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Human Cancer Biology

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A Novel Correlation between *LINE-1* Hypomethylation and the Malignancy of Gastrointestinal Stromal Tumors

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Abstract

Purpose: Gastrointestinal stromal tumors (GIST) are the most important mesenchymal tumors of the gastrointestinal tract. The vast majority of GISTs exhibit activating mutations of *KIT* or *PDGFRA*, but epigenetic alteration of GISTs is largely unknown. In this study, we aimed to clarify the involvement of DNA methylation in GIST malignancy.

Experimental Design: A total of 106 GIST specimens were studied. Levels of *LINE-1* methylation were analyzed using bisulfite pyrosequencing. In addition, methylation of three other repetitive sequences (*Alu Yb8*, Satellite-α, and *NBL2*) was similarly analyzed, and CpG island hypermethylation was analyzed using MethyLight. Array-based comparative genomic hybridization (array CGH) was carried out in 25 GIST specimens.

Results: *LINE-1* hypomethylation was significantly correlated with risk, and high-risk GISTs exhibited significantly lower levels of *LINE-1* methylation than low-risk (61.3% versus 53.2%; $P = 0.001$) or intermediate-risk GISTs (60.8% versus 53.2%; $P = 0.002$). Hypomethylation of Satellite-α and *NBL2* was also observed in high-risk GISTs. By contrast, promoter hypermethylation was relatively infrequent (*CDH1*, 11.2%; *MLH1*, 9.8%; *SFRP1*, 1.2%; *SFRP2*, 11.0%; *CHFR*, 9.8%; *APC*, 6.1%; *CDKN2A*, 0%; *RASSF1A*, 0%; *RASSF2*, 0%) and did not correlate with *LINE-1* methylation or risk. Array CGH analysis revealed a significant correlation between *LINE-1* hypomethylation and chromosomal aberrations.

Conclusions: Our data suggest that *LINE-1* hypomethylation correlates significantly with the aggressiveness of GISTs and that *LINE-1* methylation could be a useful marker for risk assessment. Hypomethylation may increase the malignant potential of GISTs by inducing accumulation of chromosomal aberrations. *Clin Cancer Res; 16(21); 5114–23. ©2010 AACR.*

Gastrointestinal stromal tumors (GIST), which consist of a spectrum of both benign and malignant tumors, constitute the most important group of primary mesenchymal tumors of the gastrointestinal tract (1, 2). Immunohistochemically, GISTs are positive for KIT and CD34 and are negative or variably positive for other neural and smooth muscle cell markers. The expression of KIT and CD34 is a characteristic feature of the intestinal cells of Cajal (ICC), which are located in the intestinal wall and regulate gastrointestinal motility. GISTs are thus thought to originate from ICCs or ICC precursors. Activating *KIT* mutations have been identified in 80% to 90% of GISTs, and mutation of the platelet-derived growth factor receptor α gene (*PDGFRA*) is observed in ~5% of GISTs (1–3). In that context, imatinib (formerly STI571) was developed as a tyrosine kinase inhibitor and has been shown to inhibit BCR-ABL, KIT, and PDGFR activities (1–3). Imatinib is currently being used for the treatment of both chronic myeloid leukemia and metastatic GISTs.

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Translational Relevance

Gastrointestinal stromal tumors (GIST) are the most important mesenchymal tumors of the gastrointestinal tract. Predicting the biological potential of GISTs is often difficult, and discovery of molecular markers to predict the malignant potential of GISTs is essential. In this study, we provide compelling evidence for the association between *LINE-1* hypomethylation and the aggressiveness of GISTs. Using quantitative bisulfite pyrosequencing analysis, we found that high-risk GISTs exhibit significantly lower *LINE-1* methylation levels than low- or intermediate-risk GISTs. We further show a novel correlation between *LINE-1* hypomethylation and increases in chromosomal losses and gains. To our knowledge, this is the first study to show that *LINE-1* methylation could be a useful marker for the risk assessment of GISTs, and that hypomethylation may increase the malignant potential of GISTs by inducing chromosomal instability.

Predicting the biological potential of GISTs is often difficult, and considerable effort has been made to define the variables that would enable more accurate identification of tumors with malignant potential. In most classification systems, key prognostic factors for estimating malignant potential are tumor size and mitotic rate, and, to a more variable degree, the proliferation index or tumor site (1, 2, 4). Other potential and promising markers of GIST malignancy are molecular alterations. As mentioned, the vast majority of GISTs exhibit activating *KIT* or *PDGFRA* mutations. By itself, however, the mutation status does not fully explain the diverse biology of GISTs, and it is believed that additional molecular alterations are required for the progression of high-risk GISTs.

Neoplasias are thought to arise through the accumulation of multiple genetic and epigenetic alterations. Two contradicting epigenetic events coexist in cancer: global hypomethylation, which is mainly observed in repetitive sequences within the genome, and regional hypermethylation, which is frequently associated with CpG islands within gene promoters (5). Hypermethylation of CpG islands is a common feature of cancer that is associated with gene silencing (5, 6). In contrast to CpG islands, repetitive DNA elements are normally heavily methylated in somatic tissues. About 45% of the human genome is composed of repetitive sequences, including long interspersed nuclear element (LINE) and short interspersed nuclear element (SINE; ref. 7), and an earlier study has shown that methylation of such repetitive elements can serve as a surrogate for global methylcytosine content (8). Moreover, *LINE-1* hypomethylation is known to occur during the development of various human malignancies (9–13), and we recently reported that *LINE-1* methylation is diminished in enlarged fold gastritis, which is a risk factor of gastric cancer (14). Hypomethylation of Alu elements and other repetitive sequences also has been observed in tumors of various origin (15–20). To date, however, only a few groups have reported epigenetic abnormalities in GISTs (21–24), and there are no published studies of *LINE-1* methylation in GISTs.

Our aim in the present study was to assess the contribution made by epigenetic alterations to the malignant potential of GISTs. We quantitatively analyzed levels of *LINE-1* methylation, and also assessed CpG island hypermethylation in a panel of tumor-associated genes in primary GIST specimens. In addition, we carried out an array-based comparative genomic hybridization (array CGH) analysis to examine the relation between chromosomal aberrations and *LINE-1* hypomethylation in GISTs.

Materials and Methods

Patients and tumor tissues

A total of 106 GIST specimens were obtained from Sapporo Medical University Hospital, Sunagawa City Medical Center, Muroran General Hospital, and Osaka University Hospital. Informed consent was obtained from all patients before collection of the specimens, and this study was approved by the respective institutional review boards. Risk grade was assessed according to the risk definition system proposed by Fletcher et al. (4). Tumors that were <2 cm in diameter with a mitotic count of <5/50 high-power fields (HPF) were categorized as very low risk. Tumors that were 2 to 5 cm in diameter with <5 mitotic count/50 HPF were considered to be low risk. Tumors that were <5 cm in diameter with a mitotic count of 6 to 10/50 HPF, or were 5 to 10 cm with a mitotic count <5/50 HPF, were considered to be intermediate risk. Tumors that were >5 cm in diameter with a mitotic count of >5/50 HPF, >10 cm in diameter with any mitotic count, or any size with a mitotic count of >10/50 HPF were considered to be high risk. Genomic DNA was extracted from formalin-fixed, paraffin-embedded tissue specimens using a QIAamp DNA FFPE Tissue kit (Qiagen). Genomic DNA was extracted from fresh-frozen tissue specimens using the standard phenol-chloroform procedure.

Bisulfite pyrosequencing

Genomic DNA (1 μg) was modified with sodium bisulfite using an EpiTect Bisulfite kit (Qiagen), and bisulfite pyrosequencing analysis was done as described previously (14). Briefly, PCR was run in a 25-μL volume containing 50 ng of bisulfite-treated DNA, 1× MSP buffer [67 mmol/L Tris-HCl (pH 8.8), 16.6 mmol/L $(NH_4)_2SO_4$, 6.7 mmol/L $MgCl_2$, and 10 mmol/L 2-mercaptoethanol], 1.25 mmol/L deoxynucleotide triphosphate, 0.4 μmol/L each primer, and 0.5 unit of JumpStart REDTaq DNA Polymerase (Sigma-Aldrich). The PCR protocol for bisulfite sequencing entailed 5 minutes at 95°C; 40 cycles of 1 minute at 95°C, 1 minute at 60°C, and 1 minute at 72°C; and a 7-minute final extension at 72°C. The biotinylated PCR product was purified, made single stranded, and used as a template in a pyrosequencing reaction run according to the manufacturer's

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instructions. The PCR products were bound to Streptavidin Sepharose HP beads (Amersham Biosciences), after which beads containing the immobilized PCR product were purified, washed, and denatured using a 0.2 mol/L NaOH solution. After addition of 0.3 μmol/L sequencing primer to the purified PCR product, pyrosequencing was carried out using a PSQ96MA system (Biotage) and Pyro Q-CpG software (Biotage). Primer sequences for *LINE-1* methylation were as described (14). Primer sequences for *Alu Yb8*, centromeric satellite-α of chromosome 1 (*Sat-α*), and *NBL2* were as described (20).

MethyLight assay

Genomic DNA (1 μg) was modified with sodium bisulfite as described above. PCR was run in a 20-μL volume containing 50 ng of bisulfite-treated DNA, 625 nmol/L each primer, 250 nmol/L Taqman-MGB probe, and 1× Taqman Fast Universal PCR Master Mix (Applied Biosystems). Fast real-time PCR was done using a 7500 Fast Real-Time PCR System according to the manufacturer's instructions (Applied Biosystems). The PCR protocol entailed 20 seconds at 95°C followed by 40 cycles of 3 seconds at 95°C and 30 seconds at 60°C. We used Alu as a normalization control reaction (25). Primers, probes, and the percentage of methylated reference (PMR) were as described previously (26, 27). We used a PMR cutoff of 4 to distinguish methylation-positive (PMR > 4) from methylation-negative (PMR ≤ 4) samples (27).

Array-based comparative genomic hybridization

Microarray-based CGH analysis was done according to the manufacturer's instructions (Agilent Technologies). Briefly, 500 ng of genomic DNA from fresh-frozen GIST specimens and gender-matched reference DNA (Promega) were digested with *Alu*I and *Rsa*I before labeling and hybridization. Using a Genomic DNA Enzymatic Labeling kit (Agilent Technologies), tumor DNA and reference DNA were labeled with Cy5 and Cy3, respectively. Before hybridization, labeled DNA was mixed with 25 μg of Cot-1 DNA (Invitrogen), denatured at 95°C for 3 minutes, and incubated at 37°C for 30 minutes to block repetitive sequences. The probe mixture was then hybridized for 40 hours at 65°C to a Human Genome CGH Microarray Kit 105A (G4412A; Agilent Technologies), which contains ~99,000 probes annotated against National Center for Biotechnology Information Build 36. After washing, the array was scanned with an Agilent G2565BA Microarray Scanner, and the fluorescent signals were acquired using Feature Extraction software (Agilent Technologies). The ADM-2 algorithm included in the DNA Analytics 4.0 software (Agilent Technologies) was used to identify DNA copy number aberrations. A copy number loss was defined as a $\log_2$ ratio <–0.5, and a copy number gain was defined as a $\log_2$ ratio >0.5. All genomic positions were defined according to the University of California Santa Cruz Human version hg18. The Gene Expression Omnibus accession numbers of the microarray data are GSM552402, GSM552403, GSM552404, GSM552405, GSM552406, GSM552407, GSM552408, GSM552409, GSM552410, GSM552411, GSM552412, GSM552413, GSM552414, GSM552415, GSM552416, GSM552417, GSM552418, GSM552419, GSM552420, GSM552421, GSM552422, GSM552423, GSM552424, GSM552425, and GSM552426, and the accession number of the Series entry is GSE22185.

Statistical analysis

Mean methylation levels were compared using *t* tests or one-way ANOVA with a post hoc Games-Howell test. Methylation levels were correlated with other biological features by calculating the Pearson's and Spearman's correlation coefficients. *LINE-1* methylation levels were categorized into four groups: greater than 1 SD (1 – SD) above the mean, plus/minus 1 – SD from the mean, and less than 1 – SD below the mean. Sex- and age-adjusted odds ratios (OR) for high-risk category were then calculated using logistic regression models. *P* values of <0.05 (two-sided) were considered significant. Statistical analyses were carried out using Statistical Package for the Social Sciences software 15.0J (SPSS, Inc.) and StatView (SAS Institute, Inc.).

Results

Clinicopathologic characteristics

The clinicopathologic features of the 106 patients with primary GISTs are summarized in Table 1. The majority of the GISTs were located in the stomach (65%) and small intestine (27%), and the mean tumor size was 6.9 cm (range, 0.5-22 cm). The risk classification proposed by Fletcher et al. (4) was available for 85 patients. Of those,

Table 1. Clinicopathologic features of the GIST samples used in this study

Age (y, median ± SD)	68.0 ± 14.1
Gender	
Male	53 (50.0%)
Female	53 (50.0%)
Tumor location	
Stomach	68 (64.8%)
Small intestine	28 (26.7%)
Omentum	4 (3.8%)
Colon	3 (2.8%)
Esophagus	2 (1.9%)
Tumor size (cm, average ± SD)	6.92 ± 41.0
Mitotic count (/50 HPF, average ± SD)	7.1 ± 11.7
Risk category (*n* = 85)	
Very low	1 (1.2%)
Low	23 (27.1%)
Intermediate	19 (22.3%)
High	42 (49.4%)
Metastasis in high-risk group (*n* = 42)	
Absent	28 (66.7%)
Present	14 (33.3%)

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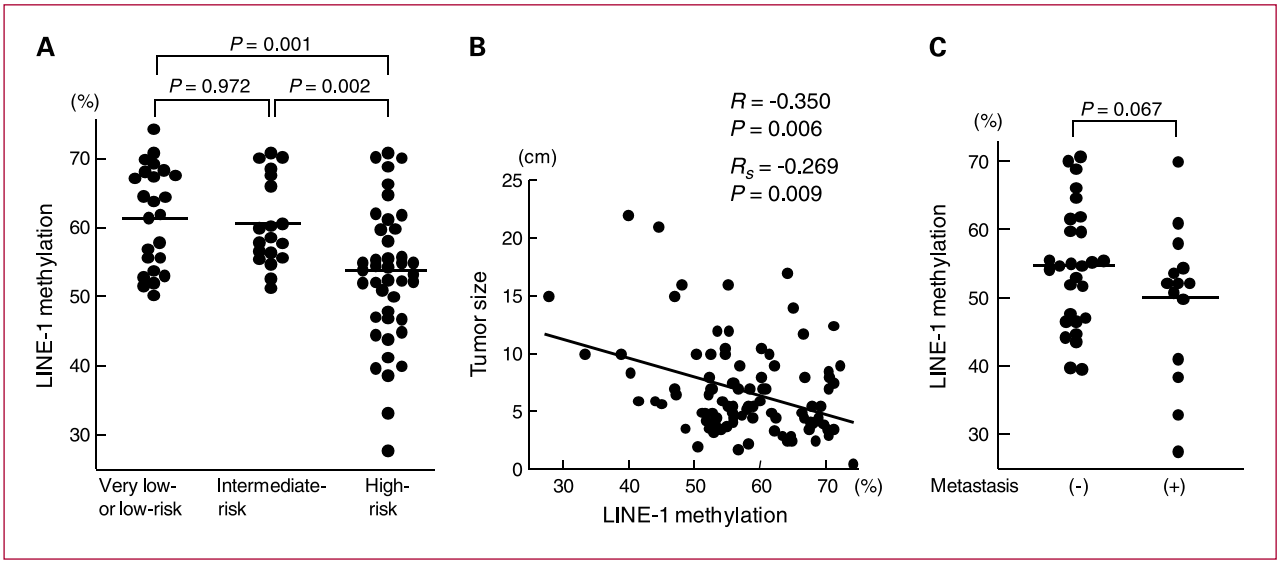

Fig. 1. Analysis of *LINE-1* methylation in GISTs. A, comparison of the levels of *LINE-1* methylation among low-risk (n = 23), intermediate-risk (n = 19), and high-risk (n = 42) GISTs. Filled circles depict the average methylation (%) at multiple CpG sites. B, correlation of *LINE-1* methylation with tumor size. The Pearson's correlation coefficient (R) and the Spearman correlation coefficient (Rs) are shown. C, comparison of *LINE-1* methylation between high-risk GISTs with and without metastasis.

1 (1.2%) was classified as very low risk, 23 (27.1%) were low risk, 19 (22.3%) were intermediate risk, and 42 (49.4%) were high risk. Among the 42 patients in the high-risk group, metastasis was found in 14 (33.3%).

Hypomethylation of *LINE-1* in GISTs

We next asked whether global DNA hypomethylation is involved in the development of GISTs. To address this question, we carried out bisulfite pyrosequencing to quantitatively analyze *LINE-1* promoter methylation as a surrogate for global methylcytosine content. All of the samples were analyzed at least twice, and the results of independent analyses were highly reproducible (Supplementary Fig. S1). We found that the mean level of *LINE-1* methylation in the 106 GIST specimens was 57.3 ± 9.3% (mean ± SD; range, 27.9-74.1%), and that the level was slightly lower in female patients than male patients, although the difference was not statistically significant (male, 58.8%; female, 55.9%; P = 0.055). We found no correlation between tumor location and *LINE-1* methylation (stomach, 58.6%; small intestine, 54.4%; esophagus, 51.9%; omentum, 57.7%; colon, 56.2%; P = 0.4136), and there was no correlation between age and *LINE-1* methylation (<60 years, 56.5%; 61-70 years, 58.8%; >71 years, 57.9%; P = 0.687).

We then compared *LINE-1* methylation with risk classification. The single very low-risk GIST specimen showed a high level of *LINE-1* methylation (74.1%). Low-risk (n = 23) and intermediate-risk (n = 19) GISTs showed similar levels of *LINE-1* methylation (61.3% versus 60.8%). By contrast, high-risk GISTs (n = 42) exhibited a significantly lower level of *LINE-1* methylation (53.2%) than GISTs in the other risk groups (Fig. 1A). Using that information, we stratified the tumors according to their level of *LINE-1* methylation, which was then correlated with the risk categories. After adjusting for age and gender, the lowest level of *LINE-1* methylation (<54.9%) was significantly associated with the high-risk category [OR, 7.5; 95%

Table 2. Correlation between *LINE-1* methylation and the GIST risk category

LINE-1 methylation (%)	All samples (N = 76)	High-risk (n = 36)	Very low-, low-, or intermediate-risk (n = 40)	P	OR* (95% CI)
>68.0	13	4	9		1.0
61.4-67.9	14	4	10	0.976	1.0 (0.2-5.5)
54.9-61.3	22	8	14	0.638	1.4 (0.3-6.5)
<54.9	27	20	7	0.009	7.5 (1.6-34.0)
				P trend < 0.001	

*Age- and gender-adjusted OR.

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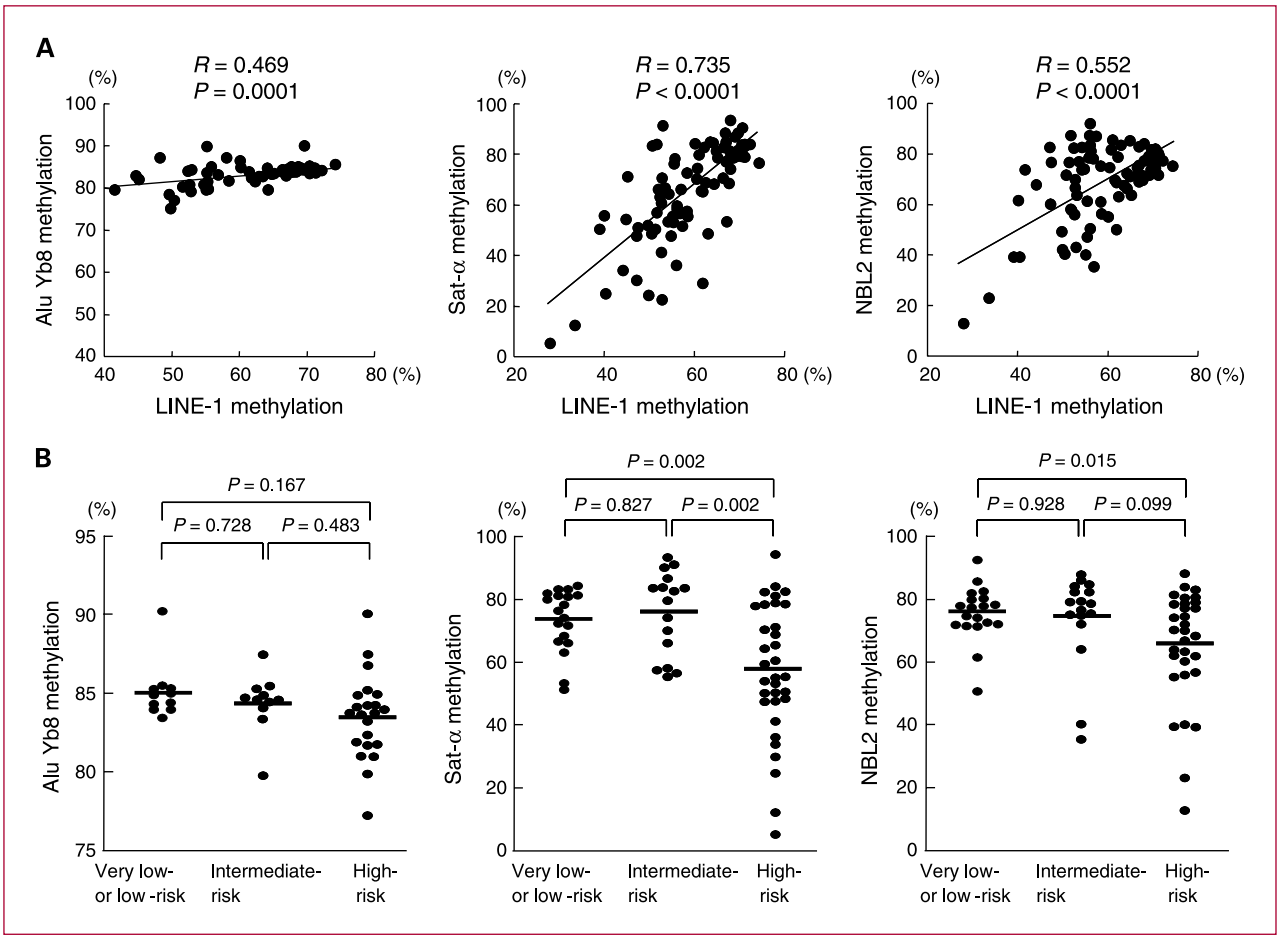

Fig. 2. Analysis of the methylation of different repetitive sequences in GISTs. A, methylation levels in three different repetitive sequences (*Alu Yb8*, *Sat-α*, and *NBL2*) were analyzed and correlated with *LINE-1* methylation. The Pearson correlation coefficients and *P* values are shown. B, comparison of the methylation of repetitive sequences among low-, intermediate-, and high-risk GISTs. Filled circles represent average methylation (%) at multiple CpG sites.

confidence interval (CI), 1.6-34.0; Table 2]. Moreover, bivariate correlation analysis revealed an inverse correlation between *LINE-1* methylation levels and tumor size (Fig. 1B). Among the high-risk GIST patients, *LINE-1* methylation was slightly lower in individuals with incidences of metastasis than in those without metastasis (50.0% versus 54.8%), although the difference was not statistically significant (Fig. 1C). However, when we divided high-risk GIST patients into two groups according to *LINE-1* methylation and did logistic regression analysis, we found that GISTs with lower *LINE-1* methylation (<55%) were significantly associated with incidences of metastasis (OR, 9.5; 95% CI, 1.5-61.2; Supplementary Table S1). These results suggest that *LINE-1* hypomethylation is strongly associated with greater risk and aggressiveness of GISTs.

Hypomethylation of other repetitive DNA elements in GIST

Previous studies showed that Alu elements and other repetitive sequences are also hypomethylated in human malignancies (15–20). We therefore carried out bisulfite pyrosequencing of *Alu Yb8* and two other tandem repeats, *Sat-α* and *NBL2*, which are reportedly hypomethylated in cancer (15, 20). We found a moderate correlation between *LINE-1* methylation and *Alu Yb8* methylation (Fig. 2A); however, levels of *Alu Yb8* methylation did not significantly correlate with risk grade (Fig. 2B). By contrast, *LINE-1* methylation strongly correlated with *Sat-α* and *NBL2* methylation (Fig. 2A). High-risk GISTs showed significantly lower levels of *Sat-α* methylation than low-risk (57.9% versus 73.9%) or intermediate-risk GISTs (57.9% versus 76.3%; Fig. 2B), and significantly lower levels of *NBL2* methylation than low-risk GISTs (65.1% versus 75.5%; Fig. 2B).

Analysis of CpG island hypermethylation of tumor-related genes

Because it was previously reported that CpG island methylation correlates with GIST malignancy (21), we next assessed the methylation levels of the CpG islands of several well-characterized tumor suppressor and tumor-related genes in the GIST specimens. Using MethyLight

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assays, we analyzed nine genes frequently methylated in gastrointestinal cancers. Unexpectedly, methylation of these genes was relatively infrequent in GISTs (*CDH1*, 12.2%; *MLH1*, 9.8%; *SFRP1*, 1.2%; *SFRP2*, 11.0%; *CHFR*, 9.8%; *APC*, 6.1%; *CDKN2A*, 0%; *RASSF1A*, 0%; *RASSF2*, 0%; Supplementary Table S2). Interestingly, however, *CDH1* tended to be methylated more frequently in higher-risk GISTs, whereas *MLH1* tended to be methylated less frequently, although these correlations were not statistically significant (Supplementary Table S2). We failed to find any significant correlation between methylation of other genes and clinicopathologic features. There was also no significant correlation between CpG island methylation and *LINE-1* hypomethylation.

Association of *LINE-1* methylation with chromosomal aberrations

The biological meaning of global hypomethylation in tumors is not yet fully understood, but it is thought to be associated with chromosomal instability (5). Consistent with that idea, earlier cytogenetic, fluorescence *in situ* hybridization, and CGH studies revealed frequent chromosomal imbalances in GISTs (28–32). This prompted us to ask whether *LINE-1* hypomethylation in GISTs is associated with chromosomal gain or loss. We addressed that question by carrying out an array CGH analysis using 25 freshly frozen GIST specimens using an Agilent 105K oligonucleotide microarray.

We found that the average number of chromosomal aberrations for a given tumor was 28.5 (range, 5-62), and genomic losses were much more common than gains. Consistent with previous CGH and array CGH studies of GIST (28–32), we observed frequent genomic losses at 14q (92%), 22q (68%), 15q (64%), and 1p (60%; Table 3; Supplementary Fig. S2; Supplementary Table S3). Total or partial losses at 14q were the most frequent chromosomal aberration (23 of 25; 92%), and 17 GISTs showed a total loss of chromosome 14. Total or partial losses of 22q were also frequently detected (17 of 25; 68%), and 11 tumors showed a loss of the whole chromosome. Losses at 1p were detected in 15 of 25 tumors (60%), and losses were generally more frequent in intestinal GISTs than in gastric ones (Table 3). Losses at 15q were often associated with 1p loss, which is consistent with an earlier observation

Table 3. Summary of the frequent chromosomal losses detected using array CGH

No.	Age/gender	Location	Risk	L-1 (%)	Chromosomal losses							
					1p	3q	4q	9p	13q	14q	15q	22q
1	67/M	Stomach	Low	71.2		Yes	Yes			Yes*	Yes	Yes
2	65/M	Stomach	Inter.	71.1			Yes	Yes		Yes*		Yes
3	64/F	Stomach	Low	70.1					Yes	Yes	Yes*	
4	50/M	Stomach	Low	69.5	Yes	Yes	Yes			Yes		
5	27/F	Stomach	Inter.	68.8		Yes	Yes			Yes*		Yes
6	68/F	Stomach	Low	68.6		Yes	Yes			Yes*		
7	61/M	Small intestine	Low	67.6	Yes*	Yes	Yes			Yes*	Yes	Yes*
8	62/F	Stomach	Low	64.7	Yes	Yes	Yes			Yes*	Yes	
9	65/F	Stomach	Low	64.0		Yes	Yes			Yes*		Yes
10	25/F	Small intestine	Inter.	60.5	Yes*	Yes	Yes		Yes	Yes*	Yes*	
11	70/M	Stomach	Inter.	60.3		Yes				Yes*		Yes
12	53/F	Stomach	High	58.3		Yes	Yes			Yes		
13	56/M	Small intestine	Inter.	56.6	Yes	Yes	Yes	Yes			Yes*	Yes*
14	65/M	Stomach	High	56.0			Yes			Yes	Yes	
15	37/M	Small intestine	High	55.2	Yes*	Yes	Yes	Yes		Yes	Yes*	Yes*
16	63/M	Stomach	Inter.	55.0	Yes	Yes	Yes	Yes		Yes*	Yes	Yes*
17	73/F	Stomach	NA	52.7		Yes	Yes	Yes		Yes		
18	68/F	Small intestine	High	52.5	Yes*	Yes	Yes	Yes	Yes*	Yes*	Yes*	Yes*
19	62/M	Small intestine	NA	51.5	Yes*	Yes	Yes				Yes*	Yes*
20	57/M	Small intestine	High	50.3	Yes*		Yes	Yes*		Yes*	Yes*	Yes*
21	49/M	Stomach	NA	49.7	Yes	Yes		Yes*	Yes	Yes*		Yes*
22	68/M	Small intestine	NA	49.5	Yes*	Yes	Yes	Yes*	Yes*	Yes*	Yes*	Yes*
23	73/F	Stomach	High	47.0	Yes			Yes		Yes*	Yes	Yes
24	87/M	Small intestine	High	44.0	Yes*	Yes	Yes		Yes*	Yes*	Yes*	Yes*
25	68/F	Stomach	High	33.4	Yes		Yes	Yes*	Yes	Yes*	Yes	Yes*

Abbreviations: L-1, *LINE-1* methylation; Inter., intermediate; NA, not available.
*Loss of the entire p- or q-arm of the chromosome.

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(Table 3; ref. 31). Partial losses at 3q were also frequently detected (19 of 25; 76%), and a minimal common overlapping region was identified at 3q26.1, although this region contains no annotated genes (Supplementary Table S3). In addition, losses at 4q13.2, the locus of *UGT2B17*/*UGT2B28*, were detected in 20 of 25 tumors (80%). Losses at 9p were found in 11 of 25 (44%) GISTs, and 4 tumors showed a loss of the whole p-arm. Among seven tumors with partial 9p losses, four showed a loss at 9p21, the locus of *CDKN2A*/*CDKN2B*.

Losses at 3q, 4q, 14q, and 22q were found to be equally distributed in tumors with all levels of *LINE-1* methylation (Table 3). By contrast, many other chromosomal aberrations correlated with *LINE-1* hypomethylation (Table 3; Fig. 3A). For example, GISTs with losses at 1p showed significantly lower *LINE-1* methylation than those without 1p loss (53.8% versus 64.1%; $P = 0.007$). *LINE-1* methylation was also much lower in tumors with loss at 9p than in those without that loss (52.1% versus 62.5%; $P = 0.005$). Bivariate correlation analysis revealed a significant inverse correlation between levels of *LINE-1* methylation and the total number of chromosomal aberrations, including both losses and gains (Fig. 3B). These results suggest a significant relationship between *LINE-1* hypomethylation and DNA copy number abnormalities in GIST.

Discussion

Although several studies have shown that genetic abnormalities, including various mutations and chromosomal imbalances, are significantly involved in the development of GISTs, little is known about the role played by epigenetic alterations in these tumors. To date, there had been only a few reports of CpG island methylation in GISTs (21–24), and levels of global DNA methylation had not yet been analyzed. In the present study, however, we found that levels of methylation of *LINE-1* and other repetitive elements are reduced in GISTs. It has been known for decades that global hypomethylation is a common feature of human cancer (33, 34), and in recent years, hypomethylation has been studied in various human malignancies using *LINE-1* and other repetitive sequences as surrogates

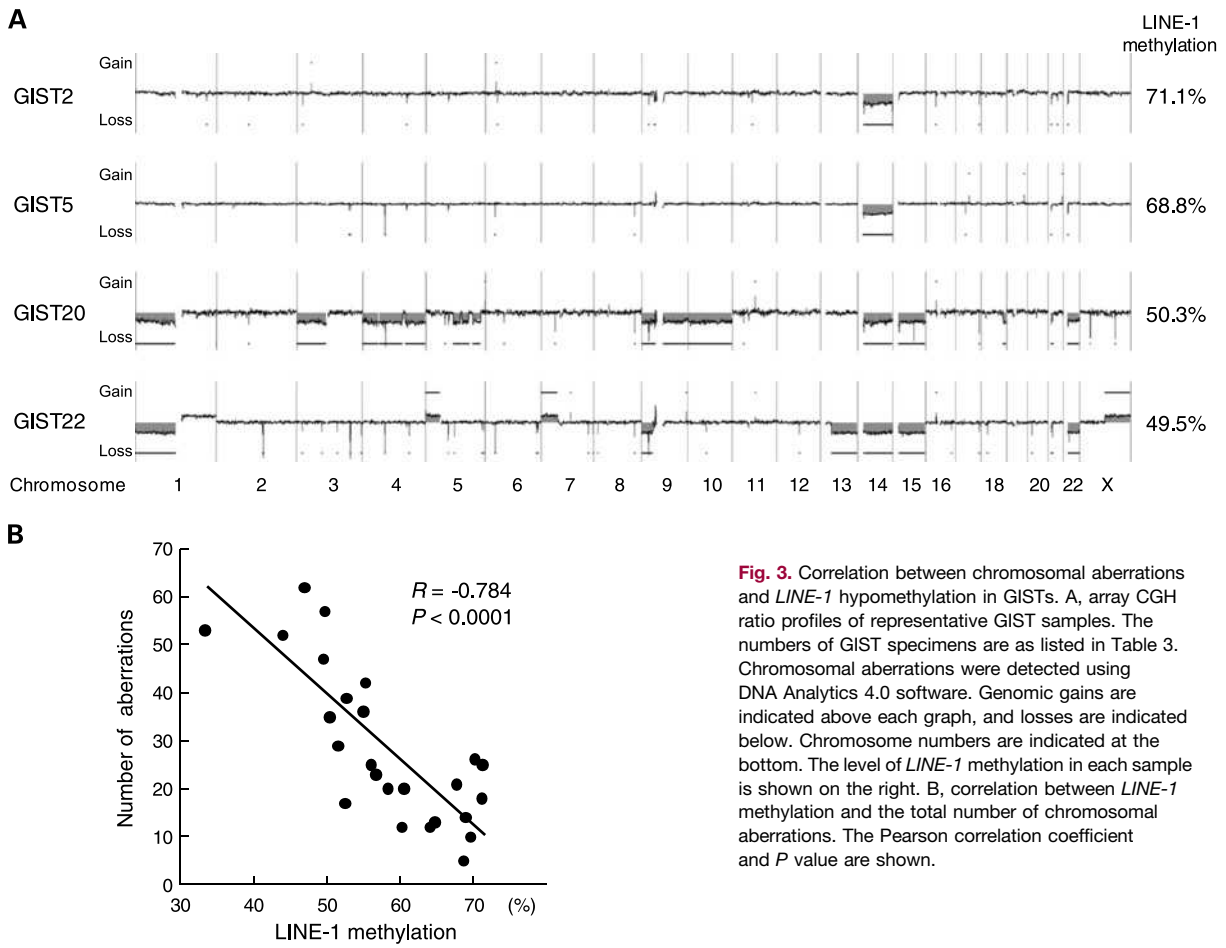

Fig. 3. Correlation between chromosomal aberrations and *LINE-1* hypomethylation in GISTs. A, array CGH ratio profiles of representative GIST samples. The numbers of GIST specimens are as listed in Table 3. Chromosomal aberrations were detected using DNA Analytics 4.0 software. Genomic gains are indicated above each graph, and losses are indicated below. Chromosome numbers are indicated at the bottom. The level of *LINE-1* methylation in each sample is shown on the right. B, correlation between *LINE-1* methylation and the total number of chromosomal aberrations. The Pearson correlation coefficient and *P* value are shown.

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(9–20). Our results confirm that GISTs exhibit a pattern of hypomethylation that is similar to those exhibited by many other human tumors.

We found that *LINE-1* hypomethylation is strongly associated with the aggressiveness of GISTs. Levels of *LINE-1* methylation were significantly lower in high-risk GISTs than in low- or intermediate-risk tumors. In addition, levels of *LINE-1* methylation inversely correlated with tumor size and mitotic counts. These results are consistent with earlier observations that a reduction in global methylcytosine content is associated with malignant potential in cancer, and that it is especially prevalent in metastatic tumors (34). *LINE-1* hypomethylation is also reportedly associated with poor prognosis in prostate (17), colon (35), and ovarian (36) cancers and in chronic myeloid leukemia (11). In normal cells, DNA methylation plays important roles in X-chromosome inactivation, genomic imprinting, and repression of repetitive elements, such as retrotransposons and endogenous retroviruses. Thus, hypomethylation may associate with tumor malignancy through a variety of mechanisms. For example, global hypomethylation is associated with genomic instability (37–40), which may confer a poor prognosis. Hypomethylation can also lead to activation of proto-oncogenes, endogenous retroviruses, or transposable elements, and such transcriptional dysregulation could affect tumor aggressiveness.

We found strong correlations between the level of LINE-1 methylation and methylation of other repetitive elements. *Sat-α* and *NBL2*, which are tandem DNA repeats, are reportedly hypomethylated in various human cancers (15, 16, 20, 41). We observed that methylation of these elements is also reduced in high-risk GISTs, which suggests that a common mechanism may induce and maintain DNA hypomethylation in tumors. On the other hand, *LINE-1* methylation correlated only moderately with *Alu Yb8* methylation, and hypomethylation of *Alu Yb8* was limited, even in high-risk GISTs. Similar modest correlation between *LINE-1* and Alu methylation was also observed in head and neck squamous cell carcinomas and neuroendocrine tumors (18, 42). This lower correlation may simply reflect a difference in assay sensitivity, although it is possible that there are functional and/or biological differences in the regulation of these two types of repetitive DNA elements.

Although global hypomethylation and regional hypermethylation of 5′ CpG islands are common features of neoplasias, the link between the two remains controversial. Recent studies using methylation of *LINE-1* and/or Alu as a marker revealed that global hypomethylation is correlated with CpG island hypermethylation in prostate cancers (17) and neuroendocrine tumors (42). In addition, we recently showed that *LINE-1* hypomethylation and CpG island hypermethylation are tightly linked in enlarged fold gastritis (14). By contrast, others did not find a similar relationship in Wilm's tumor (41), colon cancer (43), or ovarian cancer (16). Recent studies also revealed that *LINE-1* hypomethylation is inversely correlated with microsatellite instability and/or the CpG island methylator phenotype in colon cancer, suggesting that CpG island hypermethylation and global hypomethylation may reflect different tumor progression pathways (12, 13). In the present study, we failed to find a significant correlation between 5′ CpG island hypermethylation of tumor-related genes and global hypomethylation. However, this may reflect a bias toward selection of genes frequently methylated in tumors of epithelial origin, as GISTs may exhibit methylation of a different spectrum of genes.

House et al. (21) reported that hypermethylation of *CDH1* (*E-cadherin*) is positively correlated with GISTs having malignant histologic features (mitotic rate, tumor size, and necrosis) and a poor prognosis. In addition, the presence of *CDH1* methylation and the absence of *MLH1* methylation correlated with early tumor recurrence. By contrast, Saito et al. (24) reported that CpG island hypermethylation, including that of *MLH1* and *CDH1*, was frequently detected in GISTs, irrespective of their malignancy. In the present study, we found tendencies for *CDH1* methylation to occur more frequently and *MLH1* methylation to occur less frequently in higher-risk GISTs, although these correlations were not statistically significant. We also noticed that the frequencies of CpG island methylation were lower than those reported previously (21, 24). This could reflect differences in the primer sequences used in our study, but the actual reason for the discrepancy is not clear. As mentioned above, GISTs may exhibit hypermethylation of a different spectrum of genes, and further study will be needed to clarify the role of CpG island methylation in GISTs.

Global hypomethylation is strongly implicated in chromosomal instability. A study using $Nf1^{+/-}$ $p53^{+/-}$ mice has shown that introduction of a hypomorphic *Dnmt1* allele causes DNA hypomethylation that leads to significant increases in the loss of heterozygosity rate and tumor development (37). Another study has shown that hypomethylation in $Apc^{Min/+}$ mice leads to increases in microadenoma formation through loss of heterozygosity at the *Apc* locus (38). Global hypomethylation also correlates with chromosomal instability and copy number changes in human cancers (18, 19, 39, 40). Thus, to assess the potential implication of *LINE-1* hypomethylation in genomic instability in GISTs, we carried out an array CGH analysis and correlated hypomethylation with chromosomal imbalances. The results of this analysis are largely consistent with previously reported cytogenetic, CGH, and array CGH analyses of GISTs (28–32). In general, losses were more common than gains, and genomic losses frequently affected 1p, 14q, 15q, and 22q. In addition, we found losses at 3q26.1 and 4q13.2, the locus of *UGT2B17*/*UGT2B28*, in the majority of tumors tested. UGT2B17, which is a member of the UDP-glucuronosyltransferases (UGT) family, has been implicated in the metabolism of androgens, and a deletion polymorphism in *UGT2B17* is reportedly associated with the risk of prostate cancer (44), although its functional role in GISTs remains to be clarified.

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Although the most frequent aberrations (e.g., losses of 14q and 22q) were equally distributed among GISTs with all levels of *LINE-1* methylation, we found a significant correlation between many other chromosomal aberrations and DNA hypomethylation. For instance, tumors with losses at 1p or 9p showed significantly lower *LINE-1* methylation. Notably, these results are consistent with previous findings that losses at 14q and 22q are early changes in GIST development, whereas losses at 1p and 9p are associated with malignancy and poor prognosis (28–31). Moreover, total numbers of chromosomal aberrations are highly correlated with *LINE-1* hypomethylation. It thus seems that *LINE-1* hypomethylation may play an important role in inducing chromosomal aberrations and increasing the aggressiveness of GISTs. Currently, however, we have no functional evidence of a causal relationship between hypomethylation and the genomic instability of GISTs, and further studies will be required to clarify the underlying molecular mechanism.

In summary, we found that hypomethylation of repetitive elements is associated with high-risk GISTs. We also provide further evidence that *LINE-1* hypomethylation is strongly associated with chromosomal aberrations. Although the cause of DNA hypomethylation in GISTs remains unclear, *LINE-1* methylation could be a useful marker for predicting the risk and prognosis of the disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Clinical Cancer Research

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