Activation of DNA Methyltransferase 1 by EBV Latent Membrane Protein 2A Leads to Promoter Hypermethylation of PTEN Gene in Gastric Carcinoma

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Abstract

CpG island promoter methylation of tumor suppressor genes is one of the most characteristic abnormalities in EBV-associated gastric carcinoma (GC). Aberrant promoter methylation and expression loss of PTEN were evaluated in cancer tissues of GC by methylation-specific PCR and immunohistochemistry, respectively, showing that both abnormalities occurred concurrently in EBV-associated GC. PTEN abnormalities were reiterated in GC cell lines MKN-1 and MKN-7 infected with recombinant EBV, and DNA methyltransferase 1 (DNMT1) was commonly overexpressed in both cell lines. Stable and transient transfection systems in MKN-1 similarly showed that viral latent membrane protein 2A (LMP2A) upregulated DNMT1, leading to an increase in methylation of the PTEN promoter. Importantly, the level of phosphorylated signal transducer and activator of transcription 3 (pSTAT3) increased in the nuclei of LMP2A-expressing GC cells, and knockdown of STAT3 counteracted LMP2A-mediated DNMT1 overexpression. Immunohistochemistry for both pSTAT3 and DNMT1 showed diffuse labeling in the nuclei of the cancer cells in GC tissues, especially in EBV-associated GC. Taken together, LMP2A induces the phosphorylation of STAT3, which activates DNMT1 transcription and causes PTEN expression loss through CpG island methylation of the PTEN promoter in EBV-associated GC. LMP2A plays an essential role in the epigenetic abnormalities in host stomach cells and in the development and maintenance of EBV-associated cancer.

Introduction

EBV is a human oncogenic virus that has been identified in a wide variety of malignancies (1). EBV-associated gastric carcinoma (GC) is the most common among the malignant neoplasms associated with EBV, comprising ~10% of all GC cases around the world (2). EBV-associated GC exhibits global and nonrandom DNA methylation of the promoter regions of various cancer-associated genes (3–8). This high activity of CpG island methylation is referred to as the CpG island methylator phenotype (CIMP), and EBV-associated GC is remarkable among the cases of CIMP-high GC. EBV-associated GC outnumbers other cases of CIMP-high GC in the methylated gene promoters of cancer-associated genes (7, 8). Promoter methylation correlated well with p16, p73, and E-cadherin expression abnormalities in EBV-associated GC (9–11). About methylation density, all CpG sites of the p14 and p16 promoter regions were uniformly methylated in EBV-associated GC, whereas methylation was sporadic and/or variable in EBV-negative GC (9).

To further clarify the underpinning mechanism of CpG island methylation in EBV-associated GC, the present study focused on the gene expression of PTEN. The PTEN gene is a tumor suppressor on chromosome 10q23 (12), and the loss of function of PTEN leads to increases in antiapoptotic and/or mitogen signaling (13). One of the mechanisms of PTEN inactivation is epigenetic silencing of the PTEN gene, which has been also reported in breast tumors, hematologic malignancies, malignant melanoma, and carcinomas of the uterine corpus, prostate, lung, and stomach including EBV-associated GC (14–22). In the course of establishing the experimental system for this virus-associated GC, it was observed that some of the tumor-associated genes, including PTEN, are epigenetically silenced in GC cell lines infected with the recombinant EBV. This preliminary observation led to further in vivo and in vitro analyses of this characteristic abnormality of EBV-associated GC.

There have been few studies focusing on the mechanisms of promoter methylation of host genes in association with EBV infection. Tsai and colleagues (23) reported that EBV latent membrane protein 1 (LMP1) activates DNA methyltransferase 1 (DNMT1) through the activation of c-Jun NH2-terminal kinase (JNK)-activator protein-1 (AP-1) signaling in nasopharyngeal carcinoma (NPC). Seo and colleagues (24) also suggested a role for the Rb-E2F pathway in LMP1-induced DNMT1 activation in NPC cells. However, LMP1 is the hallmark for the latency II program of EBV infection, such as in NPC and Hodgkin's lymphoma. EBV-associated GC belongs to the latency I program; LMP1 or EBNA2 is not expressed and viral gene expression is restricted to several transcripts, such as EBV-determined nuclear antigen 1 (EBNA1), EBER, latent membrane protein 2A (LMP2A), and transcripts from the BamHI A region (BARF0; refs. 2, 3). Thus, the aim of the present study was to identify cellular and viral factors that exert critical roles in CpG island methylation of the PTEN promoter. As for cellular factors, the present study investigated the regulation of DNMT1, which links CpG methylation, modification of chromatin structure, and the subsequent gene silencing (25). On the other hand, as a viral factor, LMP2A has a unique NH2-terminal intracellular domain that is critical for the interaction with cellular proteins, such as tyrosine kinases (Syk and...
Lyn) and E3 protein-ubiquitin ligases (26, 27). Thus, LMP2A is a probable candidate molecule for eliciting the signaling pathway to mediate CpG island methylation of the host gene promoter. The present study firstly evaluated aberrant promoter methylation and expression loss of PTEN in cancer tissues of GC. Based on the concurrent occurrence of both abnormalities in EBV-associated GC, the underlying mechanism was investigated further using the recently characterized experimental systems, in which latency I pattern of EBV latent genesis faithfully reiterated in EBV-infected GC cell lines (28). The aim of the present study was to clarify how the host-virus interaction alters the epigenetics of the host cells, which leads to the neoplastic growth of stomach epithelial cells infected with EBV.

Materials and Methods

Materials. Protein expression was evaluated by immunohistochemistry in GC tissues, which were resected for the treatment of GC at Tokyo University Hospital between 1993 and 1997. Tissue microarray (TMA) was constructed from 10% formalin-fixed and paraffin-embedded blocks of GC tissues, as reported previously (28). All cases of GC were histologically diagnosed according to the Japanese Classification of Gastric Carcinoma (29) and Lauren's classification (30).

DNA was extracted from the sections of paraffin-embedded blocks of each GC tissue using a DNeasy Tissue kit (Qiagen) according to the manufacturer's instructions.

Immunohistochemistry and in situ hybridization. The presence of EBV in the carcinoma tissues was evaluated by in situ hybridization (ISH) targeting EBV-encoded small RNA (EBER-ISH) with an EBER-RNA probe (DakoCytomation). Immunohistochemical analyses of PTEN, phosphorylated signal transducer and activator of transcription 3 (pSTAT3), and DNMT1 were applied to TMA sections using an LSAB2 kit (Dako) with antibodies against PTEN (mouse monoclonal, clone 6H12; 1:100 dilution; Santa Cruz Biotechnology), pSTAT3 (Tyr705; rabbit polyclonal; 1:100 dilution; Cell Signaling Technology), and DNMT1 (rabbit polyclonal; 1:400 dilution; Santa Cruz Biotechnology). To obtain negative controls, the primary antibody was omitted. For criteria to evaluate the expression of these molecules, cytoplasmic positivity was seen in PTEN immunohistochemistry and nuclear positivity in pSTAT3 and DNMT1 immunohistochemistry. More than 10% positivity in carcinoma cells was considered as overexpression.

Cell lines, culture conditions, and reagents. The GC cell lines used in the study were MKN-1, MKN-7, TMK-1, and NU-GC-3. These cell lines were maintained in RPMI 1640 (Sigma-Aldrich) supplemented with 10% FCS (MP Biomedicals) at 37 °C in a 5% CO2 incubator.

EBV infection. Each GC cell line was infected with recombinant EBV using the cell-to-cell contact method (31, 32). Establishment of EBV infection was confirmed by EBER-ISH. After selection with G418 (700 µg/mL; Sigma-Aldrich), EBV-infected cells were maintained in bulk, but G418 was removed from the medium for EBV-infected GC cell lines 24 h before the experiments.

Methylation-specific PCR. The methylation status of the PTEN promoter region was determined by methylation-specific PCR (MSP) using bisulfite-modified DNA. The targets of the promoter regions were three sites (Fig. 1A): (A) (16), (B) (21), and (C) (14). The sequences of the methylated and unmethylated primer pairs were listed in Supplementary Table with other primer sequences used in the study.

DNA was modified by the bisulfite reaction using an EpiTect Bisulfite kit (Qiagen). Methylated and unmethylated genomic regions can be distinguished by PCR using sequence-specific pairs of primers. As a control, bisulfite-modified unmethylated/methylated DNA and no template control (distilled water) were included in each experiment. MSP experiments were performed at least in duplicate.

Bisulfite sequencing. To further evaluate the methylation status of site (C) in the PTEN gene promoter, bisulfite sequencing was used to analyze the frequency of CpG methylation, as described previously (10). The primers used in bisulfite sequencing were designed for the PTEN (C) region. Three clones each of MKN-1 cells with or without EBV infection were examined.

Western blot analysis. The cells were lysed in a lysis buffer consisting of 20 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 2 mmol/L EDTA, 1% NP-40, 50 mmol/L NaF, 1 mmol/L Na3VO4, 1 mmol/L phenylmethylsulfonyl fluoride, and protein inhibitor cocktail (Sigma-Aldrich). Typically, 20 µg of the protein were loaded per lane. The following primary antibodies were used: anti-PTEN (mouse monoclonal, clone A2B8; 1:100 dilution; Santa Cruz Biotechnology), anti-phosphorylated PTEN (pPTEN; rabbit polyclonal; 1:500 dilution; Cell Signaling Technology), anti-DNMT1 (goat polyclonal, clone N-16; 1:200 dilution; Santa Cruz Biotechnology), anti-STAT3 (rabbit polyclonal; 1:500 dilution; Cell Signaling Technology), anti-pSTAT3 (Tyr705; rabbit polyclonal; 1:50 dilution; Cell Signaling Technology), and anti-actin (goat polyclonal, clone 1-19; 1:200 dilution; Santa Cruz Biotechnology). After incubation with secondary antibodies, the antigen was detected using ECL Western Blotting Detection Reagents (Amersham).

Quantitative reverse transcription-PCR. Total RNA (1 µg) was reverse transcribed using a SuperScript III First-Strand Synthesis System for reverse transcription-PCR (RT-PCR; Invitrogen). PCR amplification was carried out using Platinum SYBR Green qPCR SuperMix DUG (Invitrogen) with specific primers as listed in Supplementary Table. Analysis was performed in triplicate.

Real-time quantitative MSP. Sodium bisulfite–treated genomic DNA was amplified using fluorescence-based real-time MSP (33) using Platinum SYBR Green qPCR SuperMix DUG (Invitrogen). Methylation of the PTEN gene was examined using actin as the internal control for DNA quantification. Actin contains no CpG dinucleotides and is not affected by DNA methylation status or sodium bisulfite treatment. The primers are listed in Supplementary Table. Bisulfite-treated in vitro methylated DNA (SssI methyltransferase, New England Biolabs) was used as a positive control. Each reaction was performed in triplicate.

Small interfering RNAs. Small interfering RNA (siRNA) sequences directed against STAT3 (S3) were purchased from Santa Cruz Biotechnology. A control siRNA (S'-GUCCCUGCCUGGUGCGAATCAAT'T-3', contS3) as described previously (28) was used. LMP2A-transfected MKN-1 cells were transfected with siRNAs S3 and contS3 at 5 mmol/L by transfection reagent HiPerFect (Qiagen) according to the manufacturer's instructions. Protein was collected at 72 h after each siRNA was transfected.

Plasmids and transfection. Each EBV latent gene, EBNA1, BARF0, EBER, LMP2A (LMP2A cDNA; gift from Prof. Paul J. Farrell, Imperial College, London, UK), was cloned into pcDNA3 containing FLAG tag. Each gene was confirmed by restriction analysis and DNA-sequencing. Each plasmid was transfected with LMP2A expression or control vector.

Cotransfection. Plasmid with LMP2A or FLAG tag was transfected together with siRNA-directed STAT3 (S3) into MKN-1 cell by using Lipofectamine 2000 (Invitrogen). Extraction of DNA, RNA, and proteins was performed at 72 h after transfection.

Interleukin-6 and interleukin-11 assay. The interleukin (IL)-6 and IL-11 levels of the cell culture supernatant were determined by using commercially available ELISA kit (R&D Systems) according to the manufacturer's instructions. Each measurement was performed in triplicate, and the average value was recorded (pg/mL).

Antibody neutralization. Gastric cell lines (MKN-1) with or without EBV infection were treated with 4 and 8 g/mL of IL-6 antibody (R&D Systems) for 1 h in a CO2 incubator.

Statistical analysis. Statistical analyses were performed using the χ^2 test, Fisher's exact test, or Mann-Whitney U test. Differences were considered significant in a two-tailed test for P < 0.05.

Results

PTEN methylation status and loss of PTEN expression in carcinoma tissue of surgically resected GC. MSP analysis of the
PTEN promoter was applied to DNA samples of the formalin-fixed and paraffin-embedded tissues of GC (Fig. 1A). The frequency of methylation of the PTEN gene promoter was 29%, 36%, and 64% for sites (A), (B), and (C) in EBV-associated GC, respectively, and 13%, 15%, and 30% in EBV-negative GC, respectively. The methylation of site (C) was the highest among three sites in both types of GC, and the methylation frequency of each site was significantly higher in EBV-associated GC than EBV-negative GC. The methylation of site (C) was observed in isolation or in combination with site (B) or sites (A) and (B) at nearly equal frequencies in EBV-associated GC.

Immunohistochemical staining of PTEN was applied to the sections of GC tissue specimens of GC, consisting of 13 EBV-associated and 8 EBV-negative GC. The frequencies of expression loss were significantly higher in EBV-associated GC than EBV-negative GC. Importantly, the proportion of expression loss was 67% (4 of 6) in the cases with methylation at site (C) in EBV-associated GC, whereas the rate was 7% (1 of 15) in EBV-negative GC (Fig. 1C). Methylation of sites (A) and (B) did not increase the proportion of PTEN expression loss in EBV-associated GC.

CpG island methylation and PTEN expression loss are reiterated in gastric cell lines with EBV infection. To further investigate the mechanisms underlying PTEN expression loss in EBV-associated GC, the present study screened whether these abnormalities were reiterated in GC cell lines with EBV infection. The EBV-infected GC cell lines were established by the coculture method, as applied to MKN-1, MKN-7, TMK-1, and NU-GC-3 cell lines. The methylation status of PTEN promoter site (C) was evaluated by MSP in these cell lines, with or without EBV infection, for equal passage numbers, which showed that CpG methylation occurred in two GC cell lines: EBV-MKN-1 and EBV-MKN-7 (Fig. 2A). The correlation between promoter methylation and protein down-regulation was further evaluated in both cell lines.

Figure 1. CpG island methylation of PTEN gene promoter and expression loss of PTEN in EBV-associated GC. A, MSP analysis of PTEN gene in GC with and without association of EBV. Top, map of the promoter region of PTEN gene. Horizontal lines, MSP regions studied [sites (A), (B), and (C)]; vertical bars, CpG sites. Gray box, exon 1; arrow, transcription start site. Middle, results of MSP analysis of PTEN gene in EBV-associated GC and EBV-negative GC. M and U, PCR products of methylated and unmethylated alleles, respectively; N, negative control; P, positive control. Bottom, frequencies of CpG methylation of PTEN gene promoter by MSP. B, immunohistochemical analysis of PTEN in GC. Loss (left) and retain (right) of PTEN expression in EBV-associated GC and EBV-negative GC, respectively. C, correlation between PTEN promoter methylation and PTEN expression loss in GC.
and NU-GC-3 (Fig. 2B). Methylation of sites (A) and (B) was observed in EBV-MKN-7, but the methylation of site (C) was the only common abnormality in both cell lines. The expression of pPTEN and total PTEN was negative for CpG promoter methylation and both showed similar level of PTEN protein expression. Site (C) of the PTEN promoter includes 19 CpG sites. Bisulfite sequencing of site (C) showed that almost all of the CpG sites were uniformly methylated in EBV-MKN-1 (Fig. 2C).

To further confirm that PTEN expression loss was caused by promoter methylation, GC cell lines MKN-1 and EBV-MKN-1 were incubated with 3 μmol/L 5-aza-2’-deoxycytidine. This treatment restored PTEN expression in EBV-MKN-1 (Fig. 2D).

LMP2A induces DNMT1 up-regulation and subsequent CpG methylation in EBV-infected GC cell lines. Constitutive or transient activation of DNMT is considered as a mechanism for CpG island methylation in human carcinoma. The expression of three representative DNMTs, DNMT1, DNMT3a, and DNMT3b, was then evaluated in MKN-1 and MKN-7 cell lines with or without EBV infection (Fig. 3A). Marked increase in DNMT1 expression was the common abnormality in both EBV-MKN-1 and EBV-MKN-7, although an increase in DNMT3b was also observed in EBV-MKN-7.

Table 1. Expression loss and promoter methylation of PTEN and overexpression of DNMT1 and pSTAT3 in GC with and without association of EBV

<table>
<thead>
<tr>
<th>PTEN immunohistochemistry P</th>
<th>PTEN methylation P</th>
<th>DNMT1 overexpression P</th>
<th>pSTAT3 overexpression P</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>12/16 &lt;0.001</td>
<td>18/10 &lt;0.001</td>
<td>23/5 &lt;0.001</td>
</tr>
<tr>
<td>Absent</td>
<td>9/78</td>
<td>26/61</td>
<td>36/51</td>
</tr>
<tr>
<td>Age (y)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥60</td>
<td>11/56 NS</td>
<td>21/46 NS</td>
<td>26/41 &lt;0.001</td>
</tr>
<tr>
<td>≤59</td>
<td>10/38</td>
<td>23/25</td>
<td>33/15</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>17/65 NS</td>
<td>32/50 NS</td>
<td>42/40 NS</td>
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<tr>
<td>Female</td>
<td>4/29</td>
<td>12/21</td>
<td>17/16</td>
</tr>
<tr>
<td>Locus*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper/middle</td>
<td>17/70 NS</td>
<td>37/50 &lt;0.05</td>
<td>48/39 NS</td>
</tr>
<tr>
<td>Lower</td>
<td>4/21</td>
<td>6/19</td>
<td>10/15</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early</td>
<td>5/48 &lt;0.05</td>
<td>17/36 NS</td>
<td>32/21 NS</td>
</tr>
<tr>
<td>Advanced</td>
<td>16/46</td>
<td>25/37</td>
<td>27/35</td>
</tr>
<tr>
<td>Histology†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intestinal</td>
<td>8/44 NS</td>
<td>20/32 NS</td>
<td>25/27 NS</td>
</tr>
<tr>
<td>Diffuse</td>
<td>13/50</td>
<td>24/39</td>
<td>34/29</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>10/32 NS</td>
<td>14/28 NS</td>
<td>16/26 &lt;0.05</td>
</tr>
<tr>
<td>Absent</td>
<td>11/62</td>
<td>30/43</td>
<td>42/30</td>
</tr>
</tbody>
</table>

Abbreviation: NS, no significant difference.

*Location of the stomach: upper, middle, and lower third of the stomach.
† Early: early carcinoma, in which carcinomatous invasion is restricted in the musosa and submucosa; advanced: advanced carcinoma, in which carcinoma invades beyond proper muscular layer.
‡ Histologic type of carcinoma: intestinal, intestinal type of histology of the carcinoma; diffuse, well to moderately differentiated type; diffuse, poorly differentiated type.

Viral gene expression in EBV-associated GC was restricted to EBNA1, BARF0, EBER, and LMP2A. When stable transfectants of the MKN-1 cell line with each gene were established, DNMT1 was expressed in the largest amount in LMP2A-transfected MKN-1 cells (Fig. 3B). Importantly, the constitutive overexpression of DNMT1 was accompanied by CpG methylation of the PTEN promoter and loss of PTEN expression in the transfected cells.

LMP2A-mediated DNMT1 overexpression was also observed in the transient expression system in MKN-1 cells (Fig. 3C). By quantitative RT-PCR, the mRNA expression level of the DNMT1 gene was shown to be increased 2-fold at 12 hours and 5-fold at 48 hours, and this level was sustained up to 72 hours after the transfection of LMP2A into MKN-1 cells. The mRNA expression levels of DNMT3a and DNMT3b in LMP2A gene–transfected MKN-1 cells remained at the same low level as that of control vector–transfected MKN-1 cells throughout the time course. When the amount of CpG methylation of the PTEN promoter was evaluated quantitatively (Fig. 3D), the methylated DNA was gradually elevated in MKN-1 cells up to 4.5-fold at 96 hours after transfection of the LMP2A gene. On the other hand, the increase in methylated DNA was up to 2-fold in MKN-1 cells transfected with a control vector.

LMP2A-induced STAT3 phosphorylation leads to DNMT1 overexpression. Because the promoter of the DNMT1 gene has a
STAT3 binding site (34), pSTAT3 is expected to move into the nucleus and to up-regulate DNMT1 expression. The level of total STAT3 and pSTAT3 protein in the nucleus was evaluated by immunoblotting in the stable transfectants of GC cell lines with LMP2A (LMP2A-MKN-1 and LMP2A-MKN-7; Fig. 4A). The levels of pSTAT3 (Tyr705) were significantly increased in LMP2A-MKN-1 and LMP2A-MKN-7. The amount of DNMT1 was also increased in the nuclear protein fraction of both GC cells.

In the transient transfection system as used in Fig. 3C and D, cotransfection of control siRNA (contSi) did not affect DNMT1 expression nor promoter methylation in MKN-1. On the other hand, knockdown of STAT3 by siRNA (S3Si, 5 nmol/L) counteracted the LMP2A-mediated up-regulation of DNMT1 (Fig. 4B).

To confirm the correlation between the phosphorylation of STAT3 and expression of DNMT1 in EBV-infected GC cell line, STAT3 was down-regulated with siRNA in EBV-MKN-1, which showed that the level of DNMT1 protein expression was decreased in parallel with the decrease of pSTAT3 and an increase in PTEN expression level (Fig. 4C).

To investigate the in vivo correlation between the phosphorylation of STAT3 and overexpression of DNMT1, immunohistochemistry of pSTAT3 and DNMT1 was applied to 115 clinical GC samples. As shown in Fig. 4D, pSTAT3 and DNMT1 were localized in the nuclei of carcinoma cells in 56 and 59 of 115 of GC cases (49% and 51%), respectively. In EBV-associated GC, pSTAT3(+)/DNMT1(+) cases were predominant (15 of 28, 54%), but pSTAT3(−)/DNMT1(+) cases were extremely rare (1 of 28, 4%). In EBV-negative GC, pSTAT3(+)DNMT1(+) cases were 30% (26 of 87), but pSTAT3(−)/DNMT1(−) cases were 46% (40 of 87). Thus, pSTAT3 and DNMT1 overexpression were closely correlated in GC with and without EBV infection, although much more frequently observed in EBV-associated GC (Table 1). Significant correlations other than the EBV infection were younger age and negative lymph node metastasis in both GC cases with DNMT1 and pSTAT3 overexpression.

LMP2A-induced STAT3 activation is independent of IL-6. It has been reported that IL-6 and IL-11 activate STAT3 (35). To investigate how LMP2A induces STAT3 activation, we examined IL-6 and IL-11 expression level in the culture supernatants (Fig. 5A). The IL-6 level was lower in EBV-infected MKN-1 compared with MKN-1, although the STAT3 phosphorylation level in EBV-infected MKN-1 was higher than that in MKN-1. There was no IL-11 expression in both MKN-1 and EBV-MKN-1. Antibody neutralization analysis for IL-6 (Fig. 5B) revealed that phosphorylation of STAT3 was reduced in MKN-1, whereas there was no change in the level of STAT3 phosphorylation in EBV-MKN-1.
Discussion

The carcinomas showing high activity of CpG island methylation are referred to showing CIMP, which is the most characteristic abnormality among genetic and epigenetic abnormalities that have been investigated in EBV-associated GC (8). In the present study, it was confirmed that PTEN abnormality represents this typical example. The methylation of site (C) of the PTEN promoter and the repression of PTEN were observed concurrently in EBV-associated GC but infrequently and without correlation in EBV-negative GC. The site (C), located at 5'-untranslated region (UTR) near the start codon, was the most frequent site of CpG methylation in the present study. It is reported that methylated sites in promoter CpG islands are mostly enriched in 300 bp to 1 kb upstream region of 5'-UTR and the first exon (36), and the fact is compatible with our result.

To clarify the mechanism of CpG island methylation, this study adopted an experimental system of EBV-infected GC cell lines (28) with a focus on the abnormality of PTEN expression, and it was shown that DNMT1 was markedly increased with EBV infection. Etoh and colleagues (37) observed that DNMT1 overexpression by immunohistochemistry was significantly correlated with the CIMP phenotype of GC in vivo and especially in EBV-associated GC. DNMT1 was recently suggested to play an essential role in aberrant de novo methylation in various carcinomas, some of which are associated with viral infection. Human papillomavirus-16 E7 stimulates the methyltransferase activity of DNMT1 through direct interaction (38). Hepatitis B virus X protein activates a positive circuit mechanism leading to E2F1-mediated DNMT1 up-regulation in hepatocellular carcinoma (39). In EBV-infected NPC, in addition to E2F1-DNMT1 up-regulation (24), EBV-LMP1 directly activates the DNMT1-P1 promoter via JNK-AP-1 pathway (23).

The expression of viral latency genes is determined according to the cell types and the host immune system. In latency I neoplasms, such as EBV-associated GC, the expression of viral latent genes is restricted to EBNA1, EBER, LMP2A, and BARF0 (1, 3, 40). There is no expression of LMP1, a transmembrane protein with a transforming capacity for rodent fibroblasts, in EBV-associated GC. LMP1 is the hallmark of latency II neoplasms, including NPC and Hodgkin’s lymphoma. In latency III neoplasms, EBNA2, EBNA3A, and EBNA3C are essential for immortalizing resting B lymphocytes and are additionally expressed due to the lack of effective immune mechanisms. Both EBV-associated GC and EBV-infected GC cell lines belong to the latency I group, in which LMP1 or EBNA2 is tightly repressed in neoplastic cells. In the present study, among the latent genes of the latency I
program, LMP2A was shown to be responsible for the up-regulation of DNMT1 in stomach epithelial cells by both stable and transient transfection systems. It has been shown that the expression of LMP2A is variable in infected cells. In fact, it was observed that all of the cases of EBV-associated GC expressed LMP2A but that the amount of LMP2A transcripts varies considerably in carcinoma tissues of surgically resected GC.4 However, even if LMP2A-induced DNMT1 overexpression fluctuates in its extent, DNMT1-mediated CpG island methylation can accumulate in host cell DNA, as shown in the transient transfection experiment.

The intracellular function of LMP2A and its modulation of intracellular signaling pathways have not yet been fully clarified in epithelial cells, particularly of the stomach. LMP2A affects cell growth and differentiation pathways in a human keratinocyte cell line, HaCaT, in part through activation of the phosphatidylinositol 3-kinase (PI3K)-Akt pathway (41). Downstream of the PI3K-Akt pathway, LMP2A is reported to stabilize β-catenin and activate the Wnt pathway in NPC cell lines (42). In addition, EBV uses LMP2A to activate the nuclear factor-κB-survivin pathway to rescue EBV-infected epithelial cells from serum deprivation in the present experimental system (28). Here, it was shown that LMP2A induces the phosphorylation of STAT3 and thus up-regulates DNMT1 in GC cells. Cotransfection experiments of LMP2A and siRNA of STAT3 suggested that LMP2A directly phosphorylates STAT3 rather than through autocrine IL-6 secretion (43, 44). STAT3 is a transcriptional factor, the constitutive activation of which leads to malignant transformation of various kinds of cells. STAT3 up-regulates a specific set of genes, which mediates cell proliferation (Egr-1, JunB, and cyclin D1), inhibits apoptosis (Mel-1, Bcl-2, Bcl-xl, and survivin), and promotes angiogenesis (VEGF; refs. 45–47). However, DNMT1 has also recently been shown as target of STAT3, which suggests that STAT3 can regulate gene transcription through epigenetic effects. Further studies are necessary to determine what extent the LMP2A-STAT3-DNMT1 pathway contributes to global CpG island methylation of EBV-associated GC in vivo and what conditions make it specific among other STAT3 targets.

pSTAT3 and DNMT1 expression levels were closely correlated even in EBV-negative GC in immunohistochemical studies. These findings suggest that there is a subgroup of GC with an activated STAT3-DNMT1 axis, which might correspond to CIMP-high GC in EBV-negative GC. Proximal to STAT3, IL-11 has been shown to

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4 R. Hino et al., unpublished data.
promote inflammation-associated tumorigenesis of the stomach through the cytokine receptor gp130 (35). *Helicobacter pylori* infection might affect STAT3 activity in epithelial cells through IL-6 from the inflammatory cells (48) and CagA-SHP-2 interaction in the infected cells (49). In the present study of EBV-associated GC, STAT3 activation was induced by LMP2A independent of IL-6 and IL-11 (Fig. 5C). Thus, STAT3 might be activated through several pathways in GC cells according to the pathogenetic agents or mechanisms. Further studies are also necessary to investigate the cross-talks among these pathways along the development of GC.

In conclusion, this study has shown that a latent viral protein, LMP2A, constitutively or transiently induces the phosphorylation of STAT3, which leads to the overexpression of DNMT1. The resultant CpG island methylation of the promoter of tumor suppressor genes, such as *PTEN*, may accumulate to overreach beyond the threshold of normal integrity of the stomach epithelial cells. It is also possible that DNMT1 overexpression is a kind of cellular defense to shut down viral protein expression through the methylation of viral DNA.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**Figure 5.** LMP2A-mediated signaling cascade leading to the promoter methylation of cancer-related genes, independent of IL-6 and IL-11. A, ELISA analysis of IL-6 and IL-11 in the culture supernatants in MKN-1 and EBV MKN-1. B, IL-6 antibody neutralization assay for STAT3 phosphorylation levels in MKN-1 and EBV-MKN-1. C, a model for LMP2A-mediated signaling cascade leading to the promoter methylation of cancer-related genes, independent of IL-6 and IL-11.

**References**


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Activation of DNA Methyltransferase 1 by EBV Latent Membrane Protein 2A Leads to Promoter Hypermethylation of PTEN Gene in Gastric Carcinoma

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