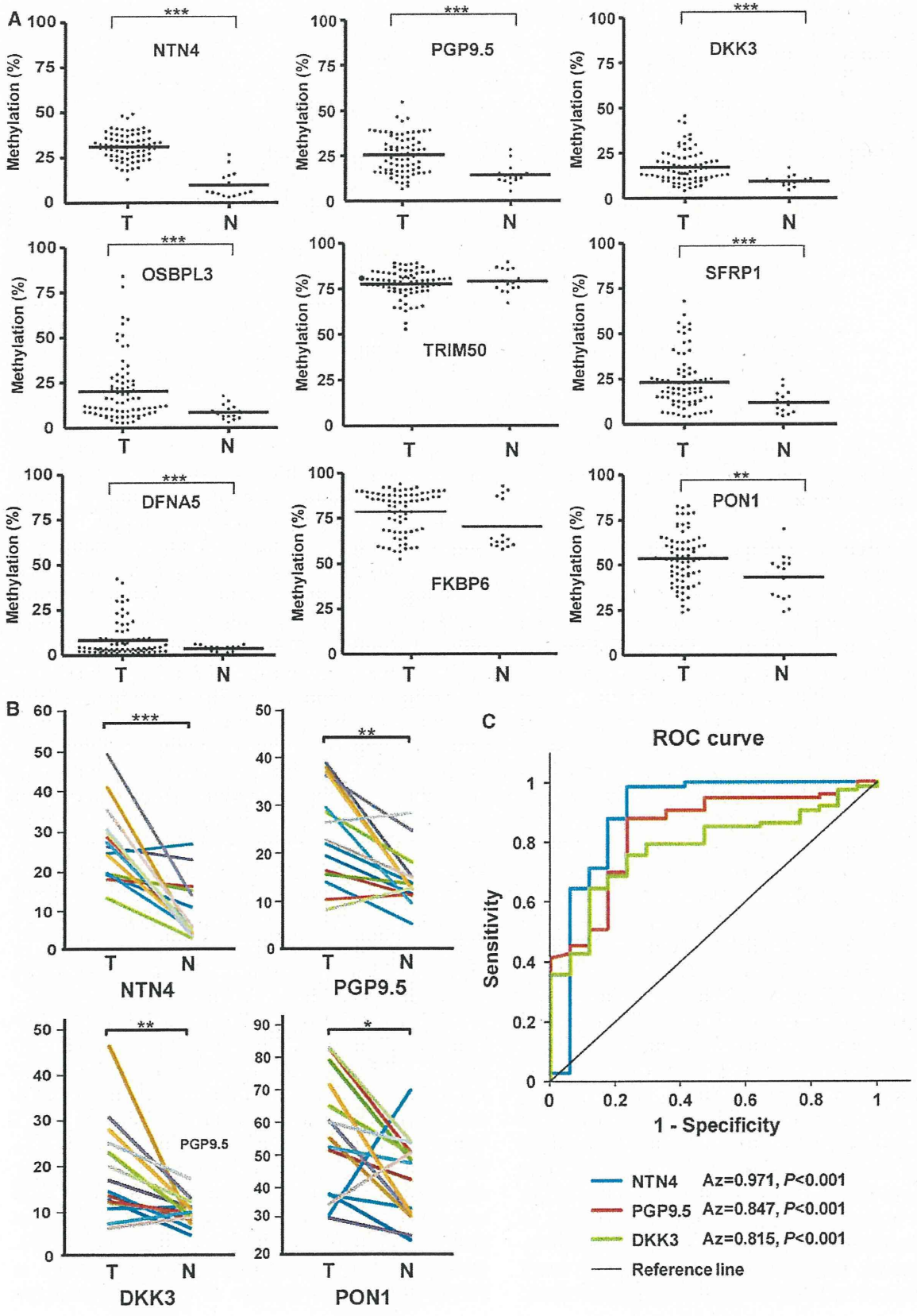
A
NTN4
PGP9.5
DKK3
OSBPL3
TRIM50
SFRP1
DFNA5
FKBP6
PON1
Methylation (%)
T
N
B
NTN4
PGP9.5
DKK3
PON1
C
ROC curve
Sensitivity
1 - Specificity
NTN4 Az=0.971, P<0.001
PGP9.5 Az=0.847, P<0.001
DKK3 Az=0.815, P<0.001
Reference line

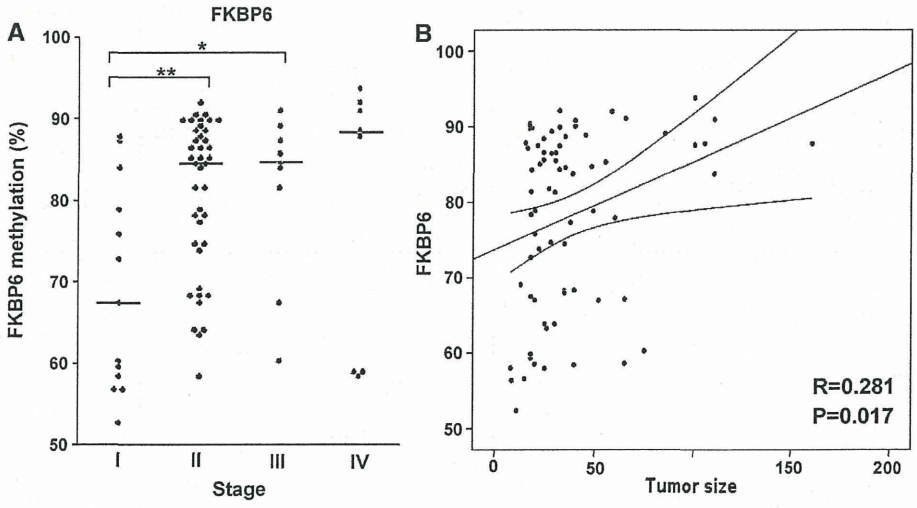

Fig. 7 Correlation between FKBP6 methylation and the clinicopathological features of breast cancer. **a** Scatter plot of FKBP6 methylation in patients with cancers of different stages. ** $P < 0.01$, * $P < 0.05$. **b** Scatter plot in which FKBP6 methylation is plotted against tumor size. *X*-axis: tumor size (mm). *Y*-axis: FKBP6 methylation level (%)

will be necessary to clarify the significance of its methylation in normal breast tissues.

Functional roles of genes silenced by DNA methylation in breast cancer

Our microarray analysis revealed several genes involved in cell signaling pathways to be methylated. SFRP1 and DKK3 are Wnt antagonists frequently silenced by methylation in colorectal and gastric cancers [29, 45, 46], and several lines of evidence suggest that activation of Wnt signaling plays a key role in breast cancer. For example, Bafico et al. [47] found that the unphosphorylated form of β-catenin is frequently present in breast cancer cell lines, and that the Wnt ligands WNT-2, WNT-3, and WNT6 are frequently overexpressed in the same cell lines. Mutation of APC or β-catenin is rare in breast cancer, however, so that the mechanism underlying the activation of Wnt signaling in this disease is not fully understood [48]. In the present study, we found that SFRP1 is inactivated by DNA methylation in MCF7 cells, and that SFRP1 is methylated in 45 of the 75 (60%) primary breast cancers tested. Perhaps inactivation of negative regulators of Wnt is involved in activating Wnt signaling in breast cancer.

DFNA5 was originally identified as a gene involved in nonsyndromic hearing impairment [49]. Since then, DFNA5 has also been identified as a gene downregulated in etoposide-resistant melanoma [50]. Although the role of DFNA5 in mediating the effects of etoposide remains unclear, Lage et al. [51] showed that introduction of the gene into tumor cells increases their susceptibility to apoptosis mediated by activated caspase-3 following etoposide treatment. In addition, DFNA5 was recently found to be inactivated by DNA methylation in gastric cancers, and introduction of DFNA5 into gastric cancer cells suppressed colony formation and induced apoptosis [26].

DFNA5 is reportedly a target gene of p53 [36]. In the present study, we found that DFNA5 is silenced by methylation in MCF7 cells and that treating the cells with DAC restored induction of DFNA5 by p53 family genes, especially p63γ. Thus, DFNA5 does not appear to be targeted selectively by p53, itself, but by p53 family member, p63γ.

Netrins are secreted molecules involved in axon guidance and angiogenesis. Among them, NTN4 is an antiangiogenic factor that acts through its receptor, neogenin [31]. Notably, expression of NTN4 is associated with a good prognosis in breast cancer [52]. In the present study, we showed for the first time that NTN4 is silenced by DNA methylation in breast cancer and that treating breast cancer cells with a demethylating agent (DAC) restores its expression. We also showed that NTN4 has tumor suppressive activity. Identification of NTN4 as a candidate tumor suppressor in breast cancer may be useful for the development of new cancer therapies [53]. The methylation of NTN4 was cancer-specific, suggesting epigenetic changes to the gene could be a useful molecular marker for diagnosis.

FK506-binding proteins (FKBPs) are immunophilins involved in protein folding and cell signaling. Among them, FKBP6 has been identified as a candidate gene underlying Williams syndrome, a developmental disorder caused by haploinsufficiency of genes at 7q11.23 [54], and expression of FKBP6 specifically localizes to meiotic chromosome cores and regions of homologous chromosome synapsis [33]. We found that methylation of FKBP6 was correlated with tumor size and stage. The role of FKBP6 in tumorigenesis remains unknown, but its methylation in normal tissue suggests that methylation of FKBP6 could be an example of so called "passenger

methylation," which has also been seen with other genes (e.g., APC). In any case, the observed correlation between FKBP6 methylation and breast cancer stage suggests that methylation of FKBP6 could be a molecular marker for advanced breast cancer.

In summary, we have screened targets of DNA methylation in breast cancers and identified nine genes silenced by methylation. NTN4, PGP9.5, and DKK3 genes are methylated in cancer-specific manner and could be useful molecular markers for diagnosing breast cancer. Introduction of NTN4 cDNA into breast cancer cells suppressed tumor growth, suggesting NTN4 could be a novel tumor suppressor in breast cancer. More broadly, identification of genes silenced by DNA methylation in breast cancer may provide valuable information that not only contributes to our understanding of the pathogenesis of the disease, but also to the development of new strategies for diagnosis and therapy.

Acknowledgments The authors thank Dr. William F. Goldman for editing the manuscript. This study was supported in part by Grants-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science, and Technology (K.I., T.T., and M.T.), Grants-in-Aid for Scientific Research (S) from Japan Society for Promotion of Science (K.I.), a Grant-in-Aid for the Third-term Comprehensive 10-year Strategy for Cancer Control, and Grant-in-Aid for Cancer Research from the Ministry of Health, Labor, and Welfare, Japan (M.T.).

References

1. Bird A (1992) The essentials of DNA methylation. Cell 70:5–8
2. Jones PA, Baylin SB (2007) The epigenomics of cancer. Cell 128:683–692
3. Jemal A, Siegel R, Ward E et al (2008) Cancer statistics, 2008. CA Cancer J Clin 58:71–96
4. Schuebel KE, Chen W, Cope L et al (2007) Comparing the DNA hypermethylome with gene mutations in human colorectal cancer. PLoS Genet 3:1709–1723
5. Herman JG, Merlo A, Mao L et al (1995) Inactivation of the CDKN2/p16/MTS1 gene is frequently associated with aberrant DNA methylation in all common human cancers. Cancer Res 55:4525–4530
6. Ferguson AT, Evron E, Umbricht CB et al (2000) High frequency of hypermethylation at the 14-3-3 sigma locus leads to gene silencing in breast cancer. Proc Natl Acad Sci USA 97:6049–6054
7. Graff JR, Herman JG, Lapidus RG et al (1995) E-cadherin expression is silenced by DNA hypermethylation in human breast and prostate carcinomas. Cancer Res 55:5195–5199
8. Krop IE, Sgroi D, Porter DA et al (2001) HIN-1, a putative cytokine highly expressed in normal but not cancerous mammary epithelial cells. Proc Natl Acad Sci USA 98:9796–9801
9. Dammann R, Yang G, Pfeifer GP (2001) Hypermethylation of the cpG island of Ras association domain family 1A (RASSF1A), a putative tumor suppressor gene from the 3p21.3 locus, occurs in a large percentage of human breast cancers. Cancer Res 61:3105–3109
10. Conway KE, McConnell BB, Bowring CE et al (2000) TMS1, a novel proapoptotic caspase recruitment domain protein, is a target of methylation-induced gene silencing in human breast cancers. Cancer Res 60:6236–6242
11. Rodriguez BA, Cheng AS, Yan PS et al (2008) Epigenetic repression of the estrogen-regulated Homeobox B13 gene in breast cancer. Carcinogenesis 29:1459–1465
12. Douglas DB, Akiyama Y, Carraway H et al (2004) Hypermethylation of a small CpGuanine-rich region correlates with loss of activator protein-2alpha expression during progression of breast cancer. Cancer Res 64:1611–1620
13. Chung W, Kwabi-Addo B, Ittmann M et al (2008) Identification of novel tumor markers in prostate, colon and breast cancer by unbiased methylation profiling. PLoS One 3:e2079
14. Ostrow KL, Park HL, Hoque MO et al (2009) Pharmacologic unmasking of epigenetically silenced genes in breast cancer. Clin Cancer Res 15:1184–1191
15. Tommasi S, Karm DL, Wu X et al (2009) Methylation of homeobox genes is a frequent and early epigenetic event in breast cancer. Breast Cancer Res 11:R14
16. Gardiner-Garden M, Frommer M (1987) CpG islands in vertebrate genomes. J Mol Biol 196:261–282
17. Clark SJ, Harrison J, Paul CL et al (1994) High sensitivity mapping of methylated cytosines. Nucleic Acids Res 22:2990–2997
18. Watanabe Y, Toyota M, Kondo Y et al (2007) PRDM5 identified as a target of epigenetic silencing in colorectal and gastric cancer. Clin Cancer Res 13:4786–4794
19. Yang AS, Estecio MR, Doshi K et al (2004) A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. Nucleic Acids Res 32:e38
20. Sasaki Y, Morimoto I, Ishida S et al (2001) Adenovirus-mediated transfer of the p53 family genes, p73 and p51/p63 induces cell cycle arrest and apoptosis in colorectal cancer cell lines: potential application to gene therapy of colorectal cancer. Gene Ther 8:1401–1408
21. Sasaki Y, Negishi H, Koyama R et al (2009) p53 family members regulate the expression of the apolipoprotein D gene. J Biol Chem 284:872–883
22. Suzuki H, Igarashi S, Nojima M et al (2009) IGFBP7 is a p53 responsive gene specifically silenced in colorectal cancer with CpG island methylator phenotype. Carcinogenesis. doi:10.1093/carcin/bgp179
23. Kim MS, Lebron C, Nagpal JK et al (2008) Methylation of the DFNA5 increases risk of lymph node metastasis in human breast cancer. Biochem Biophys Res Commun 370:38–43
24. Veeck J, Bektas N, Hartmann A et al (2008) Wnt signalling in human breast cancer: expression of the putative Wnt inhibitor Dickkopf-3 (DKK3) is frequently suppressed by promoter hypermethylation in mammary tumours. Breast Cancer Res 10:R82
25. Veeck J, Niederacher D, An H et al (2006) Aberrant methylation of the Wnt antagonist SFRP1 in breast cancer is associated with unfavourable prognosis. Oncogene 25:3479–3488
26. Akino K, Toyota M, Suzuki H et al (2007) Identification of DFNA5 as a target of epigenetic inactivation in gastric cancer. Cancer Sci 98:88–95
27. Mandelker DL, Yamashita K, Tokumaru Y et al (2005) PGP9.5 promoter methylation is an independent prognostic factor for esophageal squamous cell carcinoma. Cancer Res 65:4963–4968
28. Sato H, Suzuki H, Toyota M et al (2007) Frequent epigenetic inactivation of DICKKOPF family genes in human gastrointestinal tumors. Carcinogenesis 28:2459–2466
29. Suzuki H, Watkins DN, Jair KW et al (2004) Epigenetic inactivation of SFRP genes allows constitutive WNT signaling in colorectal cancer. Nat Genet 36:417–422

30. Wu G, Guo Z, Chang X et al (2007) LOXL1 and LOXL4 are epigenetically silenced and can inhibit ras/extracellular signal-regulated kinase signaling pathway in human bladder cancer. Cancer Res 67:4123–4129
31. Lejmi E, Leconte L, Pedron-Mazoyer S et al (2008) Netrin-4 inhibits angiogenesis via binding to neogenin and recruitment of Unc5B. Proc Natl Acad Sci USA 105:12491–12496
32. Micale L, Fusco C, Augello B et al (2008) Williams-Beuren syndrome TRIM50 encodes an E3 ubiquitin ligase. Eur J Hum Genet 16:1038–1049
33. Crackower MA, Kolas NK, Noguchi J et al (2003) Essential role of Fkbp6 in male fertility and homologous chromosome pairing in meiosis. Science 300:1291–1295
34. Marchesani M, Hakkarainen A, Tuomainen TP et al (2003) New paraoxonase 1 polymorphism I102V and the risk of prostate cancer in Finnish men. J Natl Cancer Inst 95:812–818
35. Lehto M, Mayranpaa MI, Pellinen T et al (2008) The R-Ras interaction partner ORP3 regulates cell adhesion. J Cell Sci 121:695–705
36. Masuda Y, Futamura M, Kamino H et al (2006) The potential role of DFNA5, a hearing impairment gene, in p53-mediated cellular response to DNA damage. J Hum Genet 51:652–664
37. Arakawa H (2004) Netrin-1 and its receptors in tumorigenesis. Nat Rev Cancer 4:978–987
38. Suzuki H, Toyota M, Carraway H et al (2008) Frequent epigenetic inactivation of Wnt antagonist genes in breast cancer. Br J Cancer 98:1147–1156
39. Tokumaru Y, Yamashita K, Kim MS et al (2008) The role of PGP9.5 as a tumor suppressor gene in human cancer. Int J Cancer 123:753–759
40. Yamashita K, Park HL, Kim MS et al (2006) PGP9.5 methylation in diffuse-type gastric cancer. Cancer Res 66:3921–3927
41. Karpf AR, Peterson PW, Rawlins JT et al (1999) Inhibition of DNA methyltransferase stimulates the expression of signal transducer and activator of transcription 1, 2, and 3 genes in colon tumor cells. Proc Natl Acad Sci USA 96:14007–14012
42. Issa JP, Ahuja N, Toyota M et al (2001) Accelerated age-related CpG island methylation in ulcerative colitis. Cancer Res 61: 3573–3577
43. Kwabi-Addo B, Chung W, Shen L et al (2007) Age-related DNA methylation changes in normal human prostate tissues. Clin Cancer Res 13:3796–3802
44. Shen L, Toyota M, Kondo Y et al (2007) Integrated genetic and epigenetic analysis identifies three different subclasses of colon cancer. Proc Natl Acad Sci USA 104:18654–18659
45. Nojima M, Suzuki H, Toyota M et al (2007) Frequent epigenetic inactivation of SFRP genes and constitutive activation of Wnt signaling in gastric cancer. Oncogene 26:4699–4713
46. Rattner A, Hsieh JC, Smallwood PM et al (1997) A family of secreted proteins contains homology to the cysteine-rich ligand-binding domain of frizzled receptors. Proc Natl Acad Sci USA 94:2859–2863
47. Bafico A, Liu G, Goldin L et al (2004) An autocrine mechanism for constitutive Wnt pathway activation in human cancer cells. Cancer Cell 6:497–506
48. Schlosshauer PW, Brown SA, Eisinger K et al (2000) APC truncation and increased beta-catenin levels in a human breast cancer cell line. Carcinogenesis 21:1453–1456
49. Van Laer L, Huizing EH, Verstreken M et al (1998) Nonsyndromic hearing impairment is associated with a mutation in DFNA5. Nat Genet 20:194–197
50. Thompson DA, Weigel RJ (1998) Characterization of a gene that is inversely correlated with estrogen receptor expression (ICERE-1) in breast carcinomas. Eur J Biochem 252:169–177
51. Lage H, Helmbach H, Grottke C et al (2001) DFNA5 (ICERE-1) contributes to acquired etoposide resistance in melanoma cells. FEBS Lett 494:54–59
52. Esseghir S, Kennedy A, Seedhar P et al (2007) Identification of NTN4, TRA1, and STC2 as prognostic markers in breast cancer in a screen for signal sequence encoding proteins. Clin Cancer Res 13:3164–3173
53. Toyota M, Suzuki H, Yamashita T et al (2009) Cancer epigenomics: implications of DNA methylation in personalized cancer therapy. Cancer Sci 100:787–791
54. Meng X, Lu X, Morris CA et al (1998) A novel human gene FKBP6 is deleted in Williams syndrome. Genomics 52:130–137

Published OnlineFirst October 26, 2010; DOI:10.1158/1078-0432.CCR-10-0581

Human Cancer Biology

Clinical Cancer Research

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

Shinichi Igarashi[1], Hiromu Suzuki[1,2], Takeshi Niinuma[1], Haruo Shimizu[1,8], Masanori Nojima[1,3], Hiroyuki Iwaki[9], Takayuki Nobuoka[4], Toshirou Nishida[10,11], Yasuaki Miyazaki[11], Hiroyuki Takamaru[1], Eiichiro Yamamoto[1,2], Hiroyuki Yamamoto[1], Takashi Tokino[5], Tadashi Hasegawa[6], Koichi Hirata[4], Kohzoh Imai[7], Minoru Toyota[2], and Yasuhisa Shinomura[1]

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.

Authors' Affiliations: [1]First Department of Internal Medicine, Sapporo Medical University; [2]Department of Biochemistry, Sapporo Medical University; [3]Department of Public Health, Sapporo Medical University; [4]First Department of Surgery, Sapporo Medical University; [5]Department of Molecular Biology, Cancer Research Institute, Sapporo Medical University; [6]Department of Surgical Pathology, Sapporo Medical University; [7]The Advanced Clinical Research Center, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan; [8]Department of Gastroenterology, Muroran General Hospital, Muroran, Japan; [9]Department of Pathology, Sunagawa City Medical Center, Sunagawa Japan; and [10]Department of Surgery, Osaka Police Hospital; [11]Department of Surgery, Osaka University Graduate School of Medicine, Osaka, Japan

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

S. Igarashi and H. Suzuki contributed equally to this work.

Corresponding Authors: Shinomura Yasuhisa, First Department of Internal Medicine, Sapporo Medical University, S1, W16, Chuo-ku, Sapporo 060-8543, Japan. Phone: 81-11-611-2111; Fax: 81-11-611-2282; E-mail: shinomura@sapmed.ac.jp. or Minoru Toyota, Department of Biochemistry, Sapporo Medical University, S1, W17, Chuo-ku, Sapporo 060-8556, Japan. Phone: 81-11-611-2111; Fax: 81-11-622-1918; E-mail: mtoyota@sapmed.ac.jp.

doi: 10.1158/1078-0432.CCR-10-0581

Published OnlineFirst October 26, 2010; DOI:10.1158/1078-0432.CCR-10-0581

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

Published OnlineFirst October 26, 2010; DOI:10.1158/1078-0432.CCR-10-0581

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 *AluI* and *RsaI* 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%)

Published OnlineFirst October 26, 2010; DOI:10.1158/1078-0432.CCR-10-0581

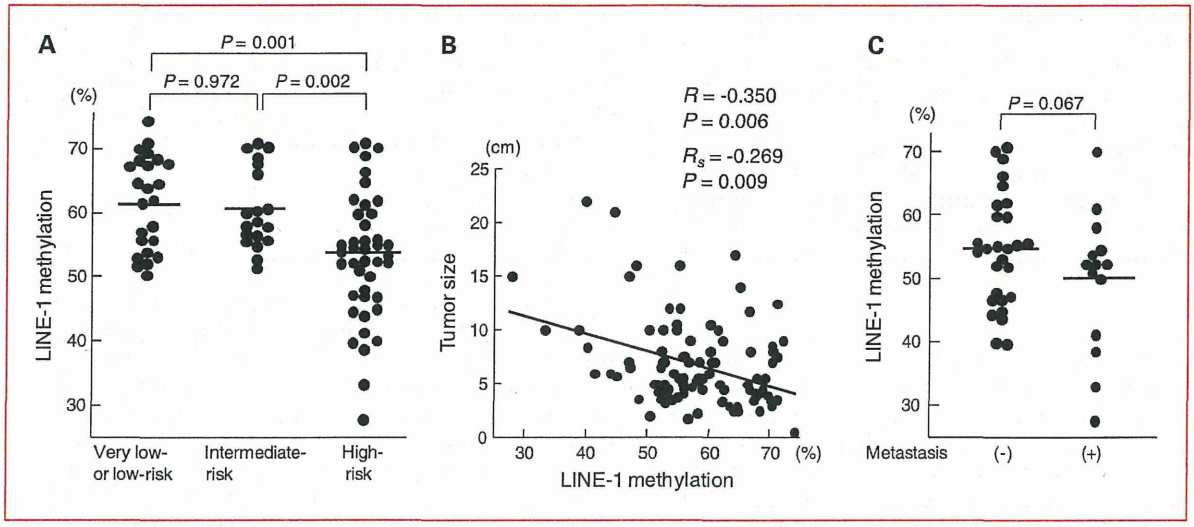

Fig. 1. Analysis of *LINE-1* methylation in GISTs. A, comparison of the levels of *LINE-1* methylation among low-risk (n = 23), intermediate-risk (n = 19), and high-risk (n = 42) GISTs. Filled circles depict the average methylation (%) at multiple CpG sites. B, correlation of *LINE-1* methylation with 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.

Published OnlineFirst October 26, 2010; DOI:10.1158/1078-0432.CCR-10-0581

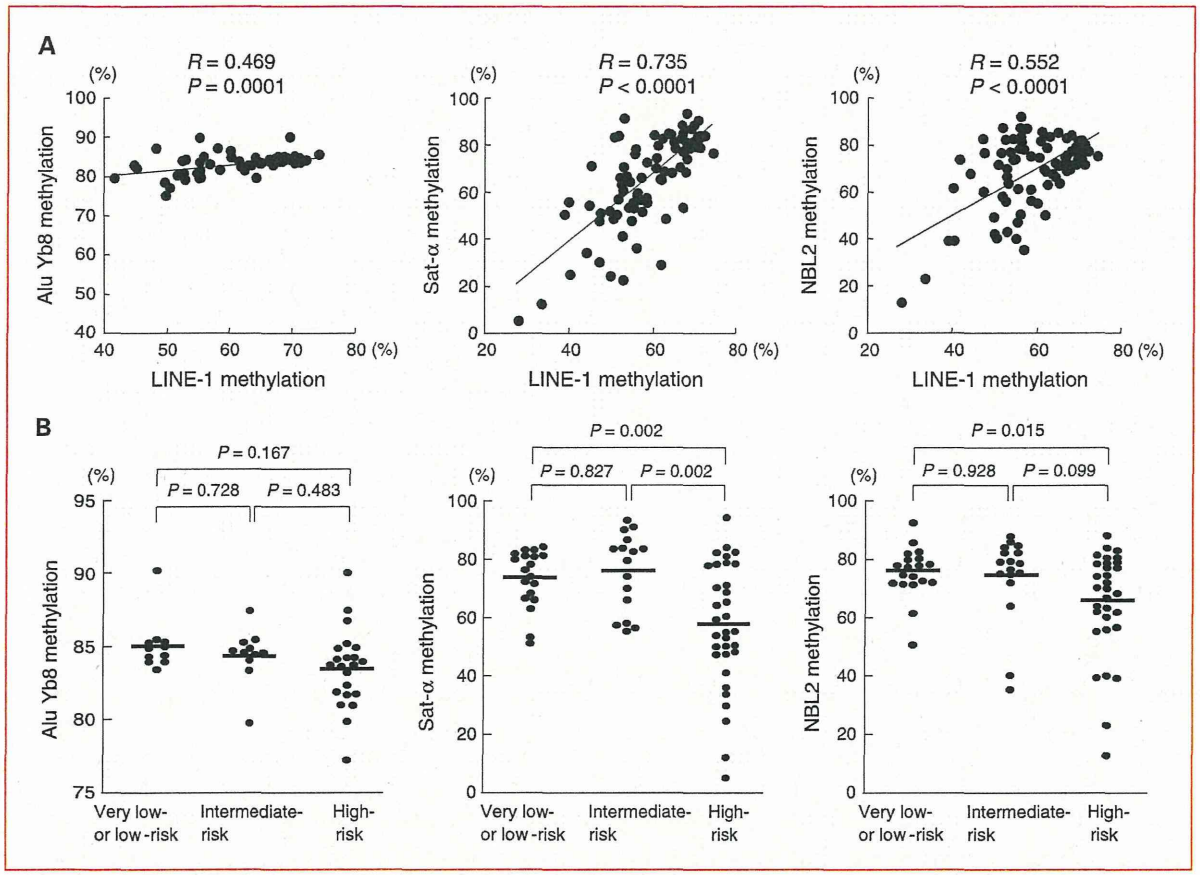

Fig. 2. Analysis of the methylation of different repetitive sequences in GISTs. A, methylation levels in three different repetitive sequences (*Alu Yb8*, *Sat-α*, and *NBL2*) were analyzed and correlated with *LINE-1* methylation. The Pearson correlation coefficients and *P* values are shown. B, comparison 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

Published OnlineFirst October 26, 2010; DOI:10.1158/1078-0432.CCR-10-0581

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.