primer sets specific to methylated and unmethylated sequences (Supplementary Table 3). As controls, fully methylated and unmethylated DNA were prepared by methylating genomic DNA with *SssI* methylase (New England Biolabs, Beverly, MA, USA) and by amplifying genomic DNA with the GenomiPhi amplification system (GE Healthcare, Buckinghamshire, UK), respectively.

Quantitative real-time MSP was performed by real-time PCR using SYBR Green I (BioWhittaker Molecular Applications, Rockland, ME, USA) and an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). Although a primer set for MSP was also used for qMSP, a specific annealing temperature in the presence of SYBR Green I was determined (Supplementary Table 3). The number of molecules in a specimen was determined by comparing its amplification with those of standard DNA that contained known numbers of molecules (10^1–10^6 molecules). Based on the numbers of methylated (M) and unmethylated (U) molecules, a methylation level was calculated as the fraction of M molecules in the total number of DNA molecules (no. of M molecules + no. of U molecules). Standard DNA was prepared by cloning PCR products of methylated and unmethylated sequences into a vector (pGEM-T Easy, Promega, Madison, WI, USA). The CIMP status in a gastric cancer was determined as described previously.[27]

Bisulfite sequencing was conducted with primers common to methylated and unmethylated DNA sequences (Supplementary Table 4). The PCR product was cloned into pGEM-T Easy, and 10–12 clones were cycle-sequenced for each specimen.

qRT–PCR

cDNA was synthesized from 1 μg of total RNA using a Superscript III (Invitrogen, Carlsbad, CA, USA). qRT–PCR was performed by real-time PCR using SYBR Green I and an iCycler Thermal Cycler. Standard DNA was prepared by serial dilution of PCR products quantified by the QIAxcel system (QIAGEN, Valencia, CA, USA) after purification using Zymo-Spin I Columns (Zymo Research, Orange, CA, USA).[41] The measured number of cDNA molecules was normalized to that of b2-microglobulin (*b2MG*). The primers and PCR conditions are shown in Supplementary Table 5.

Knockdown and cDNA introduction assays

For a knockdown assay, two pairs and one pair of oligonucleotides were designed against *FHL1* and *Luciferase* (control), respectively (Supplementary Table 6). After annealing of sense and antisense oligonucleotides, the fragment was cloned into a pGreenPuro lentiviral vector (System Biosciences, Mountain View, CA, USA). For cDNA cloning, the entire coding region of human *FHL1* was amplified by RT–PCR (Supplementary Table 7), and cloned into a pCDH-CMV-MCS-EF1-Puro lentiviral vector (System Biosciences). As a control, *copGFP* was cloned into the vector in the same manner. The mutant cDNA was synthesized using the site-directed mutagenesis technique.[42] Using complementary primers carrying mutated sequence (mutation site forward and reverse primers; Supplementary Table 7) and primers for each end of the entire coding region (entire region reverse and forward primers), RT–PCR was performed to generate two DNA fragments that had overlapping ends. These two PCR products were combined by a subsequent PCR with primers for each end of the entire coding region to obtain the mutant cDNA. The mutant cDNA was cloned into a pCDH-CMV-MCS-EF1-Puro lentiviral vector.

The viral vectors and packaging vectors (pPACKH1 HIV Lentivector Packaging Kit, System Biosciences) were cotransfected into 293TN packaging cells, and culture media-containing pseudoviral particles were retrieved. Infection of cancer cell lines with pseudoviral particles was performed according to the manufacturer's protocol (System Biosciences), and stably expressing cells were selected by puromycin without cloning.

Cell growth, migration, invasion and apoptosis analysis

Cell growth was analyzed by seeding cells in triplicate in a six-well plate (3×10^4 cells, AGS; 1×10^5 cells, HSC39) and in a 12-well plate (5×10^3 cells, HCT116). Their numbers were counted at 24, 48, 72, 96 and 120 h. Three independent cultures were performed for one experiment.

Cell migration was analyzed by a wound-healing assay.[43] Cells were seeded in triplicate in a 6-cm dish coated with type I collagen (1×10^6 cells, AGS; 4×10^6 cells, MKN28), and cultured in RPMI-1640 medium containing 1% fetal calf serum to form a monolayer. The cell monolayer was scraped in a straight line with a pipette tip. After incubation for 6 and 12 h, the migrating cells were observed under bright-field microscopy. Three independent cultures were performed for one experiment.

Cell invasion was analyzed by a Matrigel invasion assay, using a Boyden chamber with the Matrigel-precoated membrane or Matrigel-free membrane in the top chamber (BD Biosciences, Bedford, MA, USA). Cells were seeded in top chambers in serum-free RPMI1640 (5×10^4 cells, AGS; 1×10^5 cells, MKN28), and the bottom chambers were filled with RPMI1640 containing 10% fetal calf serum. After incubation for 24 and 48 h (AGS and MKN28, respectively), the area of cells invading through the top chambers was measured by ImageJ software (version 1.38, National Institutes of Health, Bethesda, MD, USA). Percent invasion was calculated as the area of cells invading through the Matrigel-precoated membrane relative to those through Matrigel-free membrane. Three independent cultures were performed for one experiment and the experiment was repeated three times.

The apoptosis of the cells was analyzed by terminal deoxynucleotidyl transferase dUTP nick end labeling assay, using an *in situ* cell death detection kit, TMRred (Roche, Basel, Switzerland).

Tumor formation assay in nude mice

Cells (8×10^6 cells, HCT116) were inoculated subcutaneously on both flanks of 7-week-old male athymic nude mice (BALB/cAJc1-nu/nu; CLEA, Tokyo, Japan). Tumor sizes were measured with calipers every 3 days and the volume was calculated as (length $\times$ width^2) $\times$ 0.5, and tumor weights were measured at their killing on day 22. All the animal experiments were approved by the Animal Experiment Ethical Committee at the National Cancer Center.

Mutation analysis

All seven exons of *FHL1* were amplified using 100 ng of genomic DNA with primers located in introns, except for one primer on exon 7 (Supplementary Table 8). The PCR products were directly cycle-sequenced with a BigDye Terminator kit (PE Biosystems, Foster City, CA, USA) and an ABI PRISM 310 automated DNA sequencer (PE Biosystems).

Statistical analysis

Differences in mean methylation levels, expression levels, cell numbers and tumor sizes were analyzed by the Welch *t*-test. Association between *FHL1* methylation and clinicopathological factors was analyzed by the χ^2 test. All the analyses were performed using SPSS (SPSS, Inc., Chicago, IL, USA), and the results were considered significant when a P value < 0.05 was obtained by two-sided tests.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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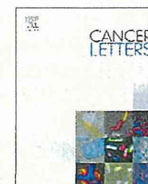

Comprehensive DNA methylation and extensive mutation analyses reveal an association between the CpG island methylator phenotype and oncogenic mutations in gastric cancers

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ABSTRACT

Recent development of personal sequencers for extensive mutation analysis and bead array technology for comprehensive DNA methylation analysis have made it possible to obtain integrated pictures of genetic and epigenetic alterations on the same set of cancer samples. Here, we aimed to establish such pictures of gastric cancers (GCs). Comprehensive methylation analysis of 30 GCs revealed that the number of aberrantly methylated genes was highly variable among individual GCs. Extensive mutation analysis of 55 known cancer-related genes revealed that 19 of the 30 GCs had 24 somatic mutations of eight different genes (*CDH1*, *CTNNB1*, *ERBB2*, *KRAS*, *MLH1*, *PIK3CA*, *SMARCB1*, and *TP53*). Integration of information on the genetic and epigenetic alterations revealed that the GCs with the CpG island methylator phenotype (CIMP) tended to have mutations of oncogenes, *CTNNB1*, *ERBB2*, *KRAS*, and *PIK3CA*. This is one of the first studies in which both genetic and epigenetic alterations were extensively analyzed in the same set of samples. It was also demonstrated for the first time in GCs that the CIMP was associated with oncogene mutations.

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1. Introduction

Both genetic and epigenetic alterations are important for human carcinogenesis [1,2]. Genetic alterations are responsible for activation of oncogenes and inactivation of tumor-suppressor genes [2]. In human gastric cancers (GCs), oncogenes activated by mutations include *CTNNB1* (β-catenin), *ERBB2*, and *PIK3CA* [3–10], and tumor-suppressor genes inactivated by mutations include *CDH1* (E-cadherin), *CDKN2A* (*p16*), *TP53*, and *ARID1A* [11,12]. Even by whole exome sequencing of GCs, the vast majority of driver genes identified were known cancer-related genes, and novel genes identified, such as *ARID1A* and *FAT4*, had only low incidences of mutations [11,12]. This indicates that extensive mutation analysis of a large number of known cancer-related genes can provide an overall picture of a cancer sample, and this is now possible with high speed and low cost by using next-generation personal sequencers [13,14].

Epigenetic alterations, namely aberrant DNA methylation of promoter CpG islands (CGIs), are also responsible for inactivation of various tumor-suppressor genes [1]. DNA methylation statuses of the entire genome can be now comprehensively analyzed using microarray technologies, and bead array technology is especially useful for its quantitative measurement [15]. In GCs, tumor-suppressor genes inactivated by promoter methylation include *CDH1*, *CDKN2A*, *FHL1*, *LOX*, *MLH1*, and *SFRP* family genes (*SFRP1*, *SFRP2*, and *SFRP5*) [16–21]. These tumor-suppressor genes are more frequently inactivated by aberrant methylation than by genetic alterations in GCs [22]. In addition, aberrant methylation is induced in gastric mucosae by *Helicobacter pylori* (*H. pylori*)

Abbreviations: GC, gastric cancer; CGI, CpG island; *H. pylori*, *Helicobacter pylori*; CIMP, CpG island methylator phenotype; EB virus, Epstein–Barr virus; TSS, transcription start site; COSMIC, Catalogue Of Somatic Mutations In Cancer.

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infection [23,24], a well-established major inducer of human GCs [25]. The frequent inactivation of tumor-suppressor genes by aberrant methylation and the deep involvement of *H. pylori* infection in its induction indicate the importance of epigenetic alterations in GCs.

Not only in GCs but also in other types of cancers, a subgroup of cancers is known to have frequent aberrant DNA methylation of CGIs, referred to as the CpG island methylator phenotype (CIMP). The CIMP was first described in colorectal cancers [26], and is associated with unique clinicopathological features. For example, the CIMP is associated with poor prognosis in colorectal cancers, lung cancers, and neuroblastomas [27–29]. In contrast, depending on the number and set of genes used for the determination of the CIMP status, the CIMP can be associated with either poor or good prognosis in GCs [30–33]. The CIMP in specific cancers is associated with genetic alterations, such as mutations of *BRAF*, *KRAS*, and *PIK3CA* in colorectal cancers [34–37], and amplification of *ERBB2* in breast cancers [38]. In contrast, little is known on a specific association between the CIMP and genetic alterations in GCs.

In this study, we aimed to establish integrated pictures of genetic and epigenetic alterations of GCs. To this end, we conducted comprehensive analysis of DNA methylation statuses using bead array technology, and extensive analysis of mutations of 55 known cancer-related genes using a next-generation personal sequencer.

2. Materials and methods

2.1. Samples

Thirty GC samples were obtained from patients who underwent gastrectomy with informed consents. Three normal gastric mucosae samples were obtained endoscopically from healthy volunteers without *H. pylori* infection with informed consents. The study was approved by the Institutional Review Boards. The samples were stored in RNAlater (Life Technologies, Carlsbad, CA) at −80 °C until the extraction of genomic DNA (GC samples and normal gastric mucosae samples) and RNA (normal gastric mucosae samples). Clinical information of the 30 GCs is shown in Supplementary Table 1. The status of Epstein–Barr (EB) virus infection was evaluated by PCR using primers specific to genomic DNA of EB virus (forward, CGGTATTATGTTTTTGGTATGTGTA; reverse, ATAACAACAACGTCATAAAAACCAC), and no infection was present in the 30 GCs.

Genomic DNA was extracted from GC and normal gastric mucosae samples by the phenol/chloroform method, and was quantified by using a Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies). Total RNA was isolated using ISOGEN (Nippon Gene, Tokyo, Japan).

2.2. Analysis of DNA methylation

Analysis of DNA methylation was performed using an Infinium HumanMethylation450 BeadChip array, which covered 482,421 CpG sites (Illumina, San Diego, CA) as described previously [39]. CpG sites with low signals (signal <500, 0.19–2.19% of total CpG sites) were excluded from further analyses. The methylation level of each CpG site was represented by β values which ranged from 0 (unmethylated) to 1 (fully methylated).

A total of 193,531 genomic "segments" were defined by their location against a transcription start site (TSS) [TSS1500 (regions between 200 bp upstream and 1500 bp upstream from TSS), TSS200 (200 bp upstream region from TSS), 5′-UTR, 1st exon, gene body, 3′-UTR, and intergenic regions] and their relative location against a CGI (N Shelf, N Shore, CGI, S Shore, S Shelf, and non-CGI). A genomic segment >500 bp was further divided into genomic "blocks". A genomic block was defined as a 500-bp region from an initial CpG site (probe), and the next genomic block started from the next CpG site (Supplementary Fig. 1). A genomic segment ⩽500 bp was counted as one genomic block. A total of 282,805 genomic blocks were produced, and 276,456 genomic blocks on autosomes were analyzed to enable comparison between males and females. A DNA methylation level of a genomic block was evaluated using the average of β value of the CpG sites within the block. A genomic block was considered as methylated when its β value was 0.4 or more, and as unmethylated when its β value was 0.2 or less.

2.3. Analysis of sequence variations

A library DNA containing 226 amplicons of 55 cancer-related genes was prepared from a sample by multiplex PCR using 50 ng of genomic DNA and an Ion *AmpliSeq* Cancer Panel Kit (Life Technologies) with 36 customized primers (Supplementary Table 2). The 226 amplicons covered the vast majority of samples with mutations reported (91.9% or more) for 15 oncogenes and the *TP53* tumor-suppressor gene (83.1%), and variable fractions of samples with mutations reported (3.3–88.5%) for 39 genes (Supplementary Table 3). Then, the entire library DNA was uniquely barcoded by using an Ion Xpress Barcode Adaptors 1-16 Kit (Life Technologies). The barcoded libraries from five to six samples were pooled, and mixed with Ion Spheres for emulsion PCR using the Ion OneTouch System (Life Technologies) with an Ion OneTouch Template Kit (Life Technologies). From the product of emulsion PCR, the complexes of Ion Spheres with amplified DNA were enriched by using Ion OneTouch ES (Life Technologies) and were loaded onto an Ion 316 chip (Life Technologies). Sequencing was performed by using Ion PGM Sequencer (Life Technologies) with an Ion Sequencing Kit (Life Technologies). Obtained sequences were mapped onto the human reference genome hg19, and sequence variations with frequencies of 10% or more were identified by using CLC Genomics Workbench 5.1 (CLC bio, Aarhus, Denmark). Common SNPs were excluded from further analysis. Reading depths of individual regions analyzed are shown in Supplementary Table 4.

2.4. Dideoxy sequencing

A region containing a sequence variation identified was amplified using 20 ng of genomic DNA with primers listed in Supplementary Table 5. The PCR product was purified by a DNA Clean and Concentrator-5 Kit (Zymo Research, Irvine, CA), and directly cycle-sequenced by using a DYEnamic ET Terminator Cycle Sequencing kit (GE Healthcare, Buckinghamshire, UK) and an ABI PRISM 310 automated DNA sequencer (PE Biosystems).

2.5. Analysis of gene expression by GeneChip oligonucleotide microarray

Gene expression levels in normal gastric mucosae were analyzed by using the GeneChip Human Genome U133 Plus 2.0 microarray (Affymetrix, Santa Clara, CA) as described [40]. Genes with signal intensities of 250 or more were defined as expressed genes.

2.6. Cluster analysis

Unsupervised hierarchical clustering analysis was performed by using R 2.15 [R Core Team (2012) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/] with the Heatplus package [Alexander Ploner (2011) Heatplus: Heatmaps with row and/or column covariates and colored clusters, R package version 2.2.0.] from Bioconductor [41]. The Euclidean distance was used as distance function both for samples and genes. Due to the limitation in the calculation algorithm for the hierarchical clustering, 25,000 elements or less were analyzed.

2.7. Survival curve

Survival curves were analyzed using the Kaplan-Meier method, and the Kaplan-Meier curve was drawn by using SPSS 13.0J (SPSS, Chicago, IL, USA).

2.8. Statistical analysis

The association between the CIMP and oncogene mutations, and that between genes aberrantly methylated in GCs and target genes of polycomb repressive complex (PRC) 2 in human embryonic stem (ES) cells were tested by the chi-square test. The differences in the survival rates among groups were evaluated using the Mantel-Cox test.

3. Results

3.1. Comprehensive analysis of DNA methylation profiles

DNA methylation levels were compared between GCs and normal gastric mucosae. First, using all the 276,456 genomic blocks, some GCs, such as S24TP, S33TP, and S37TP, had a larger fraction of aberrantly methylated blocks than other GCs, such as S2TP, S4TP, and S15TP (Fig. 1 and Supplementary Fig. 2). Second, the analysis was conducted using 6877 TSS200 CGIs unmethylated in normal gastric mucosae (genes unmethylated in normal gastric mucosae) because a TSS200 CGI is known to play a critical role in methylation-silencing [42]. The number of aberrantly methylated genes ranged from three to 1211. Third, we focused on TSS200 CGIs of genes with positive expression in normal cells but aberrantly methylated in cancer cells because this group of genes is known to frequently contain driver genes in carcinogenesis [43]. Using 263 TSS200 CGIs whose downstream genes were expressed in

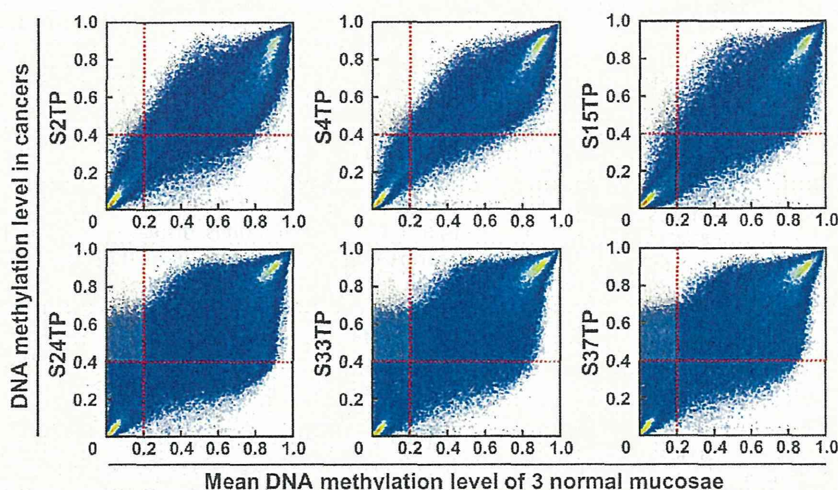

Fig. 1. Comprehensive analysis of DNA methylation profiles in GCs. DNA methylation levels were compared between GCs and normal gastric mucosae for the 276,456 genomic blocks. S24TP, S33TP, and S37TP (lower three panels) had a larger fraction of aberrantly methylated genes (yellow-colored areas) than S2TP, S4TP, and S15TP (upper three panels). The vertical and horizontal axes indicate the methylation levels in GCs and the mean methylation levels of three normal mucosae, respectively.

normal gastric mucosae and aberrantly methylated in one or more GCs (methylation-silenced genes), the number ranged from 0 to 166. These results showed that the number of aberrantly methylated genes was highly variable among individual GCs.

3.2. Extensive mutation analysis of the 55 cancer-related genes

Mutations were analyzed for the 55 cancer-related genes. Among the 30 GCs, 22 GCs had 30 sequence variations of at least one gene (Table 1 and Supplementary Table 6), and all the 30 sequence variations were confirmed by dideoxy sequencing (Supplementary Fig. 3). The confirmed sequence variations were analyzed whether or not they were somatic mutations using corresponding non-cancerous tissues. The 24 of the 30 sequence variations were shown to be somatic mutations (Fig. 2 and Table 1), and were present in 19 GCs. Among the 24 mutations, 22 were missense mutations, and two were nonsense mutations. Three GCs (S5TP, S13TP, and S33TP) had two or more mutations of different genes. Four oncogenes, *CTNNB1*, *ERBB2*, *KRAS*, and *PIK3CA*, and four tumor-suppressor genes, *CDH1*, *MLH1*, *SMARCB1*, and *TP53*, were mutated. *TP53* was most frequently mutated (43%, 13 of the 30 GCs), and *CTNNB1*, *ERBB2*, *KRAS*, and *PIK3CA* were mutated in two GCs. These results showed that 63% of GCs (19 out of the 30 GCs) had at least one somatic mutation of known cancer-related genes.

3.3. The association between the CIMP and mutations of oncogenes

Unsupervised hierarchical clustering analysis was conducted first using DNA methylation profiles of 25,000 genomic blocks randomly selected from all the 276,456 genomic blocks. However, the numbers of aberrantly methylated genes in GCs of different clusters did not appear to be different (Supplementary Fig. 4). Then, we again conducted unsupervised hierarchical clustering using DNA methylation profiles of CGIs, namely 25,000 genomic blocks randomly selected from 59,992 blocks with CGIs (Fig. 3A). This time, clusters I ($n = 3$) and IIb ($n = 13$) contained GCs with a larger number of aberrantly methylated genes than GCs in cluster IIa ($n = 14$). Among the 16 GCs in clusters I and IIb, seven GCs were shown to have mutations of oncogenes, *CTNNB1*, *ERBB2*, *KRAS*, and *PIK3CA*.

Thirdly, using DNA methylation profiles of 6877 genes unmethylated in normal gastric mucosae, two major clusters were observed (Fig. 3B). Cluster III ($n = 11$) contained GCs with a relatively large number of aberrantly methylated genes, and seven of the 11 GCs of this cluster were shown to have mutations of oncogenes, *CTNNB1*, *ERBB2*, *KRAS*, and *PIK3CA*. In contrast, cluster IV ($n = 19$) contained GCs with a relatively small number of aberrantly methylated genes, and none of the 19 GCs in this cluster had mutations of oncogenes. The difference was markedly statistically significant ($P = 7.15 \times 10^{-5}$), and GCs in cluster III and IV were considered to be the CIMP-positive [CIMP(+)] and the CIMP-negative [CIMP(−)], respectively.

Fourth, using DNA methylation profiles of the 263 methylation-silenced genes, three major clusters were produced (Fig. 3C). Cluster V ($n = 3$) contained GCs with the largest number of aberrantly methylated genes, and two of the three GCs were shown to have mutations of *PIK3CA*. Cluster VIa ($n = 8$) contained GCs with a relatively larger number of aberrantly methylated genes than GCs in cluster VIb ($n = 19$). Five of the eight GCs in this cluster were shown to have mutations of oncogenes, *CTNNB1*, *ERBB2*, *KRAS*. Clusters VIb contained the same sets of GCs as cluster IV, the previous clustering, except for one. These results showed that the CIMP(+) GCs were associated with mutations of oncogenes, such as *CTNNB1*, *ERBB2*, *KRAS* and *PIK3CA*, in GCs.

3.4. Possible association between the CIMP and good prognosis

To analyze an association between the CIMP status and prognosis of patients, Kaplan-Meier curves were drawn using overall survival (OS). Using the CIMP status based on the DNA methylation of the 6877 genes unmethylated in normal gastric mucosae, it was revealed that the prognosis of the CIMP(+) patients (Cluster III in Fig. 3B) tended to be better than that of the CIMP(−) patients (Cluster IV in Fig. 3B) ($P = 0.285$; Fig. 4). Also, using the CIMP status based on the methylation of the 263 methylation-silenced genes, the prognosis of the CIMP(+) patients (Cluster V and VIa in Fig. 3C) tended to be better than that of the CIMP(−) patients (Cluster VIb in Fig. 3C) ($P = 0.285$; Supplementary Fig. 5). These results suggested that the CIMP(+) status is possibly associated with good prognosis in GCs.

Table 1
List of somatic mutations identified in the 30 GCs.

Sample #	Sample name	Gene	Coverage	Variant frequencies	Nucleotide change	Amino acid change
1	S1TP	*CDH1*	339	10.3	c.1198G > A	p.Asp400Asn
2	S2TP	*TP53*	496	34.1	c.581T > G	p.Leu194Arg
3	S4TP	*TP53*	438	74.2	c.581T > G	p.Leu194Arg
4	S5TP	*KRAS*	1626	54.4	c.38G > A	p.Gly13Asp
		SMARCB1	50	56	c.1130G > A	p.Arg377His
5	S6TP	*TP53*	2077	24.7	c.820G > C	p.Val274Leu
6	S9TP			No mutation		
7	S11TP	*TP53*	10,211	53.4	c.844C > T	p.Arg282Trp
8	S12TP	*ERBB2*	24,516	63.8	c.2264T > C	p.Leu755Ser
9	S13TP	*TP53*	70	15.7	c.478A > G	p.Met160Val
		ERBB2	482	23.9	c.2264T > C	p.Leu755Ser
10	S14TP			No mutation		
11	S15TP	*TP53*	534	40.3	c.743G > A	p.Arg248Gln
12	S16TP	*TP53*	453	36.2	c.660T > G	p.Tyr220Ter
13	S17TP			No mutation		
14	S18TP	*TP53*	1946	26.5	c.844C > T	p.Arg282Trp
15	S19TP			No mutation		
16	S20TP			No mutation		
17	S22TP			No mutation		
18	S23TP	*TP53*	565	67.8	c.537T > A	p.His179Gln
19	S24TP			No mutation		
20	S32TP			No mutation		
21	S33TP	*MLH1*	4092	45.4	c.1744C > G	p.Leu582Val
		CTNNB1	11,994	20.5	c.101G > A	p.Gly34Glu
		PIK3CA	276	49.3	c.1633G > A	p.Glu545Lys
		TP53	1142	34.9	c.524G > A	p.Arg175His
22	S34TP	*TP53*	551	28.3	c.641A > G	p.His214Arg
23	S35TP	*KRAS*	770	41.3	c.35G > T	p.Gly12Val
24	S36TP	*TP53*	1142	34.9	c.524G > A	p.Arg175His
25	S37TP	*PIK3CA*	59	15.3	c.1624G > A	p.Glu542Lys
26	S40TP			No mutation		
27	S42TP			No mutation		
28	S43TP	*TP53*	239	74.9	c.1024C > T	p.Arg342Ter
29	S45TP			No mutation		
30	S47TP	*CTNNB1*	4591	33.7	c.121A > G	p.Thr41Ala

3.5. Association between the genes aberrantly methylated in GCs and genes targeted by PRC2 in ES cells

The fraction of genes targeted by PRC2 in ES cells was analyzed in the genes aberrantly methylated in GCs and those unmethylated in GCs because genes methylated in GCs were reported to be associated with PRC2 target genes [33]. Using the information on the PRC2 target genes in human ES cells [44,45], it was shown that the genes aberrantly methylated in GCs consisted of a larger fraction of PRC2 target genes than those unmethylated in GCs ($P = 6.64 \times 10^{-79}$) (Supplementary Fig. 6). These results confirmed that genes aberrantly methylated in GCs were associated with genes targeted by PRC2 in ES cells.

4. Discussion

In this study, we conducted comprehensive DNA methylation analysis and extensive mutation analysis of 30 GCs, and showed (1) that the number of aberrantly methylated genes was highly variable among the 30 GCs, (2) that 19 of the 30 GCs had 24 somatic mutations of 8 different genes (*CDH1*, *CTNNB1*, *ERBB2*, *KRAS*, *MLH1*, *PIK3CA*, *SMARCB1*, and *TP53*), and (3) that the CIMP was associated with mutations of oncogenes, including *ERBB2*, *CTNNB1*, *KRAS*, and *PIK3CA*, in GCs. This is one of the first studies in which both genetic and epigenetic alterations were extensively analyzed in the same set of samples, and the association between the CIMP and mutations of oncogenes in GCs was revealed here for the first time.

A similar association has been known also in colorectal cancers, but the mechanisms for this association are still unclear. As a possible mechanism, it has been proposed (1) that cancers with the CIMP can escape senescence caused by *BRAF* mutation owing to silencing of regulators of senescence by *BRAF* mutation, such as *IGFBP7* [46,47], and (2) that overexpression of the *BRAF* mutant can induce aberrant methylation at various genes, such as *MLH1* [48]. Similar possibilities can be hypothesized in GCs. As a mechanism for methylation induction by oncogenic mutation, if this applies to GCs, there is a possibility that oncogenic mutations displace factors involved in the susceptibility to methylation induction, such as RNA polymerase II [40,49–53].

Somatic mutations of four tumor-suppressor genes, *CDH1*, *MLH1*, *SMARCB1*, and *TP53*, and four oncogenes, *CTNNB1*, *ERBB2*, *KRAS*, and *PIK3CA*, were identified. Among these mutated genes, *TP53* (32%), *CDH1* (20%), *PIK3CA* (10%), *CTNNB1* (9%), *KRAS* (7%), and *ERBB2* (2%) are listed in the top 15 mutated genes in GCs in the Catalogue Of Somatic Mutations In Cancer (COSMIC) database. In contrast, mutations of *SMARCB1* have not been identified in GCs, even by whole exome sequencing [11,12], but were identified for the first time in this study, showing the usefulness of extensive mutation analysis of known cancer-related genes. *SMARCB1* encodes a component of chromatin remodeling complex, SWI/SNF, and is mutated in malignant rhabdoid tumors [54]. In GCs, the defects of components of SWI/SNF, such as mutation of *ARID1A* [11,12] and loss of BRM expression, are known [55]. Therefore, it is considered that the dysfunction of chromatin remodeling activity plays an important role in gastric carcinogenesis.

The selection of genomic blocks heavily influenced the results of unsupervised hierarchical clustering analysis. The association between the CIMP and mutations of oncogenes was clearly observed using DNA methylation profiles of the selected 6877 and 263 blocks, and some association was observed using the methylation profiles of the 25,000 blocks with CGIs. In contrast, no association was observed using the 25,000 blocks randomly selected from all the blocks. Therefore, it is considered that the selection of biologically important probes (or genes) is required to extract meaningful information from the huge amount of data obtained by comprehensive DNA methylation analysis.

We previously found that the CIMP statuses in GCs were not associated with DNA methylation statuses in background non-cancerous mucosae, contrary to expectations [30]. The presence of the CIMP(+) GCs suggested that CGIs methylated in GCs are composed of those methylated as a result of the CIMP and those methylated in background non-cancerous mucosae.

The genes aberrantly methylated in GCs here were associated with genes targeted by PRC2 in ES cells, confirming previous reports. It has been known that genes methylated in other types of cancers are associated with genes targeted by PRC2 in ES cells [49,50,53] or normal cells [40,50–52]. A recent comprehensive analysis in GCs also revealed that genes methylated in GCs were associated with genes targeted by PRC2 in ES cells [33]. EZH2, a component of PRC2, and CBX7, a component of PRC1, are known to interact with DNA methyltransferases [56,57], and these interactions seem to be a possible mechanism of the high frequency of DNA methylation of the genes targeted by PRC2.

The prognosis of the CIMP(+) patients tended to be better than that of the CIMP(−) patients. The association between the CIMP and prognosis is highly dependent upon cancer types. For example, the CIMP is associated with poor prognosis in colorectal cancers [28], lung cancers [29], and neuroblastoma [27]. In GCs, some studies showed association with good prognosis [30,31], and others showed that with poor prognosis [32,33]. The reason why the CIMP in GCs was associated with good prognosis in some studies is unknown, but it might be possible that genes involved

	Gastric cancers (sample name)																													
Gene	1	2	4	5	6	9	11	12	13	14	15	16	17	18	19	20	22	23	24	32	33	34	35	36	37	40	42	43	45	47
ABL1																														
AKT1																														
ALK																														
APC																														
ARID1A																														
ASXL1																														
ATM																														
BRAF																														
BRCA1																														
CDH1	■																													
CDKN2A																														
CSF1R																														
CTNNB1																					■									■
EGFR																														
EP300																														
ERBB2								■	■																					
ERBB4																														
FBXW7																														
FGFR1																														
FGFR2																														
FGFR3																														
FLT3																														
GNAS																														
H3F3A																														
HNF1A																														
HRAS																														
IDH1																														
JAK2																														
JAK3																														
KDR																														
KIT																														
KRAS				■																			■							
MET																														
MLH1																					■									
MLL3																														
MPL																														
MSH2																														
MSH6																														
NF1																														
NOTCH1																														
NPM1																														
NRAS																														
PDGFRA																														
PIK3CA																					■				■					
PTEN																														
PTPN11																														
RB1																														
RET																														
SMAD4																														
SMARCB1				■																										
SMO																														
SRC																														
STK11																														
TP53		■	■		■		■		■		■	■		■				■			■	■		■				■		
VHL																														

Fig. 2. Results of extensive mutation analysis of the 30 GCs. Mutations of the 55 known cancer-related genes were analyzed by Ion Torrent PGM sequencer. Among the 30 GCs, 19 had 24 somatic mutations of 8 different genes. *TP53* was mutated in 13 GCs (43%, 13 of the 30 GCs), and *CTNNB1*, *ERBB2*, *KRAS*, and *PIK3CA* were mutated in two GCs, respectively. The presence of a somatic mutation is shown by a filled square.

in tumor progression are silenced by aberrant DNA methylation in GCs with the CIMP.

In conclusion, integrated analysis of genetic and epigenetic alterations revealed that the CIMP was associated with mutations of oncogenes, including *ERBB2*, *CTNNB1*, *KRAS* and *PIK3CA*, in GCs.

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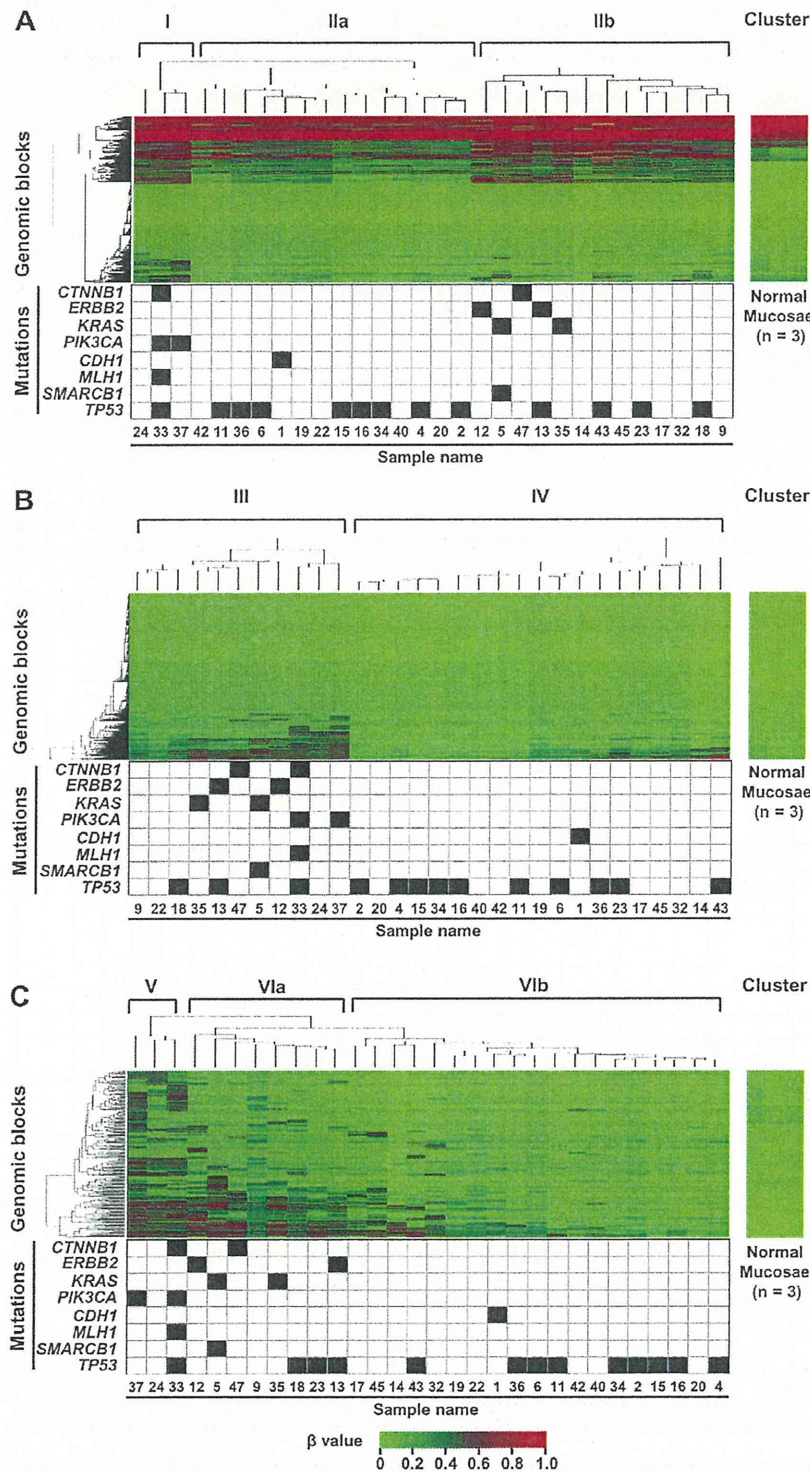

Fig. 3. The association between the DNA methylation profile and gene mutations. (A) Unsupervised hierarchical clustering analysis using DNA methylation profiles of 25,000 genomic blocks with CGIs. Clusters I ($n = 3$) and IIb ($n = 13$) contained GCs with a relatively large number of aberrantly methylated genes, and seven of the 16 GCs were shown to have mutations of oncogenes. (B) Unsupervised hierarchical clustering analysis using DNA methylation profiles of the 6877 blocks (genes) unmethylated in normal gastric mucosae. Cluster III ($n = 11$) contained GCs with a relatively large number of aberrantly methylated genes, and seven of the 11 GCs were shown to have mutations of oncogenes. (C) Unsupervised hierarchical clustering analysis using DNA methylation profiles of the 263 methylation-silenced genes. Cluster V ($n = 3$) contained GCs with the largest number of aberrantly methylated genes, and two of the three were shown to have *PIK3CA* mutations.

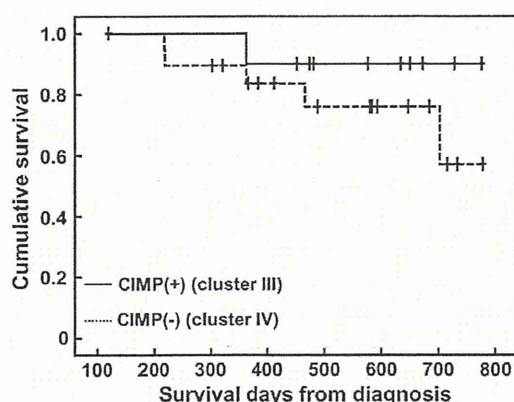

Fig. 4. The possible association between the CIMP and good prognosis. Kaplan-Meier curves were drawn using overall survival (OS). The CIMP status was determined based on the DNA methylation profile of the 6877 genes unmethylated in normal gastric mucosae. The prognosis of the CIMP(+) patients ($n = 11$) tended to be better than that of the CIMP(−) patients ($n = 19$) ($P = 0.285$).

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.canlet.2012.11.022.

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