Update on Epstein-Barr virus and gastric cancer (Review)

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Received October 10, 2014; Accepted December 29, 2014

DOI: 10.3892/ijo.2015.2856

Abstract. Epstein-Barr virus-associated gastric carcinoma (EBVaGC) is a distinct subtype that accounts for nearly 10% of gastric carcinomas. EBVaGC is defined by monoclonal proliferation of carcinoma cells with latent EBV infection, as demonstrated by EBV-encoded small RNA (EBER) in situ hybridization. EBVaGC has characteristic clinicopathological features, including predominance among males, a proximal location in the stomach, lymphoepithelioma-like histology and a favorable prognosis. EBVaGC belongs to latency type I or II, in which EBERs, EBNA-1, BARTs, LMP-2A and BART miRNAs are expressed. Previous studies have shown that some EBV latent genes have oncogenic properties. Recent advances in genome-wide and comprehensive molecular analyses have demonstrated that both genetic and epigenetic changes contribute to EBVaGC carcinogenesis. Genetic changes that are characteristic of EBVaGC include frequent mutations in PIK3CA and ARID1A and amplification of JAK2 and PD-L1/L2. Global CpG island hypermethylation, which induces epigenetic silencing of tumor suppressor genes, is also a unique feature of EBVaGC and is considered to be crucial for its carcinogenesis. Furthermore, post-transcriptional gene expression regulation by cellular and/or EBV-derived microRNAs has attracted considerable attention. These abnormalities result in significant alterations in gene expression related to cell proliferation, apoptosis, migration and immune signaling pathways. In the present review we highlight the latest findings on EBVaGC from clinicopathological and molecular perspectives to provide a better understanding of EBV involvement in gastric carcinogenesis.

1. Introduction

Epstein-Barr virus (EBV), also known as human herpes virus 4, is a gamma-herpes virus that consists of double-stranded DNA of ~170 kb in length. It is one of the most common human herpes viruses and infects >90% of the world's population by adulthood and establishes lifelong, latent infections. EBV was the first virus to be associated with human malignancy, which was discovered from a Burkitt's lymphoma cell line in 1964 (1). Subsequent studies revealed that EBV caused a number of different human malignancies, such as nasopharyngeal carcinoma (NPC), Hodgkin's lymphoma, extranodal NK/T-cell lymphoma, nasal type and lymphoproliferative disorders of immunocompromised hosts (2).

In 1990, Burke et al (3), first reported the association between EBV and gastric carcinoma with characteristic lymphoepithelioma-like histology based on polymerase chain reaction (PCR) techniques. Subsequent development of in situ hybridization (ISH) techniques to detect EBV-encoded small RNAs (EBERs) facilitated the detection of EBV in cancer tissues (4,5). In gastric carcinoma cells, EBV is not integrated into the host genome but maintained as a type of plasmid called an episome. The uniformity of the numbers of terminal repeats (TRs) among EBV positive carcinoma cells reflects the clonal origin of a tumor and suggests that EBV is a causative virus for gastric carcinoma (6).

In spite of these findings, the importance of EBV in gastric carcinogenesis has long been underestimated. The reason for this is that Helicobacter Pylori (H. pylori), discovered in 1983 by Marshall and Warren, and classified as a definite carcinogen by World Health Organization in 1994, has been regarded as the major factor in almost all gastric carcinomas worldwide (7-9). Persistent infection with H. pylori induces atrophic gastritis and intestinal metaplasia, and subsequently leads to gastric malignancies including gastric carcinoma and extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma). In addition to accumulating more case series of EBV-associated gastric carcinoma (EBVaGC), the development of comprehensive molecular

Acknowledgements

This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Japan Society for the Promotion of Science, the Japan Agency for Medical Research and Development, and the Ministry of Health, Labour and Welfare of Japan.

Conflict of interest

None declared.
analyses has provided evidence that EBVaGC is a distinct subset both in terms of its clinicopathological and molecular features.

In the present review, we first describe the clinical and histological features of EBVaGC. Furthermore, we discuss recent findings on EBV-associated gastric carcinogenesis by focusing on the roles of latent genes, epigenetic abnormalities, genomic alterations, and post-transcriptional regulation by cellular and viral microRNAs (miRNAs).

2. Definitions, epidemiology and clinical features

EBVaGC is defined by monoclonal proliferation of carcinoma cells with latent EBV infection. The gold standard for identifying EBV infection is ISH to detect EBER1, which is abundant in infected cells (up to 10^7 molecules/cell). The frequency of EBV infection in gastric carcinoma ranges from 2 to 20%, with a worldwide average of nearly 10% (10, 11). These differences in reported frequencies may be because of geographical and environmental factors, although this remains controversial. In a meta-analysis done by Murphy et al (12), the pooled estimates of EBVaGC frequency in American, European and Asian were 9.9, 9.2 and 8.3%, respectively, with an overall frequency of 8.7%. A recent meta-analysis done by Camargo et al (13) revealed a similar overall frequency (8.2%), although the frequencies they found were slightly higher in American (12.5%) and European (13.9%) cases and lower in Asian cases (7.5%). Based on the annual incidence of gastric carcinoma (934,000 cases per year), nearly 70,000-80,000 people per year are estimated to develop EBVaGC (14).

The clinical features of EBVaGC include predominance among males and a predominant location in the proximal stomach and remnant stomach after partial gastrectomy for gastric ulcer or gastric carcinoma. Most published studies have shown an association with male gender (approximately twice as many males as females), which has been confirmed by several meta-analyses (12, 15, 16). However, this male predominance decreases with age in terms of risk estimates (15). Studies conducted in the Americas have shown an association between EBV positive and younger age, although this was not confirmed in a meta-analysis (15-17).

Frequent EBV involvement in carcinomas of the remnant stomach has been reported in several studies, with frequencies ranging from 6 to 30% (10, 12, 16). EBV-positive remnant gastric carcinoma is often found at an anastomosis site in patients who underwent gastrojejunostomy and Billroth II gastric carcinoma is often found at an anastomosis site in series (to date) of 4,599 gastric carcinoma cases, EBV positive was associated with a reduced mortality rate after adjusting for the stage and other possible confounders (Hazards ratio of 0.72; 95% CI, 0.61-0.86).

3. Histopathology

By gross appearance, EBVaGC often forms ulcerated or saucer-like masses with marked thickening of the gastric wall. As noted above, a tumor is frequently located in the proximal stomach and the remnant stomach after partial gastrectomy. Multiplicity (multiple lesions occurred synchronously or metachronously in the stomach) is also a characteristic feature, as confirmed by several studies (23, 27-29). In its early stages, EBVaGC tends to form well-demarcated, nodular lesions in the submucosa with less fibrosis as compared with EBV-negative gastric carcinoma, and this is beneficial for endoscopic submucosal resection of a tumor (30).

Histologically, EBVaGC is subdivided into two types; lymphoepithelioma-like carcinoma (LELC)-type (Fig. 1) and conventional-type adenocarcinoma (Fig. 2), although there is a morphological continuum between these types. LELC-type is described as a poorly differentiated carcinoma with dense infiltration of lymphocytes, which resembles NPC. Because of the prominent lymphocytic infiltration, it is often difficult to identify individual carcinoma cells with routine hematoxylin and eosin (H&E) staining. Immunohistochemistry with antibodies to cytokeratin and EBER-ISH highlight these carcinoma cells. More than 80% of gastric carcinoma cases showing LELC-type morphology are EBV-positive (23, 31). This histological pattern is also referred to as ‘gastric carcinoma with lymphoid stroma (GCLS’ defined as carcinoma showing microalveolar, trabecular, or primitive-tubular pattern with uniformly dense and diffuse lymphoid cell infiltration, which encompasses broader morphologic variants including LELC (32).

EBVaGC with conventional-type adenocarcinoma histology shows well to moderately differentiated adenocarcinoma with variable amount of infiltrating lymphocytes, and it is classified as an intestinal type gastric carcinoma in Lauren's classification (33). Morphologically, it is almost identical to EBV-negative gastric carcinoma; therefore, EBER-ISH is necessary to identify the presence of EBV in carcinoma cells. Another subtype has been proposed called ‘carcinoma with Crohn's disease-like lymphoid reaction,’ which is defined as a tumor accompanied by three or more lymphoid follicles.
with active germinal centers at the advancing edge of the tumor, fewer lymphocytes than tumor cells, frequent tubule or gland formation and minimal or no desmoplasia (34) (Fig. 3). This type represents a morphology intermediate between the typical LELC-type and conventional-type adenocarcinoma and could be included in GCLS.

EBVaGC in its early stage shows a characteristic histology called a ‘lace pattern’ (Fig. 4). This pattern is typically observed in an intramucosal lesion, which shows irregularly anastomosing tubules and cords associated with moderate to dense lymphocytic infiltration and results in a lace-like or reticular pattern at low magnification. When this pattern is observed in biopsy specimens, EBER-ISH is recommended for diagnostic purposes (Fig. 5).
epigenetic alterations, it is currently unclear what causes the location (proximal or distal), cellular phenotype, or genomic/histological subtypes and etiological factors, anatomical studies have not demonstrated any close associations between causal role for EBV in gastric carcinogenesis. Since previous noma cells of EBVaGC are EBV-positive, which supports a with diffuse histology in Lauren's classification. is associated with intestinal histology and CLDN18 expression while in EBV-negative gastric carcinoma CLDN3 expression LELC-type and other morphologic subtypes in EBVaGC, different differentiation toward gastric cell type is common to both cells that have an intrinsic differentiation potential toward the gastric cell type, but not the intestinal type. Notably, the differentiation toward gastric cell type is common to both LELC-type and other morphologic subtypes in EBVaGC, while in EBV-negative gastric carcinoma CLDN3 expression is associated with intestinal histology and CLDN18 expression with diffuse histology in Lauren's classification.

Regardless of different morphological subtypes, all carcinoma cells of EBVaGC are EBV-positive, which supports a causal role for EBV in gastric carcinogenesis. Since previous studies have not demonstrated any close associations between histological subtypes and etiological factors, anatomical location (proximal or distal), cellular phenotype, or genomic/epigenetic alterations, it is currently unclear what causes the histological diversity of EBVaGC, which is expected to be clarified in the future studies for the better understanding of the pathogenesis of EBVaGC.

Prominent inflammatory infiltrate in the tumor is one of the characteristic features of EBVaGC. These tumor infiltrating cells are primarily lymphocytic, particularly CD8-positive or CD4-positive T cells accompanied by CD68-positive histiocytes (10,36,37). In addition, infiltration of abundant B cells, plasma cells, or neutrophils is often observed and these infiltrating cells rarely masquerade as other neoplasms. We previously reported EBVaGC with prominent Mott cell (plasma cells with multiple Russell bodies) infiltration that mimicked plasma cell neoplasms (38). Infiltrating Mott cells and B cells were negative for immunoglobulin light chain restrictions or heavy chain rearrangements, which suggested that they were reactive in nature. Another extreme variant is EBVaGC with osteoclast-like giant cells (39). These giant cells showed a histiocytic phenotype and were considered as a reaction to gastric carcinoma.

The diversity of tumor infiltrating inflammatory cells is an important feature of EBVaGC and it is also shared by other EBV-related malignancies, such as Hodgkin's lymphoma and EBV-positive diffuse large B-cell lymphoma, which are often accompanied by numerous reactive inflammatory cells. This feature reflects the immunogenicity of EBV, and some investigators have suspected that this immune response by the host is one reason for a better prognosis. Further studies are needed to determine the detailed mechanism of an immune response against EBV-positive tumor cells in order to develop an effective treatment for this disease.

One of the interesting findings on the background of EBVaGC is that it is sometimes associated with gastritis cystica profunda (GCP), which is a relatively rare, benign lesion that is characterized by polypoid hyperplasia and cystic dilatation of the gastric glands that extend into the stomach submucosa. Choi et al (40), reported that the EBV-positive rate was significantly higher in gastric carcinoma cases with GCP (31.1%) as compared to those without GCP (5.8%). It has been assumed that GCP may reflect chronic inflammation in the stomach; although, it remains unclear whether the coexistence of GCP and EBVaGC is merely coincidental, or if GCP presents as a precancerous lesion.

4. EBV infection in gastric epithelial cells and EBV latent genes

Previous studies have investigated how EBV infection is established in gastric epithelial cells which lack the expression of viral receptor CD21 (also known as CR2) through which EBV enters B lymphocytes (41,42). Since the efficiency of EBV infection is greatly improved by directly co-culturing epithelial cells with EBV-producing B lymphoblastoid cells (Akata cells) than cell-free infection, direct cell-to-cell contact with B lymphocytes is considered to be the major mode of EBV infection in epithelial cells (43). Although quite rare, EBV infection is found in a small fraction of non-neoplastic gastric mucosa in a single cell or a few glands (6,44), suggesting that EBV infection precedes the clonal growth of EBV-infected cells and subsequently develops carcinoma. Chronic gastritis in the background of EBVaGC might enhance the chance of

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**Figure 5.** EBV-associated gastric carcinoma detected in a biopsy specimen. (A) H&E staining. Ill-defined tubular structures (arrows) are observed in the mucosa accompanied by dense lymphoplasmacytic and eosinophilic infiltrates. (B) EBERISH highlights carcinoma cells.
interaction between gastric epithelial cells and B lymphocytes, and cytokines produced by inflammatory cells might support the growth of EBV-infected gastric epithelial cells.

Most in vitro studies have utilized cell-to-cell contact method to explore the role for EBV in gastric carcinogenesis by secondarily infecting gastric carcinoma cell lines with EBV. The method is quite useful in investigating how EBV infection alters the biological nature of these cells, however, these experiments have limits in that they use cell lines already transformed to malignant cells by other factors other than EBV, which should be kept in mind when applying the results to the in vivo situation.

Once EBV infection is established in B-lymphocytes or epithelial cells, it is maintained in a latent form. Latent EBV infection has three distinct forms that are determined by the expression patterns of latent genes. EBER-1 and 2, EBV-determined nuclear antigen (EBNA)-1, BamHI A region rightward transcripts (BARTs), and BART miRNAs (discussed later) are expressed in all latency types. In latency type II, latent membrane protein (LMP)-1, 2A and 2B are also expressed, and latency type III includes all of these latent genes along with EBNA-2, 3A, 3B, 3C and LP. These latency types differ among different EBV-associated malignancies as shown in Table II.

EBVaGC belongs to latency type I or II, in which EBERs, EBNA-1, BARTs and BART miRNAs are expressed and approximately half of EBVaGC cases express LMP-2A (45,46). The expression pattern of these latent genes

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<table>
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<tr>
<th>Table I. Clinicopathological features of EBV-associated gastric carcinoma.</th>
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<td><strong>Features</strong></td>
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<td>Incidence</td>
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<td>Age</td>
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<td>Microscopic features</td>
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<th>Table II. Latency type in EBV-associated malignancies.</th>
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<td><strong>Associated malignancies</strong></td>
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<tr>
<td>Burkitt’s lymphoma</td>
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<tr>
<td>Nasopharyngeal carcinoma</td>
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<td>Hodgkin’s lymphoma</td>
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<td>Immunodeficiency-associated lymphoma</td>
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is diverse among cases, and this is also true in NPCs and NK/T cell lymphomas. Two studies performed comprehensive expression profiling of viral latent and lytic transcripts in EBVaGC (47,48). EBERs were the most abundant among all latent genes, followed by BARTs. LMP-2A, 2B and EBNA-1 expressions were very low. Notably, transcription of immediate early lytic genes, BZLF1 and BRLF1 was detected in some gastric carcinoma cases without subsequent progression of lytic cycle (47). Similarly, BZLF1 expression was observed in gastric carcinoma cells in vitro under long-term cultivation after EBV infection, but very few of them progressed to late lytic phase. The authors concluded that abortive lytic replication might somehow responsible for EBV genome amplification (49). These findings may provide some clues to clarify how EBV episome is maintained in EBVaGC with low/absent EBNA-1 expression.

Although LMP-1 is a well-known oncoprotein that is essential for EBV to efficiently transform resting primary B cells into autonomously proliferating lymphoblastoid cell lines, its expression is extremely low in EBVaGC and is usually undetectable at the protein level. To investigate the oncogenic properties of latent genes other than LMP-1, the roles of latent genes expressed in gastric carcinoma have been investigated (Table III). These findings are discussed separately in the following sections.

**EBERs.** EBERs are the most abundant viral transcripts found in latently EBV-infected cells and they have been shown to have various effects with regard to cell proliferation, apoptosis-resistance, production of autocrine growth factors and interactions with host proteins to enhance cellular signaling (50). However, only a few studies have investigated the roles of EBERs in gastric carcinogenesis. Iwakiri et al. (51), demonstrated that EBV infection induced the expression of IGF-1 in an EBV-negative gastric carcinoma cell line, NUGC-3 and IGF-1 functioned as an autocrine growth factor. They showed that EBERs were responsible for these phenomena. We recently reported that EBER-1 altered cellular miRNA expression to suppress E-cadherin, which resulted in an epithelial-to-mesenchymal transition (EMT) in gastric carcinoma cell line, as will be discussed later (52). Another recent report by Banerjee et al. (53), showed that EBERs could upregulate IL-6 expression and activated its downstream regulator STAT3, which resulted in downregulation of the cell cycle inhibitors p21 and p27 in a gastric carcinoma cell line, which was associated with chemoresistance. They also showed that EBERs induced the activation of pro-metastatic molecules, pFAK and pPAK1, and the downregulation of anti-metastatic molecules, RhoGD1 and KAI-1, which promoted cell migration.

**EBNA-1.** EBNA-1 is the only viral protein that is consistently expressed in all types of EBV-associated malignancies, which is essential for the replication and stable persistence of EBV episome. Increasing evidence has demonstrated that EBNA-1 alters the cellular environment to promote genomic instability and may have the potential to act as an oncogene (54-56). However, until recently, little was known regarding a role of EBNA-1 in gastric carcinogenesis. Cheng et al. (57), reported that, the gastric cell lines SCMI and TMC1 that were transfected with EBNA-1 had enhanced tumorigenicity and growth rates in xenografts. Another report by Sivachandran et al. (58), demonstrated that AGS cells that were infected with EBV had fewer promyelocytic leukemia (PML) nuclear bodies and less PML protein than EBV-negative cells, and that these phenomena were caused by EBNA-1. These findings were also confirmed in biopsy samples. PML is a tumor suppressor protein that is associated with p53 activation. They showed that by repressing PML, EBNA-1 impaired p53 acetylation, p53-dependent p21 transcription, and apoptosis, which resulted in enhanced cell survival after DNA damage.

Considering that EBNA-1 is essential in the maintenance of EBV genome and may also act as oncogenes, EBNA-1 is one

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Table III. Roles of EBV latent genes in EBV-associated gastric carcinoma.

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<th>Latent gene</th>
<th>Related molecules (and their downstream)</th>
<th>Biological function (ref.)</th>
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<tr>
<td>EBER-1</td>
<td>IGF-1, hsa-miR-200a, 200b, (ZEB1, ZEB2, E-cadherin), IL-6 (STAT3, p21, p27)</td>
<td>Autocrine growth (51)</td>
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<td>Epithelial-to-mesenchymal transition (52)</td>
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<td>Chemoresistance (53)</td>
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<td>EBNA-1</td>
<td>p53, PML (p53, p21)</td>
<td>Tumorigenicity (57)</td>
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<td>Anti-apoptosis (58)</td>
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<tr>
<td>LMP-2A</td>
<td>NF-κB (survivin), Cyclin E1, Drp-1, STAT3 (DMT1-1, PTEN), DMP3b</td>
<td>Anti-apoptosis (63)</td>
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<td></td>
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<td>Anti-apoptosis (64)</td>
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<td></td>
<td></td>
<td>Epithelial-to-mesenchymal transition (65)</td>
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<tr>
<td>BARTs</td>
<td>Bel-2, Bax, Cyclin D1, NF-κB/cyclin D1, p21WAF</td>
<td>Chemoresistance (72)</td>
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<tr>
<td></td>
<td></td>
<td>Cell proliferation (73)</td>
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<td>Cell proliferation (74)</td>
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of the possible therapeutic targets of EBVaGC. Some previous studies have demonstrated that suppression of EBNA-1 in lymphoma cell lines inhibited cell proliferation (59-61). However, as previously mentioned, expression of EBNA-1 in EBVaGC is low, or even absent in some cases, EBNA-1-targeted therapy may not be applicable to all EBVaGCs and other therapeutic approaches should be sought.

LMP-2A. LMP-2A protein is expressed in about half of EBVaGC cases and has been relatively well investigated as compared with other latent genes with regard to gastric carcinogenesis. LMP-2A inhibited transforming growth factor (TGF) β1-induced cellular apoptosis in a gastric carcinoma cell line (62). We previously reported that, the gastric cell lines that were transfected with LMP-2A had upregulated survivin expression mediated through nuclear factor (NF)-κB activation, which resulted in resistance against serum deprivation-induced apoptosis (63). Similarly, Liu et al. (64), showed that transfecting LMP-2A into a gastric carcinoma cell line improved cell growth and reduced apoptosis, via increased cyclin E expression and the proportion of cells in S phase. Another possible function of LMP-2A is activating the Notch signaling pathway, which disrupts the mitochondrial fission-fusion equilibrium by enhancing dynamin-related protein (Drp)-1 expression, which results in increased cell migration along with the overexpression of EMT markers (65).

In addition to these direct modulating effects on cell proliferation and migration, LMP-2A has a unique function inducing epigenetic changes in the host genome. LMP-2A promotes STAT3 phosphorylation, which activates the transcription of DNA methyltransferase (DNMT) 1 (66). Upregulated DNMT1 results in the epigenetic silencing of PTEN expression through the methylation of CpG islands in its promoter region. Zhao et al. (67), demonstrated that LMP-2A caused the upregulation of DNMT3b. As will be discussed later, epigenetic changes, particularly hypermethylation of the host genome, is one of the most crucial mechanisms of EBV-induced gastric carcinogenesis, for which LMP-2A may play a part through its activation of DNMTs.

BARTs. BARTs are multi-spliced transcripts that were originally discovered by cDNA library analysis of NPC xenografts and were subsequently found to be abundantly expressed in various kinds of EBV-associated malignancies. Several of these transcripts have open reading frames (ORFs), such as BART0, BART1, RPMS1 and A73, which possibly encode for proteins. Some of these can be artificially translated into their respective proteins in vitro, although endogenous expression of these proteins has not been completely confirmed in EBV-associated cancer tissues (68-70). Several reports on BARF1 involvement in gastric carcinogenesis have been published. The BARF1 gene is expressed in nearly 100% of EBVaGC cases (71). Expression of BARF1 is also observed in NPC, while it is generally undetectable in EBV-positive B lymphocytes and lymphomas, which may be crucial in EBV-associated epithelial malignancies. Transfecting BARF1 into a gastric carcinoma cell line induces significant alterations in host gene expression, particularly those genes related to proliferation and apoptosis, and BARF1-transfected cells exhibit chemoresistance along with an increased Bcl-2-to-Bax expression ratio (72).

Wiech et al. (73), demonstrated that cyclin D1 was upregulated in BARF1-transfected HaCaT cells and that cyclin D1 was overexpressed in EBVaGC cells, by immunohistochemistry. Recently, Chang et al. (74), demonstrated that BARF1 protein was secreted into culture supernatants of gastric carcinoma cells that had been transfected with BARF1, using western blot analysis. These BARF1-expressing cells had increased cell proliferation that was mediated via upregulated NF-κB/ cyclin D1 and reduced expression of the cell cycle inhibitor p21WAF.

5. Epigenetic abnormalities

A number of studies have demonstrated that epigenetic abnormalities, such as promoter hypermethylation, play crucial roles in the carcinogenesis of EBVaGC (67,75-81). Global and non-random CpG island methylation in the promoter regions of many cancer-associated genes, particularly tumor suppressor genes, is found in EBVaGC, which results in repressing the transcription of downstream genes. Initially, assessing methylation status was performed for individual genes using methylation-specific PCR (MSP). EBVaGC exhibited promoter hypermethylation in multiple genes involved in cell cycle regulation (p14ARF, p15, p16INK4a and p73), DNA repair (hMLH1, MGMT and GSTP1), cell adhesion and metastases (CDH1, TIMP1 and TIMP3), apoptosis (DAPK and bcl-2), and signal transduction (APC, PTEN and RASSF1A). The number of reported hypermethylated genes in EBVaGC continues to increase (82).

We recently performed a comprehensive analysis of the promoter methylation status of 51 gastric carcinoma cases using an Infinium HumanMethylation27 BeadChip (Illumina), which included 27,578 CpG sites covering 14,495 genes (83). Subsequent hierarchical clustering analysis demonstrated that, based on methylation status, gastric carcinoma could be subclassified into three epigenotypes, EBV+/extensively high-methylation, EBV+/high-methylation and EBV/low-methylation, which were characterized by different sets of methylated genes: genes specific for the EBV+ type (CXXC4, TIMP2 and PLXND1); highly methylated in EBV+ and EBV+/high-methylation type (COL9A2, EYA1 and ZNF365); and frequently methylated in all epigenotypes (AMPH, SORC33 and AJAP1).

Genes that were methylated in EBV-negative carcinomas were also methylated in EBVaGC and ~270 genes were uniquely methylated in EBVaGC. Frequent MLH1 methylation (46%) was found in the EBV+/high-methylation type, but none of the EBVaGC cases showed MLH1 methylation. In addition, polycomb repressive complex (PRC)-target genes that have been reported in embryonic stem cells were enriched in genes methylated in EBV-negative types, regardless of methylation status. In contrast, aberrant methylation induced by EBV was observed not only within PRC-target genes but also within non-PRC-target genes. EBV-infection of the low-methylation type gastric cancer cell line MKN7, induced extensive methylation within 18 weeks to acquire an EBV-specific methylation epigenotype.

It is worth noting that viral latent gene expression is also suppressed by methylation. We experimentally confirmed
that viral DNA methylation preceded the methylation of host DNA by one week (unpublished data). The methylation of viral genes might be one host defense mechanism against foreign DNA for suppressing the viral gene expression. However, this might result in other outcomes. Repressing viral latent gene expression might benefit EBV by allowing it to escape a host's immune response. In addition, excessive methylation may lead to repressing tumor suppressor genes, which ultimately could give rise to carcinogenesis.

### 6. Somatic genomic alterations and gene expression

Recent advances in genome-wide, high-throughput techniques to explore genetic alterations in cancer, such as single nucleotide polymorphism (SNP) arrays, somatic copy-number analysis, whole-exome sequencing, mRNA and miRNA sequencing, array-based DNA methylation profiling, and reverse-phase protein arrays provide new means to comprehensively investigate EBVaGC at the molecular and genetic levels. These techniques have enabled researchers to identify relative unknown, infrequent genetic abnormalities that could not be found using conventional approaches (84-86) (Table IV).

Lee et al (87), used a high-throughput genotyping platform to determine the mutation status of 474 hotspots in 41 genes using 237 gastric adenocarcinomas, which included 58 EBVaGCs. Among these, 34 cases (14.3%) harbored somatic mutations, 6 of which concomitantly had two different mutations. Fourteen EBVaGC cases had mutations; 6 in PIK3CA (10.3%), 1 in p53 (1.7%), 2 in APC (3.4%), 1 in STK11 (1.7%), 3 in CTNNB1 (5.2%) and 1 in CDKN2A (1.7%). CTNNB1 mutations were significantly more frequent in EBVaGC than in EBV-negative gastric carcinomas (one of 179 cases, 0.6%). Frequent PIK3CA mutations were also reported in two subsequent studies; 16.7% (of 18 EBVaGCs) in a report by Sukawa et al (88), and 80% (of 28 EBVaGCs) in a report by The Cancer Genome Atlas (TCGA) Research Network (89). A recent report by Liang et al (90), showed several newly identified mutations in EBVaGC, including mutations in MAP3K4 (20.8%), TGFBR1 (25.0%), CCNA1 (25.0%) and AKT2 (38.2%). Among these, an AKT2 mutation was associated with poor survival (90).

Another frequently mutated gene in EBVaGC is ARID1A. ARID1A encodes for a member of the SWI/SNF chromatin remodeling family and is currently thought to function as a tumor-suppressor gene. Wang et al (91), reported that 47% of EBVaGC cases (7 of 15) harbored an ARID1A mutation by exome sequencing and 73% (11/15) had reduced ARID1A protein expression by immunohistochemical analysis. Clinically, ARID1A alterations were associated with a better prognosis in a stage-independent manner. Similarly, we demonstrated that loss of ARID1A expression was frequent in EBVaGC (23/67, 34%), while ARID1A expression was maintained in NPCs or EBV-positive lymphomas (92). Further studies are necessary to clarify the roles of these mutations in gastric carcinogenesis by EBV.

In a recent report by the TCGA Research Network, a comprehensive molecular evaluation of 295 gastric carcinomas was performed using several different modalities, including genomic alterations, gene expression profiling and proteomic analysis. They proposed a novel molecular classification to divide gastric carcinomas into four types (89), of which EBVaGC was one. The others were: ‘microsatellite instability (MSI)’ characterized by hypermutation, gastric-CIMP (CpG-island methylator phenotype) and MLH1 silencing; ‘genomically stable (GS),’ which showed diffuse histology, CDH1 and RhoA mutations, and a CDKN18-ARHGAP fusion; and ‘chromosomal instability (CIN)’ with intestinal histology, a p53 mutation, marked aneuploidy and amplification of receptor tyrosine kinases.

Frequent PIK3CA and ARID1A mutations in EBVaGC (80 and 55%, respectively) were also confirmed in that study, and frequent mutations in BCROR (BCL6 interacting co-repressor), which is a member of PRC, was also demonstrated (23%). Similar to the results in previous reports, p53 mutations were rare in EBVaGC (82,93). Both PIK3CA and ARID1A mutations were also frequent in EBV-negative, MSI-high gastric carcinoma, which was confirmed in multiple studies cited above. EBVaGC and MSI-high gastric carcinoma also shared CIMP-high features, although epigenetic silencing of mismatch repair genes (e.g. MLH1) was rarely observed in EBVaGC, as previously noted. These findings were noted in several previous reports and indicated that EBVaGC had a unique carcinogenic pathway independent of MSI (28,79,86,94).

Another novel finding characteristic of EBVaGC was recurrent amplification at 9p24.1 at the locus that includes JAK2, CD274 (encoding for PD-L1), and PDCD1LG2 (encoding for PD-L2). PD-L1 and PD-L2 are ligands of PD-1 that is expressed on T cells, B cells, monocytes and natural killer cells, as well as tumor infiltrating lymphocytes. Upon ligation with PD-L1 and PD-L2, PD-1 suppresses downstream PI3K and Akt signaling, which results in inhibiting T cell proliferation. Some malignancies have been reported to have high PD-L1 expression levels, which was associated with an aggressive behavior and poor prognosis (95). Blocking the interaction between PD-1 and PD-L1/L2 could augment an antitumor immune response, and clinical trials to investigate the efficacy of immunotherapy by targeting these molecules are under way (96).

### Table IV. Mutations in EBV-associated gastric carcinoma.

<table>
<thead>
<tr>
<th>Frequently mutated genes (ref.)</th>
<th>PIK3CA (10.3-80%) (87-89)</th>
<th>ARID1A (47-55%) (89,91)</th>
<th>AKT2 (38.2%) (90)</th>
<th>TGFBR1 (25.0%) (90)</th>
<th>CCNA1 (25.0%) (90)</th>
<th>BCROR (23%) (89)</th>
<th>MAP3K4 (20.8%) (90)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Other mutated genes &lt;10%</td>
<td>CTNNB1(87), TP53, ITH1, KRAS, NRAS, PLCE1, SHOC2, GIPC1, ITGA6, ITGB4, NRP1, PLEC, JAK2, CSF2RB, GHR, MPL, PTPN2, SOCS3, STAT5B, COL1A2, COL5A1, DCN, F2, IGF1, IGF2, IGFBP5, THBS1 (89)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

CLINICAL IMPACT:

These findings have significant implications for the development of targeted therapies in EBVaGC. The identification of PIK3CA and ARID1A as frequently mutated genes provides potential targets for treatment strategies. PIK3CA inhibitors, such as alpelisib, have shown promising results in clinical trials for other malignancies, and could be investigated in EBVaGC. ARID1A mutations could also be targeted using small-molecule inhibitors or RNAi therapies. Furthermore, the high frequency of TGFBR1 mutations suggests that targeting PI3K and Akt signaling could be effective in these tumors. The role of PD-1/PD-L1 in EBVaGC is also highlighted, and further studies are needed to explore the potential for immunotherapy in this setting.
Gene expression profiling and proteomic analysis have revealed activation in immune cell signaling and mitotic pathways, along with inactivation of the HIF-1α transcription factor network in EBVaGC (48,89,97,98). In the report by the TCGA Research Network, EBVaGC exhibited high expression of CXCL11, CXCL9, CXCL17, IDO1, CXCL1, SP100, DICER1, MICB, and low expression of CLDN3, PPP1R1B, REG4, CDH17, TFF3, SCNN1A, FUT3, MUC3A, KR7 and WFDC2 as compared to other types of gastric cancers (89). Enhanced IL-12 mediated signaling signatures were highly characteristic of EBVaGC. In the report by Kim et al (98), genes associated with cytokine activities, immune response, leukocyte migration, hormone secretion and cholesterol transport for lipoprotein clearance were deregulated in EBVaGC. Along with the evidence for PD-L1/L2 overexpression, modulating immune cell signaling may have therapeutic effects on EBVaGC (98-100).

7. miRNA abnormalities

While genetic and epigenetic alterations induce the upregulation or downregulation of cancer-associated genes at the transcriptional level, miRNAs are novel, post-transcriptional regulators of gene expression. A miRNA is a small, non-coding RNA molecule ~22 nucleotides in length and is coded in the introns or exons of encoding genes. A miRNA precursor is processed from these transcripts and is subsequently processed by Drosha and Dicer to a mature form. Mature miRNA interacts with the 3’ untranslated region (UTR) of a target mRNA and represses its translation. To date, >2,600 human miRNAs (cellular miRNAs) have been archived in miRBase (http://www.mirbase.org). An increasing number of studies have shown that the dysregulation of certain miRNAs induces carcinogenesis in various organs. Similar to oncogenes and tumor suppressor genes, miRNAs associated with carcinogenesis are called oncomiRs and anti-oncomiRs. Alterations in

<table>
<thead>
<tr>
<th>Name</th>
<th>Targets in EBV</th>
<th>Targets in host cells</th>
<th>Related malignancies (ref.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-BHRF1-1</td>
<td></td>
<td>GUF1, SCRN1</td>
<td>Lymphoma (126)</td>
</tr>
<tr>
<td>miR-BART1-5p</td>
<td>LMP-1</td>
<td></td>
<td>NPC (127)</td>
</tr>
<tr>
<td>miR-BART1-3p</td>
<td></td>
<td>CLEC2D, LU75, SP100, DICER1, MICB, CXCL11</td>
<td>Lymphoma (126)</td>
</tr>
<tr>
<td>miR-BART2-5p</td>
<td>BALF5</td>
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<td>Lymphoma (128)</td>
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<tr>
<td>miR-BART3-3p</td>
<td></td>
<td>DICER1, MICB, IPO7</td>
<td>Lymphoma (126)</td>
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<tr>
<td>miR-BART5-5p</td>
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<td></td>
<td>Lymphoma (119)</td>
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<td>LMP-1</td>
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<td>Lymphoma (116)</td>
</tr>
<tr>
<td>miR-BART6-3p</td>
<td></td>
<td></td>
<td>NPC, GC (107)</td>
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<td></td>
<td>NPC (127)</td>
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<tr>
<td>miR-BART9-3p</td>
<td>LMP-1</td>
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<td>NPC (127)</td>
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<td>miR-BART10-3p</td>
<td>BHRF1</td>
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<td>Lymphoma (116)</td>
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<td>miR-BART11-5p</td>
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<td>EBF1</td>
<td>Lymphoma (134)</td>
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<td>miR-BART13-3p</td>
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<td>CAPRIN2</td>
<td>Lymphoma (116)</td>
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<td>miR-BART15-3p</td>
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<td>NLRP3</td>
<td>Lymphoma (135)</td>
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<td>miR-BART16</td>
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<td>NPC (127)</td>
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<td>miR-BART17-5p</td>
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<td>Lymphoma (129)</td>
</tr>
<tr>
<td>miR-BART18-5p</td>
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<td>MAP3K2</td>
<td>NPC (127)</td>
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<td>miR-BART20-5p</td>
<td>BZLF1, BRLF1</td>
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<td>NPC (127)</td>
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<td>miR-BART22</td>
<td>LMP-2A</td>
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<td>NPC (127)</td>
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<tr>
<td>miR-BART miRNAs</td>
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<td>BIM</td>
<td>NPC (138)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GC (113)</td>
</tr>
</tbody>
</table>

NPC, nasopharyngeal carcinoma; GC, gastric carcinoma.

Table V. The roles of EBV miRNAs in EBV-associated malignancies.
cellular miRNAs in EBVaGC have not been intensively investigated. We previously reported that two cellular miRNAs, hsa-miR-200a and hsa-miR-200b, were downregulated in EBVaGC both in tissue samples and in cell lines (52). These miRNAs targeted the transcription repressors ZEB1 and ZEB2, which regulate E-cadherin expression. Downregulation of these miRNAs ultimately reduced E-cadherin expression and triggered the epithelial-to-mesenchymal transition. EBV latent genes, BARF0, EBNA-1 and EBERs, cooperatively suppressed hsa-miR-200a and 200b expression in cell lines; while reduced ZEB 1, ZEB2 and E-cadherin expression was significant only in EBERs-transfected cells.

Viral genomes also encode for miRNAs and EBV was the first virus in which viral miRNAs were found (101-103). To date, 25 EBV miRNA precursors and 44 mature EBV miRNAs have been registered in miRBase. EBV-encoded miRNAs fall into two major clusters: BHRF-1 and BART. The BHRF-1 cluster contains four mature miRNAs that are expressed only in lyrically infected cells or cells with latency type III infections. The BART cluster is located in the non-coding region of BARTs, and is further subdivided into subclusters 1 and 2, which include 38 mature EBV miRNAs in total, and the miRNAs ebv-miR-BART2-5p and ebv-miR-BART2-3p are located downstream of these two clusters.

Several studies profiled EBV-encoded miRNA expression in EBV-associated malignancies, including NPC and Diffuse large B cell lymphoma (DLBCL), and showed that specific viral miRNAs played roles in carcinogenesis (Table V) (104-121). Several reports investigated the expression of viral miRNAs in EBVaGC tissue samples and cell lines (97,112,122-125). EBV miRNAs were variably expressed in EBVaGC cells, among which ebv-miR-BART1-3p, 2-5p, 3-3p, 4-5p, 5-5p, 7-3p, 9-3p, 10-3p, 17-5p and 18-5p were expressed at relatively high levels. The ebv-miR-BART7-3p expression level was consistently high in other EBV-associated malignancies, although its function has not been determined. A recent study by Marquitz et al (123), reported the expression profiles of cellular and viral miRNAs in EBV-infected AGS cells. By sequencing small RNA libraries created from these cells, they showed that EBV miRNA constituted 15% of total miRNAs, and that the remainder was derived from host cells. miRNA PCR array analysis revealed that let-7 family members, miR-200 family members, and several human miRNAs were downregulated by EBV infection. In addition, EBNA-1 transfection reduced hsa-miR-143 expression in AGS cells. Another report by the same group showed that gene expression changes induced by EBV infection of AGS cells were highly enriched for genes involved in cell motility and transformation pathways, and that these genes were potentially targeted by viral miRNAs (97).

Viral miRNA involvement in EBVaGC remains largely unknown. We performed comprehensive profiling of viral miRNA expression in tissue samples of EBVaGC and identified frequently expressed viral miRNAs. In silico analysis provided potential targets of these miRNAs, including genes associated with cell proliferation, apoptosis, and migration, and the direct interaction of these target genes and specific viral miRNAs were validated in vitro (unpublished data). Further studies to clarify the roles of cellular and viral miRNAs and the regulatory mechanisms of these molecules by EBV and host cells will be needed to completely understand the carcinogenic mechanisms involved in EBVaGC.

8. Conclusions

EBVaGC is a distinct subtype of gastric carcinoma with regard to both its clinicopathological and molecular features. Recent advances in comprehensive genome-wide analysis have provided novel findings regarding the genetic and epigenetic
abnormalities that are unique to EBVaGC, and these various factors cooperate to develop carcinoma (Fig. 6). Global and non-random CpG island hypermethylation is characteristic feature of EBVaGC, and epigenetic silencing of various genes, especially tumor suppressor genes, play a key role in carcinogenesis. Furthermore, increased activation of DNA methyltransferase by LMP-2A also induces hypermethylation of EBV genome itself, resulting in limited expression of latent genes, which may benefit in escaping from immune response by the host. Viral specific transcripts including latent genes and miRNAs have oncogenic properties such as increased cell proliferation and motility, anti-apoptotic effect, and chemoresistance, which help tumor progression. Although EBVaGC is relatively genonomically stable, frequent mutations in the PIK3C and ARIDIA genes are found; and the roles of these mutations in carcinogenesis are expected to be clarified in the future studies. Furthermore, there remain questions regarding how and when these mutations occur, what triggers hypermethylation, and how host cells regulate the transcription of viral genes. Finally, the interaction between carcinoma and inflammatory cells is another key to understand its carcinogenesis, which will also benefit the development of disease-specific therapies.

References


