Table 2 CpG islands hypermethylation of tumor-associated genes in biliary fluids

	Age (yr)	Sex	Stage	*UCHL1*	*RUNX3*	*CDKN2A*	*IGF2*	*CACNA1G*	*AHRR*	*SFRP1*	*MGMT*	*APC*	*NEUROG1*
NC8	77	M		MN	MN	MN	MN	MN	MN	MN	MP	MN	MN
NC9	69	F		MN	MN	MN	MN	MN	MN	MP	MN	MN	MN
NC10	76	M		MN	MN	MN	MN	MN	MN	MN	MP	MN	MN
NC11	58	M		MN	MN	MN	MN	MN	MN	MN	MN	MN	MN
NC12	41	M		MN	MN	MN	MN	MN	MP	MN	MP	MN	MN
NC13	66	M		MN	MN	MN	MN	MN	MP	MP	MP	MP	MP
NC14	69	F		MN	MN	MN	MN	MN	MP	MP	MP	MN	MP
NC15	71	F		MN	MN	MN	MN	MN	MN	MN	MP	MP	MN
NC16	59	M		MN	MN	MN	MN	MN	MP	MP	MN	MN	MN
NC17	54	F		MN	MN	MN	MN	MN	MN	MN	MN	MP	MP
NC18	67	M		MN	MN	MN	MN	MN	MP	MP	MN	MN	MN
NC19	85	M		MN	MN	MN	MN	MN	MN	MN	MN	MN	MN
NC20	73	F		MN	MN	MN	MN	MN	MN	MP	MN	MN	MN
NC21	80	F		MN	MN	MN	MN	MN	MN	MN	MP	MN	MN
NC22	74	F		MN	MN	MN	MN	MN	MN	MN	MP	MN	MN
NC23	52	M		MN	MN	MN	MN	MN	MN	MN	MN	MN	MN
NC24	64	M		MN	MN	MN	MN	MN	MN	MP	MP	MP	MN
NC25	64	F		MN	MN	MN	MN	MN	MP	MP	MN	MN	MN
				MN	MN	MN	MN	MN	MN	MN	MN	MN	MN
GB1	75	F	ⅢB	MP	MP	MP	MP	MP	MP	MN	MN	MN	MN
GB2	76	F	ⅢB	MN	MP	MP	MP	MP	MP	MN	MN	MP	MN
GB3	62	M	ⅢA	MP	MP	MP	MP	MN	MN	MP	MP	MN	MN
GB4	67	M	ⅣA	MN	MP	MN	MN	MN	MN	MN	MN	MN	MN
GB5	59	F	ⅢB	MP	MN	MP	MN	MN	MP	MP	MN	MP	MP
GB6	63	F	Ⅱ	MP	MN	MP	MN	MN	MP	MP	MN	MP	MP
GB7	77	M	Ⅰ	MN	MP	MP	MN	MN	MN	MP	MP	MN	MN
GB8	78	M	ⅢA	MP	MN	MP	MN	MN	MN	MP	MP	MN	MN
BC1	73	M	Ⅰ	MN	MP	MN	MP	MN	MP	MP	MN	MN	MN
BC2	80	F	ⅡA	MP	MP	MN	MN	MN	MP	MP	MP	MN	MN
BC3	71	M	ⅡB	MP	MN	MN	MN	MP	MP	MP	MN	MN	MN
BC4	75	M	Ⅲ	MP	MN	MN	MN	MN	MN	MP	MP	MN	MN
BC5	77	M	ⅡB	MN	MP	MN	MN	MN	MN	MN	MP	MN	MN
BC6	65	M	ⅡA	MP	MN	MP	MN	MN	MN	MN	MN	MN	MN
BC7	72	M	ⅡA	MP	MN	MN	MN	MN	MN	MP	MN	MN	MN
BC8	73	F	Ⅳ	MP	MP	MN	MN	MN	MP	MN	MN	MN	MN
BC9	76	F	ⅡA	MP	MP	MN	MP	MP	MP	MP	MN	MN	MN
BC10	74	M	ⅡB	MP	MN	MN	MN	MN	MP	MN	MP	MN	MP
BC11	66	M	Ⅲ	MP	MN	MN	MN	MN	MN	MN	MN	MP	MN
BC12	58	F	ⅡB	MN	MP	MN	MN	MN	MN	MN	MN	MN	MN
PC1	66	F	ⅡA	MP	MN	MN	MN	MN	MN	MP	MP	MN	MN
PC5	71	M	ⅡB	MN	MP	MP	MN	MN	MN	MN	MP	MN	MN
PC7	73	F	ⅡB	MP	MN	MN	MN	MN	MP	MN	MN	MN	MN
PC8	75	M	Ⅲ	MP	MP	MN	MN	MN	MN	MN	MP	MN	MN
PC13	70	F	Ⅳ	MP	MN	MN	MN	MN	MP	MP	MP	MP	MN
PC16	59	M	ⅡA	MP	MP	MN	MN	MP	MN	MN	MN	MN	MN
PC17	80	F	ⅡB	MP	MP	MP	MP	MN	MP	MP	MN	MN	MN
PC18	67	M	ⅠB	MN	MP	MN	MN	MN	MP	MN	MP	MN	MN
PC19	63	M	ⅡA	MP	MN	MN	MN	MN	MN	MN	MP	MP	MN
PC20	78	F	ⅡB	MN	MN	MP	MN	MN	MP	MN	MN	MN	MN

NC: Non-cancer; GB: Gallbladder cancer; BC: Biliary cancer; pancreatic cancer (PC) 1, PC5, PC7 and PC8 and PC13 are identical in Tables 1 and 2. MP: Methylation-positive; MN: Methylation-negative.

number of samples analyzed was limited. It is possible that some of the pancreatic fluid samples did not contain sufficient concentrations of cancer DNA[12]. Given the relatively poor diagnostic yield of cytology in this setting, a problem that is likely to be related to the highly scirrhous nature of pancreatic ductal adenocarcinomas, sample adequacy is likely to be one of the limiting factors in the molecular analysis of these samples[12]. Serum LINE-1 hypomethylation has been reported to be a potential prognostic marker for hepatocellular carcinoma[33]. It would be interesting to analyze serum LINE-1 methylation levels in patients with pancreatobiliary cancers.

CpG island hypermethylation of tumor-associated genes was detected at various frequencies in pancreatobiliary cancers using pancreatobiliary fluids. Although genome-wide hypomethylation and regional hypermethylation of 5' CpG islands are common features of neoplasias, the link between the two remains controversial[17]. In the current study, we did not find a significant correlation between 5' CpG island hypermethylation of tumor-associated genes and global hypomethylation.

Hypermethylation of the *UCHL1* gene was cancer-

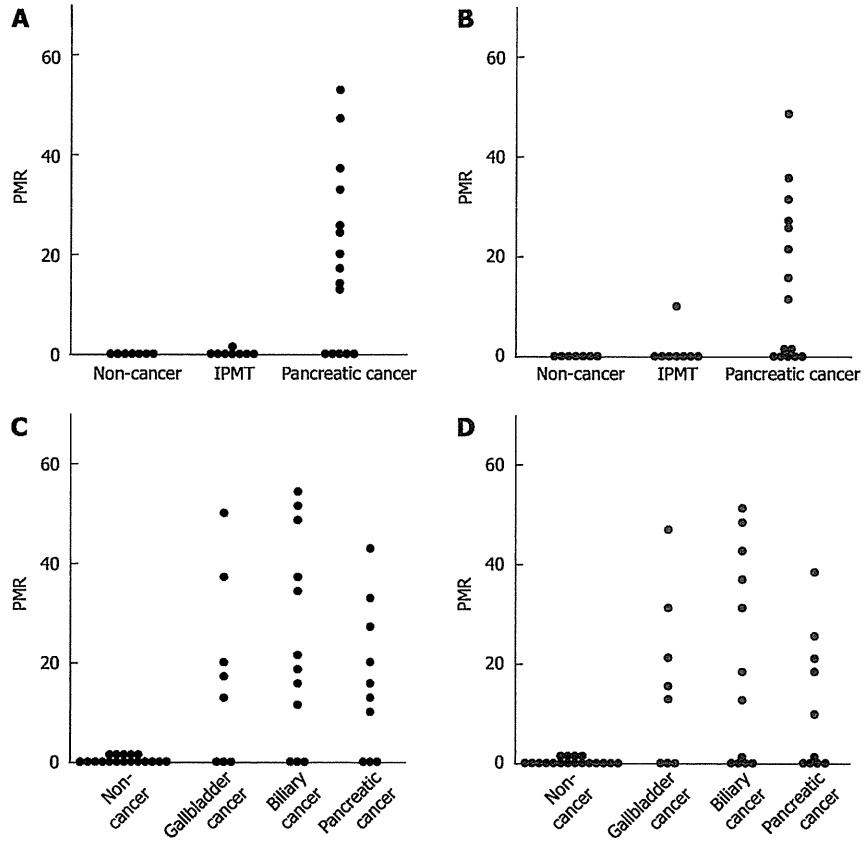

Figure 3 Analysis of methylation levels of ubiquitin carboxyl-terminal esterase L1 and runt-related transcription factor 3 using pancreatobiliary fluids. Comparison of the ubiquitin carboxyl-terminal esterase L1 (UCHL1) (A) and runt-related transcription factor 3 (RUNX3) (B) methylation levels in pancreatic fluids between pancreatic cancer and noncancerous pancreatic disease; Comparison of the UCHL1 (C) and RUNX3 (D) methylation levels in biliary fluids between pancreatobiliary cancer and noncancerous pancreatobiliary disease. PMR: Percentage of methylated reference.

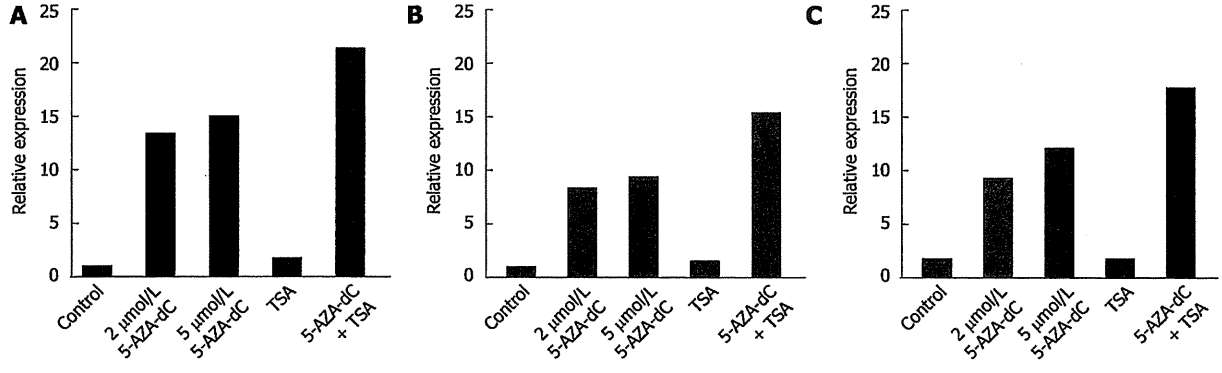

Figure 4 Reactivation of ubiquitin carboxyl-terminal esterase L1 by 5-AZA-2'-deoxycytidine and/or Trichostatin A treatment in pancreatic and biliary cancer cell lines. A: PK-1 cells; B: PK45P cells; C: TGBC1TKB cells. To examine the roles of CpG methylation and histone deacetylation in the silencing of ubiquitin carboxyl-terminal esterase L1, cancer cells were treated with 2 or 5 μmol/L 5-AZA-2'-deoxycytidine (5-AZA-dC) for 72 h or 100 nmol/L Trichostatin A (TSA) for 24 h. The cells were also treated with 2 μmol/L 5-AZA-dC for 72 h, followed by 100 nmol/L TSA for an additional 24 h.

specific and most frequently detected in pancreatobiliary cancers. Hypermethylation of the *UCHL1* gene in pancreatic and biliary fluids was the most useful single marker of pancreatic and pancreatobiliary cancers, respectively. Hypermethylation of the *UCHL1* and *RUNX3* genes in pancreatic and biliary fluids was the most useful combined marker for pancreatic and pancreatobiliary cancers, respectively. Epigenetic inactivation of UCHL1 has been reported in a variety of human cancers[34]. Epigenetic inactivation of RUNX3 is known to play an important role in the pathogenesis of pancreatobiliary cancer[35,36].

LINE-1 and SAT2 methylation levels have been reported to be significantly lower in extrahepatic cholangiocarcinoma than in normal duct and biliary intraepithelial

neoplasias (BilINs). BilINs showed a decrease of SAT2 methylation levels, but no decrease of LINE-1 methylation levels was found compared to those in normal samples[24]. Most of the cancer-specific CpG island hypermethylation is thought to occur in the BilIN stage, before LINE-1 hypomethylation. Our results also suggest that CpG island hypermethylation analyzed in pancreatobiliary fluids is more useful than LINE-1 methylation for the detection of pancreatobiliary cancer.

Importantly, the methylation patterns of 10 tumor-associated genes were similar in both the pancreatic and biliary fluids from the same patients with pancreatic cancer. Moreover, the methylation patterns of the *UCHL1* and *RUNX3* genes were identical in both the pancreatic and biliary fluids from the same patients. These results further support the notion that hypermethylation of UCHL1 and RUNX3 in pancreatobiliary fluids is a useful marker for the detection of pancreatobiliary cancer.

To confirm the role of epigenetic alterations in transcriptional repression of the *UCHL1* gene, we treated pancreatobiliary cancer cell lines, in which UCHL1 was methylated, with 5-AZA-dC alone or in combination with TSA. Treatment with 5-AZA-dC restored the UCHL1 expression in cancer cell lines. Moreover, combined treatment with 5-AZA-dC and TSA restored UCHL1 expression synergistically, indicating that CpG methylation and histone deacetylation play important roles in silencing the *UCHL1* gene.

Not only the clinical utility but also the pathobiological effects of nucleic acids in circulation (nucleosomes, DNA, RNA, microRNA *etc.*) are receiving increasing attention[37,38]. Further analysis is necessary to clarify the possible detrimental effects of nucleic acids in the tumor microenvironment, including the contribution of methylated DNA in pancreatobiliary fluids to disease progression.

In conclusion, our results suggest that hypermethylation of the *UCHL1* gene plays a key role in the pathogenesis of pancreatobiliary cancers and that detection of hypermethylation of UCHL1 and RUNX3 in pancreatobiliary fluids is useful for the diagnosis of these malignancies. Our MethyLight panel (UCHL1 and RUNX3) is simple and accurate for differentiating between neoplastic and non-neoplastic samples and compares favorably with other quantitative MSP panels and with the identification of mutant KRAS or telomerase, which have been used previously to differentiate between malignant and benign pancreatic samples[39,40]. Moreover, newer assays that can detect low concentrations of mutations in pancreatic juice[41], as well as novel assays and technologies, are likely to improve the detection of low concentrations of mutant DNA for cancer diagnosis in the future. Although we focused on epigenetic alterations in the current study, a combination of highly specific epigenetic and genetic markers might provide the best diagnostic utility.

COMMENTS

Background

Despite recent advances in diagnosis and treatment, the prognosis of patients with pancreatobiliary cancer is still poor. Elucidation of the biological characteristics of these carcinomas has become necessary to improve the prognosis of patients and to devise better treatment strategies.

Research frontiers

Roles of epigenetic alterations in pancreatobiliary cancer are receiving increasing attention. Two contradicting epigenetic alterations often coexist in cancer: global or genome-wide hypomethylation, which is mainly observed in repetitive sequences within the genome, and regional hypermethylation, which is frequently associated with CpG islands within gene promoters. Long interspersed nuclear element-1 (LINE-1) methylation status and its relationship with the hypermethylation of CpG islands in pancreatic and biliary fluids taken from patients with pancreatobiliary cancer is not known.

Innovations and breakthroughs

This is the first study to report that pancreatobiliary cancers exhibit a pattern of genome-wide hypomethylation that can be detected using pancreatic and biliary fluids. CpG island hypermethylation of tumor-associated genes was detected at various frequencies. Hypermethylation of the ubiquitin carboxyl-terminal esterase L1 (*UCHL1*) gene may play a key role in the pathogenesis of pancreatobiliary cancers.

Applications

Hypermethylation of UCHL1 and runt-related transcription factor 3 in pancreatobiliary fluids might be useful for the diagnosis of pancreatobiliary cancers. A combination of highly specific epigenetic and genetic markers might provide the best diagnostic utility.

Terminology

LINEs are 6-8 kb long, GC-poor sequences encoding an RNA-binding protein and a reverse transcriptase/endonuclease; these sequences constitute approximately 20% of the human genome. LINE-1 elements are most abundant, and over half a million copies of these elements are present in the human genome; UCHL1, which is also known as PARK5/PGP9.5, is a member of the ubiquitin carboxy terminal hydrolase family targeting the ubiquitin-dependent protein degradation pathway. With both ubiquitin hydrolase and dimerization-dependent ubiquitin ligase activities, UCHL1 plays important roles in multiple cellular processes. UCHL1 is a tumor-suppressor gene that is inactivated by promoter methylation or gene deletion in several types of human cancers.

Peer review

The presence of such high amounts of methylated DNA in pancreatobiliary fluid is intriguing. The study has translational significance.

REFERENCES

1 **Cleary SP**, Dawson LA, Knox JJ, Gallinger S. Cancer of the gallbladder and extrahepatic bile ducts. *Curr Probl Surg* 2007; **44**: 396-482 [PMID: 17693325 DOI: 10.1067/j.cpsurg.2007.04.006]

2 **Thomas MB**. Biological characteristics of cancers in the gallbladder and biliary tract and targeted therapy. *Crit Rev Oncol Hematol* 2007; **61**: 44-51 [PMID: 17164111 DOI: 10.1016/j.critrevonc.2006.07.006]

3 **Hezel AF**, Deshpande V, Zhu AX. Genetics of biliary tract cancers and emerging targeted therapies. *J Clin Oncol* 2010; **28**: 3531-3540 [PMID: 20547994 DOI: 10.1200/JCO.2009.27.4787]

4 **Kim SG**, Chan AO, Wu TT, Issa JP, Hamilton SR, Rashid A. Epigenetic and genetic alterations in duodenal carcinomas are distinct from biliary and ampullary carcinomas. *Gastroenterology* 2003; **124**: 1300-1310 [PMID: 12730870 DOI: 10.1016/S0016-5085(03)00278-6]

5 **Morris JP**, Wang SC, Hebrok M. KRAS, Hedgehog, Wnt and the twisted developmental biology of pancreatic ductal adenocarcinoma. *Nat Rev Cancer* 2010; **10**: 683-695 [PMID: 20814421 DOI: 10.1038/nrc2899]

6 **Ueki T**, Toyota M, Sohn T, Yeo CJ, Issa JP, Hruban RH, Goggins M. Hypermethylation of multiple genes in pancreatic adenocarcinoma. *Cancer Res* 2000; **60**: 1835-1839 [PMID: 10766168]

7 **Jones PA**, Baylin SB. The fundamental role of epigenetic events in cancer. *Nat Rev Genet* 2002; **3**: 415-428 [PMID:

12042769]
8 **Sato N**, Goggins M. The role of epigenetic alterations in pancreatic cancer. *J Hepatobiliary Pancreat Surg* 2006; **13**: 286-295 [PMID: 16858539]
9 **Sato N**, Fukushima N, Maitra A, Matsubayashi H, Yeo CJ, Cameron JL, Hruban RH, Goggins M. Discovery of novel targets for aberrant methylation in pancreatic carcinoma using high-throughput microarrays. *Cancer Res* 2003; **63**: 3735-3742 [PMID: 12839967]
10 **Fukushima N**, Walter KM, Uek T, Sato N, Matsubayashi H, Cameron JL, Hruban RH, Canto M, Yeo CJ, Goggins M. Diagnosing pancreatic cancer using methylation specific PCR analysis of pancreatic juice. *Cancer Biol Ther* 2003; **2**: 78-83 [PMID: 12673124]
11 **Matsubayashi H**, Canto M, Sato N, Klein A, Abe T, Yamashita K, Yeo CJ, Kalloo A, Hruban R, Goggins M. DNA methylation alterations in the pancreatic juice of patients with suspected pancreatic disease. *Cancer Res* 2006; **66**: 1208-1217 [PMID: 16424060 DOI: 10.1158/0008-5472.CAN-05-2664]
12 **Parsi MA**, Li A, Li CP, Goggins M. DNA methylation alterations in endoscopic retrograde cholangiopancreatography brush samples of patients with suspected pancreaticobiliary disease. *Clin Gastroenterol Hepatol* 2008; **6**: 1270-1278 [PMID: 18995218 DOI: 10.1016/j.cgh.2008.07.007]
13 **Cordaux R**, Batzer MA. The impact of retrotransposons on human genome evolution. *Nat Rev Genet* 2009; **10**: 691-703 [PMID: 19763152 DOI: 10.1038/nrg2640]
14 **Yang AS**, Estécio MR, Doshi K, Kondo Y, Tajara EH, Issa JP. A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. *Nucleic Acids Res* 2004; **32**: e38 [PMID: 14973332 DOI: 10.1093/nar/gnh032]
15 **Chalitchagorn K**, Shuangshoti S, Hourpai N, Kongruttanachok N, Tangkijvanich P, Thong-ngam D, Voravud N, Sriuranpong V, Mutirangura A. Distinctive pattern of LINE-1 methylation level in normal tissues and the association with carcinogenesis. *Oncogene* 2004; **23**: 8841-8846 [PMID: 15480421 DOI: 10.1038/sj.onc.1208137]
16 **Ogino S**, Kawasaki T, Nosho K, Ohnishi M, Suemoto Y, Kirkner GJ, Fuchs CS. LINE-1 hypomethylation is inversely associated with microsatellite instability and CpG island methylator phenotype in colorectal cancer. *Int J Cancer* 2008; **122**: 2767-2773 [PMID: 18366060 DOI: 10.1002/ijc.23470]
17 **Igarashi S**, Suzuki H, Niinuma T, Shimizu H, Nojima M, Iwaki H, Nobuoka T, Nishida T, Miyazaki Y, Takamaru H, Yamamoto E, Yamamoto H, Tokino T, Hasegawa T, Hirata K, Imai K, Toyota M, Shinomura Y. A novel correlation between LINE-1 hypomethylation and the malignancy of gastrointestinal stromal tumors. *Clin Cancer Res* 2010; **16**: 5114-5123 [PMID: 20978145 DOI: 10.1158/1078-0432.CCR-10-0581]
18 **Iacobuzio-Donahue CA**, van der Heijden MS, Baumgartner MR, Troup WJ, Romm JM, Doheny K, Pugh E, Yeo CJ, Goggins MG, Hruban RH, Kern SE. Large-scale allelotype of pancreaticobiliary carcinoma provides quantitative estimates of genome-wide allelic loss. *Cancer Res* 2004; **64**: 871-875 [PMID: 14871814 DOI: 10.1158/0008-5472.CAN-03-2756]
19 **Calhoun ES**, Hucl T, Gallmeier E, West KM, Arking DE, Maitra A, Iacobuzio-Donahue CA, Chakravarti A, Hruban RH, Kern SE. Identifying allelic loss and homozygous deletions in pancreatic cancer without matched normals using high-density single-nucleotide polymorphism arrays. *Cancer Res* 2006; **66**: 7920-7928 [PMID: 16912165 DOI: 10.1158/0008-5472.CAN-06-0721]
20 **Kipp BR**, Stadheim LM, Halling SA, Pochron NL, Harmsen S, Nagorney DM, Sebo TJ, Therneau TM, Gores GJ, de Groen PC, Baron TH, Levy MJ, Halling KC, Roberts LR. A comparison of routine cytology and fluorescence in situ hybridization for the detection of malignant bile duct strictures. *Am J Gastroenterol* 2004; **99**: 1675-1681 [PMID: 15330900 DOI: 10.1111/j.1572-0241.2004.30281.x]
21 **Barr Fritcher EG**, Kipp BR, Slezak JM, Moreno-Luna LE, Gores GJ, Levy MJ, Roberts LR, Halling KC, Sebo TJ. Correlating routine cytology, quantitative nuclear morphometry by digital image analysis, and genetic alterations by fluorescence in situ hybridization to assess the sensitivity of cytology for detecting pancreatobiliary tract malignancy. *Am J Clin Pathol* 2007; **128**: 272-279 [PMID: 17638662 DOI: 10.1309/BC6DY755Q3T5W9EE]
22 **Sato N**, Maitra A, Fukushima N, van Heek NT, Matsubayashi H, Iacobuzio-Donahue CA, Rosty C, Goggins M. Frequent hypomethylation of multiple genes overexpressed in pancreatic ductal adenocarcinoma. *Cancer Res* 2003; **63**: 4158-4166 [PMID: 12874021]
23 **Choi IS**, Estecio MR, Nagano Y, Kim do H, White JA, Yao JC, Issa JP, Rashid A. Hypomethylation of LINE-1 and Alu in well-differentiated neuroendocrine tumors (pancreatic endocrine tumors and carcinoid tumors). *Mod Pathol* 2007; **20**: 802-810 [PMID: 17483816 DOI: 10.1038/modpathol.3800825]
24 **Kim BH**, Cho NY, Shin SH, Kwon HJ, Jang JJ, Kang GH. CpG island hypermethylation and repetitive DNA hypomethylation in premalignant lesion of extrahepatic cholangiocarcinoma. *Virchows Arch* 2009; **455**: 343-351 [PMID: 19763613 DOI: 10.1007/s00428-009-0829-4]
25 **Yamamoto E**, Toyota M, Suzuki H, Kondo Y, Sanomura T, Murayama Y, Ohe-Toyota M, Maruyama R, Nojima M, Ashida M, Fujii K, Sasaki Y, Hayashi N, Mori M, Imai K, Tokino T, Shinomura Y. LINE-1 hypomethylation is associated with increased CpG island methylation in Helicobacter pylori-related enlarged-fold gastritis. *Cancer Epidemiol Biomarkers Prev* 2008; **17**: 2555-2564 [PMID: 18842996 DOI: 10.1158/1055-9965.EPI-08-0112]
26 **Weisenberger DJ**, Campan M, Long TI, Kim M, Woods C, Fiala E, Ehrlich M, Laird PW. Analysis of repetitive element DNA methylation by MethyLight. *Nucleic Acids Res* 2005; **33**: 6823-6836 [PMID: 16326863 DOI: 10.1093/nar/gki987]
27 **Nosho K**, Yamamoto H, Takahashi T, Mikami M, Taniguchi H, Miyamoto N, Adachi Y, Arimura Y, Itoh F, Imai K, Shinomura Y. Genetic and epigenetic profiling in early colorectal tumors and prediction of invasive potential in pT1 (early invasive) colorectal cancers. *Carcinogenesis* 2007; **28**: 1364-1370 [PMID: 17183069 DOI: 10.1093/carcin/bgl246]
28 **Weisenberger DJ**, Siegmund KD, Campan M, Young J, Long TI, Faasse MA, Kang GH, Widschwendter M, Weener D, Buchanan D, Koh H, Simms L, Barker M, Leggett B, Levine J, Kim M, French AJ, Thibodeau SN, Jass J, Haile R, Laird PW. CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. *Nat Genet* 2006; **38**: 787-793 [PMID: 16804544 DOI: 10.1038/ng1834]
29 **Ogino S**, Kawasaki T, Brahmandam M, Cantor M, Kirkner GJ, Spiegelman D, Makrigiorgos GM, Weisenberger DJ, Laird PW, Loda M, Fuchs CS. Precision and performance characteristics of bisulfite conversion and real-time PCR (MethyLight) for quantitative DNA methylation analysis. *J Mol Diagn* 2006; **8**: 209-217 [PMID: 16645207]
30 **Taniguchi H**, Yamamoto H, Hirata T, Miyamoto N, Oki M, Nosho K, Adachi Y, Endo T, Imai K, Shinomura Y. Frequent epigenetic inactivation of Wnt inhibitory factor-1 in human gastrointestinal cancers. *Oncogene* 2005; **24**: 7946-7952 [PMID: 16007117 DOI: 10.2353/jmoldx.2006.050135]
31 **Oki M**, Yamamoto H, Taniguchi H, Adachi Y, Imai K, Shinomura Y. Overexpression of the receptor tyrosine kinase EphA4 in human gastric cancers. *World J Gastroenterol* 2008; **14**: 5650-5656 [PMID: 18837080 DOI: 10.3748/wjg.14.5650]
32 **Eden A**, Gaudet F, Waghmare A, Jaenisch R. Chromosomal instability and tumors promoted by DNA hypomethylation. *Science* 2003; **300**: 455 [PMID: 12702868 DOI: 10.1126/science.1083557]
33 **Tangkijvanich P**, Hourpai N, Rattanatanyong P, Wisedopas N, Mahachai V, Mutirangura A. Serum LINE-1 hypomethyl-

ation as a potential prognostic marker for hepatocellular carcinoma. *Clin Chim Acta* 2007; **379**: 127-133 [PMID: 17303099]
34 **Tokumaru Y**, Yamashita K, Kim MS, Park HL, Osada M, Mori M, Sidransky D. The role of PGP9.5 as a tumor suppressor gene in human cancer. *Int J Cancer* 2008; **123**: 753-759 [PMID: 18512240 DOI: 10.1002/ijc.23354]
35 **Wada M**, Yazumi S, Takaishi S, Hasegawa K, Sawada M, Tanaka H, Ida H, Sakakura C, Ito K, Ito Y, Chiba T. Frequent loss of RUNX3 gene expression in human bile duct and pancreatic cancer cell lines. *Oncogene* 2004; **23**: 2401-2407 [PMID: 14743205 DOI: 10.1038/sj.onc.1207395]
36 **Nomoto S**, Kinoshita T, Mori T, Kato K, Sugimoto H, Kanazumi N, Takeda S, Nakao A. Adverse prognosis of epigenetic inactivation in RUNX3 gene at 1p36 in human pancreatic cancer. *Br J Cancer* 2008; **98**: 1690-1695 [PMID: 18475302 DOI: 10.1038/sj.bjc.6604333]
37 **Schwarzenbach H**, Hoon DS, Pantel K. Cell-free nucleic acids as biomarkers in cancer patients. *Nat Rev Cancer* 2011; **11**: 426-437 [PMID: 21562580]
38 **Mittra I**, Nair NK, Mishra PK. Nucleic acids in circulation: are they harmful to the host? *J Biosci* 2012; **37**: 301-312 [PMID: 22581336]
39 **Logsdon CD**, Simeone DM, Binkley C, Arumugam T, Greenson JK, Giordano TJ, Misek DE, Kuick R, Hanash S. Molecular profiling of pancreatic adenocarcinoma and chronic pancreatitis identifies multiple genes differentially regulated in pancreatic cancer. *Cancer Res* 2003; **63**: 2649-2657 [PMID: 12750293]
40 **Ohuchida K**, Mizumoto K, Ogura Y, Ishikawa N, Nagai E, Yamaguchi K, Tanaka M. Quantitative assessment of telomerase activity and human telomerase reverse transcriptase messenger RNA levels in pancreatic juice samples for the diagnosis of pancreatic cancer. *Clin Cancer Res* 2005; **11**: 2285-2292 [PMID: 15788678 DOI: 10.1158/1078-0432.CCR-04-1581]
41 **Bian Y**, Matsubayashi H, Li CP, Abe T, Canto M, Murphy KM, Goggins M. Detecting low-abundance p16 and p53 mutations in pancreatic juice using a novel assay: heteroduplex analysis of limiting dilution PCRs. *Cancer Biol Ther* 2006; **5**: 1392-1399 [PMID: 17106238 DOI: 10.4161/cbt.5.10.3453]

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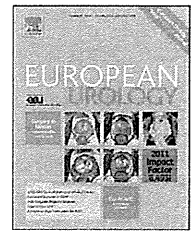

Urothelial Cancer

Methylation of a Panel of MicroRNA Genes Is a Novel Biomarker for Detection of Bladder Cancer

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DNA methylation
MicroRNA
Urinary test

Abstract

Background: Dysregulation of microRNAs (miRNAs) has been implicated in bladder cancer (BCa), although the mechanism is not fully understood.
Objective: We aimed to explore the involvement of epigenetic alteration of miRNA expression in BCa.
Design, setting, and participants: Two BCa cell lines (T24 and UM-UC-3) were treated with 5-aza-2′-deoxycytidine (5-aza-dC) and 4-phenylbutyric acid (PBA), after which their miRNA expression profiles were analyzed using a TaqMan array (Life Technologies, Carlsbad, CA, USA). Bisulfite pyrosequencing was used to assess miRNA gene methylation in 5 cancer cell lines, 83 primary tumors, and 120 preoperative and 47 postoperative urine samples.
Outcome measurements and statistical analysis: Receiver operating characteristic (ROC) curve analysis was used to assess the diagnostic performance of the miRNA gene panel.
Results and limitations: Of 664 miRNAs examined, 146 were upregulated by 5-aza-dC plus PBA. CpG islands were identified in the proximal upstream of 23 miRNA genes, and 12 of those were hypermethylated in cell lines. Among them, miR-137, miR-124-2, miR-124-3, and miR-9-3 were frequently and tumor-specifically methylated in primary cancers (miR-137: 68.7%; miR-124-2: 50.6%; miR-124-3: 65.1%; miR-9-3: 45.8%). Methylation of the same four miRNAs in urine specimens enabled BCa detection with 81% sensitivity and 89% specificity; the area under the ROC curve was 0.916. Ectopic expression of silenced miRNAs in BCa cells suppressed growth and cell invasion.
Conclusions: Our results indicate that epigenetic silencing of miRNA genes may be involved in the development of BCa and that methylation of miRNA genes could be a useful biomarker for cancer detection.

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1. Introduction

MicroRNAs (miRNAs) are a group of small noncoding RNAs that negatively regulate the translation and stability of partially complementary target mRNAs. In that way, they play important roles in a wide array of biologic processes, including cell proliferation, differentiation, and apoptosis [1]. Increasing evidence suggests that dysregulation of miRNA expression contributes to the initiation and progression of human cancer [2,3]. Altered miRNA expression is thought to play an important role in the pathogenesis of bladder cancer (BCa) and in certain tumor phenotypes. For instance, high-grade BCa exhibits upregulation of several miRNAs, including miR-21, which suppresses p53 function [4]. In addition, miR-21-to-miR-205 expression ratios are elevated in invasive BCa cells [5], while miR-200 family members regulate epithelial-to-mesenchymal transition by targeting transcription repressors ZEB1 and ZEB2 in BCa cells [6].

Although the mechanisms underlying miRNA dysregulation in cancer are not yet fully understood, recent studies have shown that the silencing of several miRNAs is tightly linked to epigenetic mechanisms, including histone modification and DNA methylation [7,8]. For example, treatment with a histone deacetylase (HDAC) inhibitor and a DNA methyltransferase (DNMT) inhibitor restored expression of various miRNAs in cancer cells [7,9], and the list of miRNA genes methylated in cancer is rapidly growing [10]. Studies have also shown that restoration of epigenetically silenced miRNAs may be an effective strategy for treating cancer and that aberrant methylation of miRNA genes could be a useful biomarker for cancer detection [10,11]. In addition, it was recently shown that the silencing of miRNA expression in BCa is associated with DNA methylation, often involving the CpG island (CGI) or CpG shore [12,13]. In an effort to identify novel biomarkers and treatment targets in BCa, we aimed to identify miRNAs epigenetically silenced in BCa cells by screening for miRNAs whose expression is upregulated by DNA demethylation and HDAC inhibition. We also investigated the methylation of miRNA genes in urine specimens and assessed its clinical usefulness as a biomarker for detection of BCa.

2. Materials and methods

2.1. Cell lines and tissue samples

BCa cell lines (T24, UM-UC-3, HT-1197, HT-1376, SW780, and 5637) and a normal urothelial cell line (SV-HUC-1) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA; Supplementary Table 1). A colorectal cancer cell line HCT116 harboring genetic disruptions within the DNMT1 and DNMT3B loci (DNMTs KO) have been described previously [8]. T24 and UM-UC-3 cells were treated first with 1 μM or 0.1 μM 5-aza-2′-deoxycytidine (5-aza-dC; Sigma-Aldrich, St Louis, MO, USA) for 72 h, and then with 3 mM 4-phenylbutyric acid (PBA; an HDAC inhibitor, Sigma-Aldrich) for 72 h, replacing the drug and medium every 24 h. A total of 83 primary BCa specimens were collected from patients who underwent radical cystectomy (RC) or transurethral resection of bladder tumor (TURBT; 66 males and 17 females; median age: 72 yr; range: 34–90 yr). Of the 83 patients, 73 underwent surgical resection after initial diagnosis, 7 received chemotherapy before surgery, and 3 are recurrent cases. Samples of nontumorous bladder tissue adjacent (<2 cm) to and distant (>2 cm) from the tumors were also collected. Six samples of normal urothelial tissue from renal cell carcinoma (RCC) patients who underwent nephrectomy were also collected. Informed consent was obtained from all patients before collection of the specimens, and this study was approved by the institutional review board. Total RNA was extracted using a *mir*Vana miRNA isolation kit (Life technologies, Carlsbad, CA, USA). Genomic DNA was extracted using the standard phenol-chloroform procedure.

2.2. Urine samples

Voided urine specimens were collected from 20 cancer-free individuals (Supplementary Table 2) and 86 BCa patients. In addition, postoperative voided urine samples were collected from 36 of the 86 patients 3–10 d after TURBT treatment. As an independent test set, preoperative urine samples were collected from 34 BCa patients, and postoperative samples were collected from 11 patients. The postoperative urine samples were collected from patients in whom tumors were successfully resected without leaving residual tumors. The urine (10 ml) was mixed with 5 ml of ThinPrep PreservCyt solution (Hologic, Bedford, MA, USA) and stored at 4 °C. Each sample was centrifuged at 3000 rpm for 10 min, and genomic DNA was extracted from the pelleted sediment using the standard phenol-chloroform procedure.

2.3. MicroRNA expression profiling

Expression of 664 miRNAs was analyzed using a TaqMan MicroRNA array v2.0 (Life Technologies). Briefly, 1 μg of total RNA was reverse-transcribed using a Megaplex Pools kit (Applied Biosystems, Foster City, CA, USA), after which miRNAs were amplified and detected using polymerase chain reaction (PCR) with specific primers and TaqMan probes. U48 snRNA (RNU48, Life Technologies) served as an endogenous control.

2.4. Quantitative real-time polymerase chain reaction of miRNA

Expression of selected miRNAs was analyzed using TaqMan microRNA assays. Briefly, 5 ng of total RNA were reverse-transcribed using specific stem-loop real-time primers, after which they were amplified and detected using PCR with specific primers and TaqMan probes. U6 snRNA (RNU6B, Life Technologies) served as an endogenous control.

2.5. Methylation analysis

Bisulfite conversion of genomic DNA, methylation-specific PCR (MSP), bisulfite sequencing, and bisulfite pyrosequencing were carried out as described previously [8]. Primer sequences and PCR product sizes are listed in Supplementary Table 3. Primer locations for methylation analysis are shown in Supplementary Figure 1.

2.6. Transfection of microRNA precursor molecules

BCa cells (1×10^6 cells) were transfected with 100 pmol of Pre-miR miRNA Precursor Molecules (Life Technologies) or Pre-miR miRNA Molecules Negative Control #1 using a Cell Line Nucleofector kit R (Lonza, Basel, Switzerland) with a Nucleofector I electroporation device (Lonza) according to the manufacturer's instructions. The viability of the miRNA precursor transfectants was analyzed using water-soluble tetrazolium salt (WST) assays [8]. Cell invasion was assessed using Matrigel invasion assays [8].

2.7. Gene expression microarray analysis

One-color microarray-based gene expression analysis was carried out according to the manufacturer's instructions (Agilent Technologies, Santa Clara, CA, USA). Briefly, 100 ng of total RNA were amplified and labeled using a Low-input Quick AmpLabeling Kit One-color (Agilent Technologies), after which the synthesized cRNA was hybridized to a SurePrint G3 Human GE microarray (G4851F; Agilent Technologies). The microarray data were then analyzed using GeneSpring GX version 11 (Agilent Technologies). The Gene Expression Omnibus accession number for the miRNA microarray data is GSE41760.

2.8. Statistical analysis

All data were analyzed using GraphPad Prism 4.0 statistical software (GraphPad Software, La Jolla, CA, USA). Quantitative variables were analyzed using a Student *t* test and one-way analysis of variance (ANOVA) with a post hoc Tukey test. Fisher exact test was used for analysis of categorical data. The Pearson correlation coefficient was used to evaluate correlations between continuous data. Receiver operating characteristic (ROC) curves for the diagnosis of BCa were constructed on the basis of the methylation levels, followed by calculation of the area under the curve (AUC). The best cut-off value for each miRNA gene was defined as the point on the ROC curve closest to the upper left corner. A diagnostic scoring system using urinary DNA methylation was constructed by analyzing the training set using the following three-step algorithm: (1) The methylation status of miRNA genes was assessed using the respective cut-off values; (2) the number of methylation-positive genes was determined, which we termed the *miR-methylation score* (M-score); and (3) the samples were classified into five groups based on the M-score. The value of $p < 0.05$ (two-sided) was regarded as significant.

3. Results

3.1. Identification of epigenetically silenced microRNA genes in bladder cancer

To identify epigenetically silenced miRNAs in BCa, we performed TaqMan array analysis using two BCa cell lines (T24 and UM-UC-3) treated with 1 μM 5-aza-dC plus 3 mM PBA. Of the 664 miRNAs examined, the drug treatment induced upregulation (more than five-fold) of 208 miRNAs in T24 cells and 200 miRNAs in UM-UC-3 cells. Of those, 146 miRNAs were upregulated in both cell lines (Supplementary Fig. 2 and 3; Supplementary Table 4). We selected 23 miRNA genes that harbored CGIs in the proximal upstream (<5 kb) of their coding regions (Supplementary Table 5), and subsequent MSP analysis revealed that the CGIs of 12 were hypermethylated in multiple BCa cell lines (Fig. 1A). These miRNAs were also induced by a low dose (0.1 μM) of 5-aza-dC plus PBA, making it unlikely that the observed induction was a secondary effect of DNA damage (Supplementary Fig. 4).

We next used bisulfite pyrosequencing to quantitatively analyze the methylation of the 12 miRNA genes showing CGI methylation in a series of BCa tissues ($n = 26$), a sample of normal urothelium tissue, and a normal urothelial cell line (SV-HUC-1). We found that four miRNA genes (miR-137, miR124-2, miR-124-3, and miR-9-3) were frequently methylated in primary tumors, though their methylation levels were limited in normal urothelium (Fig. 1B; Supplementary Fig. 5 and 6). In addition, we observed a marked

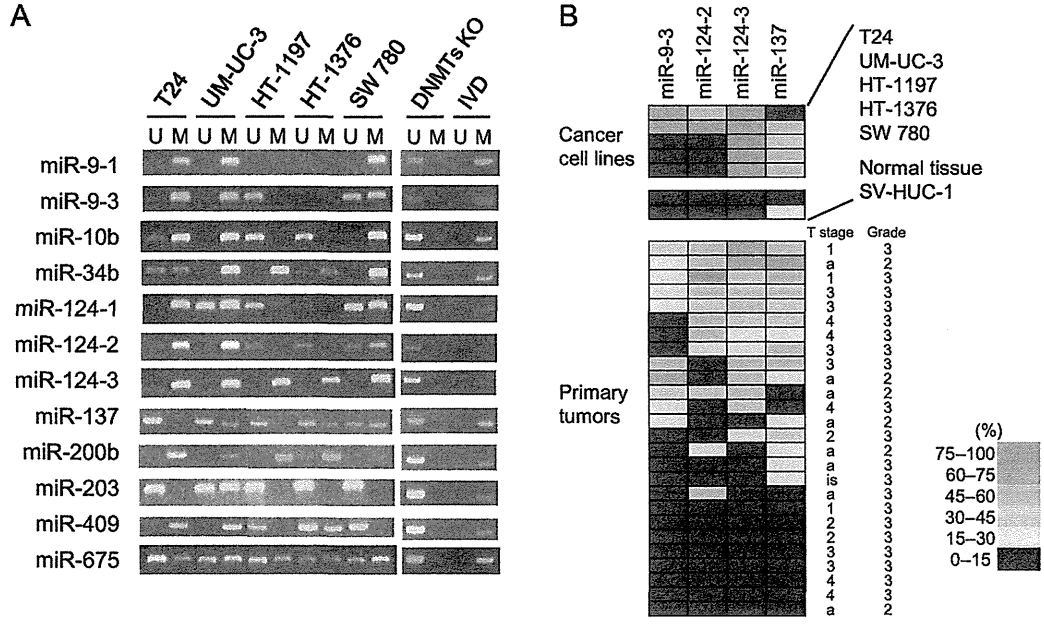

Fig. 1 – Methylation analysis of microRNA (miRNA) genes in bladder cancer (BCa). (A) Methylation-specific polymerase chain reaction (PCR) analysis of the CpG islands of 12 miRNA genes in the indicated cell lines. In vitro methylated DNA and DNA methyltransferase knockout cells served as positive and negative controls, respectively. Bands in the "M" lanes are PCR products obtained with methylation-specific primers; those in the "U" lanes are products obtained with unmethylated-specific primers. (B) Summarized results for the bisulfite pyrosequencing of miRNA genes in BCa cell lines, a sample of normal urothelial tissue, a normal urothelial cell line SV-HUC-1, and a set of primary BCa tissues ($n = 26$). Tumor stages and grades are indicated on the right.
DNMTs KO = DNA methyltransferase knockout cells; IVD = in vitro methylated DNA.

Table 1 – Correlation between microRNA gene methylation and the clinicopathologic features of bladder cancer

	(*n* = 83)	miR-137 met (%)			miR-124-2 met (%)			miR-124-3 met (%)			miR-9-3 met (%)		
		Mean	SD	*p**	Mean	SD	*p**	Mean	SD	*p**	Mean	SD	*p**
Age, yr:													
Median (range)	72 (34–90)	–	–	0.394	–	–	0.277	–	–	0.147	–	–	0.065
Gender:													
Male	66	26.7	19.9	–	25.1	23.9	–	32.0	24.4	–	19.8	17.4	–
Female	17	34.6	22.1	0.160	22.2	11.1	0.630	31.8	20.6	0.974	17.3	13.0	0.591
T stage:													
Ta	35	31.3	23.4	–	25.1	22.1	–	29.7	25.2	–	21.0	18.5	–
Tis	4	23.5	19.7	–	24.6	28.8	–	22.5	15.4	–	12.7	4.4	–
T1	8	33.4	19.2	–	33.9	29.9	–	42.6	25.2	–	24.9	16.2	–
≥T2	36	24.9	17.8	0.486	21.8	19.3	0.575	32.7	22.1	0.458	17.0	15.3	0.463
Grade:													
1	1	15.3	–	–	1.9	–	–	5.8	–	–	11.2	–	–
2	27	29.3	23.4	–	21.3	18.9	–	28.6	24.6	–	21.3	16.6	–
3	55	28.1	19.3	0.795	26.5	23.2	0.360	34.1	22.9	0.331	18.4	16.8	0.676
LN metastasis:													
N0	73	28.6	21.0	–	24.9	22.4	–	32.4	24.0	–	20.1	17.3	–
N1–N3	10	26.7	17.8	0.782	21.8	18.2	0.685	28.3	20.5	0.605	13.3	7.3	0.231

SD = standard deviation; LN = lymph node; ANOVA = analysis of variance.
* Pearson correlation coefficient, student *t* test, or ANOVA.

reduction in the methylation levels in BCa cells treated with 5-aza-dC plus PBA, which is consistent with the upregulation of miRNAs (Supplementary Fig. 7).

3.2. *Methylation of microRNA genes in primary bladder cancer*

We next examined the methylation levels of miR-137, miR-124-2, miR-124-3, and miR-9-3 in a larger set of primary tumors ($n = 83$), along with adjacent and distant nontumorous bladder tissues from the same patients (Table 1). Elevated levels of miRNA gene methylation (>15.0%) were frequently detected in primary BCa tissues (miR-137: 57 of 83, 68.7%; miR-124-2: 42 of 83, 50.6%; miR-124-3: 54 of 83, 65.1%; miR-9-3: 38 of 83, 45.8%), and the tumor tissues exhibited significantly higher methylation levels than their nontumorous counterparts (Fig. 2A). In addition, we found that levels of miRNA gene methylation were more frequently elevated in adjacent nontumorous bladder tissues (AN; miR-137: 26 of 74, 35.1%; miR-124-2: 19 of 74, 25.7%; miR-124-3: 15 of 74, 20.3%; miR-9-3: 12 of 74, 16.2%) than in more distant nontumorous tissues (DN; miR-137: 18 of 83, 21.7%; miR-124-2: 6 of 83, 7.2%; miR-124-3: 11 of 83, 13.3%; miR-9-3: 9 of 83, 10.8%). No significant correlation was found between the levels of miRNA gene methylation and the clinicopathologic characteristics of the patients (Table 1).

When we examined the methylation status of miR-137 in selected tissue specimens in more detail, we observed dense methylation in tumor tissues but only scattered methylation in nontumorous tissues (Fig. 2B). We then compared the levels of miR-137 expression determined in TaqMan assays with the methylation levels obtained by bisulfite pyrosequencing in selected pairs of tumors and corresponding distant nontumorous tissues (Fig. 2C). We found that there was an inverse relationship between the expression of miR-137 and its methylation, which suggests that CGI methylation is associated with the downregulation of miR-137 in BCa tissues.

3.3. *Detection of microRNA gene methylation in urine samples*

To assess the usefulness of miRNA gene methylation, we collected voided urine specimens from 86 BCa patients (Table 2) and 20 cancer-free individuals. Upon performing bisulfite pyrosequencing, we observed elevated methylation of miR-137, miR-124-2, miR-124-3, and miR-9-3 in the urine samples from the cancer patients (Fig. 3A) but only limited methylation of the genes in cancer-free individuals (Fig. 3B). Moreover, the methylation levels in the urine samples correlated positively with those in the corresponding tumor tissues (Supplementary Fig. 8). Notably, when we then collected postoperative voided urine samples from 36 of the 86 patients after surgical resection of their tumors, we observed dramatically reduced methylation levels (Fig. 3A; Supplementary Fig. 9).

To further evaluate the clinical usefulness of the miRNA gene methylation in urine samples, we carried out ROC curve analysis to assess its ability to distinguish preoperative from postoperative samples (Fig. 3C). The most discriminating cut-offs for miR-137, miR-124-2, miR-124-3, and miR-9-3 were 5.2% (sensitivity, 77.9%; specificity, 77.8%), 5.2% (sensitivity, 69.8%; specificity, 88.9%), 12.0% (sensitivity, 65.1%; specificity, 97.2%), and 7.2% (sensitivity, 69.4%; specificity, 86.1%), respectively (Table 3). We next compared these results with those obtained with urine cytology. Based on the urinary cytology using Papanicolaou's classification of the 86 patients, 55 (64%) were diagnosed as class I or II, 15 (17%) were class III, and only 16 (19%) were class IV or V (strongly suggestive or conclusive of malignancy), suggesting that the sensitivity of urinary methylation for detection of BCa is significantly greater than that of conventional cytology (Supplementary Table 6).

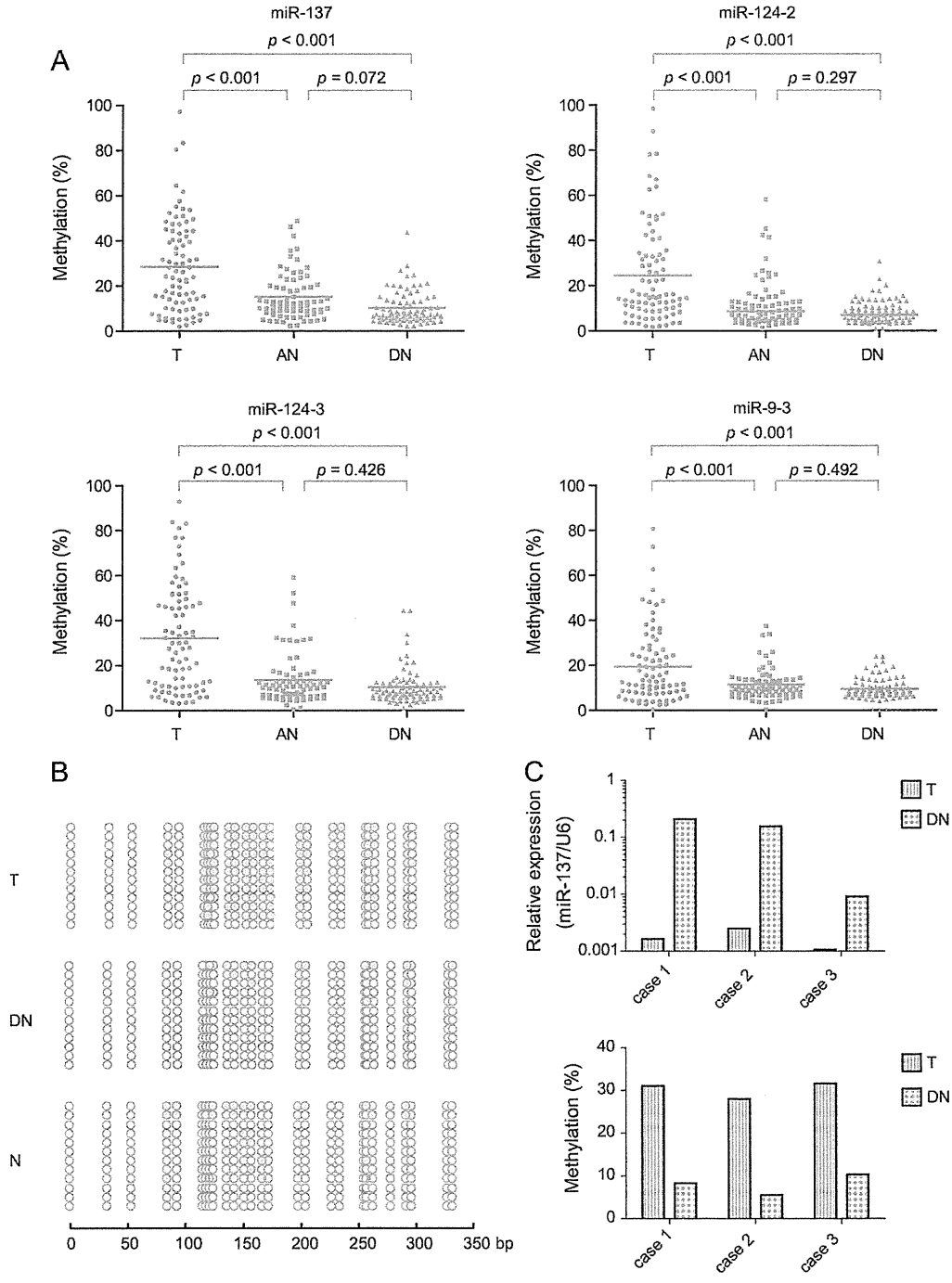

Fig. 2 – Analysis of microRNA (miRNA) gene methylation in primary bladder cancer. (A) Summarized results of bisulfite pyrosequencing of the indicated miRNA genes in primary tumors (T; *n* = 83), nontumorous bladder tissues adjacent to the tumors (AN; *n* = 74), and nontumorous bladder tissues distant from the tumors (DN; *n* = 83). $p < 0.05$. (B) Bisulfite sequencing analysis of the miR-137 CpG island (CGI) in a pair of tumor (T) and distant nontumorous tissues (DN). (C) Inverse relationship between the expression and methylation of miR-137 in three pairs of tumor (T) and distant nontumorous tissues (DN). Expression was assessed in TaqMan assays (upper panel), and methylation was determined by bisulfite pyrosequencing (lower panel).

To develop a more efficient diagnostic method for detecting BCa, we constructed a scoring system using the urinary methylation of the four methylated miRNA genes (Fig. 4). Using the cut-off value for each gene (Table 3), we classified the samples into five groups based on the M-score. A ROC curve was then constructed to evaluate the ability of the scoring system to distinguish preoperative from postoperative urine samples by plotting the sensitivity over 1-specificity at each point (Fig. 4B). We then validated the diagnostic system by analyzing an independent test set

Table 2 – Clinicopathologic characteristics of the patients in the training and test sets

	Training set (n = 86)	Test set (n = 34)
Age, yr:		
Median (range)	73 (42–90)	71 (58–93)
Gender, no.:		
Male	69	25
Female	17	9
T stage, no.:		
Ta	34	16
Tis	7	5
T1	12	8
≥T2	33	5
Grade, no.:		
1	1	0
2	28	14
3	57	20
Lymph node metastasis, no.:		
N0	81	32
N1–N3	5	2
Treatment, no.:		
TURBT	64	30
RC	22	4

TURBT = transurethral resection of bladder tumor; RC = radical cystectomy.

(Table 2). AUCs in both sets were high (training set: 0.916; test set: 0.910), confirming the accuracy of our system for detecting BCa using urinary miRNA gene methylation (Fig. 4). We also found that our scoring system could effectively detect early-stage Ta and low-grade (grades 1 and 2) BCa (sensitivity: 0.679; specificity: 0.889; AUC = 0.862), which was undetectable using urinary cytology (Supplementary Fig. 10).

3.4. *Functional analysis of microRNAs*

To test whether any of the miRNAs could act as tumor suppressors, we transfected BCa cells with an miRNA precursor molecule or a negative control, and then carried out cell viability assays. The assays showed that ectopic expression of miR-137 or miR-124 suppressed BCa cell proliferation, whereas miR-9 exerted no significant suppressive effect on growth (Supplementary Fig. 11 and 12). We then carried out Matrigel invasion assays to test the effect of the miRNAs on cell invasion. Although we detected no effect of miR-137 and miR-124 on cell invasion, ectopic expression of miR-9 suppressed the invasiveness of BCa cells (Supplementary Fig. 13).

Finally, to further clarify the effect of miRNAs, we carried out a gene expression microarray analysis of SW780 cells transfected with a miR-137 precursor or a negative control. We found that 1326 probe sets (1016 unique genes) were downregulated (more than two-fold) by ectopic miR-137 expression, including the previously reported miR-137 target genes cyclin-dependent kinase 6 (*CDK6*), cell division cycle 42 (*CDC42*), and aurora kinase A (*AURKA*) [14,15]. Among the 1016 downregulated genes, the TargetScan program predicted that 144 genes are potential targets of miR-137 (Supplementary Table 7). Moreover, Gene Ontology analysis revealed that genes related to the cell cycle were significantly enriched among the affected genes (Supplementary Table 8). Our results strongly suggest that the miRNAs in question act as tumor suppressors in BCa.

4. Discussion

We identified four miRNA genes (miR-137, miR-124-2, miR-124-3, and miR-9-3) that were frequently methylated in both cultured and primary BCa cells. Earlier studies have shown that these miRNAs are tumor-suppressive or tumor-related and that they are epigenetically silenced in cancers of various origins. Hypermethylation of miR-137 was first discovered in oral cancer [16] and has since been noted in other malignancies, including cancers of the colon [14] and stomach [3]. Within cancer cells, miR-137 targets *CDK6*, *CDC42*, and *AURKA*, which is indicative of its tumor-suppressive properties [14–16], whereas in normal cells, miR-137 regulates neuronal differentiation through targeting enhancer of zeste homolog 2 (*EZH2*) and mindbomb E3 ubiquitin protein ligase 1 (*MIB1*) [17,18]. Methylation of miR-124 family genes (miR-124-1, miR-124-2, and miR-124-3) was identified in colorectal cancer [19] and was also

Table 3 – Receiver operating characteristic analysis of microRNA gene methylation to detect bladder cancer

Gene name	Cut-off, %	Training set		
		AUC (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)
miR-137	5.2	0.782 (0.701–0.862)	77.91 (67.67–86.14)	77.78 (60.85–89.88)
miR-124-2	5.2	0.769 (0.686–0.851)	69.77 (58.92–79.21)	88.89 (73.94–96.89)
miR-124-3	12.0	0.805 (0.730–0.880)	65.12 (54.08–75.08)	97.22 (85.47–99.93)
miR-9-3	7.2	0.778 (0.697–0.860)	69.41 (58.47–78.95)	86.11 (70.50–95.33)
Gene name	**Cut-off, %**	**Test set**		
		AUC (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)
miR-137	5.2	0.816 (0.693–0.938)	79.41 (62.10–91.30)	63.64 (30.79–89.07)
miR-124-2	5.2	0.866 (0.758–0.975)	79.41 (62.10–91.30)	90.91 (58.72–99.77)
miR-124-3	12.0	0.901 (0.807–0.995)	58.82 (40.70–75.35)	100.0 (71.51–100.0)
miR-9-3	7.2	0.797 (0.660–0.934)	76.47 (58.83–89.25)	72.73 (39.03–93.98)

AUC = area under the curve; CI = confidence interval.

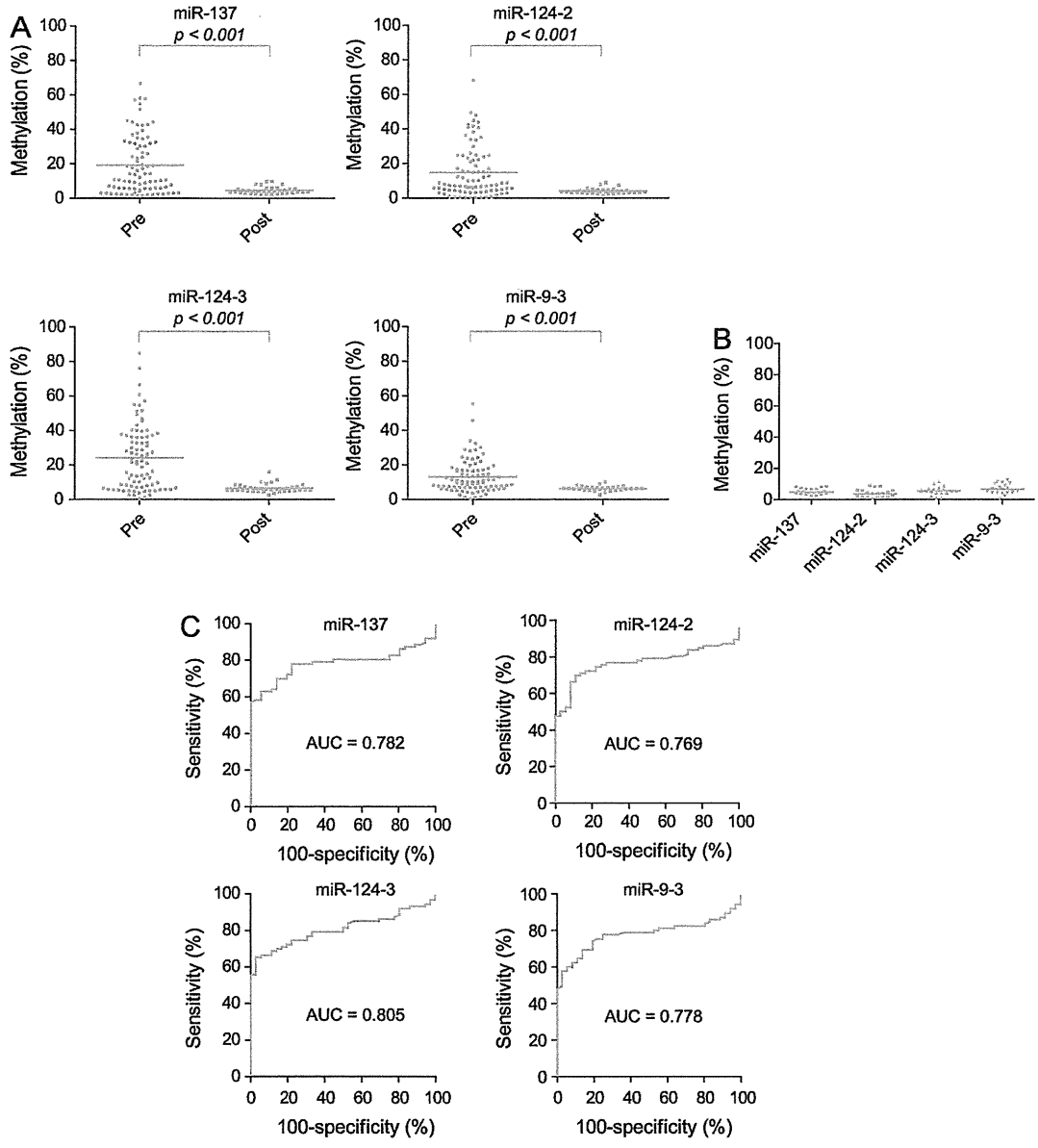

Fig. 3 – Detection of microRNA (miRNA) gene methylation in urine specimens from bladder cancer (BCa) patients. (A) Summary of bisulfite pyrosequencing analysis of the indicated miRNA genes in voided urine samples collected from BCa patients before (Pre: $n = 86$) and after surgical treatment (Post: $n = 36$). $p < 0.001$. (B) Bisulfite pyrosequencing results for miR-137, miR-124-2, miR-124-3, and miR-9-3 in voided urine samples from cancer-free individuals ($n = 20$). (C) Receiver operating characteristics curve analysis of the ability of miRNA gene methylation to distinguish preoperative and postoperative urine samples.
AUC = area under the curve.

found in gastric cancer [20], hematologic malignancies [21], and hepatocellular carcinoma [22]. In addition, screening for methylated miRNA genes in metastatic cancer cell lines also identified miR-9 family genes (miR-9-1, miR-9-2, and miR-9-3) [23].

Cumulative evidence suggests that miRNAs play important roles in the pathogenesis of BCa, and previous studies demonstrated their epigenetic silencing in the disease. For example, miR-34a, which is a direct target of p53 and a candidate tumor suppressor gene, is frequently methylated and silenced in many types of cancer, including BCa [24]. In addition, Wiklund et al. found that the silencing of miR-200 family genes and miR-205 is associated with DNA methylation in invasive BCa [12]. They also showed that reduced expression of miR-200c is associated with disease progression and poor outcome, suggesting that epigenetic silencing of miR-200 family genes could be a prognostic marker in BCa. Recently, Dudziec et al. carried out an miRNA microarray analysis after treating normal urothelium and urothelial cancer cell lines with 5-azacytidine. They identified 4 mirtrons and 16 miRNAs whose silencing was associated with DNA methylation [13]. Some of those

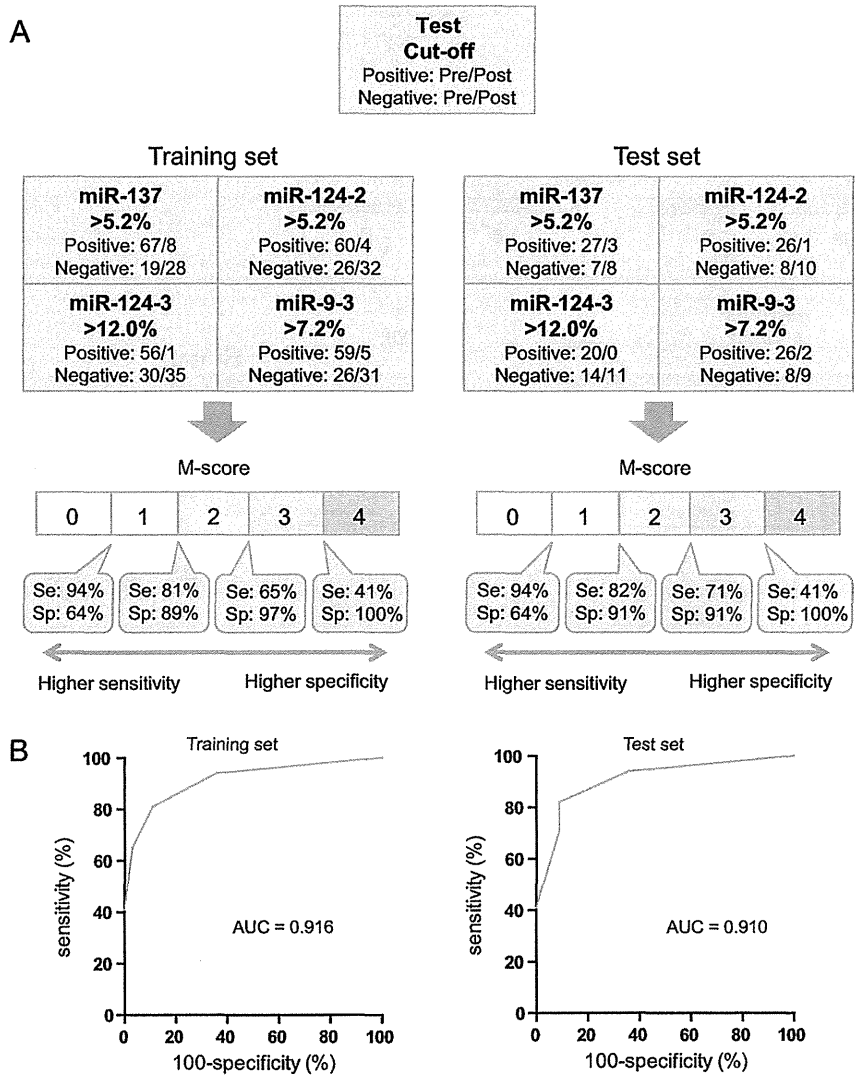

Fig. 4 – Diagnostic system for detecting bladder cancer (BCa) using urinary microRNA (miRNA) gene methylation. (A) Workflow of a system established based on the ability to distinguish preoperative from postoperative urine. Results of the training set are shown on the left; those of test set are on the right. The methylation status of miRNA genes in preoperative (training set: *n* = 86; test set: *n* = 36) and postoperative urine (training set: *n* = 34; test set: *n* = 11) was determined using the cut-off values in the respective boxes. A miR-methylation score (M-score) was determined from the number of methylation-positive genes, and samples were classified into five groups based on the M-score. The sensitivity (Se) and specificity (Sp) at each point are indicated below. (B) Receiver operating characteristic curve analysis of the training and test sets. Areas under the curve are shown in the graph. M-score = miR-methylation score; AUC = area under the curve.

mirtrons and miRNAs, including miR-9 family genes, more frequently exhibited CpG shore methylation than CGI methylation, suggesting that methylation in both the CpG shore and CGI is related to epigenetic silencing of miRNA in BCa. Interestingly, miR-9-1 and -9-2 were associated with both CGI and CpG shore methylation, whereas miR-9-3 showed only CGI methylation [13]. Consistent with those findings, we observed that among the miR-9 family genes, miR-9-3 most frequently showed CGI methylation.

Methylation of several miRNA genes is strongly related to the clinical characteristics of cancer, suggesting its potential usefulness as a biomarker. For instance, methylation of miR-9-1 and -9-3 is reportedly associated with metastatic recurrence of RCC, which is indicative of the possible role of miR-9 in cancer metastasis [25]. Despite this report, however, we did not find a significant difference in the levels of miR-9-3 methylation between noninvasive and invasive BCa tissues. Further study to clarify the functions of these miRNAs in BCa will be needed.

Recent studies have shown that miRNA levels in urine could serve as a molecular marker for detection of BCa. For instance, expression of miR-96 and miR-183 is reportedly upregulated in urothelial cancer, and their detection in urine strongly distinguished cancer patients from cancer-free patients [26]. Miah et al. also showed that evaluation of a panel of 10 miRNAs in urine is a highly sensitive method of detecting BCa [27]. DNA methylation is another potential molecular marker detectable in urine specimens. Several

protein-coding genes are targets of DNA methylation in BCa, and their urinary methylation appears to be a useful biomarker [28,29]. For instance, methylation of 11 protein-coding genes found in urine sediments revealed the presence of BCa with a high sensitivity and specificity [30], and in another study a panel of three genes (growth differentiation factor 15 [*GDF15*], transmembrane protein with EGF-like and two follistatin-like domains 2 [*TMEFF2*], and vimentin [*VIM*]) in urine could be used to accurately detect BCa [31]. In the present study, we show for the first time that methylation of miRNA genes could serve as a biomarker for detection of BCa. Methylation of miRNA genes was readily detectable in voided urine from cancer patients, and its levels were dramatically reduced after tumor resection, confirming its tumor specificity. We also showed that a combination of multiple miRNA genes could accurately distinguish between preoperative and postoperative urine samples.

Our study has several limitations. The prognostic value of miRNA gene methylation remains unclear, because the prognosis of the patients in this study is not yet available. A follow-up study in post-treatment patients will be needed to test whether urinary methylation can predict outcome or detect BCa recurrence. In addition, urinary methylation in non-BCa patients (eg, patients with other types of cancer) should be tested to evaluate the specificity of our method. Further studies to address these issues would contribute to overcoming the difficulties in translating our present findings into clinical practice.

5. Conclusions

We identified four miRNA genes that are frequent targets of epigenetic silencing in BCa. Although their specific functions in bladder carcinogenesis remain unknown, it is evident that restoration of these miRNAs may be an effective anticancer therapy. Furthermore, methylation of these miRNA genes in urine specimens could serve as a useful and noninvasive biomarker for accurate detection of BCa.

Author contributions: Hiromu Suzuki had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
Study concept and design: Suzuki, Toyota.
Acquisition of data: Shimizu, Ashida, Hatahira, Yamamoto, Maruyama, Kai.
Analysis and interpretation of data: Shimizu, Suzuki, Nojima.
Drafting of the manuscript: Shimizu, Suzuki.
Critical revision of the manuscript for important intellectual content: Taiji Tsukamoto.
Statistical analysis: Nojima.
Obtaining funding: Suzuki, Toyota, Tsukamoto.
Administrative, technical, or material support: Kitamura, Masumori, Tokino, Imai, Tsukamoto.
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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.eururo.2012.11.030.

References

[1] He L, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet 2004;5:522–31.
[2] Croce CM. Causes and consequences of microRNA dysregulation in cancer. Nat Rev Genet 2009;10:704–14.
[3] Chen Q, Chen X, Zhang M, Fan Q, Luo S, Cao X. miR-137 is frequently down-regulated in gastric cancer and is a negative regulator of Cdc42. Dig Dis Sci 2011;56:2009–16.
[4] Catto JW, Miah S, Owen HC, et al. Distinct microRNA alterations characterize high- and low-grade bladder cancer. Cancer Res 2009;69:8472–81.
[5] Neely LA, Rieger-Christ KM, Neto BS, et al. A microRNA expression ratio defining the invasive phenotype in bladder tumors. Urol Oncol 2010;28:39–48.
[6] Adam L, Zhong M, Choi W, et al. miR-200 expression regulates epithelial-to-mesenchymal transition in bladder cancer cells and reverses resistance to epidermal growth factor receptor therapy. Clin Cancer Res 2009;15:5060–72.
[7] Saito Y, Liang G, Egger G, et al. Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells. Cancer Cell 2006;9:435–43.
[8] Suzuki H, Takatsuka S, Akashi H, et al. Genome-wide profiling of chromatin signatures reveals epigenetic regulation of microRNA genes in colorectal cancer. Cancer Res 2011;71:5646–58.
[9] Suzuki H, Yamamoto E, Nojima M, et al. Methylation-associated silencing of microRNA-34b/c in gastric cancer and its involvement in an epigenetic field defect. Carcinogenesis 2010;31:2066–73.
[10] Maruyama R, Suzuki H, Yamamoto E, Imai K, Shinomura Y. Emerging links between epigenetic alterations and dysregulation of non-coding RNAs in cancer. Tumour Biol 2012;33:277–85.
[11] Esteller M. Non-coding RNAs in human disease. Nat Rev Genet 2011;12:861–74.
[12] Wiklund ED, Bramsen JB, Hulf T, et al. Coordinated epigenetic repression of the miR-200 family and miR-205 in invasive bladder cancer. Int J Cancer 2011;128:1327–34.
[13] Dudziec E, Miah S, Choudhry HM, et al. Hypermethylation of CpG islands and shores around specific microRNAs and mirtrons is associated with the phenotype and presence of bladder cancer. Clin Cancer Res 2011;17:1287–96.

[14] Balaguer F, Link A, Lozano JJ, et al. Epigenetic silencing of miR-137 is an early event in colorectal carcinogenesis. Cancer Res 2010;70:6609–18.
[15] Liu M, Lang N, Qiu M, et al. miR-137 targets Cdc42 expression, induces cell cycle G1 arrest and inhibits invasion in colorectal cancer cells. Int J Cancer 2011;128:1269–79.
[16] Kozaki K, Imoto I, Mogi S, Omura K, Inazawa J. Exploration of tumor-suppressive microRNAs silenced by DNA hypermethylation in oral cancer. Cancer Res 2008;68:2094–105.
[17] Szulwach KE, Li X, Smrt RD, et al. Cross talk between microRNA and epigenetic regulation in adult neurogenesis. J Cell Biol 2010;189:127–41.
[18] Smrt RD, Szulwach KE, Pfeiffer RL, et al. MicroRNA miR-137 regulates neuronal maturation by targeting ubiquitin ligase mind bomb-1. Stem Cells 2010;28:1060–70.
[19] Lujambio A, Ropero S, Ballestar E, et al. Genetic unmasking of an epigenetically silenced microRNA in human cancer cells. Cancer Res 2007;67:1424–9.
[20] Ando T, Yoshida T, Enomoto S, et al. DNA methylation of microRNA genes in gastric mucosae of gastric cancer patients: its possible involvement in the formation of epigenetic field defect. Int J Cancer 2009;124:2367–74.
[21] Agirre X, Vilas-Zornoza A, Jimenez-Velasco A, et al. Epigenetic silencing of the tumor suppressor microRNA Hsa-miR-124a regulates CDK6 expression and confers a poor prognosis in acute lymphoblastic leukemia. Cancer Res 2009;69:4443–53.
[22] Furuta M, Kozaki KI, Tanaka S, Arii S, Imoto I, Inazawa J. miR-124 and miR-203 are epigenetically silenced tumor-suppressive microRNAs in hepatocellular carcinoma. Carcinogenesis 2010;31:766–76.
[23] Lujambio A, Calin GA, Villanueva A, et al. A microRNA DNA methylation signature for human cancer metastasis. Proc Natl Acad Sci U S A 2008;105:13556–61.
[24] Lodygin D, Tarasov V, Epanchintsev A, et al. Inactivation of miR-34a by aberrant CpG methylation in multiple types of cancer. Cell Cycle 2008;7:2591–600.
[25] Hildebrandt MA, Gu J, Lin J, et al. Hsa-miR-9 methylation status is associated with cancer development and metastatic recurrence in patients with clear cell renal cell carcinoma. Oncogene 2010;29:5724–8.
[26] Yamada Y, Enokida H, Kojima S, et al. MiR-96 and miR-183 detection in urine serve as potential tumor markers of urothelial carcinoma: correlation with stage and grade, and comparison with urinary cytology. Cancer Sci 2011;102:522–9.
[27] Miah S, Dudziec E, Drayton RM, et al. An evaluation of urinary microRNA reveals a high sensitivity for bladder cancer. Br J Cancer 2012;107:123–8.
[28] Nishiyama N, Arai E, Chihara Y, et al. Genome-wide DNA methylation profiles in urothelial carcinomas and urothelia at the precancerous stage. Cancer Sci 2010;101:231–40.
[29] Cairns P. Gene methylation and early detection of genitourinary cancer: the road ahead. Nat Rev Cancer 2007;7:531–43.
[30] Yu J, Zhu T, Wang Z, et al. A novel set of DNA methylation markers in urine sediments for sensitive/specific detection of bladder cancer. Clin Cancer Res 2007;13:7296–304.
[31] Costa VL, Henrique R, Danielsen SA, et al. Three epigenetic biomarkers, GDF15, TMEFF2, and VIM, accurately predict bladder cancer from DNA-based analyses of urine samples. Clin Cancer Res 2010;16:5842–51.

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Association Between Genomic Alterations and Metastatic Behavior of Colorectal Cancer Identified by Array-Based Comparative Genomic Hybridization

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Colorectal cancers (CRCs) exhibit multiple genetic alterations, including allelic imbalances (copy number alterations, CNAs) at various chromosomal loci. In addition to genetic aberrations, DNA methylation also plays important roles in the development of CRC. To better understand the clinical relevance of these genetic and epigenetic abnormalities in CRC, we performed an integrative analysis of copy number changes on a genome-wide scale and assessed mutations of *TP53*, *KRAS*, *BRAF*, and *PIK3CA* and DNA methylation of six marker genes in single glands isolated from 39 primary tumors. Array-based comparative genomic hybridization (array-CGH) analysis revealed that genomic losses commonly occurred at 3q26.1, 4q13.2, 6q21.32, 7q34, 8p12-23.3, 15qcen and 18, while gains were commonly found at 1q21.3-23.1, 7p22.3-q34, 13q12.11-14.11, and 20. The total numbers and lengths of the CNAs were significantly associated with the aberrant DNA methylation and Dukes' stages. Moreover, hierarchical clustering analysis of the array-CGH data suggested that tumors could be categorized into four subgroups. Tumors with frequent DNA methylation were most strongly enriched in subgroups with infrequent CNAs. Importantly, Dukes' D tumors were enriched in the subgroup showing the greatest genomic losses, whereas Dukes' C tumors were enriched in the subgroup with the greatest genomic gains. Our data suggest an inverse relationship between chromosomal instability and aberrant methylation and a positive association between genomic losses and distant metastasis and between genomic gains and lymph node metastasis in CRC. Therefore, DNA copy number profiles may be predictive of the metastatic behavior of CRCs.

INTRODUCTION

Colorectal cancers (CRCs) develop through multiple genetic alterations, including allelic losses at chromosomal loci (e.g., 5q, 17p, and 18q) (Fearon and Vogelstein, 1990). In addition, epigenetic changes, including aberrant DNA methylation and histone modifications, are also strongly implicated in the pathogenesis of CRC, and a subset of CRCs show concurrent hypermethylation in multiple loci, which is now classified as the CpG island methylator phenotype (CIMP) (Toyota et al., 1999). Recent studies have shown that there are two types of CRCs with distinct genomic abnormalities: chromosomal instability (CIN), which accounts for 80–85% of sporadic CRCs and was originally characterized based on the presence of aneuploid/polyploid karyotypes, and microsatellite instability (MSI), also termed MIN, which accounts for 15–20% of sporadic CRCs and is characterized by mismatch repair defects and a near-diploid karyotype (Grady and Carethers, 2008). CIN cancers exhibit gains and losses at multiple chromosomal loci (copy number alterations; CNAs) (Rajagopalan and Lengauer, 2004), whereas MSI cancers show considerable

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overlap with CIMP cancers (Toyota et al., 1999; Ogino et al., 2006; Weisenberger et al., 2006).

In addition to the commonly observed CRC-related allelic losses on chromosome arms 5q, 17p, and 18q, gains and losses on many other chromosomes have been identified using conventional comparative genomic hybridization (CGH) analysis (Ried et al., 1996; Meijer et al., 1998). Diep et al. (2006) conducted a meta-analysis of the chromosomal changes in a series of 859 CRC specimens identified using CGH and reported that specific CNAs are associated with each step during the progression of CRC. Still, conventional CGH has limited resolution and can only detect CNAs of ~10 Mb or greater in length. On the other hand, array-based CGH (array-CGH) can detect genetic changes with a resolution of 1 Mb or less, making it a powerful tool with which to analyze genomic alterations (Douglas et al., 2004; Jones et al., 2005).

From a clinical viewpoint, previous studies have shown that CRCs can be categorized into distinct subgroups based on the characteristics of their CNAs (Hermsen et al., 2002), and such subtyping has predictive value with respect to prognosis (Poulogiannis et al., 2010) and the response to chemotherapy (Postma et al., 2009). Similarly, a number of studies have shown that epigenetic alterations, especially CIMP, are strongly associated with the clinical behavior of CRCs (Shen et al., 2007a; Jover et al., 2011). However, although it is recognized that CRCs develop via multiple molecular pathways, including CIN, MSI, and CIMP (Jass, 2007; Shen et al., 2007b; Issa, 2008; Hinoue et al., 2012), the associations between genetic and epigenetic abnormalities are still not fully understood. In this study, we performed an integrative analysis of copy number changes on genome-wide scale and assessed genetic mutation of *TP53*, *KRAS*, *BRAF*, and *PIK3CA* and DNA methylation of six marker genes within crypts isolated from surgically resected CRCs, and assessed their relevance to the clinicopathological characteristics.

MATERIALS AND METHODS

Patients and Tissue Samples

A total of 39 primary CRCs and corresponding normal tissue specimens were obtained from consecutive patients at the Iwate Medical University Hospital. Informed consent was obtained from all patients before collection of the specimens, and approval of this study was obtained from the Institutional Review Board of Iwate Medical University. The clinicopathological features of the patients are summarized in Table 1. Pathological diagnosis and staging were performed using a combination of the Japanese classification (Japanese Society for Cancer of the Colon and Rectum, 1997) and modified Dukes' classification (Turnbull et al., 1967). Tumor locations were classified as left- or right-sided and rectal.

TABLE 1. Clinicopathological Features of the CRC Samples Used in this Study

Age (years, median ± SD)	69 ± 11.7
Sex	
Male	24 (62%)
Female	15 (38%)
Location	
Right	14 (36%)
Left	5 (13%)
Rectum	20 (51%)
Histology	
Mod	29 (74%)
Well	7 (17%)
Pap	1 (3%)
Por	1 (3%)
Muc	1 (3%)
Dukes' stage	
A	7 (18%)
B	5 (13%)
C	13 (33%)
D	14 (36%)
Lymph node metastasis	
Positive	23 (59%)
Negative	16 (41%)

Isolation of Glands and Genomic DNA Extraction

Glands were isolated from the tumors and normal mucosae as described previously (Arai and Kino, 1989; Nakamura et al., 1994). The isolated glands were routinely processed to confirm their nature using paraffin-embedded histological sections. Contamination by other materials such as interstitial cells was not evident in the samples examined, which is consistent with previous reports (Sugai et al., 2000; Sugai et al., 2005). Genomic DNA was extracted using the standard phenol-chloroform procedure.

Analysis of *TP53*, *BRAF*, *KRAS*, and *PIK3CA* Mutations

Exons 5–8 of *TP53* were PCR amplified and then analyzed using single-strand conformational polymorphism (SSCP). PCR amplification, PCR-SSCP, and the sequencing of *TP53* were performed as described previously (Dix et al., 1994; Habano et al., 1996; Sugai et al., 2000). In addition,

mutation of codon 600 of *BRAF* and codons 12 and 13 of *KRAS* was examined by pyrosequencing using *BRAF* and *KRAS* pyro kits (Qiagen) according to the manufacturer's instructions, and exons 9 and 20 of *PIK3CA* were directly sequenced as described previously (Jhawer et al., 2008).

DNA Methylation Analysis

CpG island methylation was analyzed as described previously (Toyota et al., 2008). Briefly, genomic DNA (1 μg) was modified with sodium bisulfite using an EpiTect Bisulfite Kit (Qiagen). Pyrosequencing was carried out using a PSQ 96MA system (Qiagen) with a Pyro Gold Reagent Kit (Qiagen), and the results were analyzed using Pyro Q-CpG software (Qiagen). A cutoff value of 15% was used to define genes as methylation-positive. Tumors were defined as "tumors with frequent DNA methylation" when methylation was detected in three or more loci out of six markers (*MINT1*, *MINT2*, *MINT12*, *MINT31*, *CDKN2A*, and *MLH1*).

Array-Based CGH

Array CGH analysis was performed as described previously (Igarashi et al., 2010). Briefly, 500 ng of genomic DNA 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, respectively, labeled with Cy5 and Cy3 after being hybridized to a Human Genome CGH Microarray Kit 105A (G4412A; Agilent Technologies), which contains approximately 99,000 probes annotated against National Center for Biotechnology Information Build 36. The ADM-2 algorithm included in the Genomic Workbench software ver. 5 (Agilent Technologies) was used to identify DNA copy number aberrations. A copy number loss was defined as a log2 ratio <-0.5, and a copy number gain was defined as a log2 ratio >0.5. All genomic positions were defined according to the University of California Santa Cruz Human version hg 18. Unsupervised hierarchical analysis was performed on the log2 Cy5/Cy3 fluorescence ratio data using the Ward's linkage algorithm (JMP version 8, SAS Institute, Cary, NC).

Statistical Analysis

Continuous data was analyzed using *t* tests (for two groups) or ANOVA with a post hoc Tukey's HSD test (for more than two groups). To detect specific differences within groups, adjusted standardized residuals were calculated for the categorical data. If the absolute values of the residuals were more than 1.96, we considered them significantly different from a random distribution. *P* values <0.05 were considered significant. All statistical analyses were performed using SPSS 20 (IBM Corporation, Somers, NY) and Prism 5 (GraphPad Software, La Jolla, CA).

RESULTS

Overview of Array-CGH Analysis

The results of our array-CGH analysis of crypts obtained from 39 CRC tumors are summarized in Figure 1. Genomic losses were commonly observed at several loci, including 3q26.1 (75%), 4q13.2 (80%), 6q21.32 (83%), 7q34 (58%), 8p12-23.3 (55%), 15qcen (50%), and 18 (80%), while gains were commonly observed at 1q21.3-23.1 (41%), 7p22.3-q34 (48%), 13q12.11-14.11 (50%), and 20 (75%). Large genomic losses (>10 Mb in length) were frequently seen at 8p (54%), 18p (59%), and 18q (77%), and large gains (>10 Mb in length) were seen at 7 (41%), 13q (46%), 20p (44%), and 20q (74%) (Fig. 1, Supporting Information Fig. 1). These findings are mostly consistent with earlier results obtained using conventional CGH and array-CGH (Ried et al., 1996; Meijer et al., 1998; Douglas et al., 2004; Jones et al., 2005; Diep et al., 2006).

Mutation and Methylation Analysis

Among the 39 CRC specimens tested, *TP53* and *KRAS* mutations were found in 21 (54%) and 15 (38%), respectively, which is also consistent with earlier findings (Supporting Information Fig. 1 and Table 1) (Dix et al., 1994; Smith et al., 2002; Baldus et al., 2010). However, the frequency of samples with mutations in both *TP53* and *KRAS* (15%) was higher than previously reported (9%) (Smith et al., 2002), probably because our study included tumors at more advanced stages. By contrast, *PIK3CA* mutation was found in only four (10%) specimens, which is less frequent than previously reported (Samuels et al., 2004; Baldus et al., 2010). *BRAF* mutation was not detected in any samples.

Bisulfite-pyrosequencing analysis revealed that 9 of the 39 tumors (23%) exhibited methylation at 3 or more loci, although none showed methylation of *MLH1*. *KRAS* mutation was more prevalent among tumors with frequent DNA methylation (6/9, 67%) than among those without frequent methylation (9/30, 30%). Previous studies demonstrated that

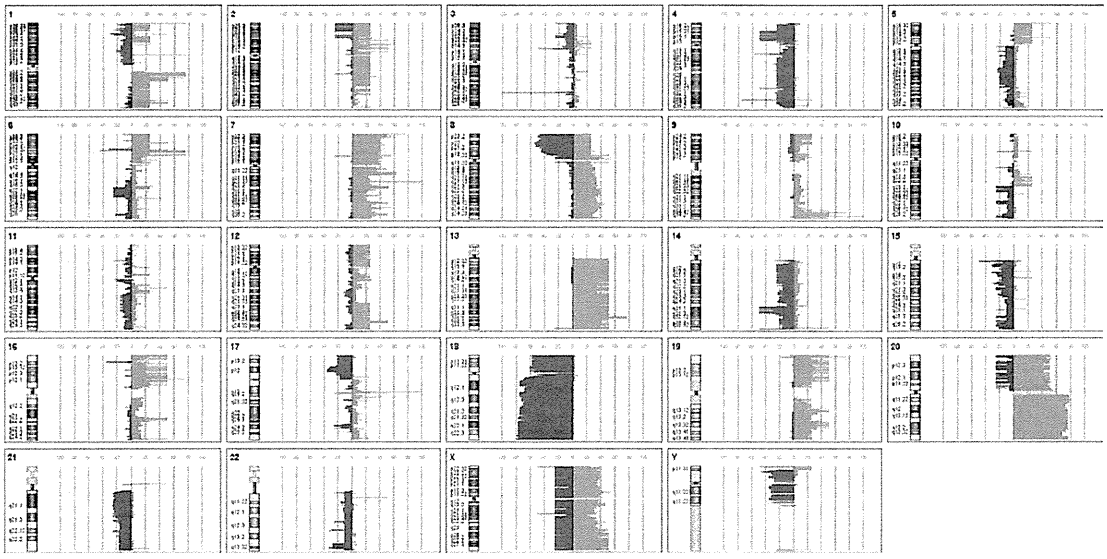

Figure 1. Summary of chromosomal aberrations and their frequencies in 39 CRC specimens determined using array-CGH analysis. Losses (green bars) are displayed on the left, and gains (red bars) are on the right. The chromosome ideogram was generated using Genomic Workbench software.

BRAF mutation and MSI are significantly more prevalent among CIMP-high (CIMP-H or CIMP1) CRCs, whereas *KRAS* mutation is more prevalent among CIMP-low (CIMP-L or CIMP2) tumors (Ogino et al., 2006; Shen et al., 2007b; Hinoue et al., 2012).

Inverse Correlation Between DNA Methylation and Chromosomal Alterations

To quantitatively evaluate copy number aberrations on a genome-wide scale, we calculated the total numbers and lengths of CNAs (losses + gains) identified by the array-CGH analysis. We observed a strong correlation between the total numbers of CNAs in the CRC samples tested and the total lengths of the CNAs (Supporting Information Fig. 2). We therefore used the total CNA length as an index representing the degree of chromosomal alteration and assessed the relationship between total CNA length and methylation status. We found that total CNA lengths were smaller in CRCs with frequent DNA methylation than in those without frequent methylation ($P = 0.033$, Fig. 2C). Interestingly, when we analyzed genomic losses and gains separately, we again observed a significant difference in the magnitude of the losses between frequent methylation-positive and -negative tumors, whereas no such difference was found for gains (Figs. 2A and 2B). We also analyzed the relationship between CNAs and mutation of *TP53* or *KRAS* but found no statistically significant correlations (Supporting Information Fig. 3).

Chromosomal Alterations and Their Association with Clinical Stage in CRC

To determine whether chromosomal alterations accumulate during the progression of CRCs, we assessed the CNA status of tumors at each Dukes' stage. When genomic gains and losses were analyzed separately, we found a tendency toward greater genomic losses in tumors at higher Dukes' stages, but the trend was not statistically significant (Fig. 3A). By contrast, Dukes' C tumors showed the greatest genomic gains, whereas Dukes' D tumors exhibited unexpectedly small gains (Fig. 3B). The total CNA lengths were greatest in Dukes' C tumors, and again Dukes' D tumors showed less chromosomal alteration than Dukes' C tumors (Fig. 3C).

We found that losses at several loci, including 3p24.3, 4p13-15.31, 5qcen-11.2, 8p11-q11, 9p21.3-21.1, 17q24.2-24.3, and 22q13.31, were prevalent in tumors with distant metastasis (Dukes' D) (Supporting Information Table 2), while gains at 11q13.1-13.2, 17q12, and 17q21.2 were prevalent among Dukes' D tumors (Supporting Information Table 3). In addition, losses at 4q21-34, 5q12.1, 5q35.3, and 11qcen-12.1 and gains at 2p15-16.1, 2p13.3, 5p13, 5p35.3, 6p21, 8q12.1-12.3, and 19q13.31 were commonly observed in tumors with lymph node metastasis (Supporting Information Tables 4, 5).

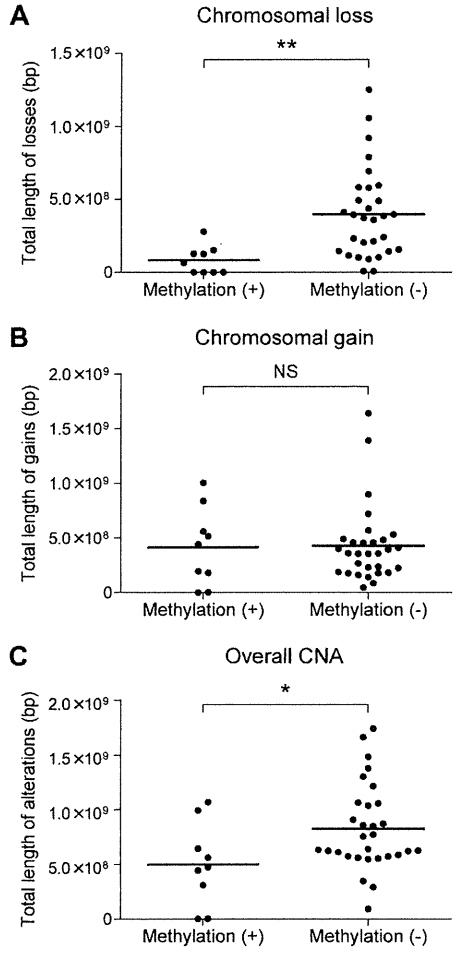

Figure 2. Association between CNAs and DNA methylation status. Total lengths of genomic losses (A) and gains (B) and overall CNAs (C) in tumors with and without frequent DNA methylation are shown. Each dot represents a single tumor. Methylation (+), tumors with frequent DNA methylation; Methylation (−), tumors without frequent DNA methylation; *$P < 0.05$; **$P < 0.01$; NS, not significant.

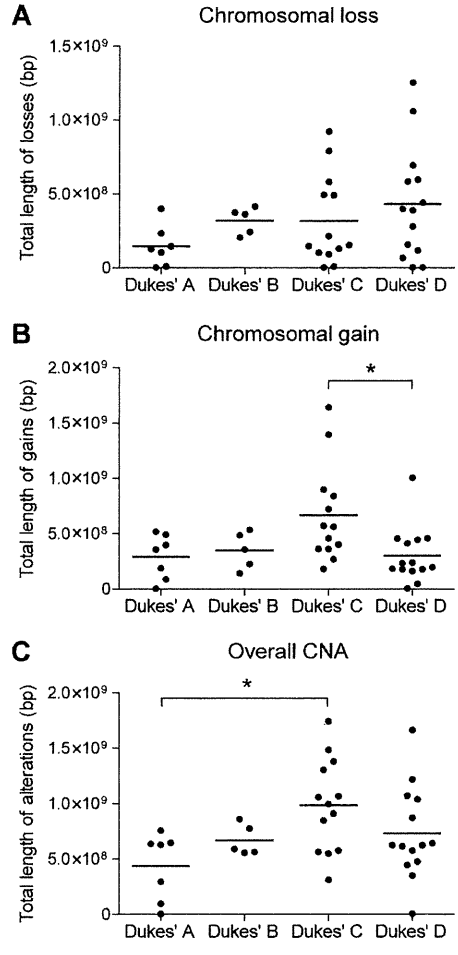

Figure 3. Association between CNAs and Dukes' stages. Total lengths of genomic losses (A), gains (B), and overall CNAs (C) in CRCs at each Dukes' stage are shown. *$P < 0.05$.

Clustering Analysis of CNAs and Their Association with Clinical Stage in CRC

Previous studies have shown that categorization of CRCs according to their chromosomal aberrations has strong relevance to their clinical behavior (Hermsen et al., 2002; Postma et al., 2009; Poulogiannis et al., 2010). For that reason, we carried out unsupervised clustering analysis using our array-CGH data (excluding the sex chromosomes) and then compared the results with genetic mutations and epigenetic alterations (Fig. 4A). We found that CRCs could be subcategorized into at least four clusters based on their CNAs. Gene mutations, DNA methylation status, and genomic alterations on representative chromosomes in each cluster are summarized in Table 2.

Tumors in cluster 1 are characterized by infrequent genomic losses and gains (Figs. 4A–4C). Losses were most prevalent among tumors in cluster 2, while gains were most prevalent among tumors in cluster 4 (Figs. 4A–4C). The total CNA lengths were greater in tumors in clusters 2 and 4 than in clusters 1 and 3 (Fig. 4D). Tumors with frequent DNA methylation were most strongly enriched in cluster 1 (5 of 9, 56%). Tumors in cluster 1 were also characterized by frequent *KRAS* mutation (6 of 10, 60%) and infrequent *p53* mutation (3 of 10, 30%), whereas *p53* mutation was most prevalent in cluster 3 tumors (10 of 15, 67%), although the difference was not statistically significant. Importantly, Dukes' D tumors were highly enriched in cluster 2, within which tumors showed the greatest genomic losses. By contrast, Dukes' C tumors were enriched in cluster 4 and