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Recent Updates on DNA Methylation in the EBVaGC Genome

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Abstract

Epstein-Barr virus (EBV)-associated gastric carcinoma (EBVaGC) is a recently recognized disease entity that is defined by the presence of EBV in gastric carcinoma cells. EBV infection causes epigenetic modifications or major epigenetic alterations in the EBV genome and its host cellular genome, suggesting that EBV acts as a direct epigenetic driver for EBV and EBVaGC. One of the major epigenetic events in the viral and cellular genomes to control is DNA hypo- or hypermethylation. In particular, it is important to understand the roles of DNA hypermethylation in determining the molecular mechanisms of EBVaGC carcinogenesis. To address the functional roles of DNA methylation, we reviewed recent literature reporting DNA hypermethylation in EBVaGC.

Keywords: DNA methylation; Epstein-Barr virus; EBV associated gastric carcinoma

Introduction

Epstein-Barr virus (EBV) is a gamma herpesvirus associated with virtually all human nasopharyngeal carcinoma (NPC) and approximately 10% of human gastric cancer (GC) worldwide [1]. Epstein-Barr virus (EBV)-associated gastric carcinoma (EBVaGC) is a recently recognized disease entity, defined by the presence of EBV in gastric carcinoma cells [2]. Annually, >80,000 patients are estimated to develop EBVaGC [2]. Characteristics of EBVaGC include predominance among males, a proximal location in the stomach, lymphoepithelioma-like histology [3], a high proportion in diffuse-type gastric carcinoma [2], and expression of several EBV latent type I genes [4].

Two distinct pathogens, *Helicobacter pylori (H. pylori)* and EBV, are associated with human gastric carcinogenesis [5]. *H. pylori* infection causes chronic inflammation of the gastric epithelium that induces aberrant polyclonal methylation, which might lead to an increased risk of gastric cancer [5]. EBV infection causes extensive methylation in EBVaGC. Such aberrant methylation extends to not only to Polycomb repressive complex (PRC)-target genes in embryonic stem cells, but also to non-PRC target genes [5]. The characteristic molecular abnormality in EBVaGC is global and nonrandom CpG-island methylation of the promoter region of many cancer-related genes, which causes downregulation of their expression [2]. Furthermore, it has been suggested that EBV plays an etiological role in gastric carcinogenesis because of the uniform presence of EBV-encoded small RNAs in tumor cells and the detection of monoclonal EBV episomes in EBVaGC [2]. Therefore, we should regard EBVaGC as a distinct entity of gastric carcinoma, even though EBVaGC accounts for a relatively small fraction of total gastric carcinoma [2].

EBV in EBVaGC showed latency type I or II, in which EBERs, EBNA-1, BARTs, LMP-2A, and BART microRNAs (miRNAs) are expressed from the EBV genome [3]. EBV LMP1 and 2A are known to induce cellular DNA methyltransferases that influence the methylation status of the viral and cellular genomes [1]. Thus, EBV infection causes epigenetic modification or major epigenetic alterations in the viral and cellular genomes [1], indicating that EBV acts as a direct epigenetic driver for EBV-associated gastric cancers [6]. A strong tendency towards CpG hypermethylation has been observed in the EBVaGC methylome [1], and it is now known to be unique for EBVaGC, resembling an EBV-specific “epigenetic signature” [1]. Global CpG island hypermethylation induces epigenetic silencing of tumor suppressor genes of EBVaGC, causing downregulation of their expression [3]. This abnormal hypermethylation of tumor suppressor gene promoter regions is crucial for EBV associated gastric carcinogenesis [7].

To address the importance of hypermethylation in EBV associated gastric carcinogenesis, we provided an update on current studies of DNA methylation in EBVaGC genes by reviewing recent literature reported since 2010.

DNA Methylation in Primary EBV Associated Gastric Carcinomas

There have been many exemplary clinical studies providing a wealth of important information; therefore, we first reviewed DNA methylation studies in human primary EBV associated gastric carcinoma **(Table 1).**

Ferrasi et al. [8] in 2010 performed *in situ* hybridization for EBER (an EBV-encoded small RNA) and methylation-specific PCR (MSP) in 54 primary gastric carcinomas (intestinal and diffuse type). Among the 54 gastric carcinoma tumors, five specimens (9.3%) were EBVaGCs. Four patients presented intestinal type adenocarcinoma and one was diffuse.

Table 1 Hypermethylated cellular genes in primary EBV-associated gastric carcinomas.

Authors & References	Samples	Hypermethylation Genes in EBVaGC	Method of analysis
Ferrasi et al. W J Gastroenterol. (2010) [8]	49 primary EBVnGC 5 primary EBVaGC	DAPK, COX-2, CDKN2A	Methylation-specific PCR (MSP)
Geddert et al. Anal Cell Pathol. (2011) [9]	18 primary EBVaGC, 74 primary EBVnGC	p16, p14, APC	
Matsusaka et al. Cancer Res. (2011) [10]	51 gastric cancer samples	CXXC4, TIMP2, PLXND1	Illumina's Infinium BeadArray
Sun et al. Virchows Arch. (2012) [11]	121 gastric carcinomas tumors	SOX9	MSP
Saito et al. J Med Virol. (2013) [12]	25 primary EBVaGC, 50 primary EBVnGC	MINTS, MINT31, p14, p16, p73, RUNX3	MSP
Liu et al. Arch Virol. (2013) [13]	23 primary EBVaGC, 25 primary EBVnGC	WNT5A	MSP
Okada et al. Cancer Sci. (2013) [14]	25 primary EBVaGC, 50 primary EBVnGC	TP73, BLU, FSD1, BCL7A, MARK1, SCRN1, and NKX3.1	MIRA-chip assay MSP
Zhao et al. Br J Cancer (2013) [15]	12 primary EBVaGC, 14 primary EBVnGC	SSRT1	Pyrosequencing
Liang et al. Gastroenterology (2014) [16]	34 primary EBVaGC, 100 primary EBVnGC	ACSS1, FAM3B, IHH, TRABD	Bisulfite sequencing MSP
Dong et al. J Microbiol. (2015) [17]	17 primary EBVaGC, 20 primary EBVnGC	IRF-5	MSP
He et al. Med Oncol. (2015) [18]	49 primary EBVaGC, 45 primary EBVnGC	p16, FHIT, CRBP1, WWOX, DLC-1	MSP

They found that the frequently hypermethylated genes in EBVaGC were DAPK, COX-2, and CDKN2A. Geddert et al. [9] in 2011 tested 92 gastric carcinomas by RNA-*in situ* hybridization for the presence of EBV using EBER. They found that 18 gastric carcinomas (19.6%) were EBVaGC out of 92 gastric carcinomas. Then, they investigated aberrant promoter methylation by methylation-specific real-time PCR for p16, p14, APC, and hMLH1. They showed that EBVaGC had significantly more frequent gene hypermethylation of p16, p14, and APC than EBV-negative gastric carcinoma (EBVnGC). They further showed that the majority of gastric carcinomas with p16 hypermethylation tended to cause a p16 protein loss (22/28). Matsusaka et al. [10] in 2011 analyzed promoter methylation in 51 gastric cancer samples using Illumina's Infinium BeadArray. They then classified these gastric cancer samples into three subgroups by hierarchical clustering analysis: EBV(-)/low methylation, EBV(-)/high methylation, and EBV(+)/high methylation. They found that the three epigenotypes were characterized by three groups of genes: genes methylated specifically in the EBV(+) tumors (EBVaGC: CXXC4, TIMP2, and PLXND1), genes methylated in EBV(+) and EBV(-)/high tumors (COL9A2, EYA1, and ZNF365), and genes methylated in all gastric cancers (AMPH, SORCS3, and AJAP1). Sun et al. [18] in 2012 reported that SOX9 expression was inversely related to advanced tumor stage, vessel infiltration, nodal metastasis, and EBV infection. Using MSP, they found that 58 (48%) of 121 gastric carcinoma tumors had a methylated SOX9 promoter in gastric carcinoma. The resultant methylated status was related to the low SOX9 expression. Saito et al. [12] in 2013 evaluated 50 EBVnGC and 25 EBVaGC that were positive for EBV-encoded small RNA 1 (EBER-1) by in situ hybridization. They analyzed the methylation status of 16 loci associated with tumor-related genes by MSP to identify genes in which DNA methylation occurred specifically in EBVaGC. They found that the frequency of methylation of six specific loci (MINTS, MINT31, p14, p16, p73, and RUNX3) was significantly higher in EBVaGC than in EBVnGC controls.

Liu et al. [13] in 2013 measured WNT5A expression and promoter methylation in 23 EBVaGC and 25 EBVnGC tissues. They found that EBVaGC had no or very low expression of WNT5A but a high level of methylation in the promoter region. In contrast, they observed that EBVnGC had higher WNT5A expression and a lower level of promoter methylation. Okada et al. [14] in 2013 verified the DNA methylation status of the genes such as TP73, BLU, FSD1, BCL7A, MARK1, SCRN1, and NKX3.1—based on the results of the MIRA-chip and MSP assays in 75 primary gastric cancer tissues from 25 patients with EBVaGC and

50 EBVnGC patients as controls. They observed that the methylation frequencies of TP73, BLU, FSD1, BCL7A, MARK1, SCRN1, and NKX3.1 were significantly higher in EBVaGC compared with those in EBVnGC. Zhao et al. [15] in 2013 analyzed the epigenetic alterations and biological function of somatostatin receptor 1 (SSTR1) in 12 EBVaGC, 14 EBVnGC and 4 normal gastric biopsies. Using a promoter methylation array, they demonstrated that the methylation level of SSTR1 was significantly higher in primary EBVaGCs compared with primary EBVnGCs (P=0.004). Liang et al. [16] in 2014 looked for genetic alterations in gastric tumor samples, with (EBVaGC, n=34) or without (EBVnGC, n=100) EBV infection, and identified 45 EBV-associated non-synonymous mutations by whole-genome sequence analysis. They found than these mutations, in genes such as AKT2, CCNA1, MAP3K4, and TGFBR1, were associated significantly with EBV-positive gastric tumors, compared with EBV-negative tumors. Furthermore, four genes (ACSS1, FAM3B, IHH, and TRABD) were selected to determine their methylation levels using bisulfite sequencing. Significantly higher methylation levels were observed in EBVaGCs as compared with EBVnGCs in ACSS1 (12.6% vs. 2.0%), FAM3B (44.6% vs. 34.0%), IHH (30% vs. 3.0%), and TRABD (20.9% vs. 3.0%). Dong et al. [17] in 2015 tested 17 EBV-positive and 20 EBVnGC samples using MSP analysis and observed IRF-5 promoter hypermethylation in EBVaGC tissues, which show type I latent infection. He et al. [18] in 2015 evaluated the global DNA methylation status in 49 EBVaGC and 45 EBVnGC tissue samples and cell lines using 5-methylcytosine immunohistochemical staining and methylation quantification. They found that global genomic DNA hypermethylation was more pronounced in EBVaGC than in EBVnGC. In particular, they demonstrated that promoter methylation of p16, FHIT, CRBP1, WWOX, and DLC-1 genes was more frequent in EBVaGC than in EBVnGC (P<0.05).

DNA Methylation in EBV Associated Gastric Carcinoma Cell Lines

We searched all literature listed in Pubmed that reported DNA methylation in EBVaGC cell lines. We found that to date, DNA methylation studies have been conducted for only two EBVaGC cell lines, AGS-EBV and SNU719. We reviewed these DNA methylation studies in AGS-EBV and SNU719 cell lines below **(Table 2).**

Table 2 Hypermethylated cellular genes in primary EBV-associated gastric carcinoma cell lines.

Authors & References	Samples	Hypermethylated Genes in EBVaGC	Method of analysis
Ryan et al. Infect Agent Cancer (2010) [19]	AGS-EBV, AGS	CDKN2A, COX2, ICAM1, TFF1	Bisulfite sequencing
Zhao et al. Cancer (2013) [20]	AGS-EBV,AGS	IL15RA, REC8, SSTR1, EPHB6, MDGA2, and SCARF2	Bisulfite sequencing
Zhao et al. Br J Cancer (2013) [15]	AGS-EBV, AGS	SSRT1	Pyrosequencing
Lu et al. Virol J (2014) [21]	AGS-EBV, AGS	GKN1, GKN2	ChIP-Seq
Liang et al. Gastroenterology (2014) [16]	AGS-EBV, AGS	ACSS1, FAM3B, IHH, NEK9, SLC7AB, TRABD	5-Aza treatment, qRT-PCR
Sun et al. Virchows Arch. (2012) [11]	SNU719, KT	SOX9	MSP
Liu et al. Arch Virol. (2013) [13]	5 EBVaGC cell lines (SNU719, GT, GT39, PT, YCCEL1), 15 EBVnGC cell lines	WNT5A	MSP
Okada et al. Cancer Sci. (2013) [14]	SNU719, KATO-III (EBVnGC cell line)	BCL7A, FSD1	MIRA-chip assay, MSP
Dong et al. J Microbiol. (2015) [17]	SNU719, AGS-BX1	IRF-5	MSP

AGS

Ryan et al. [19] in 2010 examined gene expression patterns in EBV infected and uninfected AGS gastric epithelial cell lines using a low density cDNA microarray, reverse transcription PCR, and methylation-specific DNA sequencing. Using reverse transcription PCR, they found that that EBV infection produced significant hypermethylation on factors having diverse functions, such as cell cycle regulation (IGFBP3, CDKN2A, CCND1, HSP70, ID2, and ID4), DNA repair (BRCA1 and TFF1), cell adhesion (ICAM1), inflammation (COX2), and angiogenesis (HIF1A). They then determined the methylation patterns of five genes, such as CDKN2A (p16), ID4, COX2, ICAM1, and TFF1, using bisulfite sequencing. Methylation patterns were determined for several clones of each gene with or without EBV infection, and with or without 5-aza-2'-deoxycytidine (5-Aza) treatment. Resultant bisulfite sequencing analysis revealed that the promoters of CDKN2A, COX2, ICAM1, and TFF1 were hypermethylated because of EBV infection. Zhao et al. [20] in 2013 conducted a DNA methylation study to profile EBV-driven hypermethylation in EBV-infected cells using the EBV-positive AGS gastric cancer cell line (AGS-EBV, EBVaGC) and EBV-negative AGS cell line (AGS, EBVnGC). They assessed DNA methyltransferase-3b (DNMT3b) activity and the genome-wide DNA methylation profile by EpiQuick activity assays and methyl-DNA immunoprecipitation microarray assays, respectively. They found that the expression and activity of DNMT3b was increased significantly in AGS-EBV compared with AGS. They showed that EBV infection (fold-change >2, P<0.05) in AGS-EBV tended to differentially

methylate 1065 genes in AGS-EBV compared with AGS cells. They demonstrated that the majority of the differentially methylated genes (83.2%, 886 of 1065 genes) had cytosine-guanine dinucleotide (CpG) hypermethylation in AGS-EBV (fold-change 2.43-65.2) versus that found in AGS cells. They confirmed higher levels of DNA methylation in six novel hypermethylated candidate genes (IL15RA, REC8, SSTR1, EPHB6, MDGA2, and SCARF2) in AGS-EBV cells by bisulfite genomic sequencing. Zhao et al. [15] in 2013 analyzed the epigenetic alterations and biological function of somatostatin receptor 1 (SSTR1) in EBVaGC. Using a promoter methylation array, they showed that SSTR1 was methylated preferentially in EBVaGC. They then examined promoter methylation by combined bisulfite restriction analysis (COBRA) and pyrosequencing. They detected promoter hypermethylation of SSRT1 in EBVaGC cell line (AGS-EBV) with SSTR1 transcriptional silencing, but not in EBVnGC cell line (AGS) with SSTR1 expression. They concluded that the tumor suppressive effect of SSRT1 was associated with the upregulation of cyclin-dependent kinase inhibitors (p16, p15, p27, and p21); the downregulation of oncogenes (MYC and MDM2) and key cell proliferation and pro-survival regulators (PI3KR1, AKT, BCL-XL, and MET); and inhibition of the migration/invasion-related genes (integrins, MMP1 (matrix metallopeptidase 1), PLAUR (plasminogen activator urokinase receptor), and IL8 (interleukin 8)). Lu et al. [21] in 2014 analyzed the role of EBNA1 in regulating gastrokine tumor suppressor genes in EBVaGC. They conducted chromatin-immunoprecipitation sequencing (ChIP-Seq) assays, ChIP-qPCR assays, and electrophoretic mobility shift assays (EMSA) to demonstrate that EBNA1 bound directly to the GKN1 and GKN2 promoter loci. Subsequently, they generated AGS-EBV and AGS-EBNA1 cell lines and studied the effects of EBNA1 on GKN1 (tumor suppressor gene gastrokine 1) and GKN2 (tumor suppressor gene gastrokine 2) mRNA expression, with or without 5-Aza treatment. They found that gastrokine genes such as GKN1 and GKN2 were transcriptionally silenced by DNA methylation. Furthermore, we found that both latent EBV infections caused further reductions in GKN1 and GKN2 expression in AGS gastric carcinoma cells, and short interfering RNA (siRNA) depletion of EBNA1 partially alleviated this repression. Liang et al. [16] in 2014 performed whole-genome, transcriptome, and epigenome sequence analyses of a gastric adenocarcinoma cell line (AGS cells), before and after EBV infection. Integrated analysis showed that 216 genes were hypermethylated and transcriptionally downregulated in AGS-EBV relative to AGS cells. Among these 216 genes, the hypermethylation of six randomly selected genes (ACSS1, FAM3B, IHH, NEK9, SLC7AB, and TRABD) was confirmed by demethylation treatment using 5-Aza, a methyltransferase inhibitor. These six genes were confirmed to be downregulated significantly in AGS-EBV compared with AGS using quantitative real-time reverse transcription PCR (qRT-PCR). Their downregulation was restored successfully in AGS-EBV cells by 5-Aza treatment. Finally, they observed that EBV-associated genomic and epigenomic alterations tend to affect five signaling pathways commonly (axon guidance, focal adhesion formation, interactions among cytokines and receptors, mitogen-activated protein kinase signaling, and actin cytoskeleton regulation).

SNU719 cells

Sun et al. [11] in 2012 reported that SOX9 expression was inversely related to advanced tumor stage, vessel infiltration, nodal metastasis, and EBV infection. Using MSP and RT-qPCR, they observed that three (MKN7, TMK1, and NUGC3) of six cell lines (MKN1, MKN7, MKN74, TMK1, AGS, and NUGC3) had increased methylation through EBV infection and decreased SOX9 expression. Two original EBVaGC cell lines, SNU719 and KT, showed methylated SOX9 promoters. Such downregulation by promoter methylation of SOX9 was related to gastric carcinoma progression and EBV infection. Liu et al. [13] in 2013 measured WNT5A expression and promoter methylation in five EBVaGC and 15 EBVnGC cell lines using qRT-PCR and MSP. Five EBVaGCs were GT, GT39, PT, YCCEL1, and SNU719. The 15 EBVnGCs were AGS, KatoIII, MKN45, SNU1, SNU16, YCC1, YCC2, YCC3, YCC6, YCC7, YCC9, YCC11, YCC16, SGC-7901, and HGC-27. They found that EBVaGC cell lines had no or very low expression of WNT5A but a high level of methylation in the promoter region. In contrast, they observed that EBVnGC cell lines had higher WNT5A expression and a lower level of promoter methylation. They could restore the reduced WNT5A expression by treatment with 5-Aza. Such increased expression of WNT5A *in vitro* resulted in inhibition of β-catenin expression in EBVaGC cell line (SNU719). Okada et al. [14] in 2013 studied the comprehensive DNA methylation status in the SNU719 (EBVaGC) cell line by methylated CpG island recovery on a chip assay (MIRA-chip assay) and MSP. Analysis of SNU719 cells by the MIRA-chip identified 1071 hypermethylated spots. Twenty-nine of 69 genes methylated in cancer were chosen for further examination. MSP was conducted to confirm the methylation status of the 29 genes. They then focused on seven genes—TP73, BLU, FSD1, BCL7A, MARK1, SCRN1, and NKX3.1—based on the results of the MIRA-chip and MSP assays. BCL7A, NKX3.1, and BLU were not methylated in the KATO-III EBV-negative GC cell line, but BCL7A and FSD1 were hypermethylated in SNU719. Using a combination of 5-Aza and trichostatin A treatments, they found that the expressions of the genes, except for BCL7A, were upregulated in SNU719. Dong et al. [17] in 2015 investigated the methylation status of the IRF-5 promoter-A region in EBV-associated gastric carcinoma using MSP analysis, because they had reported that IRF-5 was not detected in most of EBV-infected BL cell lines (Akata, Akata-4E3, Akata-BX1, Mutu I, Mutu III, and Rael) because of hypermethylation of the IRF-5 distal promoter (promoter-A). Hypermethylation in the IRF-5 promoter-A region was observed in EBVaGC cell lines (SNU719, AGS-BX1). By contrast, they showed hypomethylation of CpG islands in promoter-A only in EBV type III latent infected BL cell lines (LCL and Mutu III).

Conclusion

We identified several genes that are hypermethylated in EBVaGC by reviewing literature published since 2010 **(Tables 1 and 2)**. Most frequently mentioned hypermethylated genes in EBVaGC were p16 and SSRT1. These genes were reported as hypermethylated in EBVaGC in more than three studies. Next most frequently mentioned hypermethylated genes were COX2, CDKN2A, p14, SOX9, (T)p73, WNT5A, FSD1, BCL7A, ACSS1,

FAM3B, IHH, TRABD, and IRF-5. These genes were reported as hypermethylated in EBVaGC in more than two studies. Finally, hypermethylated genes in EBVaGC reported in at least one study were DAPK, APC, CXXC4, TIMP2, PLXND1, MINTS, MINT31, RUNX3, BLU, MARK1, SCRN1, NKX3.1, FHIT, CRBP1, WWOX, ICAM1, TFF1, IL15RA, REC8, EPHB6, MDGA2, SCARF2, GKN1, GKN2, NEK9, and SLC7AB. This review suggested that the highly methylated gene in EBVaGC, p16, could be considered a good target gene for novel demethylating drugs.

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