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Identification of genes specifically methylated in Epstein–Barr virus-associated gastric carcinomas

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We studied the comprehensive DNA methylation status in the naturally derived gastric adenocarcinoma cell line SNU-719, which was infected with the Epstein–Barr virus (EBV) by methylated CpG island recovery on chip assay. To identify genes specifically methylated in EBV-associated gastric carcinomas (EBVaGC), we focused on seven genes, *TP73*, *BLU*, *FSD1*, *BCL7A*, *MARK1*, *SCRN1*, and *NKX3.1*, based on the results of methylated CpG island recovery on chip assay. We confirmed DNA methylation of the genes by methylation-specific PCR and bisulfite sequencing in SNU-719. The expression of the genes, except for *BCL7A*, was upregulated by a combination of 5-Aza-2′-deoxycytidine and trichostatin A treatment in SNU-719. After the treatment, unmethylated DNA became detectable in all seven genes by methylation-specific PCR. We verified DNA methylation of the genes in 75 primary gastric cancer tissues from 25 patients with EBVaGC and 50 EBV-negative patients who were controls. The methylation frequencies of *TP73, BLU, FSD1, BCL7A, MARK1, SCRN1*, and *NKX3.1* were significantly higher in EBVaGC than in EBV-negative gastric carcinoma. We identified seven genes with promoter regions that were specifically methylated in EBVaGC. Inactivation of these genes may suppress their function as tumor suppressor genes or tumor-associated antigens and help to develop and maintain EBVaGC. (*Cancer Sci* 2013; 104: 1309–1314)

The Epstein–Barr virus is associated with a variety of tumors derived from B cells, such as Burkitt lymphoma,[1] post-transplant lymphoproliferative disease,[2] and Hodgkin's disease[3]; T cells such as peripheral T-cell lymphomas[3]; epithelial cells, including nasopharyngeal carcinoma and gastric carcinoma[1,3]; and natural killer cells, such as nasal natural killer/T-cell lymphomas.[3] Burke *et al.* first identified EBV in gastric carcinomas by PCR in 1990,[4] and since then, about 10% of gastric carcinomas have been identified as resulting from monoclonal proliferation of EBV-infected cells.[5,6] In each EBV-positive case of gastric carcinoma, almost all carcinoma cells are infected with the virus,[7–9] which suggests that EBV plays an important role in the development of gastric carcinomas.

In the EBV genome, DNA methylation has been studied intensively.[10] The expression of EBV latent genes is regulated under strict epigenetic control through DNA methylation in host cells. It is well known that Cp/Wp EBNA promoters, which can transcribe all EBNA, are methylated in Burkitt lymphoma and nasopharyngeal carcinoma, whereas the Qp promoter, which induces only EBNA1 expression, is used in these tumors.[11,12] Additionally, LMP1 expression is regulated by methylation in its promoter region in EBV-positive nasopharyngeal carcinoma.[13] In EBV-positive gastric carcinomas, there are latency patterns similar to that of Burkitt lymphoma, in which only Qp is active.[5] Thus, the latency type of EBV-positive malignancies is regulated by methylation status of the EBV genome.

The importance of the CpG Island methylator phenotype (CIMP), which is characterized by simultaneous methylation of CpG islands in multiple genes, has also been examined in gastrointestinal carcinogenesis.[14] Kusano *et al.* showed a strong association between EBV-associated tumors and CIMP-High (CIMP-H), hypermethylation of tumor-related genes, and a lack of *p53* or *K-ras* mutations.[15] Chang *et al.* also revealed that EBVaGC showed global CpG island methylation and that they comprised a pathogenetically distinct subgroup of CIMP-H gastric carcinomas.[16] Thus, hypermethylation of tumor-related genes might lead to the progression of EBVaGC, whereas methylation of viral DNA determines EBV latency type.

We studied the comprehensive DNA methylation status in the naturally derived gastric adenocarcinoma cell line SNU-719, which is infected with EBV,[17] by MIRA-chip assay. We succeeded in identifying several genes whose expression was regulated by DNA methylation in EBVaGC.

Materials and Methods

Cell cultures and drug treatment. The human gastric cancer cell lines SNU-719 (EBV-positive gastric cancer cell line), which was obtained from the Korean Cell Line Bank (Seoul, South Korea), and KATO-III (EBV-negative gastric cancer cell line) were cultured in RPMI-1640 supplemented with 10% heat-inactivated FBS (Sigma-Aldrich, St. Louis, MO, USA) at 37°C in a humidified 5% CO_2 incubator.[17]

The SNU-719 cells were split 24 h before treatment and were then given one of the following treatments: (i) DAC (5 μM) (Sigma-Aldrich) or PBS for 72 h, with the medium changed every 24 h; (ii) TSA (300 nM) or ethanol (300 nM) for 24 h; or (iii) DAC (5 μM) for 72 h, with TSA (300 nM) for the last 24 h. The medium containing DAC was changed every 24 h. The dose of DAC (5 μM) was chosen based on preliminary studies showing optimal reactivation of gene expression. The timing and sequencing of DAC and/or TSA was based on similar preliminary studies as well as previously published studies.[18]

DNA and RNA extractions. Genomic DNA and total RNA from SNU-719 was isolated with an All Prep DNA/RNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. As described previously,[19] DNA from paraffin-embedded samples was prepared. Briefly, we prepared 5-μm thick tissue sections from archival, formalin-fixed, paraffin-embedded tissue blocks. After the tissue sections were stained with H&E, the adenocarcinoma regions were microdissected with a 27-gauge needle. Tumor DNA was extracted using the

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QIAamp DNA FFPE Tissue Kit (Qiagen) following the manufacturer's protocol.

MIRA-chip assay of SNU-719. The methylated DNA was enriched with a MethylCollector Kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's protocol. Fragments of CpG-methylated DNA prepared by *Mse*I digestion specifically bind a His-tagged recombinant methyl-CpG-binding domain protein-2b. These protein–DNA complexes are captured with nickel-coated magnetic beads, and subsequent wash steps are performed with a stringent high-salt buffer to remove fragments with little or no methylation. The methylated DNA is then eluted from the beads in the presence of Proteinase K. Comparative genomic hybridization array slides (MacArray Karyo 4000; Macrogen Inc., Seoul, South Korea) were used in this study. The array was spotted with 4030 human bacterial artificial chromosome clones that covered the whole human genome at an average interval of 0.83 Mb. The experiments were performed according to the manufacturer's protocol. Briefly, arrays were prehybridized with salmon sperm DNA to block repetitive sequences in the bacterial artificial chromosomes. Then, 500-ng input DNA (reference DNA) and cancer cell line immunoprecipitated DNA (test DNA) were labeled with Cy5-dCTP and Cy3-dCTP, respectively, by randomly primed labeling. The labeled probe and human Cot-I DNA were mixed and dissolved in hybridization solution. The probe mixture was denatured, cooled, and mounted on the array. Hybridizations were performed in a sealed chamber for 72 h at 37°C. After hybridization, array slides were washed and dried. Scanning was carried out using a GenePix 4000A two-color fluorescent scanner (Axon Instruments, Union City, CA, USA), and quantification was performed using MAC viewer software (Macrogen Inc.).

Sodium bisulfite modification of DNA. We performed bisulfite treatment as reported previously.[20] In 50-μL water, 2-μg genomic DNA was denatured with 5.5-μL 2 M NaOH at 37°C for 10 min, which was followed by incubation with 30-μL 10 mM hydroquinone and 520-μL 3 M sodium bisulfite (pH 5.0) at 50°C for 16 h in darkness. Then, DNA was purified with 50-μL water and a DNA Cleanup Kit (Promega, Madison, WI, USA), which was used as recommended by the manufacturer. The DNA was incubated with 5.5-μL 3 M NaOH at room temperature for 5 min; precipitated with 1-μL 20 mg/mL glycogen, 33-μL 10 M ammonium acetate, and 260-μL 100% ethanol; washed with 70% ethanol; and finally resuspended in distilled water.

Methylation-specific PCR. Primer sequences for amplification of the *TP73, BLU, FSD1, BCL7A, MARK1, SCRN1,* and *NKX3.1* genes are listed in Table S1. The methylation status of these seven genes was determined by bisulfate treatment of DNA followed by MSP.[21] In brief, 2-μl bisulfite-treated DNA in 10-μL PCR solution was used as the template for PCR reactions with primers specific for methylated and unmethylated alleles. The DNA used as positive controls for methylated and unmethylated alleles were SssI methyltransferase-treated placental DNA (New England Biolabs, Beverly, MA, USA) and lymphocyte DNA, respectively. Next, PCR products from methylated and unmethylated reactions were electrophoresed on 3% agarose gels and visualized by ethidium bromide staining. Each MSP was repeated at least twice. The prospectively established criterion for presence of hypermethylation was detection of a methylated band in all independent assays.

Bisulfite sequencing. We performed bisulfite sequencing. The primers are listed in Table S2. The PCR amplification was performed for a total of 40 cycles. The annealing temperature of each gene is shown in Table S2. The PCR products were gel purified from agarose gel using Wizard SV Gel (Promega) and PCR Clean-Up Kit (Promega), and cloned into the T/A cloning vector pGEM-T Easy (Promega). At least six subclones were isolated and identified by direct sequencing.

Quantitative real-time PCR. The extracted total RNA was reverse-transcribed into single-stranded cDNA using a High Capacity cDNA Archive Kit (Applied Biosystems, Warrington, UK). Real-time PCR was performed using the cDNA with Power SYBR Green PCR Master Mix (Applied Biosystems). The primer sequences of *TP73, BLU, FSD1, BCL7A, MARK1, SCRN1, NKX3.1*, and *β-actin* are listed in Table S3. Quantitative PCR was performed on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Quantitative PCR parameters for cycling were as follows: 95°C for 10 min followed by 40 cycles of PCR at 95°C for 15 s and 60°C for 1 min. All reactions were done in triplicate in a 20-μL reaction volume. The mRNA expression level was determined using the $2^{-\Delta\Delta C_T}$ method.

Clinical materials. We evaluated 75 primary gastric cancer tissues from 25 patients with EBVaGC and 50 patients with EBV-negative gastric carcinoma who underwent surgical resection between 1995 and 2007 at Yamaguchi University Hospital (Ube, Japan). All patients provided informed consent, and the research project was approved by the university and institutional review boards. The samples were formalin-fixed, paraffin-embedded tissues of gastric cancer. The EBVaGC were positive for EBER1 *in situ* hybridization. Age-, sex-, histological type-, and stage-matched patients with EBV-negative gastric carcinoma were selected. The clinicopathological characteristics of the patients are summarized in Table 1.

***In situ* hybridization of EBER1.** With a digoxigenin-labeled 30-base oligomer using previously described procedures, EBER1 was detected.[7,8] Paraffin-embedded sections of 5-μm thickness were deparaffinized, rehydrated, predigested with pronase, prehybridized, and then hybridized overnight at 37°C. After the sections were washed with 0.5 × SSC, hybridization was detected using an anti-digoxigenin antibody alkaline phosphatase conjugate (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

Statistical analysis. The Mann–Whitney *U*-test was used to compare variables. Differences in methylation frequencies were evaluated with the Fisher's exact test. A *P*-value of

Table 1. Clinicopathological characteristics of EBVaGC and controls

	EBVaGC (n = 25)	Controls (n = 50)
Sex		
Men	22	44
Women	3	6
Age	64.6 ± 12.1	67.7 ± 11.1
Histological type		
Differentiated	10	20
Undifferentiated	15	30
Lymph node metastasis		
n0	15	29
n1	6	13
n2	4	7
n3	0	1
Macroscopic type		
Elevated	19	43
Depressed	6	7
Stage		
I	14	28
II	5	10
III	4	8
IV	2	4

EBVaGC, Epstein–Barr virus-associated gastric cancer.

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<0.05 was considered statistically significant. Statistical analysis was performed with GraphPad Prism (GraphPad Software, La Jolla, CA, USA) and Instat3 statistical software (GraphPad Software).

Results

MIRA-chip analysis of SNU-719. Analysis of SNU-719 by MIRA-chip showed that 1071 spots were determined to be hypermethylated, with a log ratio of >0.25. Of genes on the spots, 69 genes were known to be methylated in cancer, and 29 genes were chosen for further examination. The methylation status of the 29 genes was confirmed by MSP in SNU-719. Similar results were observed by MIRA-chip analysis and MSP in 22 of 29 genes (78.3%) (Fig. 1a). To identify genes specifically methylated in EBVaGC, we focused on seven genes, *TP73*, *BLU*, *FSD1*, *BCL7A*, *MARK1*, *SCRN1*, and *NKX3.1*, because their MSP primers were available in primary gastric cancer tissues. Methylated DNA was not detectable in *BCL7A, NKX3.1*, and *BLU* in the KATO-III EBV-negative gastric carcinoma cell line (data not shown). However, *BCL7A* and *FSD1* were hypermethylated in SNU-719 by bisulfite sequencing (Fig. 1b), and the results of MSP were validated by bisulfite sequencing. The methylation level of *TP73, BLU, FSD1, BCL7A, MARK1*, and *NKX3.1* in SNU-719 according to bisulfite sequencing is shown in Fig. S1.

mRNA expression of SNU-719 by treatment with DAC and/or TSA. SNU-719 was treated with DAC and/or TSA, and change of mRNA expression of the seven genes was evaluated by quantitative RT-PCR (Fig. 2). Treatment with DAC restored *TP73* expression, and the expression of other genes, except for *BCL7A*, was upregulated by a combination of DAC and TSA treatment. The expression levels of *BCL7A* in mock sample were higher than those in DAC and/or TSA-treated samples.

MSP analysis of SNU-719 treated with DAC and/or TSA. Analysis was performed by MSP on SNU-719 cells treated with DAC and/or TSA. After treatment with the combination of DAC and TSA, unmethylated DNA became detectable in all seven genes. The DAC was adequate to demethylate and express the *TP73* gene. In other genes except for *BCL7A*, treatment with a combination of DAC and TSA had the greatest effect on demethylating the promoter regions (Fig. 3).

Histopathology and MSP in clinical samples. A representative EBVaGC lesion is shown in Figure 4. The ulcerated carcinoma was located on the posterior wall of the upper stomach (Fig. 4a). The tumor histological type was that of a moderately differentiated adenocarcinoma, and EBER1 signals were detected in almost all cancer cells by *in situ* hybridization, suggesting EBVaGC (Fig. 4b,c).

Methylation frequencies of *TP73, BLU, FSD1, BCL7A, MARK1, SCRN1*, and *NKX3.1* were significantly higher in EBVaGC than in EBV-negative gastric carcinoma (Table 2). A representative MSP image is shown in Figure 5.

Discussion

It has been reported that hypermethylation of tumor-related genes may be involved in the development of EBVaGC.[15,16] We found that six genes, including *TP73, BLU, FSD1, MARK1, SCRN1*, and *NKX3.1*, were hypermethylated more fre-

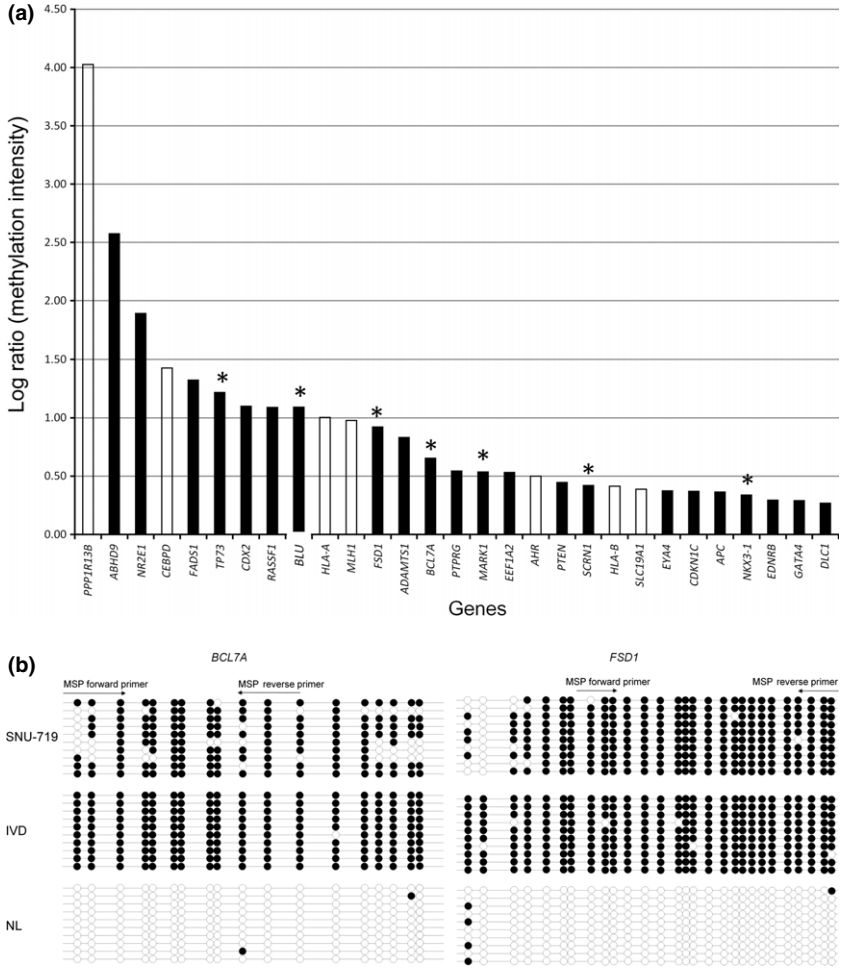

Fig. 1. (a) CpG island DNA methylation analysis of the EBV-associated cancer-specific hypermethylated genes found by the MIRA-chip approach. Methylation-specific PCR (MSP) analyses of the CpG island methylation status of 29 genes in SNU-719 and EBV-associated gastric cancer samples were performed. Of the 29 genes, 22 were methylated (black bars) and 7 were unmethylated (white bars) in SNU-719 cells. The asterisks (*) indicate the seven genes that were further examined because their MSP primers were available in primary gastric cancer tissues. (b) Methylation status of *BCL7A* and *FSD1* in SNU-719 according to bisulfite sequencing. The location of MSP primers used in this study is shown. EBV, Epstein–Barr virus; IVD, *in vitro*-methylated DNA; MIRA-chip, methylated CpG island recovery on chip; NL, normal lymphocyte.

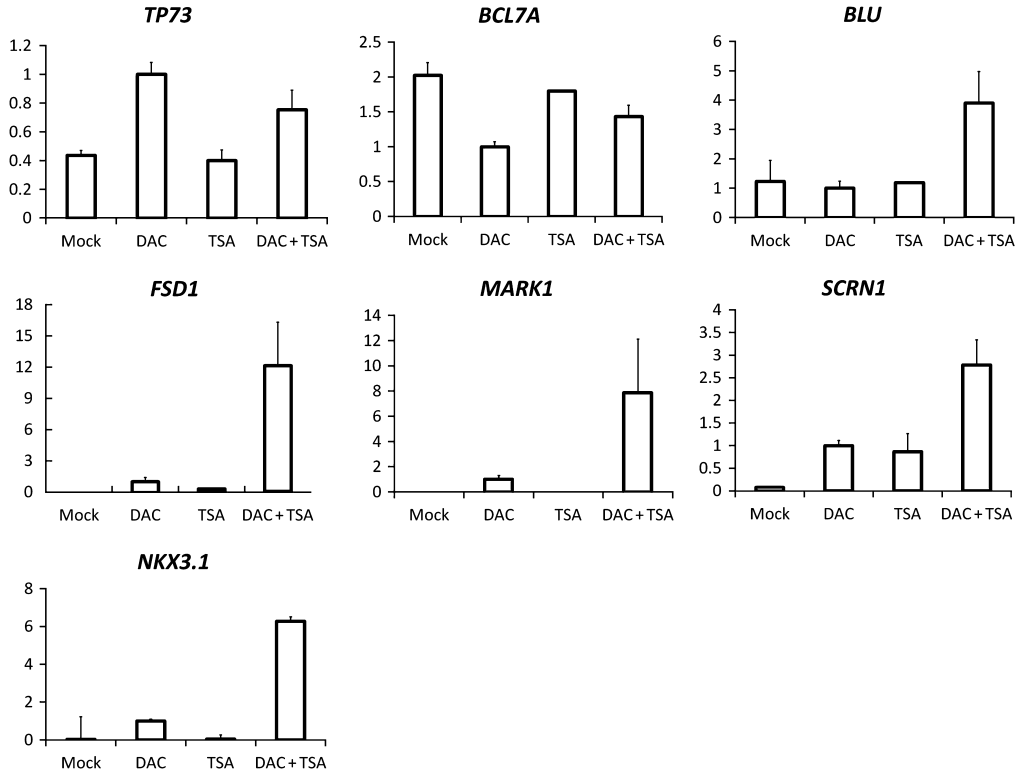

Fig. 2. Expression analysis of the EBV-associated cancer-specific hypermethylated genes found by the MIRA-chip approach. The effects of treatment with the DNA methyltransferase inhibitor DAC and/or the histone deacetylase inhibitor TSA on *TP73, BLU, FSD1, BCL7A, MARK1, SCRN1*, and *NKX3.1* expression as well as reactivation evaluated by real-time PCR are shown. The expression level of DAC-treated sample was standardized to 1 in each targeted gene. Error bars represent standard deviation. DAC, 5-Aza-2′-deoxycytidine; EBV, Epstein–Barr virus; MIRA-chip, methylated CpG island recovery on chip; Mock, treatment with PBS; TSA, trichostatin A.

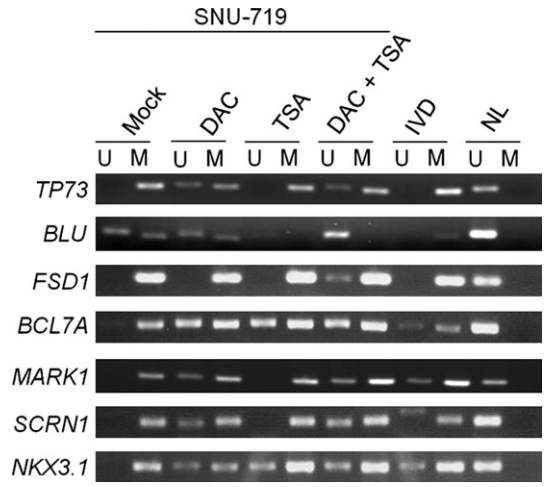

Fig. 3. Effects of treatment with the DNA methyltransferase inhibitor DAC and/or the histone deacetylase inhibitor TSA on *TP73, BLU, FSD1, BCL7A, MARK1, SCRN1*, and *NKX3.1*. Methylation evaluated by methylation-specific PCR. The presence of a PCR band indicates methylated (lane M) or unmethylated (lane U) genes. DNA from normal lymphocytes (NL) and *in vitro* methylated DNA (IVD) were used as negative and positive controls, respectively, for methylated DNA. DAC, 5-Aza-2′-deoxycytidine; TSA, trichostatin A.

quently in EBVaGC than in EBV-negative gastric carcinoma, and their expression was restored by DAC and TSA treatment in the SNU-719 EBVaGC cell line. Additionally, *TP73* has been identified as a transcription factor with structural and functional homology to a tumor suppressor. Ushiku *et al.* found that loss of *TP73* expression through aberrant methylation of the *TP73* promoter occurred specifically in EBVaGC, together with the methylation of *p14* and *p16*.[22] Transcriptional inactivation of the *TP73* gene by promoter methylation has been reported in other EBV-associated lymphoid malignancies such, as natural killer cell lymphoma and Burkitt lymphoma.[23–25] Therefore, *TP73* methylation might be an EBV-specific mechanism that leads to the development of malignancies.

The DNA methylation of *FSD1, MARK1*, and *SCRN1* in gastric carcinoma cell lines and the upregulation of the genes by 5-Aza-2′-deoxycytidine treatment have been reported by Yamashita *et al.*[26] Because the function of these genes in carcinogenesis is not fully understood, Suda *et al.* reported that *SCRN1* might be a novel immunotherapy target.[27] Thus, EBVaGC cells might evade immune reaction against *SCRN1* by downregulation of *SCRN1* expression by DNA methylation.

In the present study, *BLU* and *NKX3.1* were methylated specifically in EBVaGC as well. To date, DNA methylation of *BLU* and *NKX3.1* has not been reported in gastric carcinomas, whereas *BLU* methylation in neuroblastoma and nasopharyngeal carcinoma and *NKX3.1* methylation in prostatic cancer have been reported previously.[28–30] Because the genes act as tumor suppressor genes *in vitro*,[28–30] silencing of these genes by epigenetic mechanisms can play an important role in the development of EBVaGC.

We found that methylation of *BCL7A* was specific to EBVaGC and could be a useful marker to differentiate EBVaGC from EBV-negative gastric carcinoma. To our knowledge, this is the first report of this finding. Additionally, *BCL7A* promoter methylation has been reported in T-cell lymphoma.[31] Because SNU-719 showed *BCL7A* expression without DAC and/or TSA treatment in the current study, CpG

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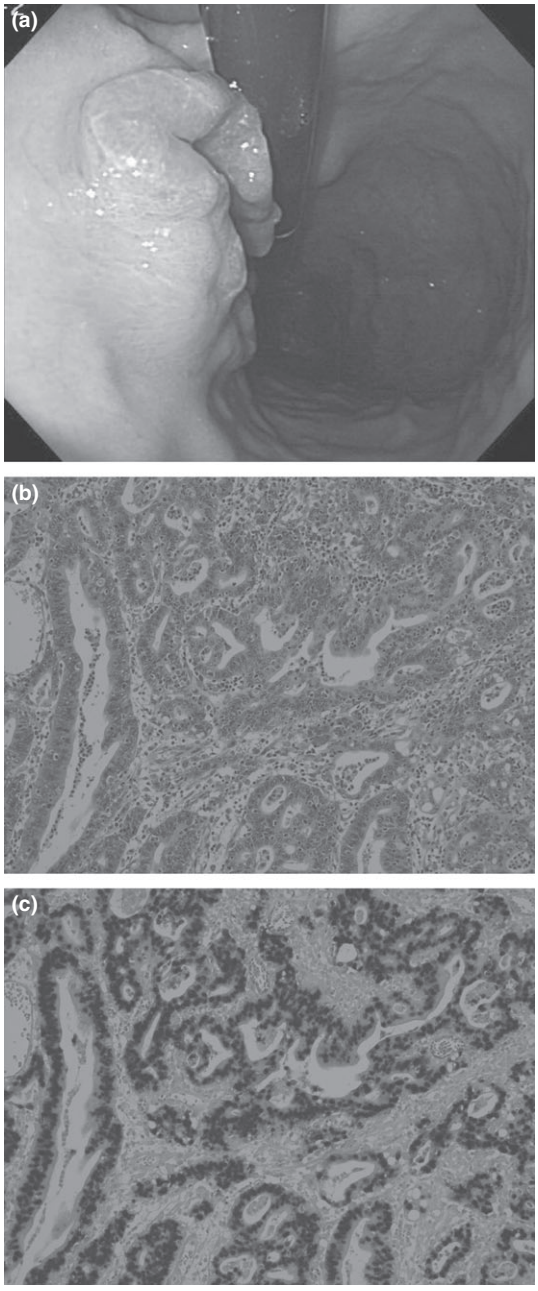

Fig. 4. Representative case of Epstein–Barr virus-associated gastric carcinoma (EBVaGC). (a) Endoscopic image of the EBVaGC. (b) Moderately differentiated adenocarcinoma was shown by H&E staining of the lesion. (c) *In situ* hybridization of EBER1. Signals of EBER1 were detected in the nucleus of almost all cancer cells. EBER1, EBV-encoded small RNA 1.

methylation of *BCL7A* could be indifferent to transcriptional silencing in clinical samples as well.

The molecular mechanism underlying EBV-associated aberrant methylation has not been elucidated. One of the EBV latent genes, *LMP2A*, could be a candidate gene to induce aberrant DNA methylation. We previously showed that *LMP2A* mRNA was detected in both EBVaGC samples and EBV-infected gastric carcinoma cell lines that we generated with recombinant EBV.[32] Hino *et al.* reported that *LMP2A* activated DNA methyltransferase 1 through *STAT3* phosphorylation and led to promoter hypermethylation of *PTEN* in gastric carcinoma.[33]

Table 2. Methylation frequency of genes in EBVaGCEBV-associated gastric carcinomas and controls

	Total (%)	EBVaGC, $n = 25$ (%)	Controls, $n = 50$ (%)	*P*-value
BCL7A	27/75 (36.0)	15/25 (60.0)	12/50 (24.0)	<0.01
SCRN1	17/74 (23.0)	12/25 (48.0)	5/49 (10.2)	<0.01
NKX3.1	22/74 (29.7)	16/25 (64.0)	6/49 (12.2)	<0.01
MARK1	23/75 (30.7)	17/25 (68.0)	6/50 (12.0)	<0.01
FSD1	17/74 (23.0)	12/24 (50.0)	5/50 (10.0)	<0.01
TP73	39/75 (52.0)	24/25 (96.0)	15/50 (30.0)	<0.01
BLU	6/74 (8.1)	5/25 (20.0)	1/49 (2.0)	0.015

EBVaGC, Epstein–Barr virus-associated gastric cancer.

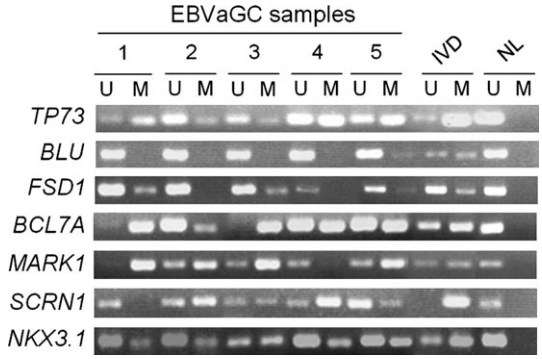

Fig. 5. Representative methylation-specific PCR analyses of *TP73, BLU, FSD1, BCL7A, MARK1, SCRN1*, and *NKX3.1* genes in Epstein–Barr virus-associated carcinomas (EBVaGC) (lanes 1–5) showing unmethylated and methylated samples. The presence of a PCR band indicates methylated (lane M) or unmethylated (lane U) genes. DNA from normal lymphocytes (NL) and *in vitro* methylated DNA (IVD) were used as negative and positive controls, respectively, for methylated DNA.

This result suggests that *LMP2A* plays an important role in the epigenetic abnormalities in the development of EBVaGC. Another EBV-latent membrane protein, *LMP1*, was reported to activate DNA methyltransferase 1 via *JNK/AP1* signaling in nasopharyngeal carcinoma,[34] but *LMP1*-positive cells are rarely seen in EBVaGC and gastric epithelium by immunohistochemical analysis. We considered the influence of treatment with DAC and/or TSA for EBV gene expression, but *LMP1* expression was not detected in SNU-719 by DAC treatment (data not shown).

In conclusion, we identified several genes with promoter regions that were specifically methylated in EBVaGC. Inactivation of these genes may suppress their function as tumor suppressor genes or tumor-associated antigens and help to develop and maintain EBVaGC.

Acknowledgment

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Disclosure

The authors have no conflict of interest.

Abbreviations

Aza	Azacitidine
CIMP	CpG island methylator phenotype
DAC	5-Aza-2′-deoxycytidine
EBER	EBV-encoded small RNA

EBNA	EBV-determined nuclear antigen 1
EBV	Epstein–Barr virus
EBVaGC	EBV-associated gastric carcinoma
LMP1	Latent membrane protein 1
LMP2A	Latent membrane protein 2A
MIRA-chip	Methylated CpG island recovery on chip
MSP	Methylation-specific PCR
TSA	Trichostatin A

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. The results of bisulfite sequencing of *TP73, BLU, FSD1, BCL7A, MARK1*, and *NKX3.1*.

Table S1. Primers for methylation-specific PCR.

Table S2. Primers for bisulfite sequence analysis.

Table S3. Primers for real-time quantitative PCR.

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