DNA methylation status of a distinctively different subset of genes is associated with each histologic Lauren classification subtype in early gastric carcinogenesis

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Abstract. DNA methylation change is known to play a crucial role in early gastric carcinogenesis. The present study aimed to identify and validate the correlation between differentially methylated regions (DMRs) and the subtypes of early gastric cancers (EGCs). Illumina Infinium methylation assay (IIMA; 450K BeadChip kit) was performed on fresh tumor and non-tumor tissues of 12 EGCs to screen the methylation status of 450,000 CpG sites. To evaluate the significance of DNA methylation in each histologic subtype, pyrosequencing assay (PA) was performed on 38 EGCs (18 intestinal-, 12 mixed- and 8 diffuse-type) using 12 genes selected from the screening. Between tumors of the intestinal-type (n=6), and diffuse- (n=4) plus mixed-types (n=2), 169 regions showed significant differences (intensity >3,000, $\Delta\beta$ >0.2) in IIMA. Hierarchical clustering using the 169 DMRs revealed distinct separation between the two groups. In PA using 12 selected genes from the IIMA results, the aberrant methylation statuses of DVL2 (p=0.0186) and ETS1 (p=0.0222) were significantly related to diffuse- and mixed-types rather than the intestinal-type, while C19orf35 (p=0.019) and CNRIP1 (p=0.0473) were related to the diffuse-type rather than intestinal-type, and GAL3ST2 (p=0.0158) and ITGA3 (p=0.0273) were related to the mixed-type rather than the other two types. The methylation of other genes, CLIP4, XKR6, CCDC57, MAML3 and SDC2, was related with age, tumor location, or *Helicobacter* infection rather than the histologic subtype. Aberrant DNA methylation of certain genes may be independently involved in each histo-logic subtype of EGC. Furthermore, mixed-type EGCs may be a distinctive histologic subtype based on the different subset of DMRs compared to those of other subtypes.

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Introduction

Despite its decreasing trend, gastric cancer (GC) still remains one of the most troublesome malignant diseases worldwide. It is the fourth most common cancer and the second leading cause of cancer-related death worldwide (1-3). In Asian countries such as Japan and Korea, it has the highest incidence among all malignancies although its overall survival rate has improved during the last few decades due to extensive screening programs (3,4). Due to the poor prognosis and limited treatment options of advanced GC, it remains a challenging task to determine effective molecular-pathological markers for early diagnosis with appropriate histological classification (5).

Lauren classification, which subgroups GCs mainly as two types, intestinal and diffuse, was first introduced in 1965 and has been generally accepted as a distinct and simple histo-logical classification of GCs by most pathologists (6). Since the two major types show distinctively different phenotypic, epidemiologic and biologic characteristics, it is believed that different genetic alterations may be implicated in each histo-logic type of the classification (7). However, a considerable number of GCs accounting for 10-15% of cases, share the histologic features of these two types and are designated as mixed-type (8,9). Although this type of tumor shows poorer prognosis than the common intestinal-type GCs, it is difficult to define and to diagnose when these tumors are encountered in a small biopsy. Furthermore, it is even more difficult to place this type of tumor into a specific category according to the WHO Classification of Gastric Tumours (2000) that was mainly founded on the Japanese Classification of Gastric Carcinoma (1981) and Lauren classification (9). For this reason, a new WHO classification has recently proposed (2010) this type of tumor as a mixed adenocarcinoma, while intestinal-type and diffuse-type are defined as tubular adenocarcinoma and poorly cohesive carcinoma, respectively (10).
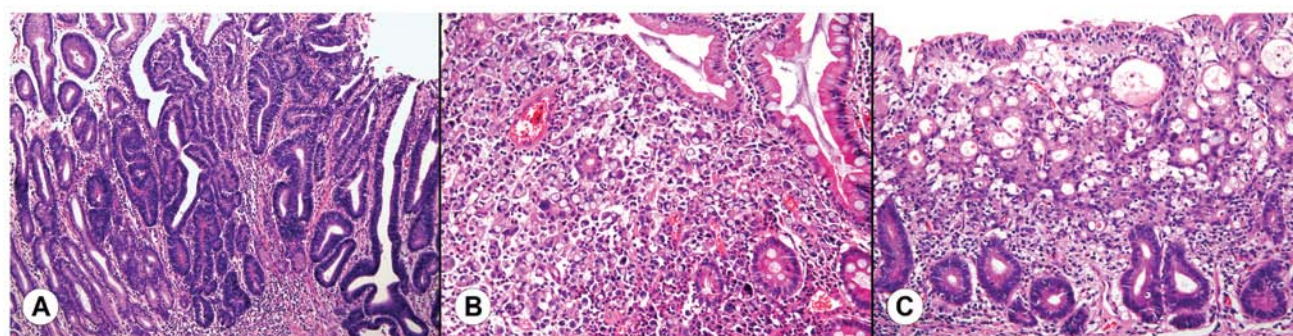
Figure 1. Histologic classification of early gastric carcinomas by Lauren classification: (A) intestinal-type, (B) diffuse-type and (C) mixed-type.

Considering that the tumor cells in diffuse-type cancers are loosely attached to each other from the early stage while intestinal-type cancers are derived from intestinal metaplasia and subsequent dysplasia, different key molecular changes may occur during the early steps of carcinogenesis in each type. For this reason, aberrant DNA methylation is being highlighted as the main change differentiating these subtypes of GCs from the very first stage (1,11,12). Accumulating evidence from over 550 studies including the authors' previous study has demonstrated that aberrant epigenetic changes of more than 100 genes play a crucial role in the early steps of gastric carcinogenesis (2,13). However, the results of these studies were based only on a small portion of GCs and at times were conflicting in regards to a certain candidate gene. That may be because most studies reported to date have focused on the presence of epigenetic alterations and their prognostic value as a screening tool without accurate consideration of their possible linkage with histologic or phenotypic features, and the stage of carcinogenesis that is mainly involved (2). Furthermore, large scale screenings of epigenetic changes in GCs have been rarely performed. Since various additional molecular changes as well as epigenetic alterations are involved throughout each stage of carcinogenesis, a careful research design is essential (11).

The present study aimed to identify key differential epigenetic changes that differentiate the histologic subtypes of GCs in the early steps of carcinogenesis. We performed a large scale screening of up to 485,000 CpG sites with Illumina Infinium methylation assay (IIMA) and selected the most promising sites out of all the differentially methylated regions (DMRs) between the groups using one of the most reliable database, KEGG pathway database (14). We subsequently verified the results of the screening by pyrosequencing assay (PA).

Materials and methods

Patient samples. The present study was approved by the Institutional Review Board of Yonsei University, Wonju College of Medicine (GR-11-009). Written informed consent to participate in the present study was properly obtained for each subject. Surgically resected early gastric cancers (EGCs) from patients treated at the Wonju Severance Christian Hospital from 2011 to 2012 were prepared using the following process. First, fresh tissue from the tumors and adjacent corresponding non-tumor mucosa (CNM) (0.5x0.5 cm-sized) were collected from each case for the methylation study. Following routine

formalin-fixed paraffin-embedded preparations and hematoxylin and eosin staining, full pathological evaluation with confirmatory histological classification according to Lauren were carried out for each case by three pathologists (Y. Chong, K. Mia-Jan and M.Y. Cho).

Diffuse-type GCs were defined when the tumor exclusively consisted of non-cohesive tumor cells diffusely infiltrating the stroma and exhibiting relatively deep infiltration with little or no gland formation (Fig. 1A). Intestinal-type GCs were defined when most part of the tumor consisted of recognizable glands similar to colonic or gastric mucosa (Fig. 1B). Poorly differentiated tumors that were difficult to categorize as either diffuse-type or mixed-type GCs were carefully excluded. Mixed-type GCs were defined when the tumor exhibited features of both typical intestinal-type and diffuse-type at the same time. In such cases, superficial parts of the tumor showed typical gland-like features as observed in intestinal-type GCs while the neck portion of the tumor consisted of irregular glands with non-cohesive singly scattered tumor cells with diffuse infiltration to the deeper part (Fig. 1C). Due to the histological similarity that mixed-type and diffuse-type GCs share and the general proportion of each subtype among overall GCs, intestinal-type GCs were designated as group A, while mixed and diffuse-type GC were designated as group B. Presence of *Helicobacter pylori* (*H. pylori*) infection and intestinal metaplasia in adjacent CNM were also evaluated. Next, 38 cases of EGC and CNM including the previous 12 cases were also enrolled for validation of DMRs using PA.

DNA extraction and bisulfite conversion. DNAs for the following studies were extracted from each sample using DNeasy Blood and Tissue kit® (cat. no. 69506; Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. The procedure included a proteinase K digestion at 55˚C overnight. DNAs were quantified by NanoDrop® UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and diluted to ~50 ng/l for IIMA. Eight hundred nanograms to 1 μg of genomic DNAs were treated with sodium bisulfite using the EZ-96 DNA methylation kit® (cat. no. D5004; Zymo Research, Orange, CA, USA) according to the manufacturer's procedure.

DNA methylation profiling by the Infinium Human Methylation-450K BeadChip kit®. The methylation assay was performed on 4 μl bisulfite-converted genomic DNA (50 ng/μl)
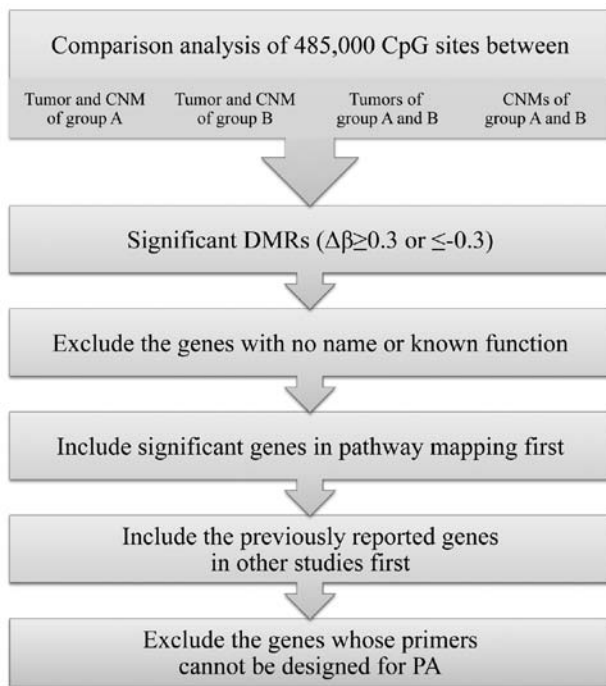
Figure 2. Determination of the eligible genes for pyrosequencing assay.

with Illumina HiScanSQ scanner (Illumina Inc., San Diego, CA, USA) according to the Infinium methylation assay protocol. Data quality was checked with the GenomeStudio™ for Methylation Module software (2010.3) and all samples passed this quality control. Uncorrected β-values were extracted with the same software. Raw data were submitted to the Gene Expression Omnibus (GPL8490) dataset.

To determine DMRs, the average β-values were computed with β-values when they had a signal intensity of >3,000. β-value is defined as the intensity of the methylated region over the sum of the intensities of the methylated and unmethylated regions plus 100, and a value of 0 designates a totally unmethylated status while the value 1 designates full methylation. Δβ-value was calculated between two comparison groups by subtracting one β-value from the other (tumor vs. CNM in group A and B; CNM of group A vs. B and tumor of group A vs. B). When this value was >0.2 or <-0.2, it was considered to be meaningful. The results were considered to be significant when the p-value was <0.05. A differential score was used for evaluating the significance of the DMRs and when it was >13 or <-13, it was considered to have the same significance as a p-value of 0.05, and when it was >22 or <-22, a p-value of 0.01 was indicated.

Validation by PA and determination of the eligible candidate genes. The steps that were used to select the most significant candidate genes eligible for PA are summarized in Fig. 2. Among the CpG sites that showed significant differential methylation between the compared groups in the hierarchical clustering of IIMA, the sites with high Δβ-values (>0.3 or <-0.3) were only selected. Next, the DMRs with no designation or known function were excluded. We then evaluated the potential functional role of selected DMRs by pathway mapping with DAVID Bioinformatics Resources 6.7 (http://

david.abcc.ncifcrf.gov/) using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (http://www.kegg.jp/kegg/pathway.html). The pathways with most DMRs were considered to be significantly implicated with the DMRs discovered by IIMA, and the DMRs in those pathways were enrolled to the list of candidate genes first. Next, we reviewed the literature and included the genes that were previously reported in other studies. Subsequently, we excluded the genes from the list whose primers cannot be designed for PA due to the inappropriate CG content in the targeted sequence.

Eligibility for designing appropriate primers for PA was checked by PyroMark® Assay Design 2.0 software (Qiagen, Hilden, Germany). The designed primers were carefully validated for proper amplification after preliminary polymerase chain reaction (PCR) tests. One microliter of the converted DNA was used as a template in each subsequent PCR. PCRs were performed with HotStarTaq® Plus Master Mix kit (Qiagen, Hilden, Germany) under the following conditions: 95°C for 15 min; 40 cycles of 95°C for 1 min; 50°C for 1 min; 72°C for 1 min and 72°C for 10 min. The success of the amplification was assessed with 2% agarose gel electrophoresis. Pyrosequencing of the PCR products was performed with the PyroMark™ Q24 system (Qiagen, Uppsala, Sweden) according to the manufacturer's instructions. Blue values (perfect calls) and yellow values (needed to be checked manually) were considered for subsequent analyses.

Statistical analyses. After initial processing of the raw data of IIMA, general statistical computing was carried out by R project. For hierarchical clustering analysis, correlation, Euclidean and Manhattan methods were applied. To search any possible correlations within the data, Pearson's correlation method was utilized.

For the PA, univariate analysis using paired t-test, correlation test, two-sample t-test, one-way ANOVA on the differences of methylation status were used for comparing the results according to Lauren classification (intestinal, diffuse and mixed, and group A and B), location (proximal and distal), age and presence of *H. pylori*. For hierarchical clustering analysis, correlation, Euclidean or Manhattan methods were applied as in the IIMA. To search for any possible correlations within the data, Pearson's correlation method was utilized. Scheffe's method for multiple comparisons was used to validate the genes that were found to have significant differences between any of the two types of the Lauren classification.

Results

Samples used for analysis. The 12 cases of EGC analyzed using IIMA consisted of 6 cases of intestinal-type, 4 cases of diffuse-type and 2 cases of mixed-type (M:F, 3:1; age range 44-77 years; median age, 60).

Thirty-eight cases of EGC used for the PA consisted of 19 cases of intestinal-type, 8 cases of diffuse-type and 11 cases of mixed-type (M:F, 3.75:1; age range 31-82 years; median age, 67).

DNA methylation profiling by IIMA
Hierarchical clustering analysis. Upon hierarchical clustering analysis of 6 cases of group A tumors and matched CNM, no
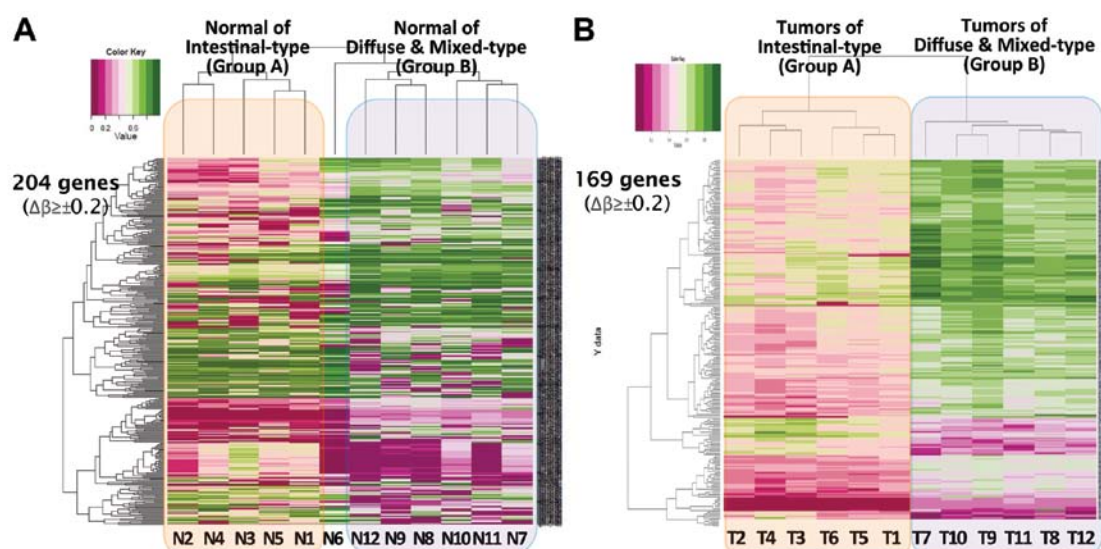
Figure 3. Hierarchical clustering analysis of IIMA. (A) In the normal tissues, a distinct grouping between group A (intestinal-type) and B (diffuse-type and mixed-type) was found with the DNA methylation patterns of 208 genes except N6 (statistically not significant). (B) In the tumors, a statistically significant distinct grouping between group A and B was found in the DNA methylation patterns of 169 genes. IIMA, Illumina Infinium methylation assay.
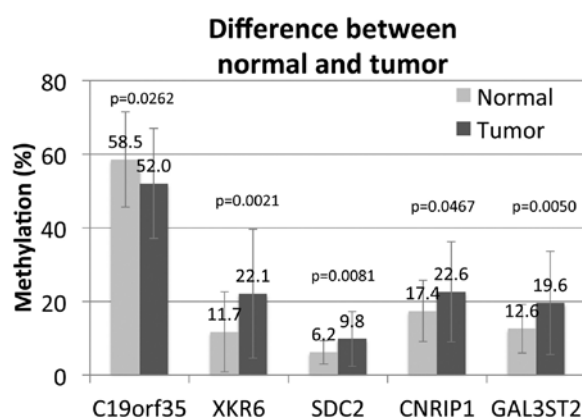

Figure 4. Methylation differences in 5 significant genes between overall normal and tumor tissues.

distinct grouping was found with DMRs by either correlation, Euclidean or Manhattan methods. Between the tumors and CNM of group B, on the other hand, a clear grouping was made by 208 genes in 3 cases, 2 cases being mixed-type, by both correlation and Euclidean methods (T7, T11 and T12 vs. N7, N11 and N12). However, it seemed inappropriate to evaluate the significance of this grouping since half of the cases were not concordantly grouped with the other 3 cases.

Hierarchical clustering analysis of CNM showed a distinct separation between group A and B (except N6) (Fig. 3A). Although it was not statistically significant, 204 genes showed a differential methylation status between the two groups with $\Delta\beta$-value of >0.2. The hierarchical clustering analysis of the tumors of group A and B showed a distinct grouping with 169 statistically significant DMRs ($\Delta\beta$-value $\geq\pm0.2$, differential score ≥13, p<0.05) (Fig. 3B). In total, >800 DMRs were found to be significantly different with a $\Delta\beta$-value of >0.2 or <-0.2 between the subgroups. After removal of the DMRs without a specific gene name or with an unspecified function,

581 genes were pooled as candidates (208 genes between tumors and CNM in group B, 204 genes found between CNM of group A and B, and 169 genes between tumors of group A and B). Among these, 197 genes were included in the list of the candidate genes with $\Delta\beta$-value >0.3 or <-0.3.

Validation of ascertained CpGs by PA
Determination of the genes eligible for PA. As a result of pathway mapping by KEGG pathway database, 18 genes out of 197 genes were found to be significantly related to the archived pathways (pathways in cancers, Notch-signaling pathway, dorso-ventral axis formation, p<0.05). Five genes, *APC*, *DVL2*, *ETS1*, *ITGA3* and *MAML3*, among them were eligible for designing primers for PA and showed a robust amplification in a pilot study. An additional 7 genes from the genes with top $\Delta\beta$-values, *C19orf35*, *XKR6*, *CLIP4*, *SDC2*, *CNRIP1*, *GAL3ST2* and *CCDC57,* were selected after checking for primer eligibility and showed a robust amplification in the pilot study. As a result, finally 12 genes from the screening result were determined as eligible for producing tailored primers for the following PA. Basic information of these 12 genes is listed in Table I. Each gene includes one or more (up to 8) CpG sites.

Genes with significantly differential methylation statuses between tumors and CNMs. Univariate analysis showed significant differences in the methylation status between all tumors and their CNM regardless of type for 5 genes: *C19orf35*, *XKR6*, *SDC2*, *CNRIP1* and *GAL3ST2* (p=0.0262, 0.0021, 0.0081, 0.0467 and 0.0050, respectively) (Fig. 4). Four of the genes showed hypermethylation in the tumor tissue when compared to the CNM (*XKR6*, *SDC2*, *CNRIP1* and *GAL3ST2*) with an average of 10.4, 3.6, 5.2 and 7.0, respectively, while *C19orf35* was significantly hypomethylated in the tumor tissue when compared to the CNM (average -6.5).

Significantly differential methylation statuses according to Lauren classification. ANOVA revealed a statistical

Table I. Primer sequences used in the pyrosequencing assay.

Gene	Ch.		Sequences	Amplicon size (bp)	CpGs
C19orf35	19	F-	AGATGGGGTATTGGGGATATTTTA	146	8
		R-	CCTCCTAAACAACTCACCC		
XKR6	8	F-	GGGGTTTTTAGTTTAGTTGG	158	8
		R-	TCCCTACCCCCTTCCACTA		
CLIP4[a]	2	F-	GGGGTGT**Y**GTAGTGTTATTTT	146	7
		R-	CTAAATCCCC**Y**ACCCCAACAACT		
SDC2[a]	8	F-	GGAGG**Y**GGTAGTGTGATTTTTAGAT	148	6
		R-	AAC**Y**CAAAAACCCTAACTTACC		
CNRIP1[a]	2	F-	GGAGGAGT**Y**GGTGAGTAGTT	153	6
		R-	CCAAACCCTC**Y**CCCAAACATA		
GAL3ST2	2	F-	GTGTGTTAGAGGTTTGTGATG	196	6
		R-	CCAAAAACCCTACAAAAATCCCC		
CCDC57	17	F-	TAGATATTGTTGTTGGGAGTAGTT	227	3
		R-	TCATTTAATCATTCTCTCCTTCAT		
APC	5	F-	ATATGTGGTTGTATTGGTG	194	3
		R-	ACATACTACAATAAACCCTATACCA		
DVL2		F-	GTTTAGGGTAGAGATAGTTTGATTG	180	2
		R-	ACTATACACTAAAACTCCCCTATC		
ETS1	11	F-	ATTTTTTAATGTTAAGGGGAGATGG	205	4
		R-	ATCTCCTTACCCTCAACC		
ITGA3	17	F-	GATTTTGTGGGTTTATGGGGATAT	201	1
		R-	CTTAAAAACTACCAAACACCTACTT		
MAML3	4	F-	GGAGTTTTTTGAGGATTTGTAAGTA	219	7
		R-	AATTCCAAAACCCCAACTACA		

Ch., chromosome number. [a]Y designates CpG sites included in the primer sequences.

difference in methylation status according to the Lauren classification in tumor tissues (*C19orf35*, *CNRIP1* and *ITGA3*; p=0.0192, 0.0373 and 0.0146, respectively) and matched CNM (*GAL3ST2*, *DVL2*, *ETS1* and *ITGA3*; p=0.0157, 0.0182, 0.0099 and 0.0259, respectively) (Fig. 5). In Scheffe's method of multiple comparisons, *C19orf35*, *CNRIP1* and *DVL2* were significantly different between the diffuse-type and intestinal-type (p=0.0195, 0.0473 and 0.0186, respectively) while *GAL3ST2*, *ETS1* and *ITGA3* were different between the mixed-type and intestinal-type GCs (p=0.0158, 0.0222 and 0.0273, respectively). Integrated interpretation of the methylation pattern of these 6 genes according to histologic subtype allowed us insight that three groups, *DVL2* and *ETS1*, *C19orf35* and *CNRIP*, *GAL3ST2* and *ITGA3*, present different transition between tumors and matched CNM. *DVL2* and *ETS1* were hypomethylated both in the tumor tissue and the CNM of intestinal-type EGCs while they were hypermethylated in the mixed-type and diffuse-type EGCs (Fig. 5A and B). On the other hand, the methylation statuses of *C19orf35* and *CNRIP* in the three histologic subtypes exhibited a similar level in the CNM while those varied in the tumors according to histologic subtype (Fig. 5C and D). Notably, *GAL3ST2* and

ITGA3 were significantly hypermethylated in the tumors and the CNM of the mixed-type rather than in the intestinal-type and diffuse-type subgroups (Fig. 5E and F).

The hierarchical clustering analysis using the methylation status of these 6 genes showed a relatively distinct grouping between each type as the results of the hierarchical clustering analysis using IIMA (Fig. 6).

Relationship between genes with significantly differential methylation status and patient age, tissue location and H. pylori infection. To determine whether changes in methylation status are related with non-tumor factors, we statistically analyzed the correlation between methylation status in the non-neoplastic mucosa with patient age, location and *H. pylori* infection (15). There was a significant correlation between patient age and the methylation status of *CLIP4* (Fig. 7A). Paired t-test revealed a significant difference in the methylation in *C19orf35*, *XKR6*, *CLIP4*, *CNRIP1*, *CCDC57* and *MAML3* between proximal and distal location (p=0.0002, 0.0088, 0.0038, 0.0003, 0.0317 and 0.0203, respectively; average difference, -14.6, 8.0, 10.5, 8.6 and 8.3, respectively) (Fig. 7B). The methylation level of all genes except that of *C19orf35*
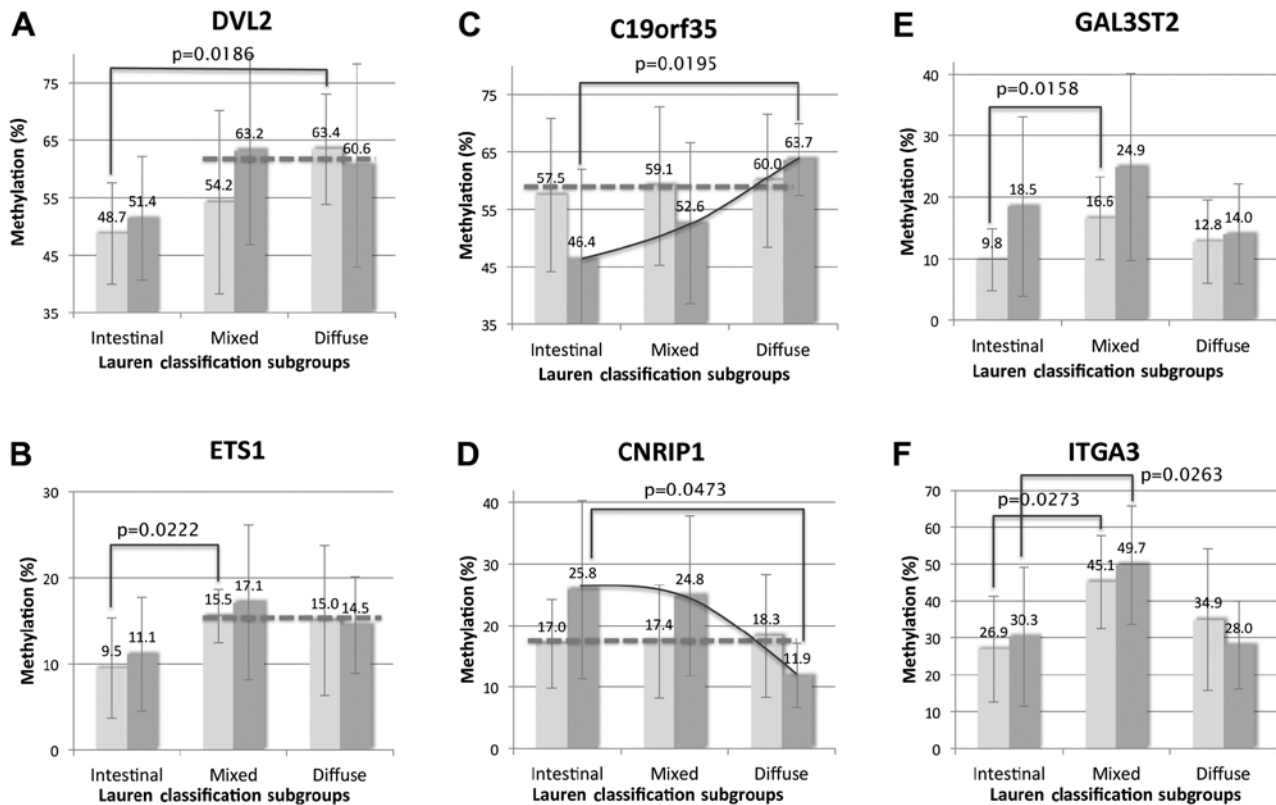
Figure 5. Differences in methylation among the three Lauren classification subtypes. Light gray bar, normal; dark gray bar, tumor tissue.

was significantly higher in the distal compared to proximal stomach. However, no significant difference was found in the tumor tissues according to their location.

In the one-way ANOVA, *SDC2* was significantly hypermethylated in CNM with *H. pylori* infection (p=0.0056, average difference, 4.4) (Fig. 7C). Aberrant methylation of *SDC2* was found to correlate with diffuse-type and mixed-type GCs (Fig. 7D and E).

Discussion

Based on methylation analysis using IIMA validated by PA, we indirectly verified that certain methylation changes are closely associated with the carcinogenesis of GC. Moreover, different subsets of aberrantly methylated genes are involved in the carcinogenesis of a specific histologic subtype of GC, logically suggesting that mixed-type GC may be a distinct subtype. Hierarchical clustering analysis of IIMA was broadly concordant with the results of PA. Tumors of group A and B in IIMA showed a sharply demarcated distinction in the methylation pattern for 169 genes (Fig. 3B). A rather clear grouping of 208 genes in 3 cases of group B indirectly denoted the molecular uniqueness of mixed-type GCs, although the data should be interpreted with extreme caution due to the limited number of cases and incomplete grouping.

Based on an integrated interpretation of the statistical analysis of the results of PA, we classified the genes with their methylation patterns according to the histologic subtypes into three groups: i) *DVL2* and *ETS1*, ii) *C19orf35* and *CNRIP1*, and iii) *GAL3ST2* and *ITGA3* (Fig. 5).

DVL2 and *ETS1* were specific to diffuse-type and mixed-type compared to the intestinal-type subgroup. Both genes showed a similar methylation pattern both in the CNM and in the tumors of the intestinal-type while they showed significantly higher methylation in the CNM as well as in the tumors of the mixed-type and diffuse-type rather than the intestinal-type (Fig. 5A and B). These results are consistent with previous findings that some aberrantly methylated genes in tumors of certain subtypes are also aberrantly methylated in CNM (16). This suggests that the aberrant methylation of these two genes may start from the CNM exclusively in mixed-type and diffuse-type subgroups. Similarly to these results, these genes showed significant difference in methylation in the CNM between group A and B in the hierarchical clustering analysis of IIMA. *Dishevelled*, symbolized as *DVL2*, is a positive mediator of Wnt/β-catenin signaling and its aberrant overexpression has been known to result in neoplastic transformation, yet the direct involvement in human cancer has not been reported to date (17). Since the function of Wnt signaling components in gastric carcinogenesis has been recently described, it seems advisable to evaluate the methylation change of this gene in relation to other components of Wnt signaling. Contrary to our screening results and the accumulated evidence on the aberrant methylation of *APC* in GC, we could not find any connection between *APC* and the parameters examined (16). The *ETS1* proto-oncoprotein is a member of the Ets family of transcription factors and has been implicated in invasive behavior in relation to angiogenesis in many types of human cancer such as astrocytomas, breast cancer and some types of colon cancer (18). It is not expressed in normal gastric epithe-
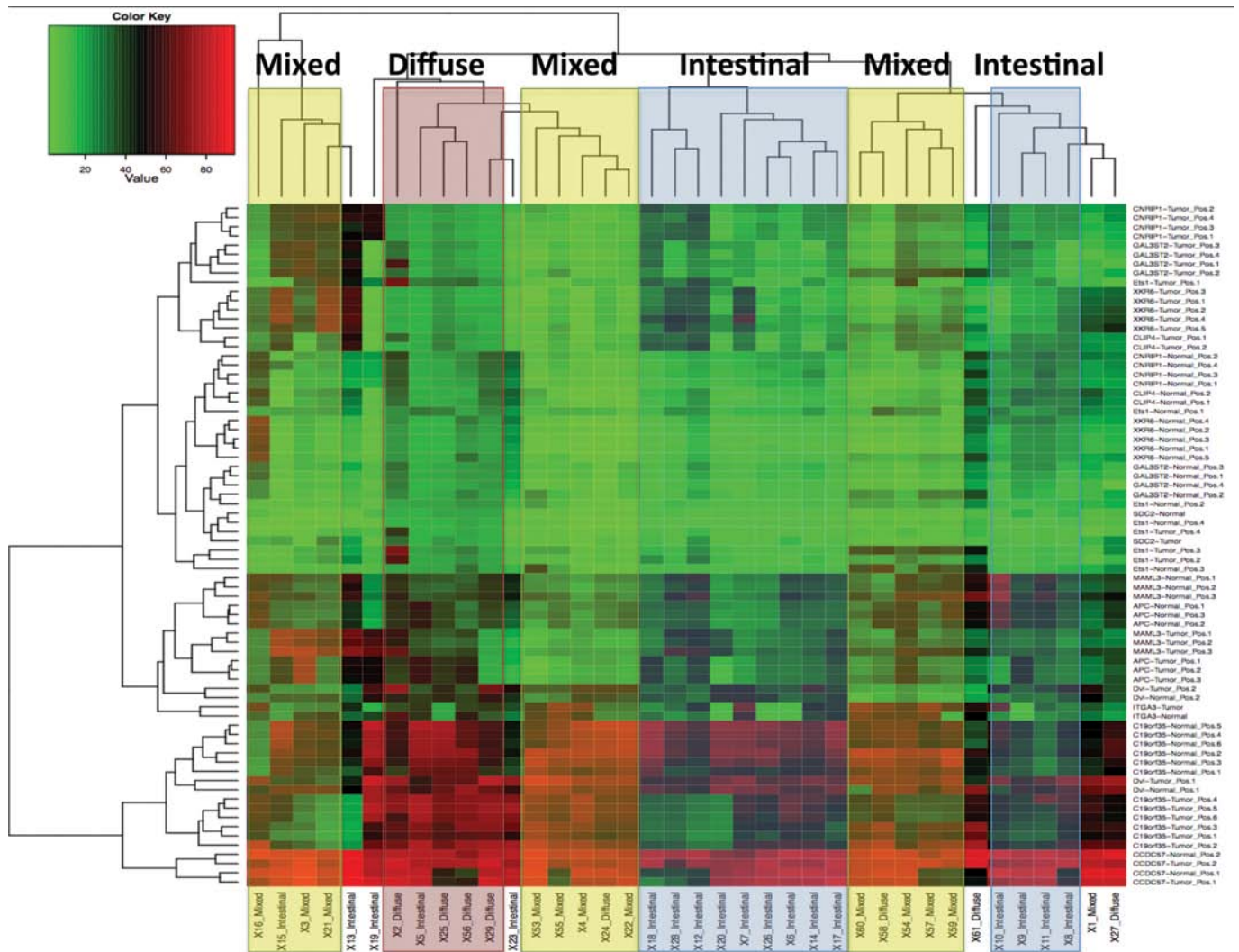
Figure 6. Hierarchical clustering analysis of the pyrosequencing assay.

lium and is overexpressed in GC, resulting in its value as a marker of poor prognosis (18-20). To our knowledge, no information exists to date on the methylation change of this gene in GC. Further evaluation for comprehensive understanding is needed.

C19orf35 and *CNRIP1* were specific to diffuse-type compared to the intestinal-type subgroups. Both revealed a similar level of methylation in CNM regardless of the histologic subtype of tumor while the methylation of the tumor tissue varied with the histologic type (Fig. 5C and D). *C19orf35* was hypermethylated but *CNRIP1* was hypomethylated in diffuse-type tumors compared to the CNM while it had an opposite trend in the tumors of the intestinal-type and mixed-type subgroups. Importantly, these findings suggest the possibility of utilizing these genes as specific markers for diffuse-type GCs. On the other hand, the function of *C19orf35* is still unknown while frequent methylation of *CNRIP1* has recently been reported in colorectal cancers and adenoma (21,22).

GAL3ST2 and *ITGA3* were specific to mixed-type compared to intestinal-type and diffuse-type subgroups. They were specifically hypermethylated in CNM as well as in the tumor tissue of mixed-type GC (Fig. 5E and F). Thus, this analogously represents the molecular unique-

ness of mixed-type GCs contrary to the idea that these are intermingled tumors of intestinal-type and diffuse-type GCs. Hierarchical clustering of IIMA also showed a distinctive methylation pattern of mixed-type compared to diffuse-type GCs, although the data were not perfect. Little is known about the functional role of *GAL3ST2*, a member of the galactose-3-*O*-sulfotransferase protein family, in human types of cancers. However, a recent series of evidence indicates that downregulation of *GAL3ST2* expression is observed in colon cancers, and *GAL3ST2* has also been implicated in the metastatic potential of breast and laryngeal cancers (23-25). Considering the mounting evidence on the central role of selectins such as galectin-3 in thyroid, pancreatic, colon and even GCs in relation to several relevant genes, the associative function of Gal:3-*O*-sulfotransferase in GCs should be emphasized and evaluated in more detail (26-29). For *ITGA3*, it has been established that α3β1 integrin is involved in the maintenance of basement membrane integrity through adhesion to laminin 5 and plays an essential role in the invasiveness and metastatic potential of tumor cells (30-32). However, there are contradictory reports regarding the significance of *ITGA3* expression in various types of cancers (33-36). Considering the results demonstrated recently that *GAL3ST3* is involved
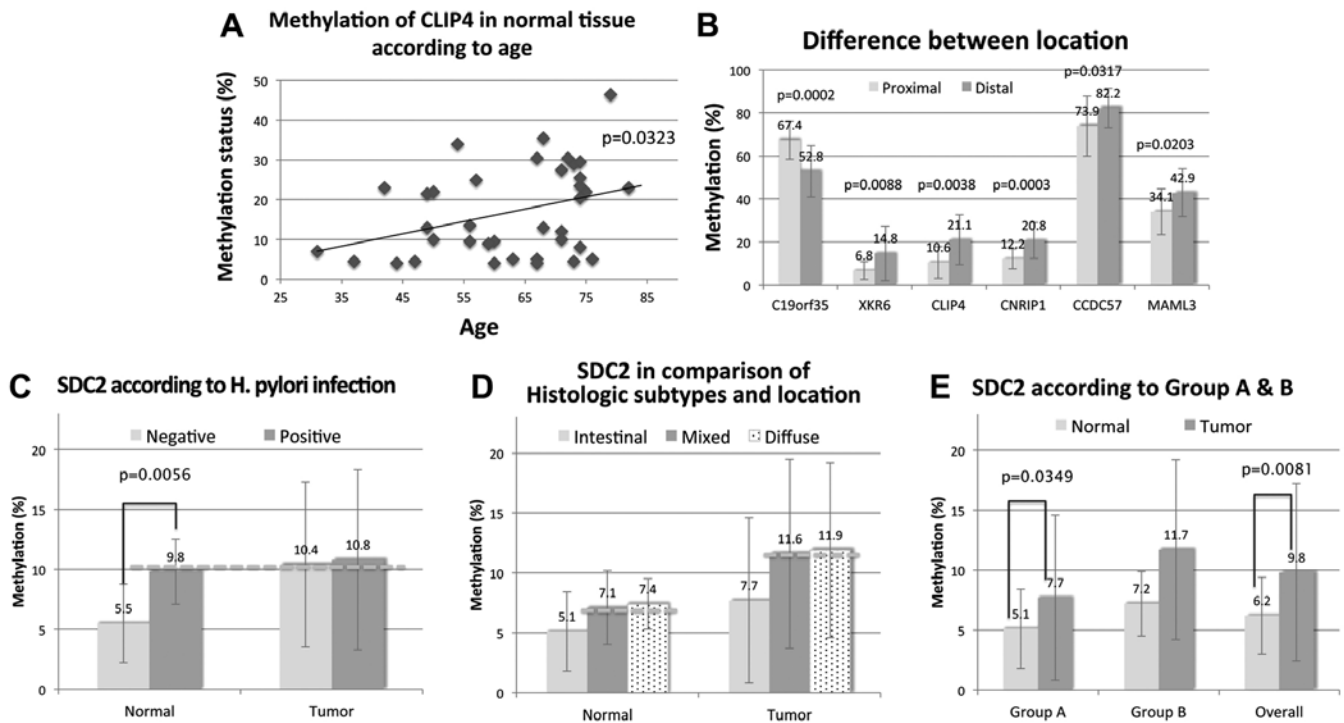
Figure 7. Methylation of various genes in relation to age, location and *H. pylori* infection in normal tissues. (A) The methylation of *CLIP4* in the normal tissues increased with the age of the GC patients in the correlation analysis. (B) The methylation of six genes, *C19orf35*, *XKR6*, *CLIP4*, *CNRIP1*, *CCDC57* and *MAML3*, in the normal tissues significantly varied according to tumor location. (C) *SDC2* exhibited significant hypermethylation in CNM with H. pylori infection. (D and E) Aberrant methylation of *SDC2* was found to correlate with diffuse-type and mixed-type GC.

in tumor metastasis by regulating the ability of adhesion to selectins and expression of integrins, more evidence is needed to clarify the relationship among these molecules (25). A recent study by Park *et al* strengthens the results of the present study by showing significantly higher frequencies of CpG island hypermethylation of 16 genes in mixed-type GCs when compared to diffuse-type and intestinal-type subgroups (37). They found no difference in the number of methylated genes between diffuse-type and intestinal-type-like components of mixed-type cancers, rejecting the hypothesis that mixed-type GCs may be the mixture of intestinal-type and diffuse-type GCs.

The heatmap made with the pyrosequencing data of the aforementioned 6 genes also demonstrated a rather clear distinction between each subtype (Fig. 6).

Regarding the results of *SDC2* methylation in relation to *H. pylori* infection, this hypothesis seems to be more credible since *SDC2* in *CNM* with *H. pylori* infection revealed significant hypermethylation compared to CNM without *H. pylori* infection up to the level of tumor regardless of *H. pylori* infection (Fig. 7C). These results are in line with the results of previous studies that some genes such as *CDH1*, known for having diffuse-type specificity, are hypermethylated in cases with *H. pylori* infection (12,16). These indirectly suggest the possible pathogenic relationship between the genes and *H. pylori* infection as well as between *H. pylori* infection and certain histologic types of GC. In our results, aberrant methylation of *SDC2* was found to correlate with diffuse-type and mixed-type GC (Fig. 7D and E). Recently, a series of genes have been implicated in gastric carcinogenesis in rela-

tion to *H. pylori* infection (38-43). However, *SDC2* was not included in the list. *SDC2* is known as one of a four-member family of transmembrane heparin sulfate proteoglycans and plays important roles in many human types of cancers such as colorectal, hepatic and pancreatic cancers (44,45). The function of *SDC2* in the gastric carcinogenesis of GC is still unclear. Interestingly, a recent study raised the possibility that upregulation of *SDC2* may play an important role in peritoneal metastasis of human GC cells (46). Taken together, these results emphasize the importance of molecular assessment of premalignant or even non-neoplastic mucosa of stomach in the presence of *H. pylori* infection.

Even in the non-tumor tissues, the methylation status of certain genes was affected by various factors such as age, location and the status of *H. pylori* infection similar to the results of previous studies (16). Similar to *CLIP4* in the present study, other genes have also been documented to show a general progressive increase in methylation in normal gastric tissues as the age of the patient increases (16). Six genes showed significantly differential methylation by PA between the anatomic locations (Fig. 7B). Considering that the component of gastric mucosa varies with location, a differential methylation status of genes according to location in normal tissues may be justifiable. Similar results in previous studies have demonstrated differences in the methylation status of genes in non-neoplastic tissues of different organs or different sites in an organ (15).

XKR6 showed significant hypermethylation in the CNM of the distal stomach (p=0.0009) as well as in overall tumor tissues (p=0.008). This suggests that certain methylation changes may be involved in making specific locations of

the stomach susceptible for carcinogenesis. Recent studies have reported that the methylation statuses of certain genes such as *CDKN2A*, *COX-2* and *HMLH1* are significantly different according to tumor location (38,39). Little is known concerning *XKR6* in human disease except that it is a member of the Kell blood group complex subunit-related family and its SNP (rs6985109) may be a potential risk factor in systemic lupus erythematosus (47).

The evaluation of the methylation status of certain genes may be helpful to distinguish between the histologic subtype of EGC. The methylation statuses of *DVL2*, *ETS1*, *C19orf35*, *CNRIP*, *GAL3ST2* and *ITGA3* were significantly different according to the histologic subtype of the EGC. We found that the methylation patterns of *GAL3ST2* and *ITGA3* were specifically different in mixed-type compared to the intestinal-type or diffuse-type subgroups, suggesting that the mixed-type is a distinct histologic subtype. The methylation of various genes that are associated with non-tumor factors such as age, location and *H. pylori* infection should be considered.

Acknowledgements

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