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Hypermethylation of the interferon regulatory factor 5 promoter in Epstein-Barr virus-associated gastric carcinoma[§]

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Interferon regulatory factor-5 (IRF-5), a member of the mammalian IRF transcription factor family, is regulated by p53, type I interferon and virus infection. IRF-5 participates in virus-induced TLR-mediated innate immune responses and may play a role as a tumor suppressor. It was suppressed in various EBV-infected transformed cells, thus it is valuable to identify the suppression mechanism. We focused on a promoter CpG islands methylation, a kind of epigenetic regulation in EBV-associated Burkitt's lymphomas (BLs) and gastric carcinomas. IRF-5 is not detected in most of EBV-infected BL cell lines due to hypermethylation of *IRF-5* distal promoter (promoter-A), which was restored by a demethylating agent, 5-aza-2′-deoxycytidine. Hypomethylation of CpG islands in promoter-A was observed only in EBV type III latent infected BL cell lines (LCL and Mutu III). Similarly, during EBV infection to Akata-4E3 cells, IRF-5 was observed at early time periods (2 days to 8 weeks), concomitant unmethylation of promoter-A, but suppressed in later infection periods as observed in latency I BL cell lines. Moreover, hypermethylation in *IRF-5* promoter-A region was also observed in EBV-associated gastric carcinoma (EBVaGC) cell lines or primary gastric carcinoma tissues, which show type I latent infection. In summary, IRF-5 is suppressed by hypermethylation of its promoter-A in most of EBV-infected transformed cells, especially BLs and EBVaGC. EBV-induced carcinogenesis takes an advantage of proliferative effects of TLR signaling, while limiting IRF-5 mediated negative effects in the establishment of EBVaGCs.

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Introduction

The interferon regulatory factors (IRFs) have important roles in innate immunity by participating in the immediate-early infection response as well as in the secondary response to cytokines (Taniguchi *et al.*, 2001; Barnes *et al.*, 2002). Although IRFs have roles in the antiviral immune response, they also participate in cell growth regulation and apoptosis. IRF-5, a member of the IRF family, was originally identified as a regulator of type I interferon (IFN) gene expression. IRF-5 has functions in anti-viral defense as well as in cell cycle arrest and apoptosis (Barnes *et al.*, 2003; Takaoka *et al.*, 2005).

Epstein-Barr virus (EBV) is a ubiquitous infectious agent that was firstly identified in Burkitt's lymphomas (BLs) (Epstein, 2001). EBV infects more than 90% of the human population, but EBV-associated malignant neoplasms are developed in a limited number of EBV-infected individuals. EBV contributes to oncogenesis as evidenced by its frequent detection in various types of B-cell lymphoma, including BL, post-transplantation lymphoproliferative disorder, Hodgkin's disease as well as in epithelial cell malignancies such as nasopharyngeal carcinoma and gastric carcinomas (Thompson and Kurzrock, 2004; Young and Rickinson, 2004). Martin *et al.* (2007) reported that upregulation of IFN-stimulated genes is driven by the interaction between EBV and B cells. Among the upregulated cellular genes were Toll-like receptor 7 (TLR7) and myeloid differentiation factor 88 (MyD88), a key participant in TLR signal transduction. Signaling by the TLR has emerged as a key oncogenic mechanism in the inflammation-related cancers (Kennedy *et al.*, 2014). Since IRF-5 is a downstream effector of TLR7, EBV infection modulates IRF-5 expression and activity at several levels (Martin *et al.*, 2007).

EBV-associated gastric carcinoma (EBVaGC) represents approximately 10% of total gastric carcinomas (Uozaki and Fukayama, 2008). EBVaGCs show monoclonal growth and EBV latency I infection, but it is not clear how EBV influences cancer progression (Fukayama and Ushiku, 2011). EBVaGC exhibits lymphoepithelioma-like phenotype with mixed inflammatory cells and global DNA methylation of the promoter regions of various cancer-associated genes (Sudo *et al.*, 2004; Kang *et al.*, 2008). TLR signal transduction as well as CpG islands' hypermethylation-induced repression of tumor suppressor genes play a role in the carcinogenesis of EBV-associated tumors (Uozaki and Fukayama, 2008; Hino *et al.*, 2009), which supports the notion that EBV-positive gastric cancers should be considered as a separate clinical

entity (Wang *et al.*, 2014). Further research is needed to discern the mechanisms during EBV-associated carcinogenesis.

In this report, we examined an epigenetic regulation of the human IRF-5 gene in BLs and EBVaGCs. We firstly evaluated with EBV positive BL cell lines. *IRF-5* expression was regulated by hypermethylation of the distal promoter region (promoter-A). Hypermethylation of CpG islands in promoter-A was observed in most of BL cell lines, which was restored by a demethylating agent, 5-aza-2′-deoxycytidine (referred to as 5-Aza). IRF-5 expression was observed only in EBV latency III BL cell lines (LCL and Mutu III). Similarly, IRF-5 was observed at early time periods (2 days to 8 weeks) during EBV infection to Akata-4E3 cells, but it was suppressed in later infection periods as observed in latency I BL cell lines. In line with the finding in EBV-BL cell lines, EBVaGC cell lines showed a hypermethylation of *IRF-5* promoter-A. In human gastric carcinomas, *IRF-5* hypermethylation is common in EBVaGCs and is a distinctive feature compared to EBV-negative gastric carcinoma.

Materials and Methods

Sample preparation

The Institutional Review Board of the College of Medicine and Severance Hospital Authority of the Yonsei University approved the use of archival gastric carcinoma tissues without the informed consent according to the international guidelines as applied previously (Wang *et al.*, 2011). Gastric carcinoma tissues and paired unaffected gastric tissues were collected from gastrectomy specimens from Severance Hospital, Yonsei University. DNA was extracted from tumor tissues which were confirmed to be greater than 80% in tumor content and paired unaffected gastric tissues which were confirmed to be tumor free. Genomic DNA was extracted using QIAGEN kits according to the manufacturer's instructions, then submitted for methylation-specific PCR (MSP) and bisulfite sequencing. The presence of EBV in cancer tissues was analyzed by EBV-encoded RNA *in situ* hybridization as described previously (Chong *et al.*, 2002; Kang *et al.*, 2008)

Cell lines

SNU-719, EBV-positive gastric carcinoma cell line was obtained from the Korea Cell Line Bank (Korea). AGS and AGS-BX-1 were kindly gifted from L. Hutt-Fletcher (Borza and Hutt-Fletcher, 2002). BL cell lines (Akata, Akata-4E3, Akata-BX1, Mutu I, Mutu III, and Rael) and LCL-1 cells (Martin *et al.*, 2007) were maintained RPMI-1640 supplemented with 10% heat-inactivated FBS (Hyclone) at 37°C in a humidified CO_2 incubator. SNU-719, AGS and AGS-BX-1 were also maintained as BL cell lines.

EBV harvest and infection

EBV green fluorescent protein (GFP)-BX1 virus was obtained by treating Akata-BX1 cells with 50 μg/ml anti-IgG (Cappel). Virus supernatant, which was harvested from 3 days to 1 week, was concentrated by using Centricon Plus-80 filters (Millipore). Akata-4E3 cells were infected with concentrated virus for 3 h and were cultured in RPMI medium 1640 plus 10% FCS.

Reverse transcription PCR (RT-PCR)

RNA was extracted from EBV-infected BL cells using the RNeasy kit (QIAGEN) and treated with DNase using the DNA-free kit (Ambion). Reverse transcription was performed using avian myeloblastosis virus reverse transcriptase (Promega). The amount of cDNA added to each PCR mixture was normalized relative to GAPDH levels. PCR was performed using PCR Master Mix (Promega) or Platinum PCR Master Mix (Invitrogen). The primers used were: *IRF-5* (sense, 5′-AATTATTCTGCATCCCCTGG-3′; antisense, 5′-GCTCCAGGACCTCAGAGAGA-3′), *LMP1* (sense, 5′-GTGATTCTGACGAAGCCAGAG-3′; antisense, 5′-CGTGGGGCGCCCCAGGCACCA-3′), *LMP2A* (sense, 5′-TGACGGGTTTCCAAGACTATCC-3′; antisense, 5′-GTGATTCTGACGAAGCCAGAG-3′), and *EBNA2* (sense, 5′-AGAGGAGGTGGTAAGCGGTTC-3′; antisense, 5′-TGACGGGTTTCCAAGACTATCC-3′) (Kempkes *et al.*, 1995). The internal control was *GAPDH* cDNA amplified by RT-PCR using the following specific primers: (sense, 5′-CATGACAACTTTGGTATCGTG-3′; antisense, 5′-GTGTCGCTGTTGAAGTCGTCAGA-3′).

5-Aza-2′-deoxycytidine (5-Aza) treatment

Cells were plated at a density of 1×10^6 cells/150-mm dish and treated with freshly prepared 10 μM 5-Aza (Sigma Chemical Co.) for 24 h. RNA extraction was performed using RNeasy kits (QIAGEN). Two independent 5-Aza treatment experiments were performed.

Methylation-specific PCR (MSP) analyses

Genomic DNA (2 μg) was modified using EZ DNA methylation-direct kits (Zymo Research). The primer sequences for MSP analyses were as follows: 5′-AGTAGTAGTTGTTTAGGGGTGG-3′ (forward) and 5′-CCTAAACTTAAAAACAATAACCAAC-3′ (reverse) for unmethylated *IRF-5* promoter and 5′-GGTATCGCGTCGTTTGGTAT-3′ (forward) and 5′-GAACACTTCCGCGTCTTACC-3′ (reverse) for methylated *IRF-5* promoter. The 20 μl reaction mixture contained 2 μl modified DNA, 0.2 μM each primer, 0.2 μM dNTPs, 1 unit Taq polymerase (TaKaRa) and 2× GC buffer (TaKaRa). PCR reactions were performed at 95°C for 5 min, followed by 35 cycles of 95°C for 45 sec, 65°C for 45 sec and 72°C for 45 sec, with a 10 min final extension at 72°C. PCR products were analyzed on 2.5% agarose gels stained with ethidium bromide and visualized by ultraviolet illumination.

Bisulfite sequencing

For bisulfite sequencing, we used the same DNAs as for the methylation-specific PCR analyses. The primer sequences for bisulfite sequencing of *IRF-5* promoter A were as follows: A-1, 5′-TATTTATGAGAAGGAATAGGAGGTG-3′ (A-1 forward) and 5′-AATCACTCTATACCTTTCTCATCCTC-3′ (A-1 reverse), and A-2, 5′-ATTTTAGATTGTTAAAAGAGTTAG-3′ (A-2 forward) and 5′-TATAATCCAAACCAACCTCCAACG-3′ (A-2 reverse). The 20 μl reaction mixture

contained 2 μl modified DNA, 0.2 μM each primer, 0.2 μM dNTPs, 1 unit Taq polymerase (TaKaRa) and 2× GC buffer (TaKaRa). The resulting amplification pools were cloned into the pCR II vector using the TA cloning kit (Invitrogen). Ten individual clones per PCR reaction were isolated and sequenced. The clones were sequenced using both T7 and Sp6 primers.

Results

IRF-5 expression is regulated by promoter CpG islands methylation, which was influenced by latency of EBV infection

IRF-5 expression was examined in BL cell lines, most of which were already known to show EBV type I latent infection (Klein *et al.*, 2007). RT-PCR for the *IRF-5* transcript revealed that *IRF-5* was not present or was barely detected in most of EBV BL cell lines (Mutu I, Rael, Akata, and Akata-BX1), but it was detected only in the latency III BL cell lines (LCL-1 and Mutu III) (Fig. 1A). Two discrete promoters of *IRF-5* including promoter-A and promoter-B regulate the expression of alternative spliced *IRF-5* isoforms (Martin *et al.*, 2007). We observed dense CpG islands around promoter-A region by MethPrimer tool (Fig. 1B) (Li and Dahiya, 2002). To determine whether latency-specific *IRF-5* expression or suppression is associated with methylation status of CpG islands, MSP analysis was performed (Fig. 1C). Target region of promoter-A was methylated in *IRF-5* negative cells (Akata, Akata-4E3, and Akata-BX1) and was unmethylated in *IRF-5* positive cells (LCL-1 and Mutu III). IRF5 promoter-A is partially methylated in Mutu I and Rael cells, which were shown to have weak IRF-5 expression (Fig. 1C). To confirm that EBV latency influences methylation status in *IRF-5* promoter-A region, we observed IRF-5 expression and methylation status during EBV infection to Akata-4E3 cells, EBV-negative BL cell line, which showed latency III or latency I in early or late infection periods, respectively (Supplementary data Fig. S1A). Interestingly, *IRF-5* transcripts were observed only in early infection period (Supplementary data Fig. S1B). Moreover, unmethylation of promoter-A region was observed at the same period (Supplementary data Fig. S1C). IRF-5 was gradually decreased and promoter methylation was increased as time passed to late period (Supplementary data Fig. S1B and C). These were in line with the previous findings in EBV BL cell lines.

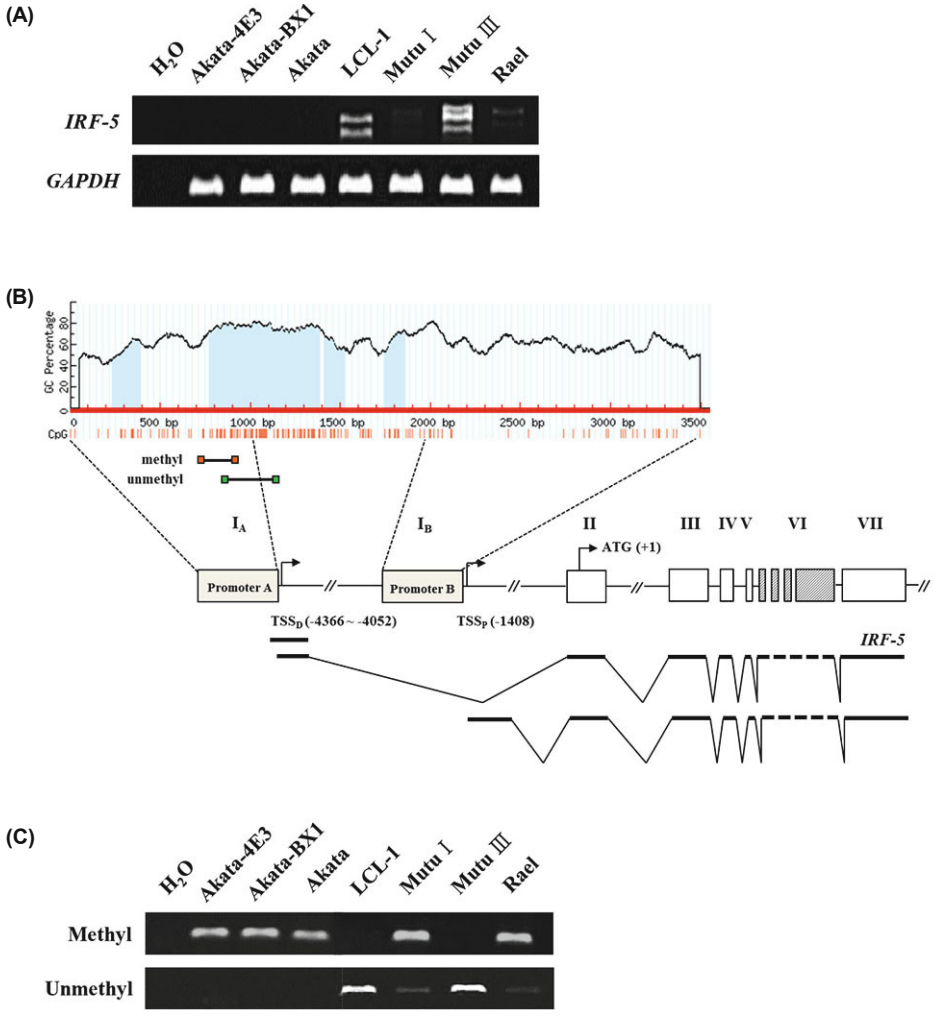


Fig. 1. *IRF-5* expression was regulated by promoter CpG islands methylation in various EBV-infected Burkitt's lymphoma cell lines. (A) RT-PCR was performed to compare IRF-5 expression in an EBV-negative BL cell (Akata-4E3), type I latently infected BL cells (Akata, Akata-BX1, Mutu I, and Rael), and type III latently infected BL cells (LCL and Mutu III). (B) Analysis of CpG islands around two discrete promoters using the MethPrimer database (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi). The methyl- and unmethyl-primers for MSP are indicated. (C) MSP analyses of the IRF-5 promoter-A region in various EBV-associated BL cell lines.

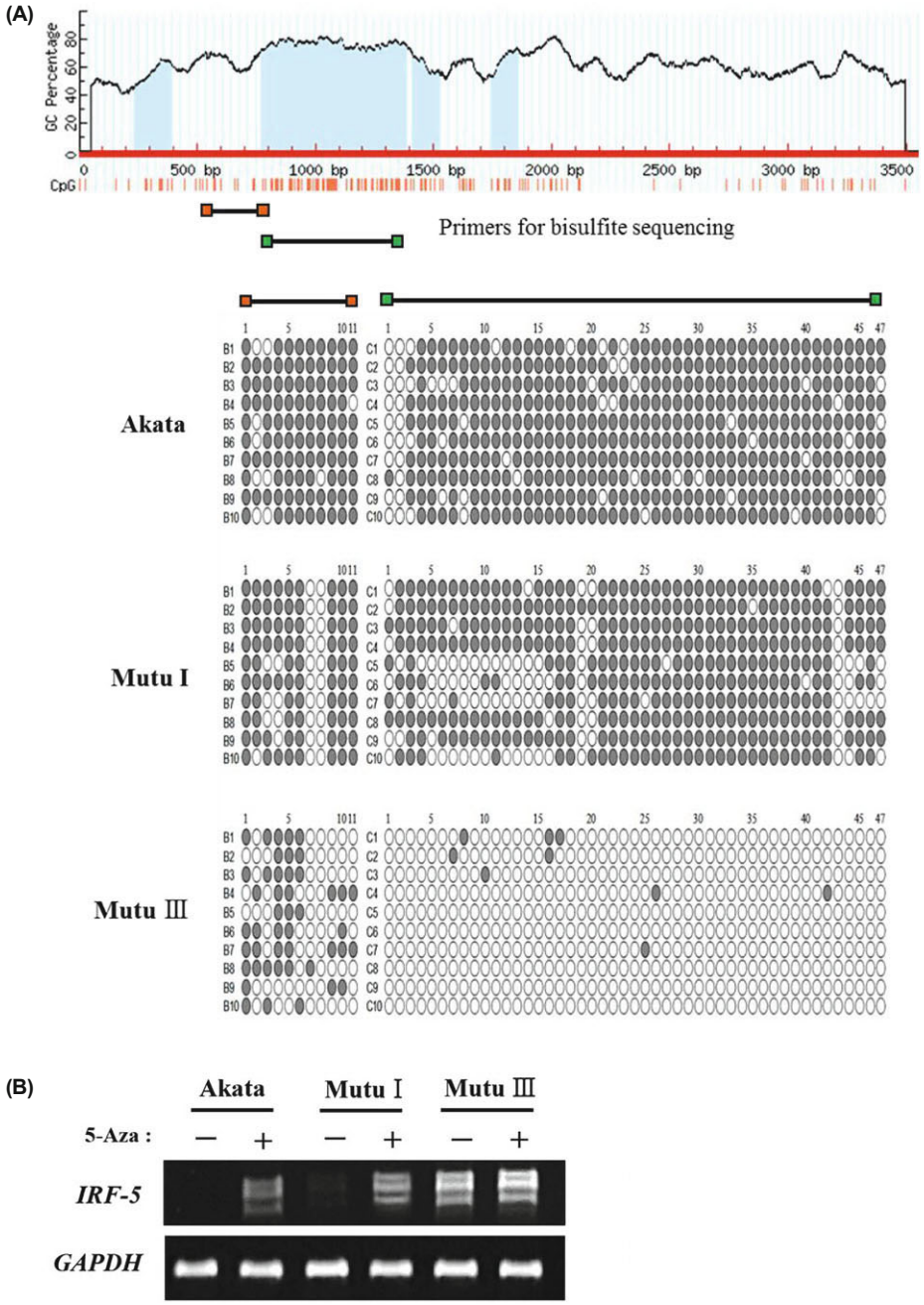


Fig. 2. The correlation between epigenetic modification of *IRF-5* promoter-A region and *IRF-5* expression. (A) Methylated CpG islands status of *IRF-5* promoter-A region in EBV-infected BL cell lines (Akata, Mutu I, and Mutu III) was evaluated by bisulfite sequencing. Two CpG islands as indicated in (A) were used to determine DNA methylation status. Ten clones of two amplicons from each cell were sequenced, then the methylation status was illustrated. (● , methylated cytosine of CpG; ○ , unmethylated cytosine of CpG) (B) BL cells (Akata, Mutu I, and Mutu III) (10^6 cells/plate) were treated with 10 μM 5-Aza. RT-PCR was performed on RNA samples from individual cells before (-) and after (+) 5-Aza treatment. *GAPDH* was used as a loading control.

CpG islands methylation of promoter-A as a critical determinant of *IRF-5* expression

The degree of methylation in the CpG islands of the *IRF-5* promoter-A region was analyzed by bisulfite sequencing with indicated primers (A-1 and A-2) in Fig. 2A. A high frequency of methylated cytosine was detected in Akata, and Mutu I cells but not in Mutu III cells. Decreased methylation of CpG sites in *IRF-5* promoter-A was correlated with upregulation of IRF-5 expression in Mutu III cells (Fig. 2). To validate the contribution of methylation in IRF-5 expression, we applied DNA methyl-transferase inhibitor, 5-Aza, which induced or upregulated IRF-5 in all tested cells (Fig. 2B). These results confirmed that hypermethylation of CpG sites in the promoter-A region is related to *IRF-5* down-regulation in EBV latency I BL cells.

IRF-5 expression in gastric cancer cell lines

We next explored this finding in human gastric carcinoma-derived cell lines. *IRF-5* transcripts were detected in AGS, an EBV-negative gastric carcinoma (EBVnGC) cell line, whereas it was not present or significantly decreased in EBVaGC cell lines such as SNU-719 and AGS-BX1 (Fig. 3A). The relationship of *IRF-5* expression and its epigenetic regulation were examined to define the regulation of *IRF-5* caused by

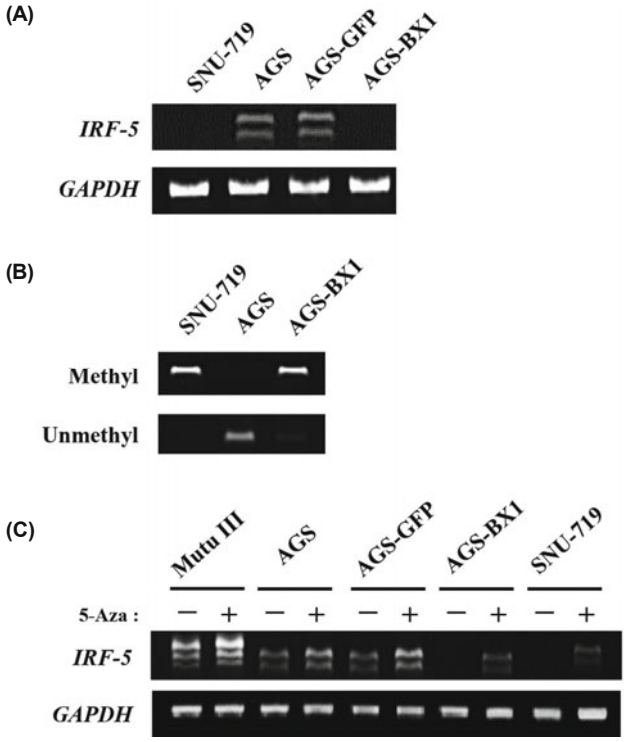
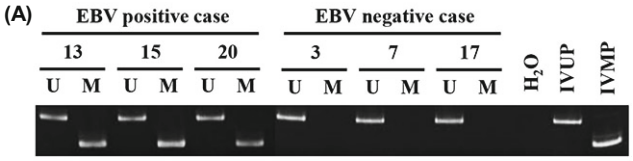


Fig. 3. Regulation of IRF-5 expression by 5-Aza treatment in EBVaGC. (A) RT-PCR was performed to compare *IRF-5* expression in EBVaGC cell lines (SNU-719, AGS-BX1) and EBVnGC cell lines (AGS and AGS-GFP). *GAPDH* was used a loading control. (B) MSP analyses of the IRF-5 promoter-A region in gastric carcinoma cell lines. (C) 5-Aza treatment of Mutu III and EBVaGC cell lines. AGS-GFP, AGS-BX1, and SNU-719 cells (10^6 cells/plate) were treated with 10 μM 5-Aza. RT-PCR was performed on RNA samples of individual cells before (-) and after (+) 5-Aza treatment. *GAPDH* was used as a loading control.

EBV infection in human gastric carcinomas. Down-regulation of *IRF-5* in AGS-BX1 and SNU-719 cells was correlated with methylation of target region in promoter-A as observed in BL cells (Fig. 3B). Moreover, *IRF-5* expression in SNU-719 and AGS-BX1 cells dramatically recovered upon 5-Aza treatment (Fig. 3C).

CpG methylation of *IRF-5* promoter-A region in primary gastric cancer tissues

We finally examined the CpG islands methylation status of the *IRF-5* promoter-A region in primary gastric carcinoma tissues. We tested 17 EBV-positive and 20 EBV-negative gastric carcinoma samples using MSP analysis (Fig. 4A). CpG islands methylation of this region was detected in 43.2% (16/37) of primary gastric cancers, which made no significance in gastric cancers (Fig. 4B). However, when we analyzed *IRF-5* promoter methylation under the consideration of EBV infection status, *IRF-5* promoter methylation was identified in 76.5% (13/17) of EBV-positive GC and 15% (3/20) of EBV-negative GCs (EBVnGC) (Fig. 4B). Moreover, unmethylation of promoter-A in EBVnGC was 85% (17/20). Thus, there is a significant correlation between *IRF-5* promoter methylation and EBV infection in gastric cancers (p=0.0002).



(B)

		promoter-A of *IRF-5*		Chi-square test
		Methyl	Unmethyl	
Gastric cancer		43.2% (16/37)	56.7%(21/37)	
EBV	Positive (17)	76.5% (13/17)	23.5% (4/17)	P = 0.0002
	Negative (20)	15% (3/20)	85% (17/20)	

Fig. 4. *IRF-5* Promoter CpG islands methylation in primary gastric carcinoma samples. (A) MSP analyses of the *IRF-5* promoter-A region in primary gastric carcinomas. Representative cases (EBV-positive case No.: 13, 15, 20; EBV-negative case No.: 3, 7, 17) were selected from gastric carcinoma tissues. (U, unmethylated promoter-A sequence; M, methylated promoter-A sequence; IVUP, Mutu III as *in vitro* unmethylation control; IVMP, Akata as *in vitro* methylation control) (B) Quantitation of promoter-A methylation in primary gastric carcinomas.

Discussion

EBV manipulates B cell signaling pathways in order to establish latent infection. TLR signaling has been reported to contribute to B cell activation and is potentially a target for EBV modulation (Joseph *et al.*, 2000; Thorley-Lawson and Gross, 2004). TLRs are known to stimulate the innate immune response as well as connecting innate and adaptive immunity. In a previous study, TLR7 and its downstream effector IRF-5 were induced in EBV-infected naïve B cells (Martin *et al.*, 2007). IRF-5 was detected in type III latently infected BL cell lines and was not present (or barely detectable) in type I BL cell lines and in EBV-negative BL cell lines (Epstein, 2001). EBNA2 has functions as a transcriptional activator to regulate the expression of EBV latency genes and cellular genes. EBNA2 activates CBF1 bound promoters by competing away the co-repressor complex (Young and Rickinson, 2004). In the current study, we investigated another regulation mechanism for IRF-5 expression in EBV-infected cells. We obtained some evidences for epigenetic regulation of *IRF-5*, which was influenced by the latency type of EBV infection.

Methylation of promoter CpG islands appears to play a role in EBV-associated carcinogenesis. In the EBV life cycle, latent viral gene expression is controlled by CpG islands methylation of EBV-DNA via cellular DNA methylation mechanisms (Iizasa *et al.*, 2012; Kaneda *et al.*, 2012). DNA methylation may be regarded as a host defense mechanism against foreign DNA to suppress its expression; however, it can also be utilized by viruses for their survival and propagation. There are some reports of alternative epigenetic silencing through promoter hypermethylation of cancer-related genes such as APC, p16, MINT1, and MLH1 in EBV-infected cancer cells (Ryan *et al.*, 2010; Geddert *et al.*, 2011).

In the present study, we found altered *IRF-5* expression caused by CpG islands hypermethylation in one specific promoter region (promoter-A). *IRF-5* has two promoter regions.

The TATA-less promoter-A has prominent CpG islands, which suggests that epigenetic alterations via promoter methylation may be a regulatory mechanism for controlled *IRF-5* expression. MSP analyses and bisulfite sequencing experiments of *IRF-5* promoter-A revealed that the methylation status of *IRF-5* promoter-A was inversely correlated with IRF-5 expression (Figs. 1 and 2). Moreover, in subsequent examinations of EBV primary infection of Akata-4E3 cells, we noted *IRF-5* induction by promoter-A demethylation after EBV infection (Supplementary data Fig. S1B and C). *IRF-5* induction coincided with the time when EBNA2 was induced (Supplementary data Fig. S1A). These results suggest that epigenetic regulation of *IRF-5* is a major factor during EBV infection.

Moreover, IRF-5 in type III BL cells may be negatively regulated by competing with IRF-4 for interaction with MyD88 (Xu *et al.*, 2011). We previously described that IRF-4 is also present in type III BL cells which have high IRF-5 expression (Fig. 1A) (Martin *et al.*, 2007). IRF-4 expression was induced by LMP1 (Xu *et al.*, 2008), a latency gene that is regulated by EBNA 2. *LMP1* and *IRF-5* expression/promoter-A demethylation were observed simultaneously (~3 days) after EBV infection (Supplementary data Fig. S1). Thus, in addition to modulation of IRF-5 expression by promoter-A methylation, IRF-5 activation in response to TLR signaling might be negatively modulated by cellular and viral molecules (IRF-4, LMP1, and EBNA2) in type III latent infection.

Although the molecular mechanism of gastric carcinoma is unclear, a causal correlation has been well established between infection-triggered inflammation and cancers (Tye *et al.*, 2012). Extensive studies of gastric carcinoma also revealed that CpG islands methylation in promoter regions of tumor suppressor genes, such as p16 INK4A, p73, and E-cadherin made the expression of those genes decreased (Chang *et al.*, 2006; Kusano *et al.*, 2006; Xu *et al.*, 2011; Kaneda *et al.*, 2012). In particular, Methylation of promoter region in cancer-related genes including APC, p16 INK4A, MINT1, and MLH1 was more frequently observed in EBVaGCs compared to uninfected gastric carcinomas (Qu *et al.*, 2013; Saito *et al.*, 2013). In our study, primary gastric carcinomas showed no significance in methylation status of *IRF-5* promoter-A region in gastric carcinomas, whereas significant methylation was observed in EBVaGCs (Fig. 4). Since EBVaGCs are latency I neoplasms, detection of frequent *IRF-5* hypermethylation in EBVaGC may be linked to the mechanism in EBV-associated BLs. The precise molecular mechanism of host DNA methylation during EBV infection of the gastric epithelium is not fully understood. It was reported that DNMT1, activated by phosphorylation of STAT3, causes loss of PTEN expression through CpG islands methylation in its promoter region (Hino *et al.*, 2009; Geddert *et al.*, 2011). According to recent report, TLR2-induced STAT3 activation promotes tumorigenesis of gastric cancer (Tye *et al.*, 2012). Further investigations must be performed to determine the precise mechanisms related with EBV infection, methylation, transformation, and selection of the predominant clone within the stomach mucosa.

Our results suggest that EBV affect cancer cell proliferation and survival via an epigenetic regulation, especially the regulation of promoter CpG islands methlylation. The underlying molecular mechanisms are unclear, so further investigation must be performed to apply these epigenetic modifications as a therapeutic target in EBV-associated cancers.

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