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CpG Island Methylation According to the Histologic Patterns of Early Gastric Adenocarcinoma

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Background: Although the importance of aberrant DNA methylation in the development of gastric adenocarcinoma has been described, the mechanism of pathogenesis has not been revealed yet. We quantitatively analyzed methylation of four CpG islands and one repetitive DNA element, according to the histologic features of adenocarcinoma with precursor lesions. **Methods:** We divided the cases as adenocarcinoma with intestinal type precursors (type A, n=19 cases) and adenocarcinoma with diffuse type precursors (type B, n=19 cases). We micro-dissected tumor cells and matched non-neoplastic gastric mucosa from the hematoxylin and eosin-stained slides. **Results:** A total of 20 CpG sites of long interspersed nucleotide element-1 (*LINE1*), RAR-related orphan receptor alpha (*RORA*), Kruppel-like factor 7 (*KLF7*), mutL homolog 1 (*MLH1*), *MINT25,* and *CD133* were analyzed. Methylation was determined by bisulfate-pyro-sequencing, and hypomethylation of *LINE1* and *CD133* was noted in the tumors, compared to the levels in the non-neoplastic gastric mucosa (p=0.014 and p=0.015, respectively). A statistically different methylation pattern of CpG sites at *CD133* and *KLF7* was noted only in type B lesions, compared to that in matched non-neoplastic gastric mucosa (p=0.027 and p=0.043, respectively). **Conclusions:** Given that aberrant methylation occurs in a relatively early phase of carcinogenesis, different patterns of methylation may determine the carcinoma phenotype. However, further large-scale study is required to clarify the significance of this difference.

Key Words: Adenoma; CpG Islands; DNA methylation; Dysplasia; Stomach

Gastric cancer is one of the most common forms of malignancy and is associated with high mortality, ranked third for cancer death in Korea in 2009.[1] Fatality has been reported in 23% of patients, which is probably due to patient presentation with advanced disease.[2] In spite of a steady decline in both the incidence and mortality of gastric carcinoma, the incidence of diffuse carcinoma localized to the proximal stomach has been increasing.[3]

It has been suggested that epigenetic abnormalities might play a critical role in the earliest steps of cancer initiation. Recent studies suggested that the abnormal epigenetic silencing of genes occurs frequently during the early stages of the neoplastic process, such as the precancerous stage of tumor development.[4] Specifically, gene silencing by aberrant methylation of CpG dinucleotide-rich areas, known as CpG islands (CGIs), has been suggested during oncogenesis.[5] Moreover, frequent CGI methylation in early gastric carcinogenesis has been documented in the literature.[6]

Two major histological types of gastric adenocarcinoma, i.e., the intestinal and diffuse types, are based on the Lauren's classification.[7,8] Different pathogeneses and genetic alterations have been proposed for these two distinctive histological types. The more frequent intestinal type is often preceded by sequential steps of precancerous changes, whereas the diffuse type of gastric carcinoma tends to arise *de novo*, and is less commonly associated with dysplasia or adenoma. Similarly, two morphologically distinct gastric precancerous lesions of adenoma and flat dysplasia have been documented. In European countries and North America, adenomatous lesion usually refers to the neoplastic proliferation that produces a discrete, protruding lesion, whereas in Japan adenoma includes all types.[3] On the basis of the similarities between the morphological and genetic aspects of colorectal and gastric cancers, it has been postulated that some gastric carcinoma may arise from either gastric adenomas or flat dysplasias, similar to the colorectal adenoma-carcinoma sequence.[9] Given that the epigenetic regulation of carcinogenesis plays a role during a relatively early phase, different genetic and epigenetic alterations separating the two lesions are expected to exist.

Findings from several studies indicated the necessity of considering the histologic type of gastric adenocarcinoma, because

of the different patterns of methylation-related gene regulation.[10,11] For example, expression of transcription factor 4 (TCF4), a member of the class A sub-family of bHLH transcription regulators, is known to be silenced by DNA methylation, and to play a role in the development and progression of gastric carcinoma.[10] In that study, a different level of DNA methylation was noted between intestinal and diffuse types.[10] A recent study by Nakamura *et al.*[11] revealed that the amount of methylation was significantly correlated with carbonic anhydrase 9 (*CA9*), and the methylation value of a -74 bp site in the *CA9* promoter was significantly lower in the intestinal type, compared with diffuse-type lesions. Thus, we can hypothesize that analysis without considering the histologic type of carcinoma may either dilute or over-exaggerate the role of epigenetic regulation by methylation in certain carcinoma subgroups with different carcinogenesis, since heterogeneity of histology within a studied group may mask the potential contribution of the methylation pattern to the regulation of gene expression.

The aim of the current study was therefore to quantitatively analyze the promoter methylation of multiple tumor-related genes, according to two different histological types of early gastric adenocarcinoma associated with precursors.

Table 1. Clinicopathological characteristics of patients

		Type A	Type B
Age (mean ± SD, yr)		67.2 ± 8.1	56.2 ± 12.6
Sex	M	16	12
	F	3	7
Helicobacter pyroli infection	Positive	12	11
	Negative	6	7
	Unknown	1	1
Intestinal metaplasia	Present	17	16
	Absent	2	3
Histological type			
Adenocarcinoma	Well	11	0
	Moderate	7	5
	Poor	1	7
Signet ring cell carcinoma		0	7
Mixed		0	2
Gross type	I	3	1
	IIa	7	2
	IIb	7	4
	IIc	2	12
	III	0	0
Location	Upper 1/3	0	0
	Mid 1/3	4	3
	Lower 1/3	15	16
Node metastasis	Yes	1	4
	No	7	11
	Unknown	11	4

SD, standard deviation; M, male; F, female.

MATERIALS AND METHODS

Case selection

This study was approved by the Institutional Review Board of Yonsei University College of Medicine, Wonju Christian Hospital. Thirty-eight cases of early gastric carcinoma in 38 patients from Yonsei University Wonju Christian Hospital were selected, including 25 cases of gastrectomy and 13 cases of mucosectomy. To minimize the selection bias of this study in a limited number of cases, the patients' age and lesion location were controlled. For the control group, we used matching non-neoplastic gastric mucosa from the surgical resection margin. According to the histological pattern of growth in the gastric adenocarcinoma with precursor, the tumors were divided into two types: intestinal type adenocarcinomas associated with their precursors (type A, n = 19 cases) and diffuse type adenocarcinomas with their precursors (type B, n = 19 cases). Clinicopathological features of the cases are summarized in Table 1. Representative histological features of the two groups are shown in Fig. 1. Type A lesions were defined as early adenocarcinoma with an intestinal precursor lesion mimicking tubular adenoma, whereas type B lesions were defined as diffuse type adenocarcinoma with precursors. In the latter type, the morphological criteria included two types of precursor lesions, defined according to the World Health Organization (WHO) classification of digestive system tumors and the lesion previously known as tubular neck dysplasia.[12-14] Specifically, the first is described as *in situ* signet ring cell carcinoma (Tis), corresponding to the presence of signet ring cells within the basal membrane, replacing normal epithelial cells. The second is a pagetoid spread pattern of signet ring cells located below the preserved epithelium of glands and foveolae, but within the basal membrane.[14] Two pathologists (J. Choi and M. Y. Cho) reviewed the slides and concordant findings.

DNA extraction

Micro-dissection for paired neoplastic and non-neoplastic gastric mucosa from each case was performed under microscopy of hematoxylin and eosin-stained slides of formalin-fixed, paraffin-embedded tissue. Transitional areas of early invasive gastric carcinoma arising from precursor lesions were included for study. We scraped the tissue under the microscope and extracted DNA from each sample using a DNeasy Blood and Tissue kit (cat. No. 69506, Qiagen, Valencia, CA, USA), according to the man-

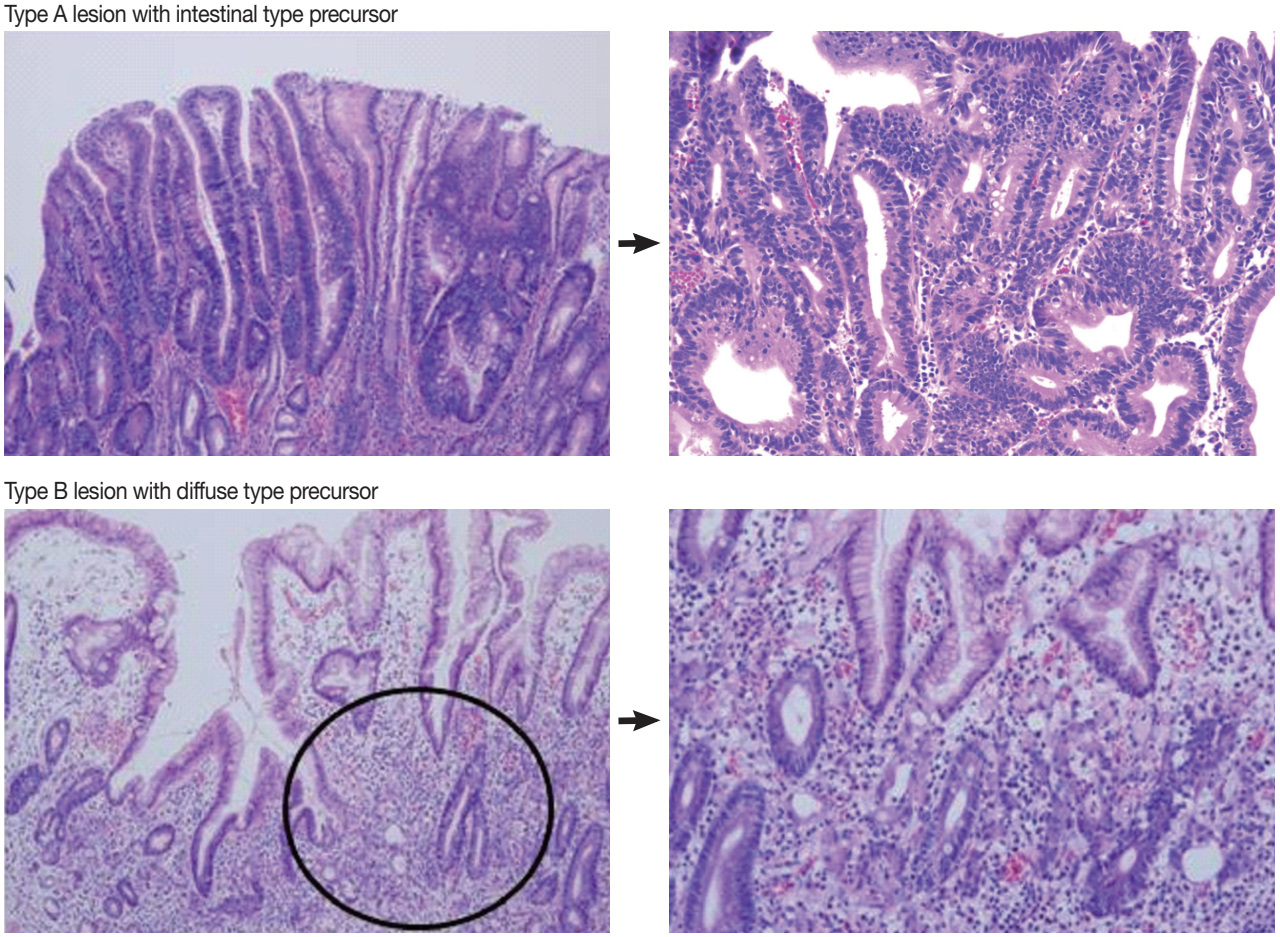

Fig. 1. Representative images of type A and type B lesions. Type A lesion is the classical intestinal type of adenocarcinoma with precursor. Type B lesion is a diffuse type of adenocarcinoma, accompanied by tubular neck cell dysplasia and/or *in situ* signet ring cell carcinoma.

ufacturer's instructions.

Selection of repetitive DNA elements and CpG sites of related genes

Methylation statuses of 20 CpG sites of repetitive DNA elements (long interspersed nuclear element-1 [*LINE1*]) (n = 3) and five selected genes, including RAR-related orphan receptor alpha (*RORA*) (n = 3), Kruppel-like factor 7 (*KLF7*) (n = 3), mutL homolog 1 (*MLH1*) (n = 2), *MINT25* (n = 5) and *CD133* (n = 4), previously suggested or potentially related to early gastric carcinogenesis, were assessed using pyro-sequencing-based methylation analysis.[15]

Bisulfite conversion and pyro-sequencing analysis of DNA methylation

Genomic DNA was modified with sodium bisulfate using the EpiTect® bisulfatite kit (cat. No. 59104, Qiagen), according to the manufacturer's instructions. All primers for pyro-sequencing were designed with Pyrosequencing Assay Design 2.0 (Qiagen/Biotage, Uppsala, Sweden). Bisulfite-treated genomic DNA was used as a template in subsequent polymerase chain reaction. All of the primers and polymerase chain reaction (PCR) conditions used for amplifying CpG DNA fragments of methylation marker genes are listed in Table 2. For each gene, a 30 μL PCR reaction was carried out with HotStarTaq plus mast mix (cat. No. 203645, Qiagen), to label bisulfate-converted DNA. After PCR, the biotinylated strand was captured on streptavidin-coated beads (Amersham Bioscience, Uppsala, Sweden) and incubated with sequencing primers. Pyrosequencing was performed with PSQ HS 24 Gold single-nucleotide polymorphism reagents on a PSQ HS 24 pyrosequencing machine (Biotage, Uppsala, Sweden). The protocol for pyrosequencing has been described in detail previously.[15] Pyrosequencing quantitatively measures the methylation status of several CpG sites in a

Table 2. Primer sequences and polymerase chain reaction (PCR) conditions for methylation analysis

Gene		Primers	PCR conditions	Product size (bp)	CpG sites
LINE1	F	TTTTGAGTTAGGTGTGGGATATA	95℃, 30 sec	146	3
	RU	GGGACACCGCTGATCGTTTAAAAATCAAAAAA TTCCCTTTC	56℃, 30 sec × 45 cycles		
	S	AGTTAGGTGTGGGATATAGT	72℃, 30 sec		
	BU	Biotin-GGGACACCGCTGATCGTTTA			
RORA	F	TTTGGTATTATAGAGTTGTTTTGAAAATAGAA	95℃, 30 sec	105	3
	R1	ACCCAAACTAACTCCATATTTTTTCC	56℃, 30 sec × 45 cycles		
	RU	GGGACACCGCTGATCGTTTAACCCAAACTAACTCCATATTTTTTCC	72℃, 30 sec		
	S	TGAAAATAGAAGATAGAGGGA			
KLF7	F	GGTTTTTAGTGATTTTATGAGTTTTTTGTTTATT	95℃, 30 sec	185	3
	R	CCACCCCATCCTTACTAATTTATAATCT	56℃, 30 sec × 45 cycles		
	RU	GGGACACCGCTGATCGTTTACCTTTTCTCCTCCTCCTACTCTTC	72℃, 30 sec		
	S	TTTTTGTTTATTTTATATGGTTA			
MINT25	F	TGTTTGTAAAGGGTTGGAATTATT	95℃, 30 sec	175	5
	R	CCCRCCAAAACAACTTTA	55℃, 30 sec × 45 cycles		
	RU	GGGACACCGCTGATCGTTTACCCRCCAAAACAACTTTA	72℃, 30 sec		
	S	TAGTTTATTATTTTTAAGAG			
hMLH1	F	GGGAGGGAYGAAGAGATTTAGTAA	95℃, 30 sec	165	2
	R	CTTCAACCAATCACCTCAATACC	57℃, 30 sec × 45 cycles		
	RU	GGGACACCGCTGATCGTTTA CTTCAACCAATCACCTCAATACC	72℃, 30 sec		
	S	AGTTATAGTTGAAGGAAGAA			
CD133	F	GGAGTAGGGATATGGGGGTATAAA	95℃, 30 sec	163	4
	R	Biotin-AAACACCCCAATTCTCCATCT	54℃, 30 sec × 45 cycles		
	S	GGGATATGGGGGTATAAA	72℃, 30 sec		

LINE1, long interspersed nucleotide element-1; *RORA,* RAR-related orphan receptor alpha; *KLF7,* Kruppel-like factor 7; *hMLH1*, human mutL homolog 1; F, forward; R, reverse; U, unmetylated; B, biotin; S, streptavidin.

given promoter. These adjacent sites usually showed highly concordant methylation. Therefore, the mean percentage of methylation at detected sites was used as a representative value for the degree of methylation in each gene.

Statistical analysis

The data was statistically processed using SPSS ver. 18.0 (SPSS Inc., Chicago, IL, USA). For comparison between type A and type B lesions in the methylation of a certain gene, the Mann-Whitney U test was performed. Wilcoxon signed rank test was used for comparison of methylation degree between paired normal and tumor tissues. Statistical significance was assumed when $p < 0.05$.

RESULTS

Hypomethylation of *LINE1* and *CD133* was observed in tumors, compared to the levels in non-neoplastic gastric mucosa

Thirty-eight cases of early carcinoma were selected for analysis. We randomly selected the cases and performed age and lesion location adjustments to minimize selection bias. Since the status of *Helicobacter pylori* infection and the intestinal metaplasia of normal stomach tissue may potentially affect the results, such information was also provided. Nineteen cases each of type A and type B lesions were examined.

A total of 20 CpG sites of *LINE1, RORA, KLF7, MLH1, MINT25,* and *CD133* were quantitatively analyzed by pyrosequencing. First, we compared the methylation degree of non-neoplastic gastric mucosa and lesions in the type A and type B groups. In all tested genes, there was no statistically significant difference in the degree of methylation between non-neoplastic mucosa of type A and type B groups. We then analyzed the difference in methylation between non-neoplastic gastric mucosa and tumors, including both type A and type B. When analyzed by the non-parametric test, significant hypomethylation of *LINE1* and *CD133* was noted in tumors ($p = 0.014$ and $p = 0.015$, respectively) (Table 3).

A statistically significant aberrant methylation pattern was noted in *KLF7* and *CD133* genes only in type B lesions

When individual analyses were performed, different patterns of aberrant methylation were noted in type A and type B lesions. When compared with matching non-neoplastic gastric mucosa, only type B lesions showed hypermethylation at the

Table 3. Methylation profiles of matched non-neoplastic gastric mucosa and tumor tissues

Gene	Normal	Tumor (types A and B)	p-value
	Methylation (%)[a]	Methylation (%)[a]	
LINE1	67.44 (13.28)	63.79 (7.28)	**0.014**
RORA	33.44 (20.27)	32.68 (24.03)	0.695
KLF7	4.78 (8.22)	8.02 (12,87)	0.07
hMLH1	4.84 (8.57)	2.87 (2.71)	0.557
MINT25	19.43 (12.78)	27.19 (22.21)	0.128
CD133	11.32 (11.61)	6.12 (4.66)	**0.015**

Hypomethylation of *LINE1* and *CD133* is noted in the tumors.
[a]Values are presented as mean (standard deviation).
LINE1, long interspersed nucleotide element-1; *RORA,* RAR-related orphan receptor alpha; *KLF7,* Kruppel-like factor 7; *MLH1,* mutL homolog 1.

CpG sites of *KLF7*, whereas those of *CD133* showed significant hypomethylation ($p = 0.043$ and $p = 0.027$, respectively). A tendency for hypomethylation at CGIs of *LINE1* was reported in both types, but the difference was not statistically significant, probably due to the small sample size (Table 4). Methylation profiles of each locus within candidate genes are shown in Fig. 2. When we compared paired non-neoplastic mucosa and tumor samples, there was no statistically significant difference in the DNA methylation of *RORA, MLH1,* and *MINT25* genes examined in this study set.

DISCUSSION

Epigenetic alterations in cancer have been highly regarded as crucial regulators of gene expression without affecting the primary DNA sequence. Three types of changes, including chromatin modification, DNA methylation and genomic imprinting, have been described in cancer cells. Among these, the importance of DNA methylation has been established and well described in several human cancers, including colorectal, pancreatic and uterine cervical carcinomas.[4,16-18] Epigenetic gene silencing can occur during the early phases of carcinogenesis, even in pre-invasive lesions, and can involve disruption or overactivation of cardinal cell signaling pathways, leading to aberrant early clonal expansion of cells.[4] In the gastric carcinoma, promoter methylation seems to be related to carcinogenesis in certain groups.[19] Lee *et al.*[6] showed that concurrent promoter methylation was an early and frequent event in gastric carcinoma, both in highly unstable and stable microsatellite neoplasms. In addition, a marked increase in methylated genes from non-metaplastic mucosa to intestinal metaplasia, as well as from precursor lesions to carcinoma has been suggested.[6]

Table 4. Methylation profiles of type A and type B lesions

Gene		Type A		Type B	
		Methylation (%)[a]	p-value	Methylation (%)[a]	p-value
LINE1	N	69.24 (9.19)	0.116	65.63 (16.46)	0.067
	T	64.54 (7.94)		63.37 (6.73)	
RORA	N	34.89 (20.78)	0.286	31.98 (20.20)	0.687
	T	30.42 (23.1)		34.95 (25.34)	
KLF7	N	5.48 (10.31)	0.444	4.08 (5.61)	**0.043**
	T	3.79 (3.95)		12.26 (16.95)	
MLH1	N	4.84 (8.57)	0.778	2.87 (2.71)	0.243
	T	3.61 (3.70)		4.92 (7.53)	
MINT25	N	21.03 (13.90)	0.481	17.82 (11.69)	0.171
	T	27.92 (22.70)		26.46 (22.31)	
CD133	N	10.44 (8.10)	0.199	12.21 (14.49)	**0.027**
	T	7.15 (5.48)		5.08 (3.51)	

Statistically significant aberrant pattern of methylation is noted in *KLF7* (hypermethylation) and *CD133* (hypomethylation) only in type B lesions.
[a]Values are presented as mean (standard deviation).
LINE1, long interspersed nucleotide element-1; *RORA,* RAR-related orphan receptor alpha; *KLF7,* Kruppel-like factor 7; *MLH1,* mutL homolog 1; N, non-neoplastic; T, tumor.

The present results showing hypomethylation of *LINE1* and *CD133* in tumors confirmed the results of previously reported cases.[20,21] Although performed in a limited numbers of cases, our analysis according to histological type revealed different patterns of aberrant methylation. Thus, hypomethylation of *CD133* and hypermethylation of *KLF7* seems to be more related to the development of type B lesions, i.e., the diffuse type of early adenocarcinoma, in this study set. Our results support the idea that quantitative analysis of DNA methylation in lesions with different histology types should be performed separately, considering the heterogeneity of cellular and molecular mechanisms in the subgroups of gastric carcinoma. To understand the biological implication of the epigenetic control of DNA methylation, a targeted group of patients should be selected and included in the analysis.

Our initial hypothesis was that the tubular neck portion of type B lesions serves as an initiating point of carcinogenesis. Accordingly, a subset of cells may express stem-like features, and the expressions of related gene products may be regulated by a certain epigenetic mechanism, such as DNA methylation. The pre-invasive lesions of the intestinal type of gastric carcinoma have been well recognized, whereas those of the diffuse type have not been studied thoroughly.[13] Tubule neck dysplasia (TND) was described as a neoplastic precursor lesion of diffuse gastric carcinomas, and has been reported in some patients with hereditary gastric carcinoma.[22] Kumarasinghe *et al.*[13] described TND as architecturally abnormal tubular neck areas lined by cytologically abnormal epithelial cells, expressing gastric mucin cou-

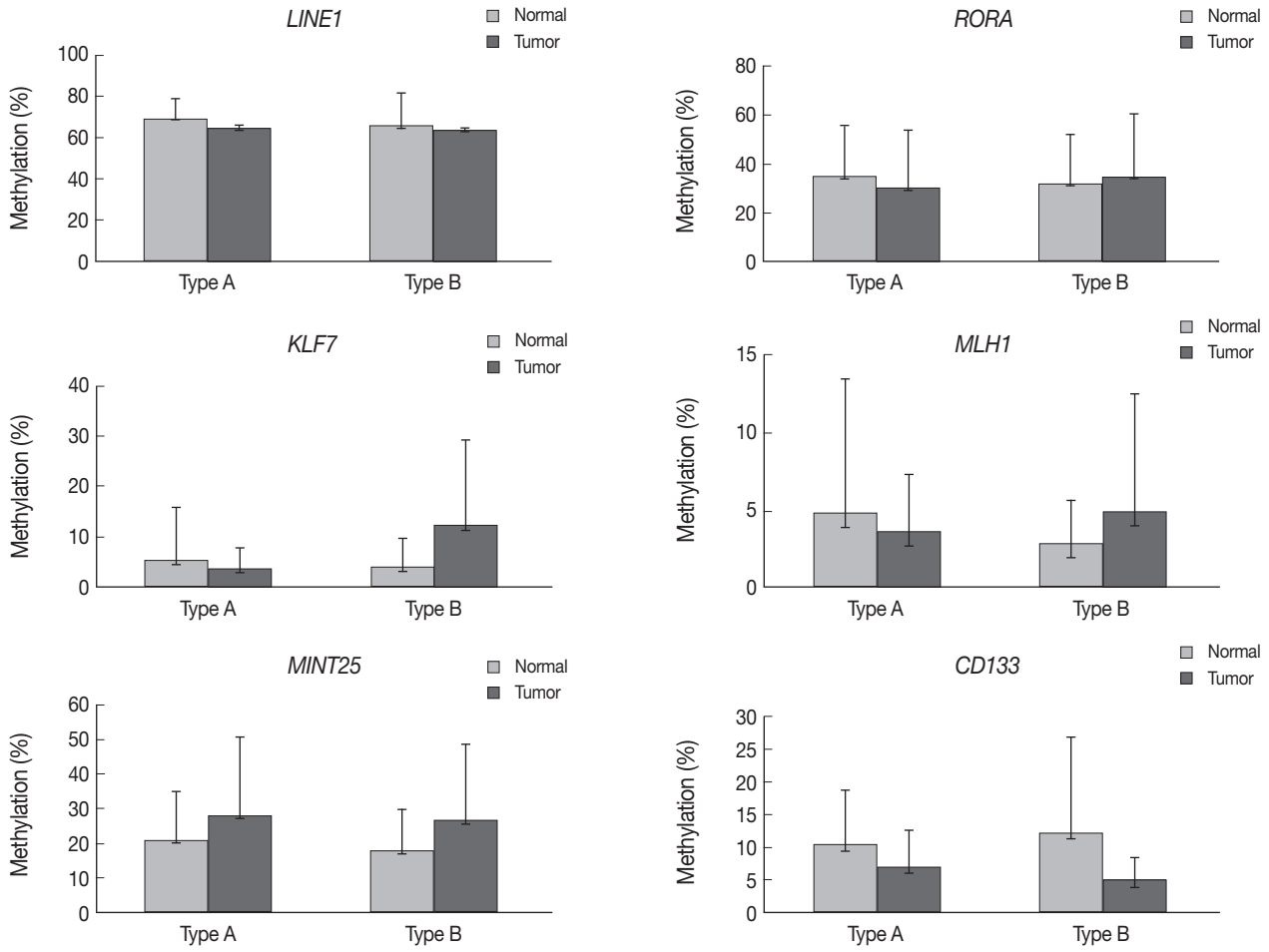

Fig. 2. Different methylation profiles are noted in type A and type B lesions (Table 4). Statistically different methylation patterns of both *KLF7* and *CD133* are noted only in type B lesions (error bar, standard deviation).
LINE1, long interspersed nucleotide element-1; *RORA,* RAR-related orphan receptor alpha; *KLF7,* Kruppel-like factor 7; *hMLH1,* human mutL homolog 1.

pled with enhanced MIB-1 and p21. Morphologically, the described precursor lesion was well correlated with precursors of the type B lesion in this study. In the setting of hereditary diffuse gastric carcinoma, most of the studies have focused on *CDH1* hypermethylation, but another line of evidence showed that global hypomethylation of a long interspersed nuclear element may contribute to gastric tumorigenesis via an unknown mechanism.[20] Accordingly, we suggest that aberrancy in the methylation of certain groups of genes contributes to the regulation of gene expression during early gastric carcinogenesis, especially in carcinomas with diffuse type precursors.

Recently, demethylation of the *CD133* gene was reported in gastric neoplasm.[21] The authors examined 36 patients of early gastric carcinoma using quantitative methylation-specific polymerase chain reaction and demonstrated demethylation of *CD133* in 14 cases, indicating that *CD133* may be frequently demethylated in early gastric carcinoma.[21] They analyzed the association of several clinicopathological features and demethylation, but there was no statistically significant difference in methylation between well/moderately differentiated histology and poorly differentiated histology cases. They did not analyze the cases according to the accompanying precursor lesions.[21] A recent study revealed that aberrancy in *CD133* methylation is associated with aberrant gene silencing in both colorectal carcinoma and glioblastoma, and it is likely that the dynamic nature of *CD133* methylation contributes to the regulatory mechanisms of *CD133* expression and gastric carcinogenesis, as in the colon.[23] Notable ovarian cancer findings showed that DNA methylation at the active promoter site was inversely correlated with *CD133* transcription.[24] Given that *CD133* expression is known to be closely related to the tumor initiating properties in certain groups of carcinoma, further studies on the correlation between

CD133 expression and methylation status should be performed.

KLFs are DNA-binding transcriptional regulators involved in differentiation and development.[25] Only few published studies are currently available regarding the biological implication of *KLF7* in carcinogenesis. Interestingly, *KLF4* has been suggested as a putative tumor suppressor in the gastrointestinal tract, and aberrant methylation of the *KLF4* gene was observed in a subset of stomach cancers.[26,27] Since KLFs share homology and serve as transcription regulatory factors for the differentiation of the cells, the potential role of *KLF7* in the regulation of early gastric carcinogenesis should be elucidated.

Several studies revealed the association of DNA methylation and *Helicobacter pylori* infection with intestinal metaplasia in the stomach, which may potentially affect the methylation status in each lesion.[28,29] When analyzed statistically, the levels of infection and intestinal metaplasia did not show differences between the two groups, but we refrained from any conclusion, due to the small number of analyzed samples. Nevertheless, we analyzed the methylation status of the neoplastic lesions with matched non-neoplastic mucosa of each patient and performed paired analysis, so that changes in methylation within an individual were properly assessed.

In summary, we identified an aberrant pattern of methylation in both *CD133* and *KLF7* genes only in early gastric carcinoma accompanied with a diffuse type of precursor lesion (type B). The difference in the epigenetic regulation suggests the presence of different pathways of early carcinogenesis in two morphologically distinguished phenotypes. Given the fact that differences in clinical behavior and prognosis exist between the two groups, further study on their clinical implications should be considered in a large scale sample.

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