Classification of Epstein–Barr Virus–Positive Gastric Cancers by Definition of DNA Methylation Epigenotypes

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Abstract
Epstein–Barr virus (EBV) is associated with Burkitt lymphoma, nasopharyngeal carcinoma, opportunistic lymphomas in immunocompromised hosts, and a fraction of gastric cancers. Aberrant promoter methylation accompanies human gastric carcinogenesis, though the contribution of EBV to such somatic methylation changes has not been fully clarified. We analyzed promoter methylation in gastric cancer cases with Illumina’s Infinium BeadArray and used hierarchical clustering analysis to classify gastric cancers into 3 subgroups: EBV+/low methylation, EBV+/high methylation, and EBV+/high methylation. The 3 epigenotypes were characterized by 3 groups of genes: genes methylated specifically in the EBV+ tumors (e.g., CXXC4, TIMP2, and PLXND1), genes methylated in both EBV+ and EBV−/high tumors (e.g., COL9A2, EYA1, and ZNF365), and genes methylated in all of the gastric cancers (e.g., AMPH, SORCS3, and AJAP1). Polycomb repressive complex (PRC) target genes in embryonic stem cells were significantly enriched among EBV+/high-methylation genes and commonly methylated gastric cancer genes (P=2×10−15 and 2×10−34, respectively), but not among EBV+ tumor-specific methylation genes (P=0.2), suggesting a different cause for EBV+-associated de novo methylation. When recombinant EBV was introduced into the EBV−/low-methylation epigenotype gastric cancer cell, MKN7, 3 independently established subclones displayed increases in DNA methylation. The promotors targeted by methylation were mostly shared among the 3 subclones, and the new methylation changes caused gene repression. In summary, DNA methylation profiling classified gastric cancer into 3 epigenotypes, and EBV+ gastric cancers showed distinct methylation patterns likely attributable to EBV infection. Cancer Res; 71(23); 7187−97. ©2011 AACR.

Introduction
Gastric cancer is the second leading cause of cancer-related deaths in the world (1). It has been reported that 2 pathogens, Helicobacter pylori and Epstein–Barr virus (EBV), participate in gastric cancer development (2, 3). H. pylori is a gram-negative spiral-shaped bacterium infected in the stomach of about half of the world’s population (4, 5), and the recent prospective cohort studies have shown that H. pylori infection played an essential role in gastric carcinogenesis (6). The other pathogen, EBV, belongs to the Herpesviridae family causing infectious mononucleosis in initial infection, and more than 90% adult people finally become EBV carriers (7). EBV is involved in several kinds of malignant tumors, such as Burkitt lymphoma (8), nasopharyngeal carcinoma (9), and opportunistic lymphoma in immunocompromised host (10, 11). EBV+ gastric cancer was discovered in 1990 (3) and proved to be distributed all over the world without regional or racial differences at the rate of 7% to 15% (12, 13).

During multistep carcinogenesis, gastric cancer is thought to arise through accumulation of epigenetic alterations and genetic alterations including p53 and CDH1 mutation (14−16). Aberrant DNA methylation of gene promoter region is one of the most important epigenetic alterations resulting in gene silencing, and gastric cancer is one of the tumors exhibiting high frequency of aberrant promoter methylation, for example, in CDKN1A/p16, RUNX3, and LOX (17, 18). It was reported that aberrant methylation in MLH1 had close correlation with microsatellite instability in gastric cancer similar to colorectal cancer (19, 20).

Kang and colleagues reported that EBV+ gastric cancer was involved in aberrant promoter methylation more frequently than EBV− gastric cancer (21). Although frequency of MLH1 methylation was significantly less in EBV− than EBV+ gastric cancer (21−23), TP73 and HOXA10 were reported to be methylated more in EBV− gastric cancer (24, 25). The number of analyzed genes, however, has been limited to known cancer-associated genes in previous reports of EBV+ gastric cancer. It
was also reported that an EBV+ gastric cancer tissue had monoclonal EBV genome in each cancer cell as an epismal latent form without integration into the host genome (26, 27). The monoclonality suggested that EBV infection might occur at a very early stage of carcinogenesis and that EBV infection might play a role in cancer development, including induction of the specific aberrant DNA methylation phenotype if any.

To clarify methylation epigenotypes specific to EBV+ gastric cancer, we analyzed DNA methylation status of promoter regions in 51 clinical gastric cancers, including 11 EBV cancer, we analyzed DNA methylation status of promoter regions in 51 clinical gastric cancers, including 11 EBV cases, with Infinium HumanMethylation27 BeadChip (Infinium, Illumina) for 27,578 individual CpG dinucleotides, which covers 14,495 gene promoter regions. Gastric cancer was clustered into 3 epigenotypes by unsupervised 2-way hierarchical clustering: low- and high-methylation epigenotypes in EBV− cancer and EBV+−specific markedly higher methylation epigenotype. Using Akata system of recombinant EBV (rEBV) infection into monolayer cells in vitro (28), EBV infection per se was shown to induce the EBV+−specific methylation epigenotype in EBV+ gastric cancer cell.

Materials and Methods

Detailed information regarding materials and methods is provided in Supplementary Information.

Clinical samples and cell lines

Primary gastric cancer samples were obtained from patients undergoing gastrectomy at the University of Tokyo, with written informed consent. These samples were immediately frozen with liquid nitrogen and stored at −80°C. The frozen materials were microscopically examined for determination of cancer cell contents by 2 independent pathologists and were dissected to enrich cancer cells when necessary. Fifty-one samples containing more than 40% of cancer cells were used. DNA was extracted by QIAquick DNA mini-kit (QIAGEN). Gastric cancer cell line MKN7 or AGS, to coculture for 3 days. The floating Akata cells. The rEBV-infected gastric cancer cell line, MKN7 or AGS, to coculture for 3 days. The dish was thoroughly washed 4 times with PBS on day 3 to remove floating Akata cells. The rEBV-infected gastric cancer cells were then selected with geneticin. Three rEBV− clones were established independently in different dishes to avoid selecting the same clones.

In situ hybridization

To determine EBV infection status, in situ hybridization targeting EBER (EBER-ISH) was carried out as reported (27) with formalin-fixed, paraffin-embedded specimens in all clinical samples and blocks of cell lines.

Immunohistochemistry

Formalin-fixed, paraffin-embedded cell block was analyzed by immunohistochemistry using anti-cytokeratin antibody (#M3515; DAKO) as epithelial cell marker and anti-CD45 antibody (#M0701; DAKO) as leukocyte marker.

Plasmid construction and transfection

LMP2A cDNA was a kind gift from Prof. Paul J. Farrell (Imperial College, London, UK). As for small RNAs, EBER1 and EBER2, pcDNA3 containing 10 tandem repeats of EBER1 and EBER2 open reading frames was previously established (34). MKN7 was transfected with pcDNA3 or pcDNA5/T vector (Invitrogen) containing cDNA of LMP2A, EBER1, EBER1/2, and on the basis of CpG ratio and GC content within 500-bp region (from −250 bp to +249 bp) around the probe site. Genes in X and Y chromosomes were excluded to avoid gender difference, so the whole genes analyzed were 13,897. Infinium data are available at Gene Expression Omnibus (GEO) data sets (GSE31789).

Pyrosequencing analysis

Quantitative validation for methylation was carried out by pyrosequencing as described previously (31). Primers were designed by Pyro Q-CpG Software (QIAGEN) to amplify bisulfite-treated DNA region containing several CpG sites. Primer sequences are listed in Supplementary Table S1. Methylation control samples (0%, 25%, 50%, 75%, and 100%) were prepared as previously described (32) and used to check quantitatitivity of pyrosequencing assays (Supplementary Fig. S1).

Gene Ontology analysis

Gene annotation enrichment analysis was conducted for Gene Ontology (biologic process, cellular component, and molecular function) using the Functional Annotation tool at DAVID Bioinformatics Resources (33).
BARF0 or mock vector, with Lipofectamine 2000 (Invitrogen), and selected with 200 μg/mL genetin or hygromycin B. Cells were cultured for 6 weeks after transfection and collected for extraction of genomic DNA, total RNA, and protein.

Expression microarray analysis
mRNA expression in gastric cancer cell lines was analyzed by Affymetrix GeneChip Human Genome U133 plus 2.0 oligonucleotide arrays. For global normalization, the average signal in an array was made equal to 100. Expression array data are available at GEO data sets (GSE31789).

Reverse transcription PCR
Reverse transcription PCR (RT-PCR) analysis was conducted for EBV latent genes (LMP2A, EBNA1, EBER1, EBER2, and BARF0) and ACTB. Ten microliters of each PCR product was separated on 2% agarose gels and stained with ethidium bromide. Primer sequences are listed in Supplementary Table S2.

Quantitative real-time PCR
Real-time PCR analysis using iCycler Thermal Cycler (Bio-Rad Laboratories) was carried out as previously described (18). For real-time RT-PCR, the quantity of mRNA of each gene was normalized to that of ACTB. For PCR of genomic DNA, the quantity of BZLF1 region in EBV genome was normalized to that of CRP region in host genome (35). Primer sequences are listed in Supplementary Tables S2 and S3.

Immunoblot analysis
Aliquots of protein were subjected to SDS-PAGE followed by immunoblot analysis with antibodies against DNMT1 (N-16; Santa Cruz) and actin (I-19; Santa Cruz), and the resulting immunoblots were visualized using Amersham ECL Plus (GE Healthcare).

Statistical analysis
Statistical analyses were conducted using χ² test, t test, ANOVA, or Kolmogorov–Smirnov test. Unsupervised 2-way hierarchical clustering was carried out based on correlation distance and average linkage clustering algorithm in sample directions and probe directions using Cluster 3.0 software (36).

Result

Probe selection in Infinium analysis
To analyze Infinum data, the following criteria were set up: (i) probe with the highest CpG ratio was selected for 1 promoter when multiple probes were designed; (ii) SD of β value about 51 gastric cancer and 2 NGM samples was calculated for each probe, and probes with SD less than 0.05 were excluded for hierarchical clustering; (iii) genes with intermediate- and high-CpG probes were used in hierarchical clustering to analyze promoter with high CpG density (e.g., CpG islands), and genes with low-CpG probe were separately shown in the heat map. Based on these criteria, 4,249 probes were selected (Fig. 1).

Unsupervised 2-way hierarchical clustering
Unsupervised hierarchical clustering analysis of 51 clinical gastric cancer and 2 NGM samples classified gastric cancer into 3 subgroups: low- and high-methylation epigenotypes (Low- and High-epigenotype hereafter) in EBV− cases, and markedly higher methylation epigenotype that was completely matched to EBV+ cases (EBV+-epigenotype hereafter; P = 3.0 × 10−6; Fig. 1, Table 1). Two NGM samples were less methylated and clustered in a fourth subgroup together with 2 normal PBC samples, and the PBC samples showed different methylation pattern in low-CpG probes compared with other 53 stomach samples.

The EBV+-epigenotype had the following features (Figs. 1 and 2, Table 1): (i) EBV+-epigenotype had approximately 270 excessively methylated genes which were not methylated in either low- or high-epigenotypes; (ii) genes methylated in EBV− gastric cancers were mostly methylated in EBV− cases, except MLH1, in which methylation specific to EBV− cancer was seldom detected; (iii) whereas high-epigenotype showed frequent methylation of MLH1 (46%), EBV−-epigenotype did not show MLH1 methylation at all (P = 0.037, Table 1); and (iv) polycomb repressive complex (PRC)-target genes reported in ES cells (38) were enriched in genes methylated in low-epigenotypes and high-epigenotypes, but not in genes methylated specifically in EBV−-epigenotype (Figs. 1 and 2).

Clinicopathologic information was summarized in Table 1, and EBER-ISH results of clinical samples were shown in Table 1 and representatively in Fig. 3A.

The methylation profiles of 3 gastric cancer cell lines, MKN7, AGS, and SNU719, and one xenograft tumor, KT, were also analyzed by Infinum (Fig. 1). SNU719 and KT, derived from EBV− gastric cancer, showed markedly higher methylation epigenotype similar to EBV− clinical gastric cancer samples, indicating that EBV+-epigenotype was not epigenotype due to contamination of infiltrated blood cells but due to EBV+ gastric cancer cells themselves. Interestingly, though AGS was not EBV+ cell (Fig. 3B, Supplementary Fig. S2), AGS was considered to be EBV−-epigenotype because its markedly higher methylation pattern was similar to SNU719 and KT (Figs. 1 and 2A).

Extraction of marker genes for 3 epigenotypes
Three groups of marker genes characterizing 3 epigenotypes were extracted (see detailed criteria in Supplementary Table S4): 72 common-marker genes, methylated commonly in low-, high-, and EBV−-epigenotypes but unmethylated in NGM; 79 high-marker genes, methylated in EBV− and high-epigenotypes but unmethylated in low-epigenotype and NGM; 53 EBV−-marker genes, methylated in EBV−-epigenotype specifically (Fig. 2A, Supplementary Tables S4 and S5).

Whereas the ratio of PRC target genes among the whole 13,897 genes was 8.7% (1,211 of 13,897), PRC target genes were significantly enriched in common- and high-marker genes, 50.0% (36 of 72, P = 2 × 10−4) and 34.2% (27 of 79, P = 2 × 10−6), respectively (Fig. 2A and B). PRC target genes in EBV−-epigenotype were, however, only 15.1% and not significantly enriched at all (8 of 53 genes, P = 0.2), suggesting a possible
methylated mechanism different from common- and high-marker genes (Fig. 2B).

Validation by pyrosequencing

Methylation status obtained by Infinium was quantitatively validated by pyrosequencing. Nine marker genes were randomly chosen to include 3 markers each for common-, high- and EBV⁺-marker groups (Fig. 2C, Supplementary Table S1). Although each Infinium probe determines methylation status at a single CpG site, pyrosequencing data not only validated the methylation at probe site, but also guaranteed the methylation status of surrounding multiple CpG sites as a DNA region.

rEBV infection experiment

To analyze whether EBV infection per se could induce the EBV⁻-epigenotype, rEBV was infected into 2 gastric cancer cell lines, MKN7 and AGS, using Akata system. Although AGS was considered to be EBV⁻-epigenotype, MKN7 was considered to be low-epigenotype because high- and EBV⁺-markers were generally unmethylated and common-markers were methylated in MKN7. It was confirmed that the 3 rEBV-infected clones obtained were not contaminated with Akata cells (Fig. 3C, Supplementary Fig. S3). These clones (MKN7_EB#1, #2, #3) and AGS_EB#1, #2, #3) were compared with the parent cells (MKN7_p and AGS_p) and mock cells cultured for the same period of 18 weeks without rEBV infection (MKN7_EB⁻ and
EBV+). EBV existence in rEBV-infected clones was confirmed by EBER-ISH (Fig. 3A, Supplementary Fig. S2) and by real-time PCR for the EBV genome (Fig. 3D). Expression of the EBV latent genes in the 3 clones was confirmed by real-time RT-PCR (Fig. 3E).

DNA methylation profiles of the clones with or without EBV infection were analyzed using Infinium. All the MKN7_EB#1, #2, and #3 showed marked de novo DNA methylation into EBV+ and high-marker genes that were not methylated in MKN7_p (Fig. 4A), and thus were considered to acquire the EBV+ epigenotype. The methylation status was again validated by pyrosequencing (Fig. 4B). The genes 2,215, 1,917, and 2,073 showed de novo methylation in MKN7_EB#1, #2, and #3, respectively, and were well overlapped (Fig. 5A), indicating that this methylation induction is a stable phenomenon.

Interestingly, de novo methylation was induced in highmarkers as well as in EBV−markers, but was not in MLH1 (Fig. 4A). These results supported the features in clinical samples in which EBV−epigenotype gastric cancer showed methylation in all of common-, high-, and EBV−markers, but not in MLH1. The de novo methylation was not detected in MKN7_EB−, confirming that the methylation was not due to 18-week culture during clone establishment but due to EBV infection. As for AGS, most of EBV+ and high-marker genes had already been methylated in AGS_p, and the methylation profile did not change in any of clones with or without rEBV infection.

### Evaluation of gene silencing

To analyze whether the de novo methylation contributed to gene repression, gene expression changes and methylation increase in rEBV− clones compared with MKN7_EB+ were analyzed (Fig. 5B). In all the clones of MKN7_EB#1, #2, and #3, there was a significant correlation between decreased expression and increased β value (P<0.01; Kolmogorov–Smirnov test).

### Induction of EBV latent genes

EBV latent genes, LMP2A, EBNAA1, EBER1/2, and BARF0, were reported to be expressed in EBV+ gastric cancer (39, 40). MKN7 was transfected with expressing vector containing each EBV latent gene, and expression of the induced gene was confirmed by RT-PCR (Supplementary Fig. S4A). DNA methylation analysis by Infinium showed no remarkable changes of DNA methylation profile in any EBV latent gene−induced MKN7.
Figure 2. A, marker extraction. EBV⁺-markers were 53 genes methylated in EBV⁺-epigenotype only. High-markers were 79 genes methylated in EBV⁺- and High-epigenotypes, but not in low-epigenotype or NGM. Common-markers were 72 genes methylated commonly in EBV⁺-, high-, and low-epigenotypes. Right, PRC target genes in ES cells (black bar; ref. 38). B, the ratio of PRC target genes. PRC target genes were significantly enriched in high-markers ($P = 2 \times 10^{-15}$) and common-markers ($P = 2 \times 10^{-34}$), but not in EBV⁺-markers ($P = 0.2$), compared with the ratio in the whole 13,897 genes. C, validation of 9 markers by pyrosequencing. Top, Infinium result. Bottom, pyrosequencing result. Although Infinium probe determines methylation status of single CpG site, pyrosequencing data not only validated the methylation status of the probe site (red number in each marker), but also analyzed multiple surrounding CpG sites quantitatively (see also Supplementary Fig. S1). E-epigenotypes, EBV⁺-epigenotypes; H-epigenotypes, high-epigenotypes; L-epigenotypes, low-epigenotypes; NS, not statistically significant.
compared with mock-transfected cells (Supplementary Fig. S4B). To analyze DNA methyltransferase level, Western blotting analysis was conducted. The DNMT1 level in rEBV⁺ MKN7 clone was increased to approximately 2-fold compared with MKN7_EB⁺/C0 (Supplementary Fig. S4C), which is relevant to the markedly high methylation epigenotype. However, increase in the DNMT1 level was not detected in any EBV latent gene–induced MKN7 compared with mock MKN7 cells (Supplementary Fig. S4D).

Discussion

We here clarified through unsupervised hierarchical clustering analysis, using methylation data of thousands of genes, that clinical gastric cancer was classified into 3 epigenotypes and that EBV⁺ cases completely matched with the markedly high methylation epigenotype. However, increase in the DNMT1 level was not detected in any EBV latent gene–induced MKN7 compared with mock MKN7 cells (Supplementary Fig. S4D).

Figure 3. EBV infection. A, hematoxylin and eosin staining and EBER-ISH for gastric cancer tissue and nonneoplastic lesion. EBER-ISH showed EBV existence in cancer cells. Tumor stroma of EBV⁺ gastric cancer showed lymphocyte infiltration, and these lymphocytes were negative for EBER-ISH (top right). B, EBER-ISH for cell lines. Although SNU719 was positive for EBV, MKN7 parental cells (MKN7_p) and AGS parental cells (AGS_p) were negative. Clones infected with rEBV were respectively shown (MKN7_EB#1 and AGS_EB#1), and other clones were shown in Supplementary Fig. S2. C, immunohistochemical analysis of EBV⁺ MKN7 clones and Akata cells. Although cell block of Akata cells was positive for CD45 and negative for cytokeratin, MKN7_EB#1 was positive for cytokeratin and negative for CD45, indicating no contamination of Akata cells in rEBV-infected clones. Other clones are shown in Supplementary Fig. S3. D, real-time RT-PCR for EBV genome. Copy number of EBV genomes was measured quantitatively by real-time RT-PCR of BZLF gene, normalized to CRP of host genome. E, real-time RT-PCR of EBV genes. Expression of EBV genes, EBER1, EBER2, and EBNA1, was measured quantitatively, normalized to ACTB. Their expression in infected clones was confirmed. p, parental cell.
in EBV+ gastric cancer. This excessive methylation in EBV+ gastric cancer was also suggested to imply a unique mechanism of aberrant methylation.

The methylation of MKN7 was similar to low-epigenotype, and EBV infection induced marked de novo methylation both in EBV+-marker and high-marker genes, clearly resulting in resemblance to the EBV+-epigenotype. The de novo methylation caused gene repression as well. MLH1 was not methylated in MKN7, and MLH1 methylation was not induced in EBV-infected MKN7 clones (Fig. 4A), which also resembled the EBV+-epigenotype. These data indicated that EBV infection per se could cause markedly higher methylation epigenotype in EBV+ gastric cancer and may partly account for the reason why there was no case in which EBV+-marker genes were
Figure 5. A, overlapping of genes with de novo methylation in MKN7_EB#1, #2, and #3. Genes in which β value in MKN7_p was less than 0.2 and showing increase of β value by more than 0.2 compared with MKN7_p were counted. B, enrichment of genes with de novo methylation in repressed genes. From the whole 13,897 genes analyzed, 6,170 genes were extracted by excluding genes not on expression array and genes having no methylation in repressed genes. Of these 6,170 genes, 1,519 genes showed GeneChip score less than 100 in all of MKN7_EB clones. Moreover, EBV⁺-marker CDKN1C was not a PRC target gene in ES cells (38). Though EBV infection in nonneoplastic epithelium of the stomach was detected rarely, monoclonality of EBV genome suggested that the phase of EBV infection into the epithelial cells should occur at the initial or a very early stage of carcinogenesis (26, 27). These data and reports may suggest that EBV infection might play a role in gastric carcinogenesis by causally inducing excessive DNA methylation, even into non-PRC target EBV⁺-marker genes, for example, CDKN1C.

In other EBV-associated neoplasms, such as nasopharyngeal carcinoma (45, 46) and natural killer cell lymphoma (47), DNA hypermethylation in the limited number of promoter regions has been reported. DNA methylation of the double-strand DNA virus genome itself was also observed in the host cells, not only for EBV, but also other oncogenic viruses, such as hepatitis B virus and human papilloma virus (48). Although methylation of viral genome may be induced by defense reaction of host cells against foreign invasion, viruses could take advantage by suppressing adverse expression of viral genes to escape from host immunity and maintain latent infection and therefore maintain virus-positive cancer cells. The host defense system might induce aberrant methylation in host cellular genome, too, as a side effect.

In the latent EBV infection, only EBNAA1, EBER1, EBER2, LMP2A, and BARF0 genes are expressed (40). To gain insight into which gene expression could contribute to methylation induction, each latent gene was introduced in MKN7, and cells were cultured for 6 weeks. Overexpression of any single gene did not induce de novo methylation, at least at the CpG sites analyzed by Infinium. Several possibilities are raised. First, whereas rEBV⁺ clones were established through 18-week culture, 6 weeks may not be long enough to acquire de novo methylation. Second, combination of 2 to 5 latent genes might be necessary to induce methylation. Third, some mechanism in host cells may also have to be activated through virus infection. Considering these possibilities, further analyses, for example, infection of rEBV with a specific gene knockout, might clarify which gene expression should be critical in methylation induction. Interestingly, an EBV⁺ cell line AGS showed the EBV⁺-epigenotype, and rEBV infection could not induce any more methylation. This might suggest that host cells themselves have a methylation mechanism and causes other than EBV infection could also trigger the high methylation epigenotype, though it might be rare and mostly triggered by EBV infection. In any case, the host genome methylation cannot be explained simply by methylation expansion from inserted foreign DNA to surrounding sequences on a chromosome because EBV virus exists as an extrachromosomal circular molecule in the nucleus of cancer cell (40).

In summary, gastric cancer was classified into 3 DNA methylation epigenotypes: EBV⁺ gastric cancers showed markedly high methylation epigenotype expanding to the non-PRC target genes and EBV infection per se could cause the methylation of EBV⁺-epigenotype.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
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