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

Rumi Hino,[1] Hiroshi Uozaki,[1] Noriko Murakami,[1] Tetsuo Ushiku,[1] Aya Shinozaki,[1] Shumpei Ishikawa,[1] Teppei Morikawa,[1] Takeo Nakaya,[1] Takashi Sakatani,[1] Kenzo Takada,[2] and Masashi Fukayama[1]

[1]Department of Pathology, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan and [2]Department of Tumor Virology, Institute for Genetic Medicine, Hokkaido University, Sapporo, Japan

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) up-regulated 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. [Cancer Res 2009;69(7):2766–74]

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 underlying 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 brain 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 NH_2-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 *Bam*HI 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 NH_2-terminal intracellular domain that is critical for the interaction with cellular proteins, such as tyrosine kinases (Syk and

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Masashi Fukayama, Department of Pathology, Graduate School of Medicine, The University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-0033, Japan. Phone: 81-3-5841-3341; Fax: 81-3-3815-8379; E-mail: mfukayama-tky@umin.ac.jp.

doi:10.1158/0008-5472.CAN-08-3070

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 genes is 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 6H2.1; 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% CO_2 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. 1*A*): (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 each 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[3] 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% NP40, 50 mmol/L NaF, 1 mmol/L Na_3VO_4, 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 A2B1; 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 I-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 (*Sss*I 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* (S3Si) were purchased from Santa Cruz Biotechnology. A control siRNA (5′-GUGCGCUGCUGGUGCCAACdTdT-3′, contSi) as described previously (28) was used. *LMP2A*-transfected MKN-1 cells were transfected with siRNAs S3Si and contSi at 5 nmol/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*, or *LMP2A* (LMP2A cDNA; gift from Prof. Paul J. Farrell, Imperial College, London, UK), was cloned into pcDNA3 containing FLAG tag. MKN-1 was transfected with each expression vector by lipofection. The expression of each gene was confirmed by RT-PCR. MKN-7 cells were also transfected with the LMP2A expression or control vector.

Cotransfection. Plasmid with LMP2A or FLAG tag was transfected together with siRNA-directed STAT3 (S3Si) 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 CO_2 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

[3] The primer sequences used in the study are presented in Supplementary Table.

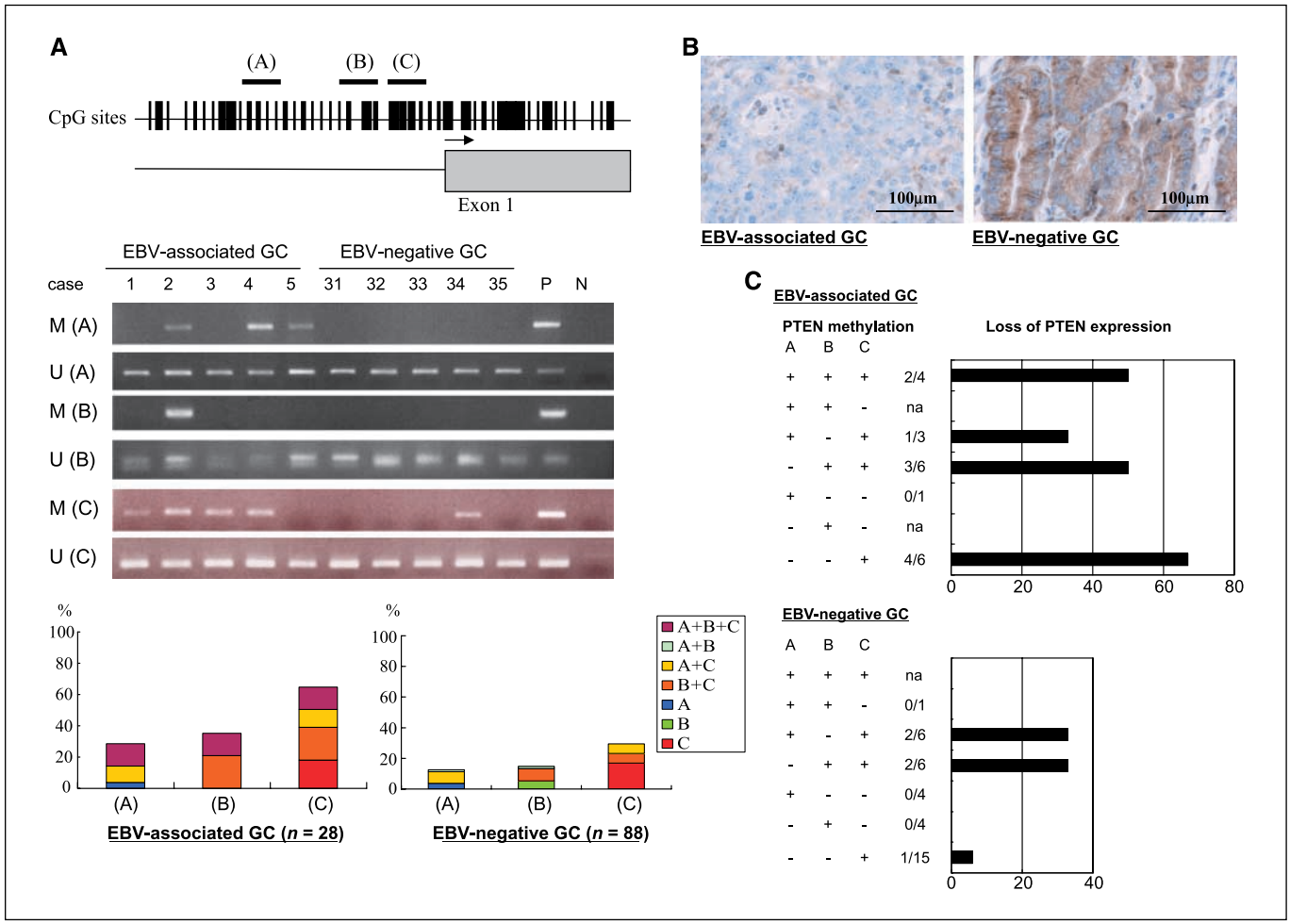

Figure 1. CpG island methylation of *PTEN* gene promoter and expression loss of PTEN in EBV-associated GC. *A,* MSP analysis of *PTEN* gene in GC with and without association of EBV. *Top,* map of the promoter region of *PTEN* gene. *Horizontal lines,* MSP regions studied [sites (A), (B), and (C)]; *vertical 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.

PTEN promoter was applied to DNA samples of the formalin-fixed and paraffin-embedded tissues of GC (Fig. 1*A*). 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 tissues (Fig. 1*B*). PTEN expression was lost in 21 of 116 tumor 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 (43%) than EBV-negative GC (10%; $P < 0.01$; Table 1).

As for the relationship between CpG island methylation of the *PTEN* promoter and protein expression of PTEN, the expression loss was only observed in cases showing the methylation of site (C) in any combination in both types of 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. 1*C*). 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. 2*A*). The correlation between promoter methylation and protein down-regulation was further evaluated in both cell lines

and NU-GC-3 (Fig. 2*B*). 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 similarly decreased in both EBV-MKN-1 and EBV-MKN-7. On the other hand, NU-GC-3 with or without EBV infection 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. 2*C*).

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. 2*D*).

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. 3*A*). 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.

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. 3*B*). 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. 3*C*). 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. 3*D*), 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

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

	PTEN immunohistochemistry	*P*	PTEN methylation	*P*	DNMT1 overexpression	*P*	pSTAT3 overexpression	*P*
	Loss/retain		+/−		+/−		+/−	
EBV								
Present	12/16	<0.001	18/10	<0.001	23/5	<0.001	19/9	<0.001
Absent	9/78		26/61		36/51		37/50	
Age (y)								
≥60	11/56	NS	21/46	NS	26/41	<0.001	27/40	<0.05
≤59	10/38		23/25		33/15		29/19	
Gender								
Male	17/65	NS	32/50	NS	42/40	NS	40/42	NS
Female	4/29		12/21		17/16		16/17	
Locus*								
Upper/middle	17/70	NS	37/50	<0.05	48/39	NS	46/41	NS
Lower	4/21		6/19		10/15		9/16	
Stage †								
Early	5/48	<0.05	17/36	NS	32/21	NS	31/22	NS
Advanced	16/46		25/37		27/35		25/37	
Histology ‡								
Intestinal	8/44	NS	20/32	NS	25/27	NS	26/26	NS
Diffuse	13/50		24/39		34/29		32/33	
Lymph node metastasis								
Present	10/32	NS	14/28	NS	16/26	<0.05	12/31	<0.001
Absent	11/62		30/43		42/30		44/28	

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.

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. 4*A*). 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. 3*C* 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. 4*B*).

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. 4*C*).

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. 4*D*, 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 levels in the culture supernatants (Fig. 5*A*). 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. 5*B*) 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.

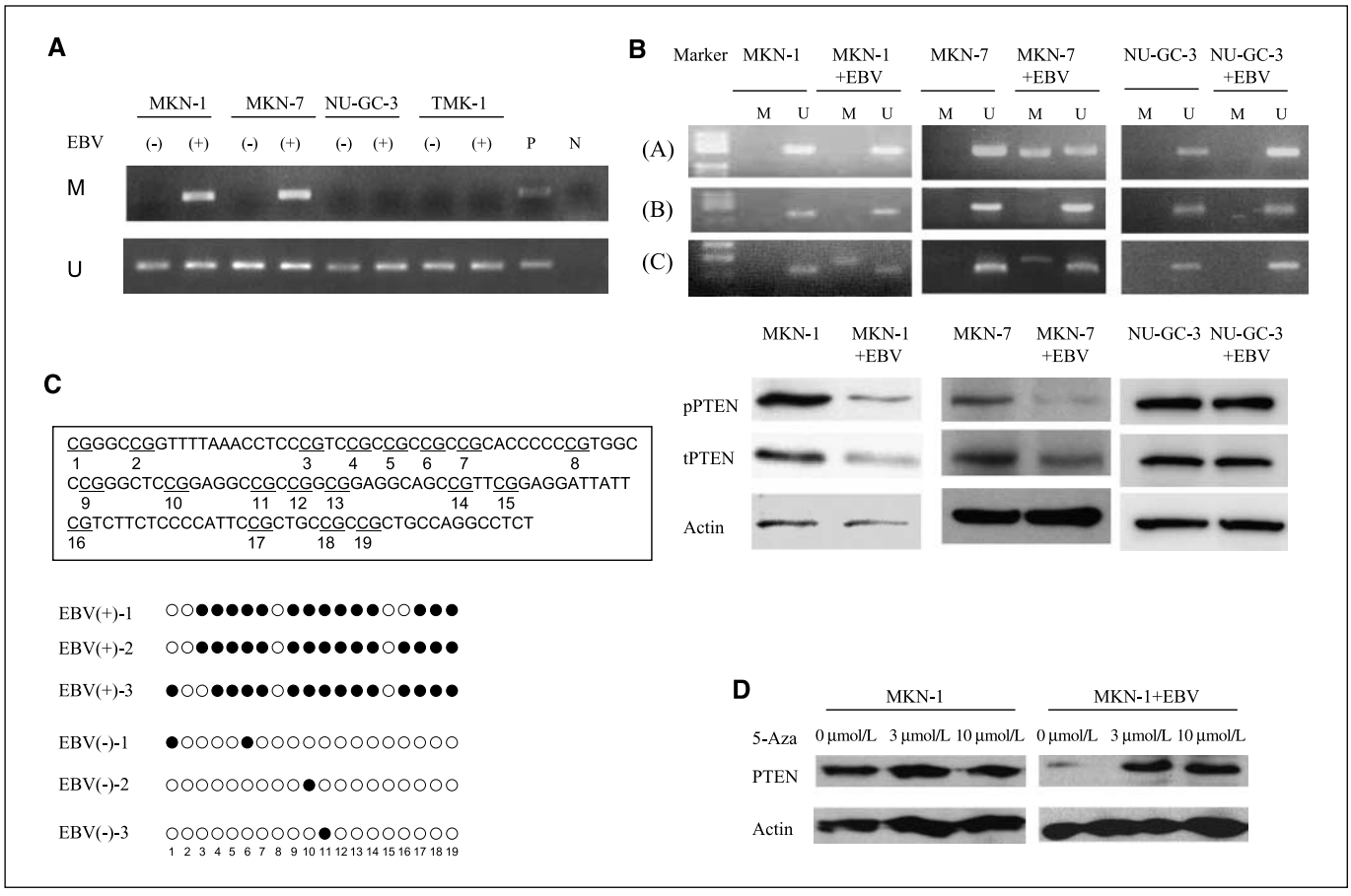

Figure 2. CpG island methylation of *PTEN* gene promoter and expression loss of PTEN in EBV-infected GC cell lines. *A,* MSP analysis of the site (C) of *PTEN* promoter in GC cell lines (MKN-1, MKN-7, NU-GC-3, and TMK-1) with and without infection of the recombinant EBV. *B,* methylation status of *PTEN* promoter and PTEN expression loss in MKN-1, MKN-7, and NU-GC-3 with EBV infection. *Top,* MSP analysis of the sites (A), (B), and (C) of *PTEN* promoter in GC cell lines; *bottom,* Western blot of PTEN and pPTEN in these GC cell lines. *C,* bisulfite sequencing evaluation of CpG island methylation of the site (C) of *PTEN* promoter in EBV-MKN-1 and MKN-1. *Top,* location of 19 CpG sites in the site (C), −404 to −198 relative to the start codon of the *PTEN* gene. *Open circles,* unmethylated CpG; *closed circles,* methylated CpG. *D,* treatment with 5-aza-2′-deoxycytidine (*5-Aza*) restoring PTEN expression in EBV-MKN-1.

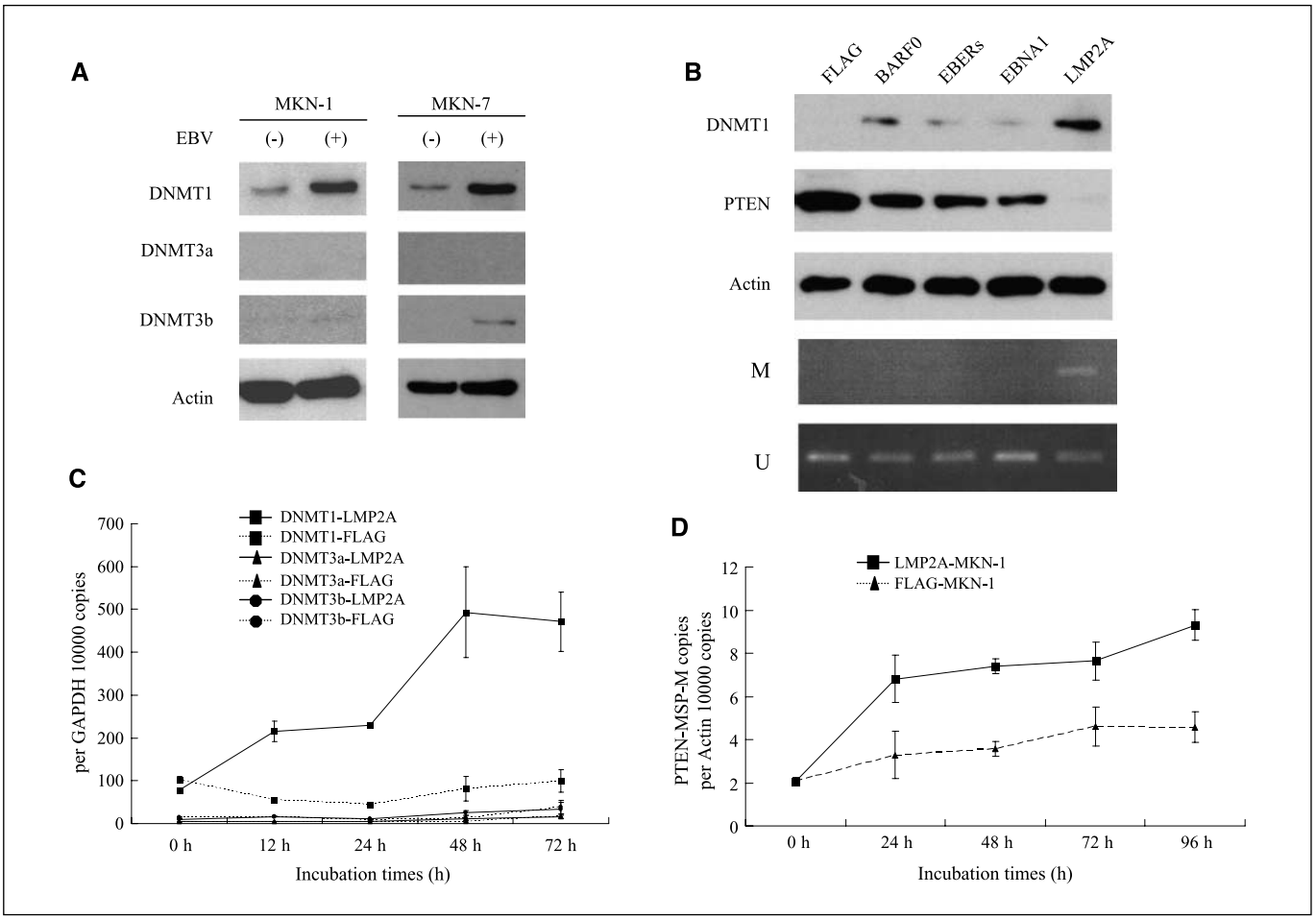

Figure 3. LMP2A-induced DNMT1 is responsible for *PTEN* promoter methylation in EBV-infected GC cell lines. *A,* immunoblot analysis of DNMT1, DNMT3a, and DNMT3b in MKN-1 and MKN-7 with EBV infection. *B,* Western blot analysis of DNMT1 and PTEN, and MSP analysis of *PTEN* promoter methylation in MKN-1 transfected with LMP2A. *C,* quantitative real-time analysis of DNMT1, DNMT3a, and DNMT3b in MKN-1, induced by transient transfection of *LMP2A* gene. *D,* quantitative MSP analysis of DNA methylation of *PTEN* promoter site (C) in MKN-1, induced by transient transfection of *LMP2A* gene.

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

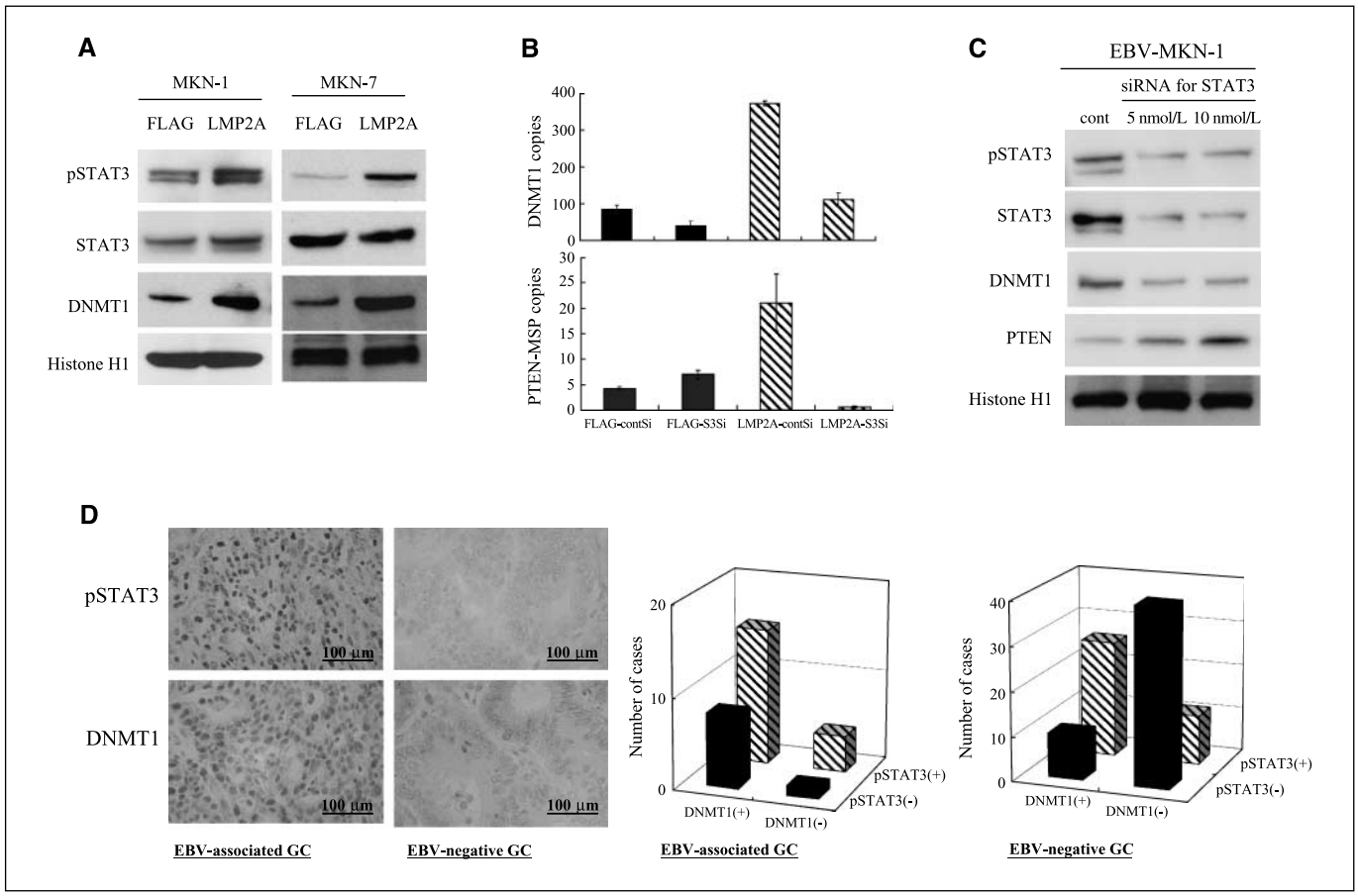

Figure 4. Constitutive phosphorylation of STAT3 up-regulates DNMT1 in LMP2A-expressing, MKN-1–infected and EBV-infected GC *in vitro* and *in vivo*. *A,* immunoblot analysis of nuclear pSTAT3 and DNMT1 expression in MKN-1 and MKN-7 stably transfected with LMP2A (LMP2A-MKN-1 and LMP2A-MKN-7). *B,* cotransfection analysis of *STAT3*-siRNA and LMP2A in MKN-1, suppressing LMP2A-induced *PTEN* promoter methylation. Knockdown of STAT3 by siRNA (S3Si, 10 nmol/L) counteracts up-regulation of DNMT1, preventing the promoter methylation. *C,* immunoblot analysis of nuclear protein extracts of EBV-MKN-1 with or without siRNA of STAT3. *D,* immunohistochemical analysis of pSTAT3 and DNMT1 in GC *in vivo*. The frequencies of immunoreactivities for pSTAT3 and DNMT1 in EBV-associated GC and EBV-negative GC *in vivo* show close correlation between pSTAT3 and DNMT1 expression.

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 (*Mcl-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

[4] R. Hino et al., unpublished data.

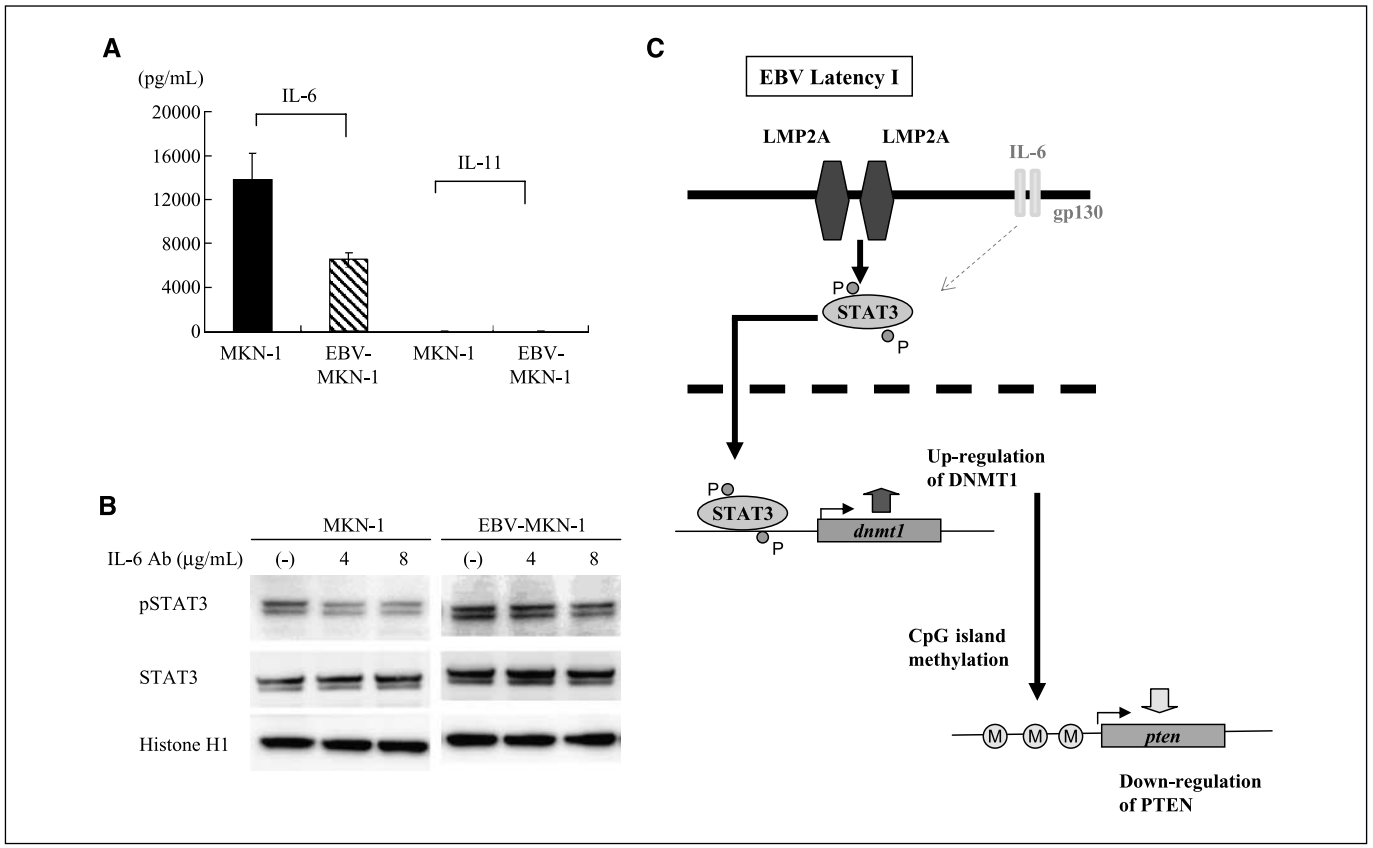

Figure 5. LMP2A-mediated signaling cascade leading to the promoter methylation of cancer-related genes, independent of IL-6 and IL-11. *A,* ELISA analysis of IL-6 and IL-11 in the culture supernatants in MKN-1 and EBV MKN-1. *B,* IL-6 antibody neutralization assay for STAT3 phosphorylation levels in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

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. 5*C*). 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.

Acknowledgments

Received 8/12/08; revised 12/28/08; accepted 1/8/09; published OnlineFirst 3/10/09.

Grant support: Grants-in-Aid for Scientific Research on Priority Areas (20249022) from the Ministry of Education, Science, Sports, and Culture of Japan.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Yukiko Watanabe (Niigata University) and Katsuhiko Hashimoto (University of Tokyo) for technical support.

References

1. Fukayama M, Hayashi Y, Iyasaki Y, et al. Epstein-Barr virus-associated gastric carcinoma and Epstein-Barr virus infection of the stomach. Lab Invest 1994; 71:73–81.
2. Fukayama M, Chong JM, Kaizaki Y. Epstein-Barr virus and gastric carcinoma. Gastric Cancer 1998;1:104–14.
3. Fukayama M, Chong JM, Uozaki H. Pathology and molecular pathology of Epstein-Barr virus-associated gastric carcinoma. Curr Top Microb Immunol 2001;258: 91–102.
4. Kaizaki Y, Sakurai S, Chong JM, Fukayama M. Atrophic gastritis, Epstein-Barr virus infection, and Epstein-Barr virus-associated gastric carcinoma. Gastric Cancer 1999; 2:101–8.
5. Iwasaki Y, Chong JM, Fukayama M, et al. Establishment and characterization of a human Epstein-Barr virus-associated gastric carcinoma in SCID mice. J Virol 1989;72:8321–6.
6. Tokunaga M, Uemura Y, Okamura A, et al. Epstein-Barr virus related gastric cancer in Japan: a molecular patho-epidemiological study. Acta Pathol Jpn 1993;43: 574–81.

7. Fukayama M, Hino R, Uozaki H. Epstein-Barr virus and gastric carcinoma: virus-host interactions leading to carcinoma. Cancer Sci 2008;99:1726–33.
8. Chang MS, Uozaki H, Fukayama M, et al. CpG island methylation status in gastric carcinoma with and without infection of Epstein-Barr virus. Clin Cancer Res 2006;12:2995–3002.
9. Sakuma K, Chong JM, Fukayama M, et al. High-density methylation of p14ARF and p16INK4A in Epstein-Barr virus-associated gastric carcinoma. Int J Cancer 2004; 112:273–8.
10. Ushiku T, Chong JM, Fukayama M, et al. p73 gene promoter methylation in Epstein-Barr virus-associated gastric carcinoma. Int J Cancer 2006;120:60–6.
11. Sudo M, Chong JM, Fukayama M, et al. Promoter hypermethylation of E-cadherin and its abnormal expression in Epstein-Barr virus-associated gastric carcinoma. Int J Cancer 2004;109:194–9.
12. Li J, Yen C, Liaw D, et al. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science 1997;275:1943–7.
13. Isabelle S, William RS. The biology and clinical relevance of the PTEN tumor suppressor pathway. J Clin Oncol 2004;22:2954–63.
14. Salvesen HB, MacDonald N, Das S, et al. PTEN methylation is associated with advanced stage and microsatellite instability in endometrial carcinoma. Int J Cancer 2001;91:22–6.
15. Mirmohammadsadegh A, Marini A, Hengge UR, et al. Epigenetic silencing of the PTEN gene in melanoma. Cancer Res 2006;66:6546–52.
16. Soria JC, Lee HY, Khuri FR, et al. Lack of PTEN expression in non-small cell lung cancer could be related to promoter methylation. Clin Cancer Res 2002; 8:1178–84.
17. Roman-Gomez J, Jimenez-Velasco A, Torres A, et al. Promoter hypermethylation of cancer-related genes: a strong independent prognostic factor in acute lymphoblastic leukemia. Blood 2004;104:2492–8.
18. Wiencke JK, Zheng S, Stokoe D, et al. Methylation of the PTEN promoter defines low-grade gliomas and secondary glioblastoma. Neuro Oncol 2007;9:271–9.
19. Cairns P, Okami K, Sidransky D, et al. Frequent inactivation of PTEN/MMAC1 in primary prostate cancer. Cancer Res 1997;57:4997–5000.
20. Kang GH, Lee S, Ro JY, et al. Epstein-Barr virus-positive gastric carcinoma demonstrates frequent aberrant methylation of multiple genes and constitutes CpG island methylator phenotype-positive gastric carcinoma. Am J Pathol 2002;160:787–94.
21. Furuta J, Umebayashi Y, Ushijima T, et al. Promoter methylation profiling of 30 genes in human malignant melanoma. Cancer Sci 2004;95:962–8.
22. Kang YH, Lee HS, Kim WH. Promoter methylation and silencing of PTEN in gastric carcinoma. Lab Invest 2002;82:285–91.
23. Tsai CL, Li HP, Chang YS, et al. Activation of DNA methyltransferase 1 by EBV LMP1 involves c-Jun NH(2)-terminal kinase signaling. Cancer Res 2006;66: 11668–76.
24. Seo SY, Kim EO, Jang KL. Epstein-Barr virus latent membrane protein 1 suppresses the growth-inhibitory effect of retinoic acid by inhibiting retinoic acid receptor-β2 expression via DNA methylation. Cancer Lett 2008;270:66–76.
25. Kimura H, Shiota K. Methyl-CpG-binding protein, MeCP2, is a target molecule for maintenance DNA methyltransferase, Dnmt1. J Biol Chem 2003;278: 4806–12.
26. Fukuda M, Longnecker R. Epstein-Barr virus (EBV) latent membrane protein 2A regulates B-cell receptor-induced apoptosis and EBV reactivation through tyrosine phosphorylation. J Virol 2005;79:8655–60.
27. Winberg G, Matskova L, Pawson T, et al. Latent membrane protein 2A of Epstein-Barr virus binds WW domain E3 protein-ubiquitin ligases that ubiquitinate B-cell tyrosine kinases. Mol Cell Biol 2000;20:8526–35.
28. Hino R, Uozaki H, Fukayama M, et al. Survival advantage of EBV-associated gastric carcinoma: survivin up-regulation by viral latent membrane protein 2A. Cancer Res 2008;68:1427–35.
29. Japanese Gastric Cancer Association. The Japanese Research Society for Gastric Cancer: Japanese classification of gastric carcinoma. 2nd English ed. Gastric Cancer 1998;1:10–24.
30. Lauren P. The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. An attempt at a histo-clinical classification. Acta Pathol Microbiol Scand 1965;64:31–49.
31. Imai S, Nishikawa J, Takada K. Cell-to-cell contact as an efficient mode of Epstein-Barr virus infection of diverse human epithelial cells. J Virol 1998;72:4371–8.
32. Shimizu N, Yoshiyama H, Takada K. Clonal propagation of Epstein-Barr virus (EBV) recombinants in EBV-negative Akata cells. J Virol 1996;70:7260–3.
33. Robert TP, Sheng ZM, O'Leary TJ, et al. Methylation profiling of mesothelioma using real-time methylation-specific PCR. Diagn Cytopathol 2007;35:498–502.
34. Zhang Q, Wang HY, Woetmann A, Raghunath PN, Odum N, Wasik MA. STAT3 induces transcription of the DNA methyltransferase 1 gene (DNMT1) in malignant T lymphocytes. Blood 2006;108:1058–64.
35. Judd LM, Bredin K, Kalantzis A, Jenkins BJ, Ernst M, Giraud AS. STAT3 activation regulates growth, inflammation, and vascularization in a mouse model of gastric tumorigenesis. Gastroenterology 2006;131:1073–85.
36. Hayashi H, Nagae G, Aburatani H, et al. High-resolution mapping of DNA methylation in human genome using oligonucleotide tiling array. Hum Genet 2007;120:701–11.
37. Etoh T, Kanai Y, Hirohashi S, et al. Increased DNA methyltransferase 1 (DNMT1) protein expression correlates significantly with poorer tumor differentiation and frequent DNA hypermethylation of multiple CpG islands in gastric cancers. Am J Pathol 2004;164: 689–99.
38. Burgers WA, Blanchon L, Pradhan S, de Launoit Y, Kouzarides T, Fuks F. Viral oncoproteins target the DNA methyltransferases 1. Oncogene 2007;26:1650–5.
39. Jung JK, Arora P, Pagano JS, Jang KL. Expression of DNA methyltransferase 1 is activated by hepatitis B virus X protein via a regulatory circuit involving the p16INK4a-cyclin D1-CDK 4/6-pRb-E2F1 pathway. Cancer Res 2007;67:5771–8.
40. Oh ST, Cha JH, Shin DJ, Yoon SK, Lee SK. Establishment and characterization of an *in vivo* model for Epstein-Barr virus positive gastric carcinoma. J Med Virol 2007;79:1343–8.
41. Scholle F, Bendt KM, Raab-Traub N. Epstein-Barr virus LMP2A transforms epithelial cells, inhibits cell differentiation, and activates Akt. J Virol 2000;74: 10681–9.
42. Morrison JA, Raab-Traub N. Roles of the ITAM and PY motifs of Epstein-Barr virus latent membrane protein 2A in the inhibition of epithelial cell differentiation and activation of β-catenin signaling. J Virol 2005;7:2375–82.
43. Stewart S, Dawson CW, Young LS, et al. Epstein-Barr virus-encoded LMP2A regulates viral and cellular gene expression by modulation of the NF-κB transcription factor pathway. Proc Natl Acad Sci U S A 2004;101: 15730–5.
44. Buettner M, Haussinger N, Neidobitek G. Expression of Epstein-Barr virus (EBV)-encoded latent membrane proteins and STAT3 activation in nasopharyngeal carcinoma. Virchows Arch 2006;449:513–9.
45. Yu H, Jove R. The STATs of cancer—new molecular targets come of age. Nat Rev Cancer 2004;4:97–105.
46. Bromberg JF, Wrzeszczynska MH, Darnell JE, Jr., et al. Stat3 as an oncogene. Cell 1999;98:295–303.
47. Frank DA. STAT3 as a central mediator of neoplastic cellular transformation. Cancer Lett 2007;251:199–210.
48. Jackson CB, Judd LM, Giraud AS, et al. Augmented gp130-mediated cytokine signalling accompanies human gastric cancer progression. J Pathol 2007;213: 140–51.
49. Hatakeyama M. Oncogenic mechanisms of the *Helicobacter pylori* CagA protein. Nat Rev Cancer 2004; 4:688–94.