Review Article

Epstein-Barr Virus and Gastric Carcinoma – Viral Carcinogenesis through Epigenetic Mechanisms

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Abstract: Epstein-Barr virus (EBV)-associated gastric carcinoma (GC) is the monoclonal growth of EBV-infected epithelial cells, and the entity was recognized only recently. EBV-associated GC is distributed worldwide and more than 90,000 patients are estimated to develop GC annually in association with EBV (10% of total GC). EBV-associated GC occurs in two forms in terms of the histological features, i.e., lymphoepithelioma-like GC and ordinary type of GC. Both share characteristic clinicopathological features, such as the preferential occurrence as multiple cancer and remnant stomach cancer. While the expression of EBV-latent genes is restricted to several in the infected cells (Latency I), EBV-associated GC shows gastric cell phenotype, resistance to apoptosis, and the production of immunomodulator molecules. Recently, global and non-random CpG island methylation of the promoter region of many cancer-related genes has been demonstrated with their decreased expression, such as p16 INK4A, p73 and E-cadherin. This abnormality is accompanied by methylation of the EBV genome itself, suggesting a process of virus-driven hypermethylation in the development of neoplastic cells. Further studies are necessary to determine the precise sequence of EBV infection, methylation, transformation and selection of the predominant clone within the stomach mucosa. Future studies are also desirable for the target and strategy of therapy, such as initiating viral replication or reversing the DNA methylation of cellular genes.

Key Words: Epstein-Barr virus, gastric cancer, DNA methylation, viral oncogenesis, histology, chronic inflammation

Introduction

Epstein-Barr virus (EBV) is a herpes virus, which was discovered from a Burkitt lymphoma cell line in 1963 [1]. It was the first virus identified from a human neoplastic cell, followed by human papilloma virus (HPV), hepatitis virus B and C, human T-lymphotropic virus 1 (HTLV1), and human herpes virus type 8 (HHV8). Subsequent studies have demonstrated two important facts in EBV-associated malignant neoplasms. First, more than 90% of the world population is infected with EBV before adolescence, but EBV-associated malignant neoplasms develop in a limited number of patients in an endemic or non-endemic manner. Secondly, EBV is associated with the transformation of various types of cells, such as lymphoid, dendritic, smooth muscle and epithelial cells (**Table 1**), although latent infection in healthy individuals is observed in a small number of lymphoid cells. EBV-associated lymphoproliferative disorders include infectious mononucleosis and chronic EBV infection, and various types of lymphomas of B-cell origin, such as Burkitt lymphoma, Hodgkin lymphoma and B-cell lymphomas in immunocompetent (e.g. pyothorax-associated lymphoma) or immuno-compromised hosts, and T and NK/T cell lymphomas. Epithelial cell malignancies are nasopharyngeal carcinomas (NPC), and some carcinomas at extra-nasal sites, such as the salivary gland, thymus, and stomach.

The pathogenic role of EBV appears to vary in these tumors, and accumulation of the precise knowledge of each neoplasm is indispensable for thorough understanding of the role of EBV in human neoplasms. In the current review, we focused on EBV-associated gastric carcinoma (GC), since this entity was recognized only recently and is most common among EBV-associated malignant neoplasms. EBV infection in a gastric carcinoma was first reported by Burke et al in 1990 [2] using PCR, followed by other groups in many countries [3-5]. In contrast to the endemic nature of NPC,

Table 1 EBV-associated malignancies

Normal counterpart	Malignancy	Immuno-compromised host*
Lymphocytes		
B cells	Burkitt lymphoma	
	Classical Hodgkin lymphoma	
	Post-transplant lymphoproliferative disorder	+
	AIDS-associated B-cell lymphomas	+
	Pyothorax-associated lymphoma	
T/NK cells	Extranodal NK/T cell lymphoma, nasal type	
	Virus-associated hemophagocytic syndrome T-cell lymphoma	
Epithelial cells		
	Nasopharyngeal carcinoma	
	Part of gastric carcinoma	
	Lymphoepithelioma-like carcinoma (salivary, thymus, lungs and stomach)	
Mesenchymal cells		
Dendritic cells	Follicular dendritic cell sarcoma	
Smooth muscle	Leiomyosarcoma	+

*, + indicates that the tumor develops in immunocompromised host.
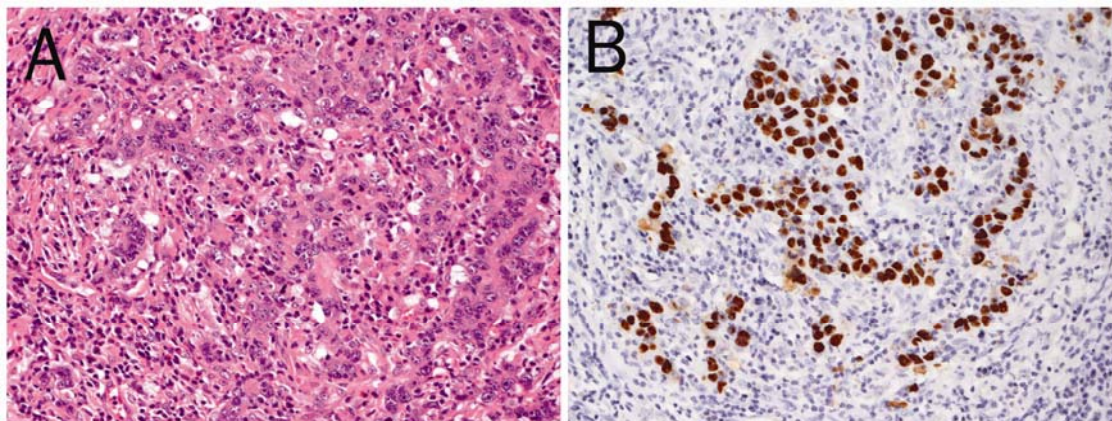

Figure 1 Histology of an EBV-associated GC. This tumor is similar to lymphoepithelial carcinoma. Lymphocytes infiltrate massively among cancer cells (**A**, H&E stain). Lymphocytes are mainly CD8-positive T-cells. All tumor cells are positive for EBNA, whereas infiltrating lymphocytes are negative (**B**, EBER in situ hybridization).

EBV-associated GC is distributed worldwide and more than 90,000 patients are estimated to develop gastric carcinoma annually in association with EBV (approximately 10% of total gastric cancer) [6].

Clinicopathological Features

EBV-associated GC is defined by the presence of EBV in the neoplastic cells of GC; EBV DNA or its abundant small RNAs, EBERs, are always present in almost all neoplastic cells when it is present in tumor tissues of GC (**Figure 1**).

General Histopathological and Clinical Features

EBV-associated GC occurs in two forms in terms of histological features, i.e., lympho-epithelioma-like GC (**Figure 1**) and the ordinary type of GC (**Figure 2**). The relative frequency of the two types is roughly 1:4. Lympho-epithelioma-like GC has a typical histology of poorly differentiated carcinoma with dense infiltration of lymphocytes, which was also named as GC with lymphoid stroma by Watanabe and Enjoji [7]. More than 80% of
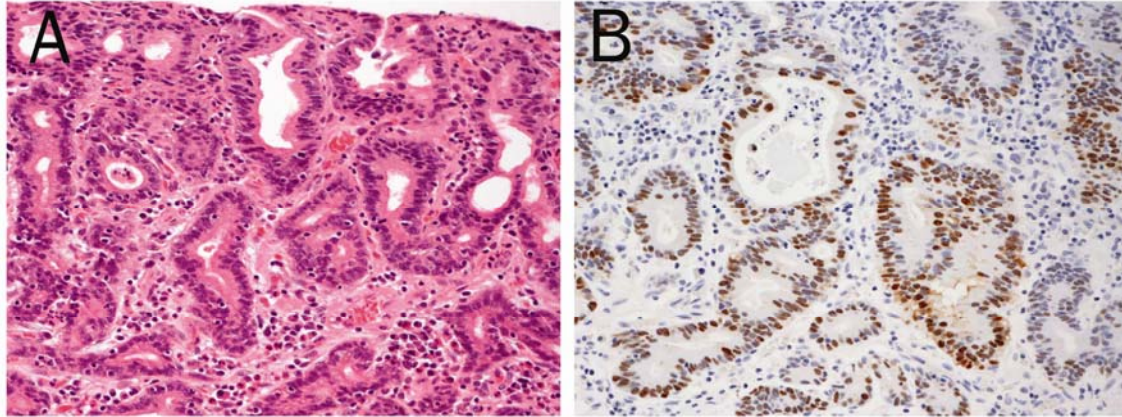
Figure 2 Histology of an ordinary GC with EBV infection. About 5-10% of ordinary GCs are infected with EBV. EBV-associated GC has more lymphocyte infiltration than EBV-negative GC; HE sections (**A** for an example) give no suggestion of EBV infection. EBER in situ hybridization reveals EBV infection (**B**).

Figure 3 Macroscopic appearance of EBV-associated GC. EBV-associated GC often appears in the upper part of the stomach. This case is in the advanced stage. It is ulcerated without definite limits.

lymphoepithelioma-like GC is infected with EBV [4, 8-10]. On the other hand, the ordinary type, comprising 5-10% of total GC, shows a moderately or poorly differentiated type of adenocarcinoma with various degrees of lymphocytic infiltration [11]. Both types of carcinoma, however, share clinicopathological features, such as the preponderant location at the proximal stomach.

Some EBV-associated GC show a "lace pattern" at the stage of intramucosal carcinoma, which consists of the connection and fusion of neoplastic glands at the intermediate zone of the proper mucosa [12]. Further infiltration of the carcinoma into the submucosa is occasionally accompanied by the infiltration of massive lymphocytes, which are predominantly CD8-positive cytotoxic T lymphocytes [13, 14]. EBV infection is sometimes observed in a very limited number of these lymphocytes.

The clinical features of EBV-associated GC are male predominance and relatively younger age compared to EBV-negative GC [15, 16]. In endoscopical examination, EBV-associated GC often appears as superficial depressed or ulcerated lesions in the upper part of the stomach (**Figure 3**). In the advanced stage, it is ulcerated without definite limits [17]. On endoscopic ultrasonography, EBV-associated GC shows hypoechogenic submucosal nodules and the ratio of maximal thickness to width is significantly larger than EBV-negative GC [18], and these features are also well illustrated on computerized tomographic scan of the stomach [Maeda E et al, in press].

EBV-associated GC shows a lower rate of lymph node involvement, especially in its early stage in the submucosa [16, 19]. Even if lymph node involvement is radiologically suspected, many cases are pathologically proven to be negative for metastasis [20]. EBV-associated GC as well as lympho-epithelioma-like GC has a favorable prognosis compared to EBV-negative GC [10, 21, 22].

Particular Features, Focus of Interest

The incidence of multiple carcinomas is higher in EBV-associated GC than in EBV-negative carcinomas, as suggested by Arikawa et al
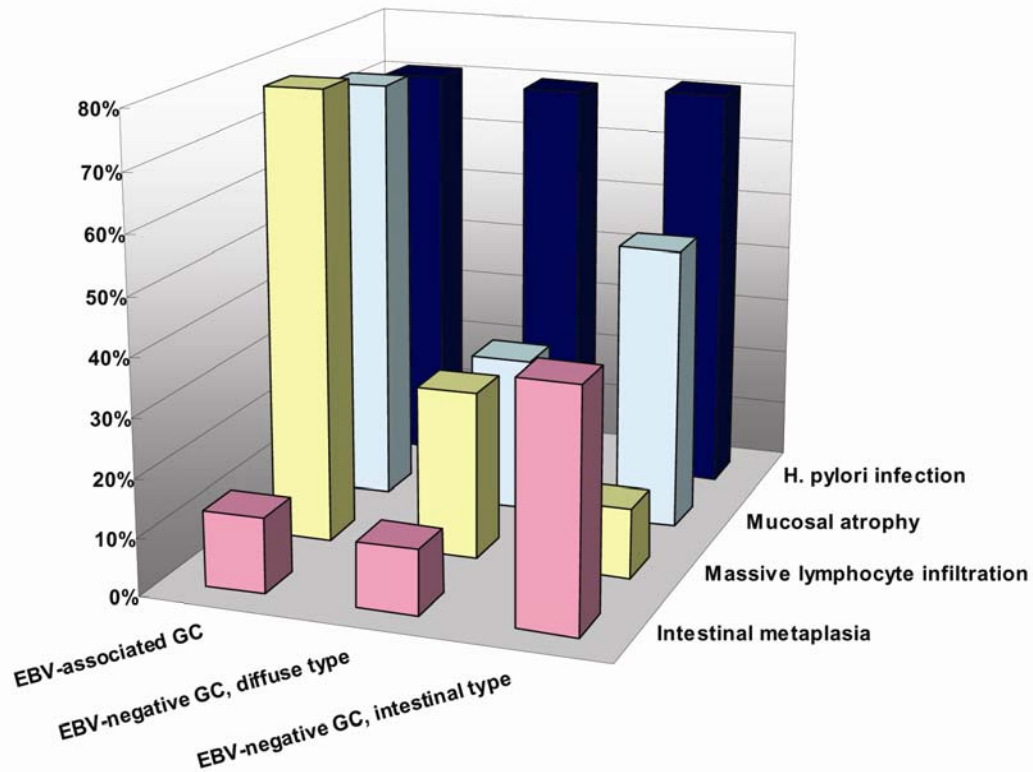
Figure 4 Features of non-neoplastic mucosa around GCs. EBV-associated GC is accompanied with atrophic gastritis and massive lymphocyte infiltration compared to both EBV-negative GC, diffuse type and intestinal type. H. pylori infection rate was not related to EBV infection.

[23] and Matsunou et al [10]. Clonal analysis of EBV, as mentioned below, demonstrates different EBV clones in independent foci of the same stomach, indicating that EBV-associated GC develops at multiple sites independently. Thus, a stomach with EBV-associated GC may have been conditioned to develop gastric carcinomas by EBV infection (field cancerization). The background gastric mucosa in EBV-associated GC is characterized by atrophic gastritis and lymphocyte infiltration [24, 25] (**Figure 4**). Some researchers have reported the frequent presence of EBV in the epithelial cells with intestinal metaplasia in the non-neoplastic stomach; however, the findings are not reproducible, and it is now considered that EBV infection in the non-neoplastic epithelium is extremely rare [26].

Another risk factor is the remnant stomach. The proportion of EBV involvement is 27-42%

in gastric remnant cancer after a primary gastrectomy for benign gastric diseases [27-29], which is significantly higher than 10% in *de novo* GC. Gastritis cystica polyposa, which is frequently observed in the remnant stomach, especially with Billroth II anastomosis, might be a prerequisite for the development of EBV-associated GC [30].

A recent case report by Au et al might illustrate the possible sequence of mucosal events [31]. A 52-year-old man, who received hemato-poietic stem cell transplantation for the treatment of multiple myeloma, developed an EBV-associated GC after immunosuppression of three months and mucosal damage caused by graft-versus-host disease (GVHD). Retrospective analysis of the patient's serum revealed a surge of circulating EBV DNA peaking at the time of gastritis, when EBV infection is likely to occur in the damaged
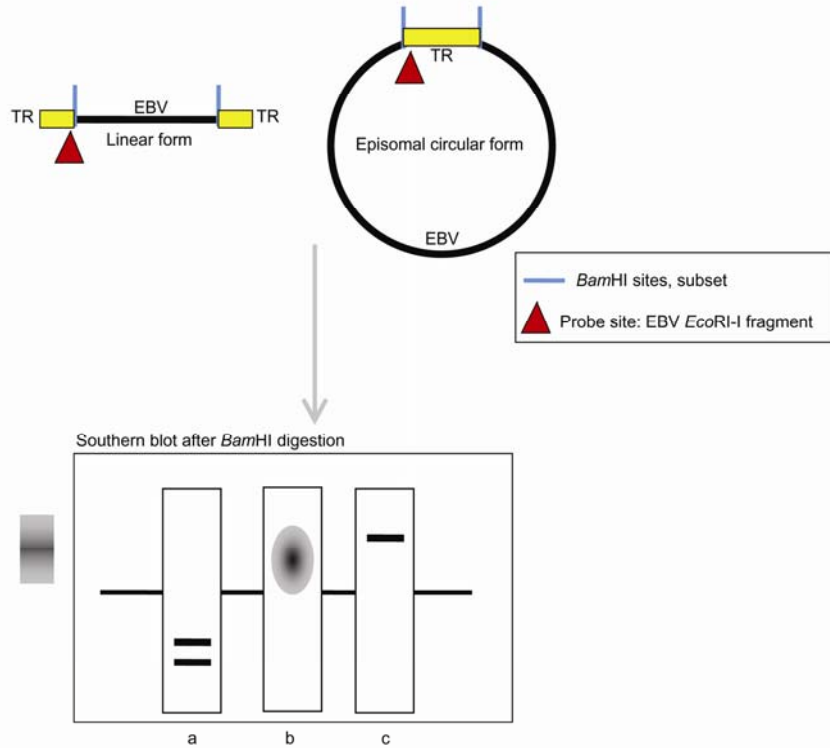
Figure 5 Linear and circular episomal forms of EBV. EBV takes 2 forms, linear and circular. EBV replicates itself in the linear form, whereas EBV is in the state of latent infection. EBV binds at the terminal repeat (TR) and changes itself into the episomal circular form. Southern blotting after BamHI digestion with probes to TR reveals the different forms of EBV. The detected bands are short when EBV takes the linear form (**a**). When various clones of EBV infect the tumor, smearing signals are found (**b**). The band is single when monoclonal infection of EBV is established in the tumor (**c**).

epithelial cells of the stomach. Another possible explanation may be the development of EBV-associated GC from EBV-infected stem cells, which are derived from bone marrow, although such an idea is totally controversial at present.

Virology of EBV

There are two approaches to investigate the pathogenesis of virus-associated human neoplasms, their virology and pathology, and this is also the case in EBV-associated GC.

EBV in Carcinoma Tissue

EBV is a double-stranded DNA virus and takes a linear form in viral particles. After EBV enters infected cells, it becomes circular in the nuclei by fusing both ends of viral DNA, which consist of repetitive 500bp structures (terminal repeat, TR). Southern blotting analysis of the specific structure of EBV TR in infected cells provides information about the clonality of EBV, viral integration, and the state of viral activation, i.e., replicating (linear configuration) versus latent (episomal circular forms) (**Figure 5**). In EBV-associated GC, analysis has demonstrated single fragments larger than 6kb in carcinoma tissues, indicating that monoclonal EBV is present in an episomal form without integration into the host genome, and that infection is latent with no viral replication.

When this analysis was applied to carcinomas in intramucosal and early invasive stages (**Figure 6**), single or bi-clonal EBV was observed in carcinoma tissues at the intramucosal stage, and always monoclonal at the stage of submucosal invasion. Nearly all of the carcinoma cells in all cases of EBV-associated GC showed a positive signal in EBER (EBV-encoded poly (A)-RNAs)-in situ hybridization (ISH) whether in the intramucosal or early invasive stage [5, 23, 26]; therefore,
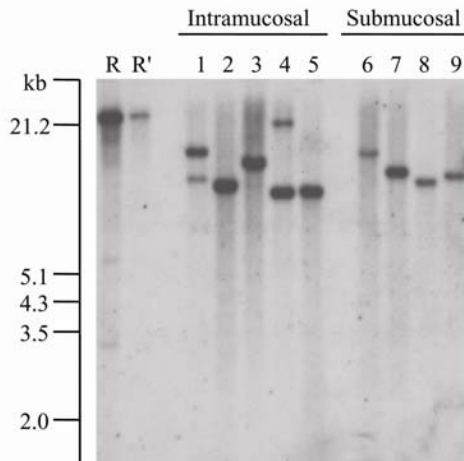
Figure 6 EBV clonality assay by Southern blot. DNA is cut with *Bam*HI restriction enzyme, electrophoresed, and hybridized with an EBV-TR-specific probe. Five cases of intramucosal EBV-associated GCs and 4 cases of submucosal GC. Among intramucosal GC, 2 showed bi-clonal EBV infection and 3 showed monoclonal infection. All submucosal tumors showed monoclonal infection. EBV infection occurred at the initial or very early stage of carcinoma development.

EBV infection occurs at the initial or very early stage of carcinoma development.

EBV in Non-Neoplastic Tissue

The tissue distribution of EBV DNA in immunocompetent individuals has been investigated by PCR and other morphological methods, such as EBER ISH. PCR study applied to systemic tissues revealed the frequent presence of EBV DNA in the oral mucosa, esophagus, and stomach, but absence in the small intestine and colon [32]. Real-time PCR of EBV DNA in biopsy tissues of the gastric mucosa demonstrated that EBV was detected in 66% (23 of 35 cases) and most frequently in cases showing moderate chronic atrophic gastritis (92%; 12 of 13 cases) [33]. By ISH targeting EBER, cells showing positive signals are lymphocytes, but not epithelial cells. The reported frequencies of EBER-positive lymphocytes are none [34], rare in only nine of 242 patients with chronic gastritis, and up to 25% in 24 of 97 patients with GC [35].

EBV status in non-neoplastic gastric epithelium is still uncertain. The methods used in early reports demonstrating positivity in normal gastric epithelium with anti-latent membrane protein (LMP)1 antibody [36] or EBV DNA ISH [37] are neither concrete nor reproducible. By EBER ISH, positivity was reported in a few gastric glands with intestinal metaplasia [38]. Our own observation was EBER-1 positivity in several shedding epithelial cells at the fundic gland mucosa of patients with EBV-associated GC [26].

Direct contact of lymphocytes with epithelial cells is considered essential for EBV entry into epithelial cells, but its mechanism remains uncertain. Infection of B cells and epithelial cells occurs by different routes, and the subset of envelope proteins involved in mediating virus fusion with two cell types is different. Following attachment via gp350 and CD21 [39, 40], fusion with a B cell requires glycoprotein gB and an oligomeric three-part complex of glycoproteins, gH, its chaperone gL, and gp42. The gp42 interacts with HLA class II on the B-cell surface [41], and this interaction is essential for triggering fusion machinery into an active state. On the other hand, neither gastric epithelium nor GC cells express CD21 molecules and anti-CD21 antibody does not inhibit EBV infection to GC cells [42]. Interaction between gp42 and HLA class II is not used for triggering fusion machinery of gHgL and gB in epithelial cells. Instead, gH, as part of a two-part gHgL oligomer, interacts directly with an as-yet-unknown molecule(s) in the epithelial membrane [43].

Epidemiology: Virus Strains/Host Reaction

EBV shows some differences in the DNA sequence, and certain types of EBV are found preferentially in areas where EBV-related malignancies occur at remarkably high incidence (**Table 2**) [26, 44-51]. Well-known examples are EBV type B in the EBV-determined nuclear antigen (EBNA) 2 region in equatorial Africa where Burkitt lymphoma is endemic, and EBV type C in the *BamHI*-I region in southern China, and the f variant in the *BamHI*-F region in NPC patients in southern China. Such analyses of EBV-associated GC have demonstrated that the prevalent EBV strains are type A, C and F, the same as from throat washings of healthy individuals [26, 52, 53]. The 30bp deletion of COOH terminal LMP1 variant has been identified in NPC and various EBV-associated lymphoid tumors. It

Table 2 EBV genotypes, regional distributions and malignancies

Viral gene or location	Genotype	Regional prevalence	Related disease	References
EBNA2	A/B	A in Asia, North Africa, USA, France and Germany B in equatorial Africa	Unknown	[44]
BamHI W1* to I1* fragment	C/D	C in Southern China and Japan D in United States	Unknown	[26, 45]
BamHI-W1/I1	I/i	Unknown	"i" in EBV-associated GC	[46]
BamHI F region	F/f	F in Southern China F in Japan	f in NPC, F in normal mucosa in Southern China F in NPC among Caucasian	[45, 47]
EBNA-3C	1/2	Unknown	Unknown	
LMP1	30bp deletion	Unknown	No relation	[48-50]
LMP2A	SNPs(codon 23, 348, 444)	Unknown	EBV-associated GC	[51]

has been suggested that 30bp deletion variants of LMP1 induce a more aggressive transformation of epithelial cells, and a 30bp deletion variant was associated with the clinical aggressiveness of EBV-associated Hodgkin lymphoma. However, 30bp deletion variants of LMP1 were similarly frequent in cancer tissues, and lymphocytes from individuals with carcinoma, and throat washings from the general population [48-50]. Lee et al reported that 30bp deletion variants are not associated with tumor progression, lymph node metastasis or profiles of protein expression in EBV-associated GC [54]. Corvaln et al recently reported that polymorphisms of the BamHI-W1/I1 boundary region and the XhoI restriction site of LMP1 might be correlated with a higher risk of EBV-associated GC in South America [46]. The candidate viral genes in the vicinity of these polymorphic sites are BARF1, EBER-1 and –2, and LMP2A. According to Tanaka et al, LMP2A in EBV-associated GC shows several amino acid substitutions, including one from serine to threonine at codon 348, which is within one of the target epitopes of EBV-specific cytotoxic T-lymphocytes [51].

The balance between the host reaction and the virulence of EBV is important when we consider the interrelationships among infective organisms, chronic inflammation and carcinogenesis. The host reaction is partly dependent on cytokine production, which is influenced by a single nucleotide polymorphism (SNP) of cytokine genes. SNPs of certain cytokines have been reported to modify the outcomes of chronic gastritis and the risk of gastric cancer in association with H. pylori infection. High-producer alleles (-308A) in the TNF-alpha gene are significantly more frequent among patients with EBV-associated GC (23.3% vs 12.0%, P<0.05), while the frequency of high-producer alleles (-1082G) in the IL-10 gene was significantly higher among EBV-negative GC patients compared with the control (6.3% vs 3.0%, P<0.05) [55]. The SNP of IL1β-511/-31 was associated with GC risk in the Dutch population, but not with EBV status [56]. In our studies of Japanese, there was no significant influence of IL-1β-511/+3953 gene polymorphism in EBV-associated GC, and no correlation was observed between H. pylori infection and IL-1β gene polymorphism in GC; however, the cancer risk of the gastric corpus in Japanese is influenced by IL-1β+3953 polymorphisms [57].

Serological typing for major histocompatibility complex class I and class II antigens suggested that a deficiency of DR11 and a

Table 3 Viral gene expression in 3 latency patterns of EBV infection

	EBERs	EBNA1	EBNA2,3s,LP	LMP1	LMP2A	BARFs	Malignancies
Latency I	+	+	-	-	±*	+	BL,GC and HL
Latency II	+	+	-	+	+	+	NPC, NK/T and AIDS-associated lymphomas
Latency III	+	+	+	+	+	+	PTLDs

*, LMP2A is expressed in about half of EBV-associated GC. BL, Burkitt lymphoma; HL, Hodgkin lymphoma; GC, gastric cancer; NPC, nasopharyngeal carcinoma; PTLDs, posttransplant lymphoproliferative disorders

high frequency of DQ3 may be markers for a population at greater risk of EBV-associated gastric carcinoma [58].

Viral Genes and Carcinogenesis

EBV takes the form of latent infection without replication of viral particles in EBV-associated malignant neoplasm, in which the expression of EBV genes is restricted to various latent genes. These tumors are classified into three types, latency I, II, and III, according to the expression profile of EBV-latent genes (**Table 3**). EBV-associated GC, as well as Burkitt lymphoma, belongs to latency I, in which the expression of viral latent genes is restricted to EBNA1, EBERs, LMP2A and transcripts from the BamHI A region (BARF0 and BARF1) [59-61]. Latency II neoplasm includes NPC and Hodgkin lymphoma, and is characterized by the expression of LMP1, a transmembrane protein with transforming capacity for rodent fibroblasts. EBNA2, 3A and 3C, essential for immortalizing resting B-lymphocytes, are additionally expressed due to the lack of effective immune mechanisms in patients with latency III neoplasms, such as lymphomas in AIDS or organ-transplant patients.

Since latency I neoplasms arise in non-immunodeficient patients without the expression of LMP1 or EBNA2, a question has been raised about the role of EBV in these neoplasms. In the case of Burkitt lymphoma, however, loss of EBV from the EBV-positive lymphoma cell line, Akata, abolishes colony-forming capacity in soft agar, and tumorigenicity in nude mice, demonstrating that EBV contributes to the tumor growth of latency I-type neoplasms. In EBV-associated GC, the following findings about the role of latent genes have also been demonstrated.

EBER: EBER-1 and -2 are non-coding small RNA, abundantly produced in infected cells.

EBER1 upregulates insulin-like growth factor (IGF) expression, and secreted IGF-I acts as an autocrine growth factor in a gastric cancer cell line, NU-GC-3 [62].

BARF1: BARF1 can transform rodent fibroblasts and immortalizes primary monkey epithelial cells [63, 64]. BARF1 has an anti-apoptotic role in GC cells through an increase of the bcl-2 to bax ratio, thus promoting cancer cell survival [65].

LMP2A: LMP2A greatly affects cell growth and differentiation pathways in epithelial cells (a human keratinocyte cell line, HaCaT), in part through activation of the phosphatidylinositol-3-kinase (PI3K)-Akt pathway [66]. LMP2A inhibits transforming growth factor-β1-induced apoptosis in a GC cell line [67]. Downstream of the PI3K-Akt pathway, LMP2A is reported to stabilize β-catenin and to activate the Wnt pathway [68]. Recently, we also identified that LMP2A confers resistance to serum deprivation-induced apoptosis through the upregulation of cellular survivin in GC cell lines with EBV infection (Hino et al, in submission).

Cellular and Molecular Features

The other approach for EBV-associated GC is to investigate the cell biology and molecular biology of neoplastic epithelial cells of the stomach with EBV infection.

Cellular Abnormalities

Abnormalities of p16INK4A [69, 70], p73 [6] and E-cadherin [71,72] are observed in relation to aberrant promoter methylation, and described separately.

Cellular differentiation: Immunohistochemistry using antibodies against various molecules associated with cellular differentiation has characterized tumor cells of EBV-associated

GC compared to those of EBV-negative GC; lower level of expression of keratin molecules (CK7, 18, and 19) [73, 74], and null or gastric phenotype of mucin molecules [75,76]. These findings suggest that certain specific types of epithelial cells, with restricted capability of cellular differentiation, are the targets of EBV infection and subsequent transformation.

Proliferation and apoptosis: An important cellular abnormality in EBV-associated GC is its resistance to apoptosis. While the proportion of proliferative cells is significantly lower, the frequency of apoptosis is significantly lower in EBV-associated GC than in EBV-negative GC [77, 78].

As for the expression of apoptosis-related proteins, the data is conflicting; EBV-associated GC showed a higher expression of bcl-2 [77, 79] with a lower expression of bak protein [79], but the opposite results were also reported [80]. P53 overexpression, such as more than 50% positive cells indicating p53 gene mutation, is less frequent in EBV-associated GC (less than 10%) in marked contrast to EBV-negative GC (30-40%) [81-84]. Elevated p53 expression was also observed by a non-mutational mechanism in these tumors [71, 82]. Kijima Y et al reported that c-met amplification was observed, but was not specific to EBV-associated GC [85].

Adhesion molecules and immunomodulator molecules: EBV-associated GC express variant isoforms 3-5 and 6 of CD44 molecules [86]. EBV infection may influence CD44 expression by interacting with cytokine genes, such as those for TNF-α and IL10, which are known to modulate CD44 expression.

The significance of CD8-positive T-lymphocytes in EBV-associated GC has not been clarified yet. The infiltration contains a component that reacts against EBV-infected epithelial cells [87]. Alternatively, infiltrating T-cells are the result of induction by immunomodulator molecules, such as interleukin 1-beta (IL1-β). According to GeneChipTM (a high-density oligonucleotide array) analysis of an EBV-associated GC strain, which is transplantable to SCID mice (KT-tumor), the expression of higher amounts of IL1-β was demonstrated [88]. IL1-β may also exhibit an autocrine effect on carcinoma cells.

Global analysis of expression profiles:

Recently, global analysis of gene expression profiles has been applied to various carcinomas, including GC. In one such study [89], the expression levels of 326 genes showed a significant association with EBV infection, some of which appeared to be related to the lymphoid infiltrate. The analysis also confirmed that EBV-associated GC showed a gastric-like gene expression phenotype. Another approach to the global analysis of protein expression using tissue microarray has demonstrated the possibility of stratifying patients with EBV-associated GC into prognostically different subgroups [54].

Genetic Abnormalities

Cytogenetics: There are few studies of EBV-associated GC with comparative genomic hybridization, showing inconsistent results. According to Chan et al, gains in chromosome 11 and losses in 15q15 are more common in EBV-associated GC [90, 91]. zur Hausen et al reported that loss of chromosome 4p and of 11p was exclusively restricted to EBV-associated GC and that loss of 18q was also significantly more frequent in this type of GC [92].

Gene mutation and microsatellite instability: We investigated the deletion of 5q and/or 17p and microsatellite instability (MSI) using PCR-restriction fragment length polymorphism (PCR-RFLP) [93]. These abnormalities were extremely rare in EBV-associated GC in contrast to their high frequency in EBV-negative GC, especially the intestinal type. Chang et al reported the absence of MSI in EBV-associated GC among 549 GC cases [15], suggesting that the contribution of EBV and MSI is mutually exclusive in gastric carcinogenesis.

Aberrant DNA Methylation as a Key Abnormality

Consistently negative findings in earlier studies of genetic abnormalities led us to the hypothesis that an epigenetic alteration, such as DNA hypermethylation, plays a primary role in the genesis of EBV-associated GC. DNA hypermethylation of cytosines in CpG dinucleotides at promoter regions silences the gene expression. Interestingly, such epigenetic silencing is observed in both EBV and EBV-infected host cells.
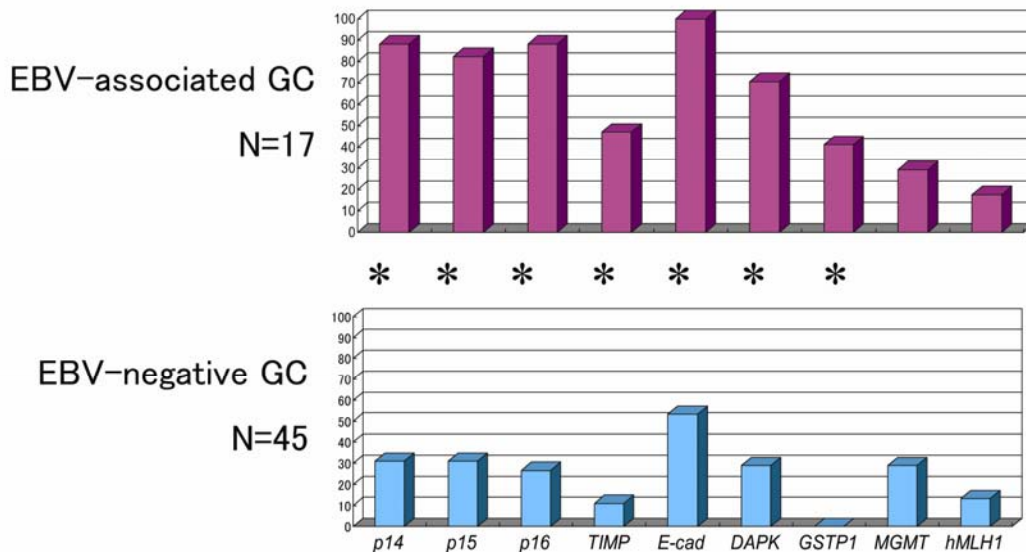
Figure 7 Methylation rates of each gene examined by MSP. Seventeen cases of EBV-associated GC and 45 cases of EBV-negative GC were examined. EBV-associated GC showed a higher methylation rate of p14ARF, p15, p16INK4A, TIMP3, E-cad, DAPK, GSTP1 than EBV-negative GC.

DNA Hypermethylation in EBV and Host Genomes

EBV gene silencing through methylation has been recognized and intensively investigated since the middle of the 1990s [59, 94]. EBV W promoter (Wp) is active immediately after initial infection and Wp-derived EBNA2 activates EBV C promoter (Cp). Wp is then soon silenced by *de novo* methylation [95]. Cp is also silenced by methylation in prolonged infection, when latent genes are driven by Q promoter (Qp). EBV in virtually all tumors has methylated Cp except for lymphoid tumors in immunocompromised hosts [96]. Since this epigenetic silencing of EBV genes results in hiding infected cells from cytotoxic T cells, silencing through methylation is considered to be a "stealth technology" of EBV [95]. LMP1 is also a methylation-sensitive gene and is silenced in a host cell-dependent manner [97].

DNA hypermethylation is also remarkable in neoplastic epithelial cells of EBV-associated GC. Using methylation-specific PCR (MSP), Kang et al [69], Vo et al [98] and our group [99] have demonstrated that promoter hypermethylation of various tumor-related genes occurs much more frequently in EBV-associated GC (**Figure 7**). Importantly, the

subsequent reduction of gene expression has been confirmed in EBV-associated GC in p16INK4A , E-cadherin, and p73 [6, 69, 70, 72, 98], but such a linkage between promoter methylation and gene silencing is not consistent in EBV-negative GC. One possible explanation is that these findings suggest the presence of mechanisms of *de novo* and maintenance methylation specific to EBV-associated GC. When the methylation state of p14ARF and p16INK4A was evaluated by bisulfite sequencing, methylation was observed in all 29 CpG sites of p14ARF and all 16 sites of p16INK4A with equally high densities in EBV-associated GC. On the other hand, in EBV-negative GC, the methylation profiles differed between the 2 genes. Promoter methylation was sporadic and variable in p14ARF, and only the last position of CpG in p14ARF was methylated at high frequency. High-density methylation in p16INK4A was observed in a subset of GC, but the first position of CpG was never methylated in EBV-negative GC [100].

CpG Island Methylator Phenotype

CpG island methylator phenotype (CIMP) was proposed in colorectal cancer to describe carcinomas showing high activity of CpG island

methylation, using MINT genes as an indicator [101]. CIMP high is defined by the number of methylated loci, such as three or more loci among the five loci [102]. CIMP high is closely related to microsatellite instability phenotype (MSI high) through hypermethylation of the hMLH1 promoter in colorectal carcinomas [103]. In gastric carcinomas, Kaneda et al identified five genes, the promoter region of which is densely methylated, by a genome scanning technique, methylation-sensitive representational difference analysis [104]. Adopting these genes as indicators: LOX (lysyl oxidase), HRASLS (HRAS-like suppressor), FLNC (gamma filamin), HAND1 (basic helix-loop-helix transcription factor), and THBD (thrombomodulin), we classified GC into CIMP none, intermediate, and high (CIMP-N, I, and H) by the numbers of methylated loci, 0, 1-3, and 4 or more, respectively [105]. As a result, nearly all (14 of 15) EBV-associated GC exhibited CIMP-H and showed frequent methylation of p15, p16INK4A and other cancer-related genes (p14ARF, p73, TIMP3, E-cadherin, DAPK, GSTP1, hMLH1, and MGMT). EBV-associated GC showed significantly higher frequencies of methylation of cancer-related genes (mean number $\pm$ SD = 6.9 $\pm$ 1.5) even if compared with EBV-negative/CIMP-H GC (3.5 $\pm$ 1.8). Thus, in view of the quantitative aspects, CpG island methylation is characteristically globally in EBV-associated GC; however, since the methylation frequency of the hMLH1 promoter region was not different in EBV-associated and EBV-negative GC, the methylation process was not random, and might be specific to EBV infection to stomach epithelial cells.

Methylation of EBV and Host Genomes

Considering that methylation occurs in both EBV and host genomes in EBV-associated GC, aberrant DNA methylation may start as a host defense system against EBV, but the mechanism might be overdriven to act on the host genome. The methylation of CpG dinucleotide is caused by DNMTs (DNMT1/3a/3b). Etoh et al reported that EBV-associated GC shows increased expression of DNMT1 [106]. Further studies are necessary to clarify the mechanisms of this unique hypermethylation status of EBV-associated GC. Virus-driven hypermethylation has been observed in HTLV1, and might be one distinct mechanism for human viruses for survival and propagation.

The methylation status of non-neoplastic mucosa was evaluated in stomachs with CIMP-H GC and EBV-associated GC. According to Lee et al, EBV-negative CIMP-H GC showed frequent methylation of MINT loci in the surrounding mucosa, where intestinal metaplasia or dysplasia/adenoma was observed [107]. In our own study, however, the methylation frequencies of p14ARF, p15, p16INK4A, and p73 promoters were low in the adjacent mucosa of early GC with and without EBV infection [6]. Further studies are necessary to determine the precise sequence of EBV infection, methylation, transformation and selection of the predominant clone within the stomach mucosa.

Study Model for EBV-associated GC

There are two strains of EBV-associated GC, which is transplantable to SCID mice. KT strain, which we established, preserves the feature of the original EBV-associated GC and can be propagated only in SCID mice [108]. SNU-0719 is a cell line of GC, in which EBV infection was identified [109], and is transplantable in SCID mice [110].

Recombinant EBV with the neomycin gene enables to establish stable cell lines infected with EBV. The rate of EBV infection to the GC cell line is very low, but a cell-to-cell contact co-culture method with recombinant EBV is efficient to obtain an EBV-infected GC cell line [111]. Studies on GC cell lines with each viral gene expression will clarify the viral gene responsible for carcinogenesis or maintenance of EBV-associated GC.

Conclusion

EBV-associated GC is a unique type of GC, consisting of neoplastic cells with monoclonal EBV. The most characteristic abnormality is global and non-random CpG island methylation of the promoter region of many cancer-related genes, which causes downregulation of their expression. This abnormality is accompanied by methylation of the EBV genome, suggesting virus-driven hypermethylation. Since the latent genes are limited, future studies will disclose critical interactions of viral proteins with the cellular proteins responsible for this abnormality.

The possible sequence of events within the mucosa is EBV infection of certain gastric stem
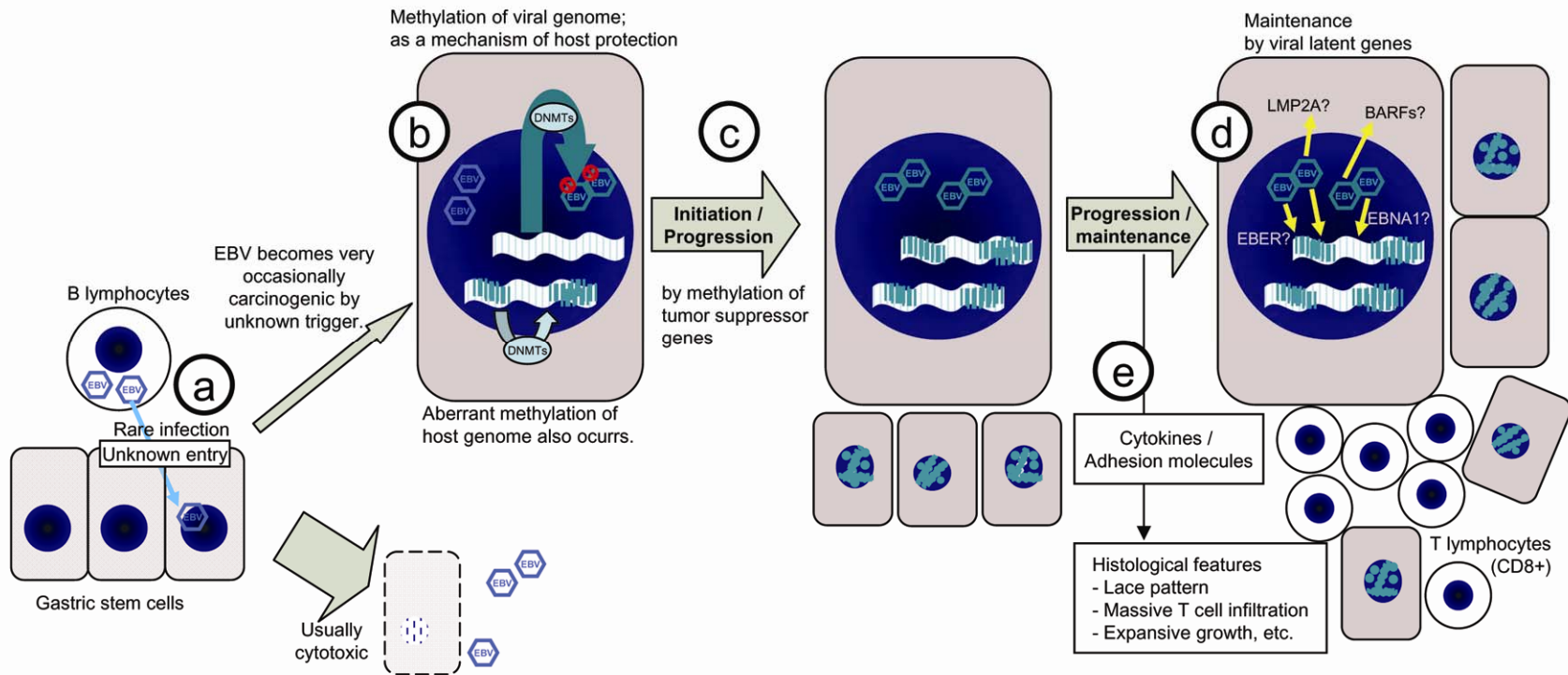
Figure 8 Hypothesis of gastric carcinogenesis and maintenance by EBV. In healthy individuals, EBV infection of gastric epithelial cells is a very rare phenomenon (**a**). The entry point remains unclear. Even if EBV infected the gastric epithelium, usually EBV is cytotoxic and cannot persistently infect the gastric epithelium. Once persistent infection is established by an unknown trigger, the gastric epithelial cell methylates the EBV genome as a mechanism of host protection (**b**). The mechanism of DNA methylation works on the EBV genome, but also on the host genome in part. Some cells are methylated at the promoter region of tumor suppressor genes, and such cells dominantly proliferate (**c**, cancer initiation and progression). EBV infects tumor cells latently after EBV-associated GC is established. Latent genes of EBV maintain GC (**d**) through an anti-apoptotic effect or stimulation on growth factors. Some such effect makes the characteristic features of EBV-associated GC, such as a lace pattern, massive T cell infiltration, and expansive growth (**e**).

cells, methylation, transformation and selection of the predominant clone (**Figure 8**). Further study will disclose the target and strategy of therapy, such as initiating viral replication by gene transfection and reversing the DNA methylation of cellular genes by DNA-modifying agents.

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