, 2015; Accepted January 29, 2016

DOI: 10.3892/mmr.2016.5087

Abstract. Epstein-Barr virus (EBV) infection is associated with the development of T cell lymphoma, nasopharyngeal cancer (NPC), and EBV-associated gastric cancer (EBVaGC). This study assessed the expression of the EBV-associated proteins latent membrane protein 1 (LMP1) and *Bam*HI-A rightward frame 1 (BARF1) in NPC and EBVaGC tissue specimens and determined their association with clinicopathological data, microvessel density (MVD) and micro-lymphatic vessel density (MLVD). This study collected 600 gastric cancer and 75 NPC tissue samples. EBV infection was assessed using *in situ* hybridization, and LMP1 and BARF1 expression was assessed using immunohistochemistry. The levels of MVD and MLVD were assessed using immunostaining of vascular endothelial growth factor (VEGF)-C, CD34, and lymphatic vessel endothelial receptor 1 (LYVE-1). Among the 600 gastric cancer cases, 30 were positive for EBV infection, which was shown to be associated with the age of patients (P=0.073), tumor differentiation (P<0.0001), tumor location (P<0.0001) and lymph node metastasis (P<0.0001). In these 30 EBVaGC cases, only one case was weakly positive for LMP1, but 17 cases were BARF1 positive. BARF1 expression was associated with lymph node metastasis of EBVaGC and the level of MLVD. Furthermore, 61 (81%) of 75 NPC patients were EBV positive, among which 38 cases were LMP-1 positive (62.3%) and LMP1 expression was associated with tumor-node-metastasis stage (P=0.011) and lymph node metastasis (P=0.041). MLVD was significantly higher in LMP1-positive cases than LMP1-negative cases. There were only 8 (13.3%) cases positive for BARF1 expression. In conclusion, EBV infection exhibits a role in gastric cancer and NPC development; however, expression of EBV-associated proteins LMP1 and BARF1 have differential functions during tumorigenesis of these two types of cancer.

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Key words: Epstein-Barr virus infection, nasopharyngeal cancer, Epstein-Barr virus-associated gastric cancer, biomarker, microvessel density, micro-lymphatic vessel density, latent membrane protein 1, *Bam*HI-A rightward frame 1

Introduction

Epstein-Barr virus (EBV) infection contributes to human carcinogenesis, particularly in the development of lymphoma, nasopharyngeal cancer (NPC) and gastric cancer (1). EBV is a γ-herpes virus and infects B cells of the immune system and epithelial cells. EBV latently persists in the host B cells for life (2) and thus, EBV is estimated to infect >90% of the population worldwide (2). In a small number of infected cell populations, EBV infection can transform B cells to lymphoma cells (3) or epithelial cells to nasopharyngeal cancer cells (4). Furthermore, ~10% of gastric cancer worldwide was associated with EBV infection and this subtype of gastric cancer is termed EBV-associated gastric cancer (EBVaGC) (5,6). However, the molecular mechanism by which EBV infection causes these malignancies remains to be defined.

Molecularly, the *Bam*HI-A rightward frame 1 (BARF1) is an EBV gene that is expressed early in the EBV lytic cycle and shares 38% primary amino acid sequence homology with the bcl-2 proto-oncogene product (7). The constitutive expression of BARF1 protein was able to immortalize lymphoblast cells and prolong cell survival (8). The carcinogenic activity of the oncogene c-myc is obligatory for the development of Burkitt lymphoma and previous studies suggested that BARF1 expression is required to inhibit c-myc-induced apoptosis and exhibits a synergic role in mediating the effect of Bcl-2 and c-myc during B cell transformation (9-13). BARF1 protein is also involved in the regulation of microvessel density (MVD) and micro-lymphatic vessel density (MLVD) (14,15). Furthermore, MVD and MLVD are strongly associated with cancer metastasis and with the survival of cancer patients (14). In addition, during EBV latency, there are >8 EBV encoded proteins and several non-coding RNAs expressed in cells (e.g.,

two EBV encoded small RNAs, termed EBER1 and EBER2), nuclear antigens and membrane proteins (16). Latent membrane protein 1 (LMP1) and LMP2A are two EBV latent membrane proteins, which function as constitutively active receptors independent of ligand binding and manipulate the signaling pathways for B cell activation and differentiation in order to sustain the long-life of EBV-positive cells (17). The carboxyl terminus of LMP1 contains consensus tumor necrosis factor receptor-associated factor (TRAF)-binding domains, which can constitutively activate signal transducers and activators of transcription (STAT), janus kinase (JNK), and nuclear factor (NF)-κB pathways for cell survival and growth (18). Although, the oncogenic role of LMP1 is well established, its roles in microvessel and micro-lymphatic vessel formation are less clear. Thus, the present study assessed the EBV infection in NPC and gastric cancer tissue samples, and then analyzed the levels of MVD and MLVD to identify their association with the clinicopathological features of the patients. This study may provide a novel insight into the oncogenic role of EBV in NPC and gastric cancer.

Patients and methods

Patients and tissue samples. A total of 600 gastric cancer and 75 NPC tissues were collected. All tumor tissue specimens were histologically confirmed and retrieved from the Department of Pathology, The Shandong Provincial Institute of Cancer Prevention and Research (Jinan, China) and The Affiliated Hospital of Jining Medical College (Jining, China) between 2008 and 2012. The present study was approved by the Institutional review boards of The Shandong Provincial Institute of Cancer Prevention and Research and The Affiliated Hospital of Jining Medical College. All patients provided informed consent to participate in this study.

Tissue specimens from each patient were fixed in 10% buffered formalin and embedded in paraffin (Shijiazhuang Chemical Technology Co., Ltd., Shijiazhuang, China), and then cut into serial sections (5 μm). One of the consecutive sections was stained with hematoxylin and eosin (H&E; Shanghai Biological Technology Co., Ltd., Shanghai, China) for confirmation of diagnosis, while others were subjected to *in situ* hybridization to detect EBV RNA or immunohistochemistry to detect the expression of LMP1, BARF1, vascular endothelial growth factor-C (VEGF-C), lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) and CD34.

In situ hybridization and immunohistochemistry. For *in situ* hybridization, an ISH-5022 EBER kit was used (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.; OriGene Technologies, Inc., Beijing, China). A labeled oligonucleotide probe complementary to EBER1 was used to detect the EBER1 as positive REBV infection. Briefly, tissue sections were deparaffinized and rehydrated, and then hybridized to a labeled probe according to the manufacturer's instructions. Tissue sections without probe hybridization were used as negative controls. For immunohistochemistry, tissue sections were deparaffinized by 2 times incubation in xylenol for 10 min at room temperature and dehydrated in a series of ethanol (100, 75 and 50%). Sections were then incubated in 3% H_2O_2 for 10 min at room temperature and subsequently subjected twice to antigen repair in 0.01 M acid buffer (pH 6.0) using a microwave at 92-98˚C for 5 min, with a 10 min break between incubations. Sections were washed in phosphate-buffered saline (PBS), incubated with normal goat serum for 15 min at room temperature, and further incubated with primary antibodies at 4˚C overnight. The primary antibodies used were rabbit monoclonal anti-LMP1 (cat. no. ZM-0386), rabbit monoclonal anti-BHRF1 (cat. no. ZA-0627), rabbit monoclonal anti-VEGF-C (cat. no. ZA-0266), mouse monoclonal anti-LYVE-1 (cat. no. ZA-0483) and mouse monoclonal anti-CD34 (cat. no. ZM-0046) (dilution used for all was 1:200; Zhongshan Golden Bridge Biotechnology Co., Ltd.; OriGene Technologies, Inc.). The sections were washed 3 times with PBS and incubated with biotinylated anti-mouse (cat. no. BA1001) or anti-rabbit (cat. no. BA1003) secondary antibodies (dilution used for all was 1:200; Boster Biological Technology, Ltd., Beijing, China) for 30 min at room temperature, and subsequently visualized by incubation of tissue sections with a 3,3'-diaminobenzidine solution (Zhongshan Golden Bridge Biotechnology Co., Ltd.; OriGene Technologies, Inc.). Sections were counterstained with H&E, mounted with a coverslip and visualized using the Olympus CX23 light microscope (Olympus Corporation, Tokyo, Japan).

Review and scoring of stained tissue sections. All tissue samples were reviewed and scored blindly by two pathologists, and the pathology of each tissue section was confirmed. To assess the *in situ* hybridization data, negative controls were indicated as the sections without any staining, and positive controls were indicated as the stained sections with appropriate nuclear localization. To score immunohistochemical data, the two pathologists reviewed at least ten x400 fields and counted staining intensity and percentage of positive cells vs. total cells. The staining intensity was judged as no staining, -; light brown, +; brown, ++; and strong brown, +++. The percentage of positive cells <10% was considered as negative and the detailed information is as follows: <10%, -; 11-25%, +; 26-50%, ++; >50%, +++. These two scores were added together to form a total score, high (score ≤2+) vs. low expression of a protein.

Quantitative measurement of MVD and MLVD levels in tissue specimens. MVD and MLVD were visualized using immunostaining of the tissue sections with CD34 and LYE-1 antibodies, respectively, to perform morphometric analysis. The stained sections were reviewed and scored by two pathologists in a blind manner, under a CX23 light microscope. Briefly, the pathologists reviewed the sections under a x100 magnification and then selected 5 high power fields (x400 magnification) to capture images. CD34 immunostaining was used to visualize microvascular endothelial cells, while LYVE-1 is specifically expressed in microlymphatic endothelial cells. Under the CX23 light microscope, any brown colored endothelial cells or microvascular endothelial cells observed in a cluster was counted as one microvessel. Their branches were also counted as a microvessel as long as the structures were not connected. However, if the lumen had >8 red blood cells or the vessel had a muscular and vascular wall, this vessel was not counted. MVD was calculated as the mean number of microvessels from five x200 microscopic fields. Furthermore, micro-lymphatic

Table I. Association of EBV infection with clinicopathological characteristics from patients with gastric cancer.

Factor	No. of cases (n=600)	No. of EBV-positive cases (n=30)	No. of EBV-negative cases (n=570)	χ^2	P-value
Gender					0.049
Male	384	25	359	3.87	
Female	216	5	211		
Age (years)					0.073
<45	32	4	32	2.24	
45-60	223	13	210		
>60	345	13	334		
Tumor differentiation					<0.0001
Well	68	8	60	15.69	
Moderate	124	12	112		
Poor	408	10	398		
Tumor location					<0.0001
Gastric cardia region	102	12	90	14.99	
Gastric body region	139	10	129		
Gastric antral region	359	8	351		
Lymph node metastasis					0.0001
Yes	580	21	559	40.39	
No	20	9	11		

EBV, Epstein-Barr virus.

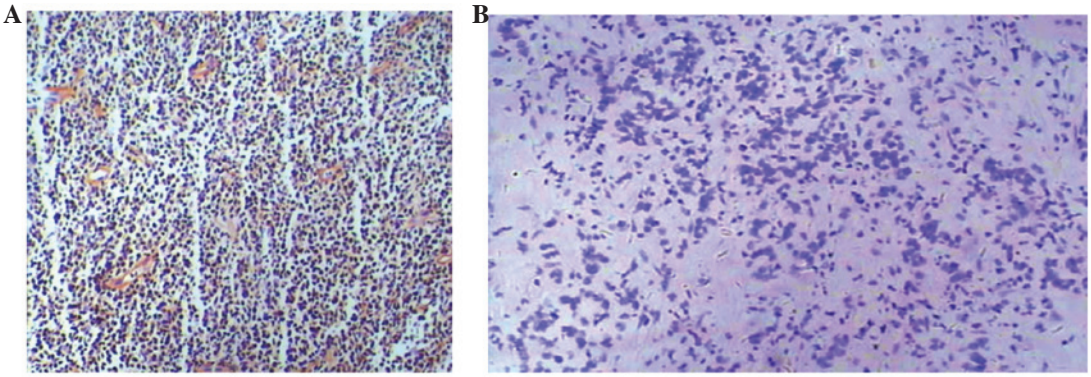

Figure 1. *In situ* hybridization detection of EBV-encoded small RNA 1 to indicate Epstein-Barr virus infection in gastric cancer and nasopharingeal carcinoma tissues. (A) Positive and (B) negative expression.

vessels were labeled by anti-LYVE-1 antibody and reviewed and counted as same as MVD.

Statistical analysis. All statistical analyses were performed using SPSS software, version 18.0 (SPSS Inc., Chicago, IL, USA). All data were analyzed using the Student's t-test. P<0.05 was considered to indicate a statistically significant difference. Count data were presented as frequency and percentage, and analyzed by χ^2 analysis or Fisher's exact test. Measurement data were presented as the mean/median ± standard deviation. Data with normal distribution were analyzed by an F-test or t-test. Data with uncommon distribution were analyzed by a non-parametric test or Wilcoxon rank test.

Results

EBV infection is associated with clinicopathological characteristics of patients. EBV infection has been linked to EBVaGC and NPC (5,19); thus, *in situ* hybridization was performed to detect EBV-encoded small RNA 1 (EBER1) as the indication of EBV infection. Among the 600 gastric cancer tissue specimens, 30 positive cases of EBV infection were identified (Fig. 1A and Table I). Compared with negative staining, ISH positive particles had deep purple blue staining present in the nuclei (Fig. 1B). EBV infection was shown to be correlated with the age of the patient (P=0.073), tumor differentiation (P<0.0001), tumor location (P<0.0001) and

Table II. Association of EBV infection with clinicopathological characteristics of patients with nasopharingeal carcinoma.

Factor	No. of cases (n=75)	No. of EBV-positive cases (n=61)	No. of EBV-negative cases (n=14)	χ^2	P-value
Gender					0.79
Male	59	50	9	0.070	
Female	16	11	5		
Age (years)					0.311
<45	17	14	3	2.333	
45-60	36	35	1		
>60	22	11	11		
Pathology					
Keratinizing squamous cell carcinoma	8	7	10	0.016	0.90
Non-keratinizing carcinoma	67	54	24		

EBV, Epstein-Barr virus.

Table III. Association of MVD and MLVD levels with clinicopathological characteristics of patients with Epstein-Barr-associated gastric cancer.

Factor	n	MVD	P-value	MLVD	P-value
Gender			0.408		1.000
Male	25	32±2.3		3.1±0.9	
Female	5	33±3.1		3.1±1.4	
TNM stage			0.402		0.128
I/II	10	32±3.1		2.5±0.9	
III/IV	20	33±3.0		3.5±1.9	
Lymph node metastasis			0.111		1.000
Negative	9	32±3.4		3.2±0.9	
Positive	21	34±2.9		3.0±1.7	

MVD, microvessel density; MLVD, micro-lymphatic vessel density; TNM, tumor-node-metastasis.

lymph node metastasis (P<0.0001; Table I). The data suggest that EBV infection only occurs in a small percentage of gastric cancers (5%).

Furthermore, in 75 patients with NPC, 61 patients were positive for EBV infection (Table II). EBV infection was not shown to be correlated with any of the clinicopathological characteristics investigated (Table II).

Expression of the EBV-associated proteins LMP1 and BHFR1 is correlated with MLVD rather than MVD in patients with EBVaGC. Expression of the EBV-associated proteins LMP1 and BHFR1 and markers of MVD and MLVD (CD34 and LYVE-1) was then further analyzed in the tissue specimens. Morphometric image analysis of CD34 and LYVE-1 was used to visualize MVD and MLVD. The data showed that MVD and MLVD were not associated with TNM stage and lymph node metastasis in patients with EBVaGC (Table III). In the 30 patients with EBVaGC, only one case showed weak LMP1 expression, but 17 cases (56.7%) showed BARF1 expression (Fig. 2 and Table IV). BARF1 expression was significantly associated with lymph node metastasis of EBVaGC. Among the 30 patients with EBVaGC, 18 patients (60%) were VEGF-C positive (data not shown). The expression of VEGF-C was not associated with lymph node metastasis. BARF1 expression was associated with MLVD but not MVD. These data suggest that EBV could infect lymphatic vessels and induce micro-lymphatic vessel formation. Expression of VEGF-C was associated with MVD and MLVD (data not shown).

Expression of the EBV-associated proteins LMP1 and BHFR1 was correlated with MLVD rather than MVD in patients with NPC. Among 61 EBV positive NPC patients, there were 38 cases that were LMP1-positive (62.3%, Fig. 3). LMP1 expression was associated with TNM stage (P=0.021) and lymph node metastasis (P=0.046). By contrast, BARF1 was only expressed in 8 cases (13.3%) and BARF1 expression was not identified to be associated with the analyzed factors. Moreover, VEGF-C was expressed in 52 cases (85.2%) and VEGF-C expression

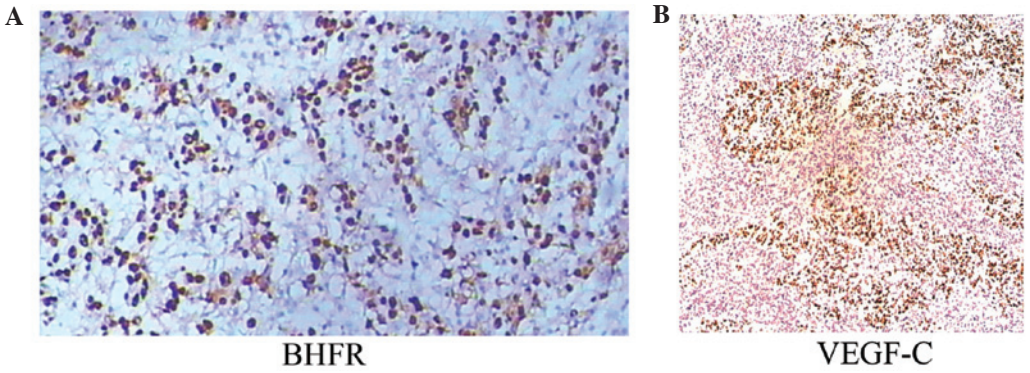

Figure 2. Expression of BARF1 and VEGF-C in Epstein-Barr-associated gastric cancer (x100 magnification). (A) Positive expression of BARF1; (B) Positive expression of VEGF-C. VEGF-C, vascular endothelial growth factor-C, BARF1, *Bam*HI-A rightward frame 1.

Table IV. Association of BARF1 and VEGF-C expression with MVD and MLVD level in Epstein-Barr-associated gastric cancer.

Expression	n	MVD	P-value	MLVD	P-value
BARF1			1.000		0.000
Negative	13	29±3.1		1.0±0.6	
Positive	17	29±2.3		3.4±0.9	
VEGF-C			0.000		0.000
Negative	12	23±4.8		1.1±0.8	
Positive	18	31± 4.1		3.1±0.4	

BARF1, *Bam*HI-A rightward frame 1; VEGF-C, vascular endothelial growth factor-C; MVD, microvessel density; MLVD, micro-lymphatic vessel density.

Table V. Association of BHRF1, LMP1 and VGGF-C expression with the clinicopathological data of patients.

		LMP1 expression			VEGF-C expression			BHRF1 expression		
Factor	n	Negative	Positive	P-value	Negative	Positive	P-value	Negative	Positive	P-value
Gender				0.931			0.893			0.914
Male	50	20	30		7	43		43	7	
Female	11	3	8		2	9		10	1	
TNM stage				0.021			0.31			0.943
I/II	27	18	9		6	21		23	4	
III/IV	34	5	29		3	31		30	4	
LN metastasis				0.046			0			0.752
Negative	14	10	2		7	7		12	2	
Positive	47	13	36		2	45		41	6	

LMP1, latent membrane protein 1; VEGF-C, vascular endothelial growth factor-C; BARF1, *Bam*HI-A rightward frame 1; TNM, tumor-node-metastasis; LN, lymph node.

was associated with lymph node metastasis (P<0.0001). The MVD level was not shown to be significantly different between LMP1-positive and -negative cases (Tables V and VI). However, MLVD in the LMP1-positive group was significantly higher than the LMP1-negative group. This suggests that LMP1 may contribute to micro-lymphatic formation. MVD and MLVD in the VEGF-C-positive group were higher than in the negative group suggesting that VEGF-C may contribute to microvessel and micro-lymphatic formation in NPC.

Discussion

EBVaGC is a recently identified cancer type that may be caused by EBV infection (19). The present study further

Table VI. Association of LMP1 and VEGF-C expression with MVD and MLVD levels in nasopharyngeal carcinoma.

Expression	n	MVD	P-value	MLVD	P-value
LMP1			1.000		0.046
Negative	23	59±3.1		5.0±2.0	
Positive	38	59±5.3		6.4±2.9	
VEGF-C			0.000		0.000
Negative	9	53±4.8		4.1±1.8	
Positive	52	61±4.1		8.1±3.0	

LMP1, latent membrane protein 1; VEGF-C, vascular endothelial growth factor-C; MVD, microvessel density; MLVD, micro-lymphatic vessel density.

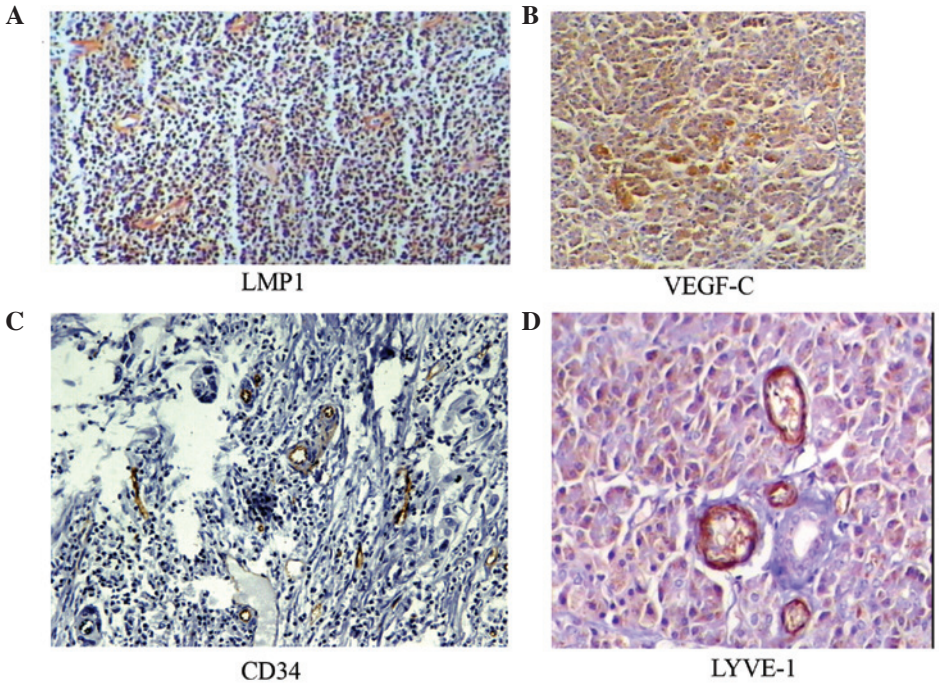

Figure 3. Expression of LMP1, VEGF-C, CD34 and LYVE-1 in nasopharyngeal tissues (x100 magnification). (A) Positive expression of LMP1; (B) positive expression of VEGF-C; (C) positive expression of CD34; and (D) positive expression of LYVE-1. LMP1, latent membrane protein 1; VEGF-C, vascular endothelial growth factor-C, LYVE-1, lymphatic vessel endothelial receptor 1.

demonstrated that EBV infection contributes to the development of a small percentage (5%) of gastric cancers, although previous studies have shown that 10% of worldwide gastric cancers were EBVaGC (6,20). By contrast, the frequency of EBV infection in NPC was notably higher, effecting 85% of the patients observed. Although the frequency of EBV infection in EBVaGC and NPC was different, the molecular mechanism of EBV infection in different types of cancer may be the same. Thus, in this study expression of the EBV-associated proteins LMP1 and BARF1 was further assessed in NPC and EBVaGC tissue specimens for association with clinicopathological data and with MVD and MLVD. It was demonstrated that BARF1 expression was associated with lymph node metastasis and MLVD in patients with EBVaGC. In NPC, LMP1 expression was associated with TNM stage (P=0.011) and lymph node metastasis (P=0.041). Only 13.3% cases were BARF1 positive and MLVD was significantly higher in LMP1-positive cases than in LMP1-negative cases. The data from the current study indicate that although EBV infection is involved in the development of gastric cancer and NPC, expression of EBV-associated proteins LMP1 and BARF1 may have differential functions during the tumorigenesis of these two types of cancer.

Generally, primary EBV infection occurs via the oral route and establishes a lifelong virus carrier state, termed latent infection (21). In latent infection, infected cells only express a limited set of viral genes, but can provide a survival advantage to the infected cell (22). The latent infection can be further divided into different subgroups due to specific viral proteins (23). EBVaGC is considered as a latency I EBV infection, while NPC can be both latency I and II EBV infections (24,25). Latency I infection is characterized by expression of EBV nuclear antigen 1 (EBNA1), EBER1 and 2, and *Bam*HI-A rightward transcripts (BART) (4,26).

In addition to latency I transcripts, latency II infection can also express latent membrane protein 1 (LMP1) (4,26). Our current data are consistent with previous findings (4,26). Only one patient with weak LMP1 expression was identified in the 30 EBVaGC cases. By contrast, 38 NPC tissues in these 61 NPCs exhibited LMP1 expression. This confirmed that the latency of EBV infection between EBVaGC and NPC was different. Therefore, due to the different expression of viral proteins, oncogenic mechanisms of EBV in EBVaGC and NPC may differ.

Furthermore, tumor metastasis is the leading cause of cancer-related mortality (27,28). A greater understanding of the molecular mechanism underlying tumor metastasis may aid the development of effective cancer therapies. The initial site of cancer metastasis is usually the regional lymph nodes (29,30). Clinical and experimental data suggested that lymphoangiogenesis can greatly facilitate the migration of tumor cells into the lymph nodes (31-33). The present study demonstrated that MLVD was associated with NPC lymph node metastasis and that EBV infection was associated with MLVD. The association between EBV infection and MLVD was consistent in EBVaGC and NPC. These data suggest that EBV infection may have a common effect on the regulation of lymphoangiogenesis, although the exact molecular mechanisms remain unknown. Further investigation is required to clarify how EBV infection contributes to an increase in MLVD.

EBV-positive epithelial malignancies show selective and abundant expression of a viral gene that encodes BARF1 protein (34). BARF1 expression was usually low in lymphomas, but more frequent in EBV-associated carcinomas. BARF1 may function as an oncogene in NPC, parallel to the more widely investigated viral protein LMP1 (35). In EBV-positive gastric cancer, BARF1 was expressed in the absence of LMP1, possibly functioning as the predominant EBV oncogene in this disease (36). BARF1 expression was able to immortalize and transform epithelial cells of different origins by acting as a mitogenic growth factor, inducing cyclin-D expression and upregulating anti-apoptotic Bcl-2, and in turn stimulating host cell growth and survival (37). In the current study, 13% of NPCs were positive for BARF1, whereas BARF1 was expressed in 56.7% of EBVaGCs. This finding suggests that BARF1 and LMP1 may have redundant functions in promoting tumorigenesis in gastric cancer and in NPC.

The current study does have certain limitations. For example, it is only a proof-of-principle descriptive study and additional mechanistic data are required to support the current findings. It remains to be determined how these two viral proteins function differentially in these two types of human cancer or whether EBV is involved in the development of EBV-positive gastric cancer since EBV is estimated to infect >90% of the worldwide population and EBV infection may be just bystander in these gastric types of cancer.

Acknowledgements

This study was partially supported by the 973 Projects of China (grant no. 2011CB504302) and the Natural Science Foundation of China (grant no. 30872318). The authors would also like to acknowledge the valuable comments from other members of the laboratories.

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