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Table 1. Epigenetically silenced miRNA genes in HCT116

Name	miRNA position	Strand	Gene/EST	DKO H3K4me3	Distance	CpG	Methylation
hsa-mir-137	chr1:98284213-98284315	–	*AK311400*	chr1:98282607-98285011	<2 kb	CGI	M
hsa-mir-488	chr1:175265121-175265204	–	*ASTN1*	chr1:175267182-175269163	<2 kb	–	
hsa-mir-205	chr1:207672100-207672210	+	*LOC642587*	chr1:207668045-207668780	2–5 kb	–	
hsa-mir-10b	chr2:176723276-176723386		EST: BQ722165	chr2:176720798-176721389	<2 kb	CGI	M
hsa-mir-885	chr3:10411172-10411246	–	*ATP2B2*	chr3:10723790-10724643	>10 kb	–	
hsa-mir-944	chr3:191030404-191030492	+	*TP63*	chr3:190831254-190831806	>10 kb	–	
hsa-mir-146a	chr5:159844936-159845035	+	*DQ658414*	chr5:159827186-159829843	>10 kb	–	
hsa-mir-218-2	chr5:168127728-168127838	–	*SLIT3*	chr5:168127234-168128479	<2 kb	–	
hsa-mir-9-2	chr5:87998426-87998513	–	*LOC645323*	chr5:87997315-87999774	<2 kb	–	
hsa-mir-548b	chr6:119431910-119432007	–	*FAM184A*	chr6:119440287-119442581	5–10 kb	CGI	M
hsa-mir-129-1	chr7:127635160-127635232	+		chr7:127628134-127629349	5–10 kb	CGI	M
hsa-mir-153-2	chr7:157059788-157059875	–	*PTPRN2*	chr7:157061089-157064064	<2 kb	CGI	M
hsa-mir-596	chr8:1752803-1752880	+		chr8:1751984-1754090	<2 kb	CGI	M
hsa-mir-124-1	chr8:9798307-9798392	–		chr8:9798198-9799439	<2 kb	CGI	M
hsa-mir-598	chr8:10930125-10930222	–	*XKR6*	chr8:11094125-11097386	>10 kb	CGI	M
hsa-mir-486	chr8:41637115-41637183	–	*ANK1*	chr8:41660039-41661630	>10 kb	–	
hsa-mir-124-2	chr8:65454259-65454368	+	*BX537900*	chr8:65452660-65453362	<2 kb	CGI	M
hsa-mir-876	chr9:28853623-28853704	–	EST: DA506041	chr9:29200954-29205760	>10 kb	CGI	M
hsa-mir-873	chr9:28878876-28878953	–	EST: DA506041	chr9:29200954-29205760	>10 kb	CGI	M
hsa-mir-146b	chr10:104186258-104186331	+		chr10:104185067-104186052	<2 kb	–	
hsa-mir-129-2	chr11:43559519-43559609	+	EST: BI964058	chr11:43556836-43561086	<2 kb	CGI	M
hsa-mir-708	chr11:78790713-78790801	–	*ODZ4*	chr11:78825491-78830366	>10 kb	CGI	M
hsa-mir-34b	chr11:110888872-110888956	+	*BC021736*	chr11:110887701-110889527	<2 kb	CGI	M
hsa-mir-34c	chr11:110889373-110889450	+	*BC021736*	chr11:110887701-110889527	<2 kb	CGI	M
hsa-mir-337	chr14:100410582-100410675	+		chr14:100407328-100408197	2–5 kb	–	
hsa-mir-431	chr14:100417096-100417210	+		chr14:100417110-100420818	<2 kb	–	
hsa-mir-433	chr14:100417975-100418068	+		chr14:100417110-100420818	<2 kb	–	
hsa-mir-127	chr14:100419068-100419165	+		chr14:100417110-100420818	<2 kb	CGI	M
hsa-mir-432	chr14:100420572-100420666	+		chr14:100417110-100420818	<2 kb	–	
hsa-mir-136	chr14:100420791-100420873	+		chr14:100417110-100420818	<2 kb	–	
hsa-mir-211	chr15:29144526-29144636	–	*TRPM1*	chr15:29160209-29160816	>10 kb	–	
hsa-mir-190	chr15:60903208-60903293	+	*TLN2*	chr15:60769608-60770528	>10 kb	–	
hsa-mir-9-3	chr15:87712251-87712341	+	*CR612213*	chr15:87711049-87714084	<2 kb	CGI	M
hsa-mir-195	chr17:6861657-6861744	–	EST: DA285925	chr17:6862382-6864312	<2 kb	–	
hsa-mir-497	chr17:6861953-6862065	–	EST: DA285925	chr17:6862382-6864312	<2 kb	–	
hsa-mir-193a	chr17:26911127-26911215	+		chr17:26910360-26912430	<2 kb	CGI	M
hsa-mir-152	chr17:43469525-43469612	–	*COPZ2*	chr17:43468887-43470981	<2 kb	CGI	M
hsa-mir-196a-1	chr17:44064850-44064920	–	EST:AI222881	chr17:44065082-44066831	<2 kb	CGI	M
hsa-mir-142	chr17:53763591-53763678	–	*AK311311* (antisense)	chr17:53762545-53765705	<2 kb	–	
hsa-mir-338	chr17:76714277-76714344	–	*AATK*	chr17:76753247-76754663	>10 kb	CGI	M
hsa-mir-371	chr19:58982740-58982807	+		chr19:58982297-58983364	<2 kb	–	
hsa-mir-372	chr19:58982955-58983022	+		chr19:58982297-58983364	<2 kb	–	
hsa-mir-373	chr19:58983770-58983839	+		chr19:58982297-58983364	<2 kb	–	
hsa-mir-1-1	chr20:60561957-60562028	+	*C20orf166*	chr20:60557701-60559681	2–5 kb	CGI	M
hsa-mir-133a-2	chr20:60572563-60572665	+	*C20orf166*	chr20:60557701-60559681	>10 kb	CGI	M
hsa-mir-124-3	chr20:61280296-61280383	+		chr20:61276185-61277804	2–5 kb	CGI	M
hsa-mir-155	chr21:25868162-25868227	+	*MIR155HG*	chr21:25855844-25857551	>10 kb	CGI	M

Abbreviations: gene/EST, overlapping gene or EST; distance, distance between pre-miRNA coding region and presumed promoter; CGI, CpG island positive at the promoter; M, CGI methylated.

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noted that a small number of non-CGI miRNAs acquired more active chromatin states upon DNA demethylation than did CGI-methylated miRNAs. For instance, miR-146a is characterized by a lack of active histone marks and enrichment of H3K27me3, but it showed restoration of both H3K4me3 and H3K79me2 in DKO cells (Fig. 3C). We observed similar upregulation of both active marks in miR-142 (Supplementary Fig. S7E). Weak basal expression of these miRNAs, detectable by TaqMan assay but not by microarray, and robust upregulation after DNA demethylation indicate that the silencing of these miRNAs is less stringent than that of miRNAs with methylated CGIs (data not shown).

DNA demethylation significantly upregulated the expression of mature miRNAs derived from 47 silenced pre-miRNAs (Fig. 3D). In addition, expression data from 13 host genes of the silenced miRNAs were obtained from Agilent gene expression microarray analysis (6), and we observed a strong tendency for the host genes to be upregulated by DNA demethylation (Fig. 3E). Recent studies have shown that genes marked by polycomb (PcG) group proteins in ES cells have a predisposition toward DNA hypermethylation in cancer (18, 19). By comparison with previously published results (9), we found that miRNAs with SUZ12 binding and H3K27me3 marks in human ES cells are significantly enriched in CGI-methylated miRNAs in colorectal cancer (Fig. 3E).

We further analyzed CGI methylation in a series of colorectal cancer cell lines using MSP and bisulfite pyrosequencing and found that they are methylated to varying degrees (Fig. 4A, Supplementary Fig. S8). We also confirmed inverse relationships between methylation and expression of selected miRNAs in colorectal cancer cell lines and normal colonic tissue (Fig. 4B). To determine the extent to which these miRNA genes are aberrantly methylated in primary tumors, we carried out bisulfite pyrosequencing of 18 miRNA promoter CGIs in primary colorectal cancer tumors ($n = 90$) and normal colonic tissue obtained from colorectal cancer patients ($n = 20$; Supplementary Fig. S9). Most of the miRNA genes were methylated in a tumor-specific or tumor-predominant manner. The two exceptions were miR-153-2 and miR-196a-1, which were methylated to similar degrees in both normal colon and tumor tissues, as well as in various normal human tissues (Supplementary Figs. S9 and S10). Elevated levels of miRNA gene methylation (>15.0%) were frequently detected in primary colorectal cancer tumors (miR-1-1, 77.8%; miR-9-1, 57.8%; miR-9-3, 89.9%; miR-34b/c, 89.7%; miR-124-1, 87.7%: miR-124-2, 96.6%; miR-124-3, 100.0%; miR-128-2, 73.6%; miR-129-2, 40.0%; miR-137, 100.0%; miR-193a, 28.7%; miR-338, 15.6%; and miR-548b, 47.8%), whereas a small number of genes were rarely methylated in primary tumors (miR-152, 4.4%; miR-155, 6.7%; and miR-596, 2.3%).

MiR-1-1 is a candidate tumor suppressor gene in colorectal cancer

Among the epigenetically silenced miRNAs, we next focused on miR-1-1 because it has received relatively little attention in colorectal cancer despite its frequent hypermethylation in that disease. Using bisulfite pyrosequencing, we detected elevated levels (>15.0%) of miR-1-1 methylation in both primary colorectal cancer tumors and colorectal adenomas (54 of 78, 69.2%), suggesting that its methylation is an early event in colorectal tumorigenesis (Fig. 5A). In contrast, levels of miR-1-1 methylation were relatively low (<15.0%) in the normal colonic tissues tested (Fig. 5A). We carried out bisulfite sequencing analysis to confirm the methylation results in selected tissue specimens and colorectal cancer cell lines (Fig. 5B, Supplementary Fig. S11A and B). We also confirmed that DNA demethylation could restore expression of the primary transcript of miR-1-1 (pri-miR-1-1) in colorectal cancer cells (Supplementary Fig. S11C).

To determine whether miR-1-1 serves as a tumor suppressor in colorectal cancer, we transfected colorectal cancer cell lines with a miR-1 precursor molecule or a negative control and then carried out a series of MTT assays. Forty-eight hours after transfection, we observed that ectopic expression of miR-1 moderately suppressed growth in all 3 cell lines (Fig. 5C). Colony formation assays also revealed reduced colony formation by colorectal cancer cells transfected with a miR-1-1 expression vector (Fig. 5D).

To further clarify the effect of the miRNA, we next carried out a gene expression microarray analysis of HCT116 cells transfected with a miR-1 precursor molecule or a negative control. We found that 2,769 probe sets were downregulated (>1.5-fold) by ectopic miR-1 expression, and gene ontology analysis revealed that "extracellular regions," "membrane," and "response to wounding" genes were significantly enriched among the downregulated genes (Supplementary Table S4). The genes downregulated by miR-1 included a number of predicted miR-1 targets (Supplementary Table S5). Among them, we noted 2 genes, Annexin A2 (*ANXA2*) and brain-derived neurotrophic factor (*BDNF*), which have been implicated in tumor growth and metastasis (20–22). Reduction of their expression by miR-1 in colorectal cancer cells was confirmed by Western blotting and real-time reverse transcriptase PCR (RT-PCR; Fig. 5E, Supplementary Fig. S12A). Reporter assays using luciferase vectors containing the putative miR-1 binding sites revealed that cotransfection of a miR-1 precursor molecule markedly reduced luciferase activities and that such reductions were not induced by a negative control or an irrelevant miRNA molecule (Fig. 5F and G, Supplementary Fig. S12B and S12C). Finally, we carried out wound-healing and Matrigel invasion assays to test the effect of miR-1 expression on colorectal cancer cell migration and invasion. We found that wound closure by HCT116 cells transfected with the negative control was complete within 28 hours whereas miR-1–expressing cells migrated toward the wound at a much slower rate (Fig. 5H). We also observed significant inhibition of cell invasion by miR-1 in HCT116 cells (Fig. 5I). These results strongly suggest that miR-1 acts as a tumor suppressor in colorectal cancer.

Discussion

In the present study, we provide a comprehensive view of the epigenetic regulation of miRNA genes in colorectal cancer cells. Because of the poor annotation of primary miRNA genes, the precise locations of the promoters and TSSs are not fully understood yet. To overcome these difficulties, earlier studies have searched for specific genomic features including RNA

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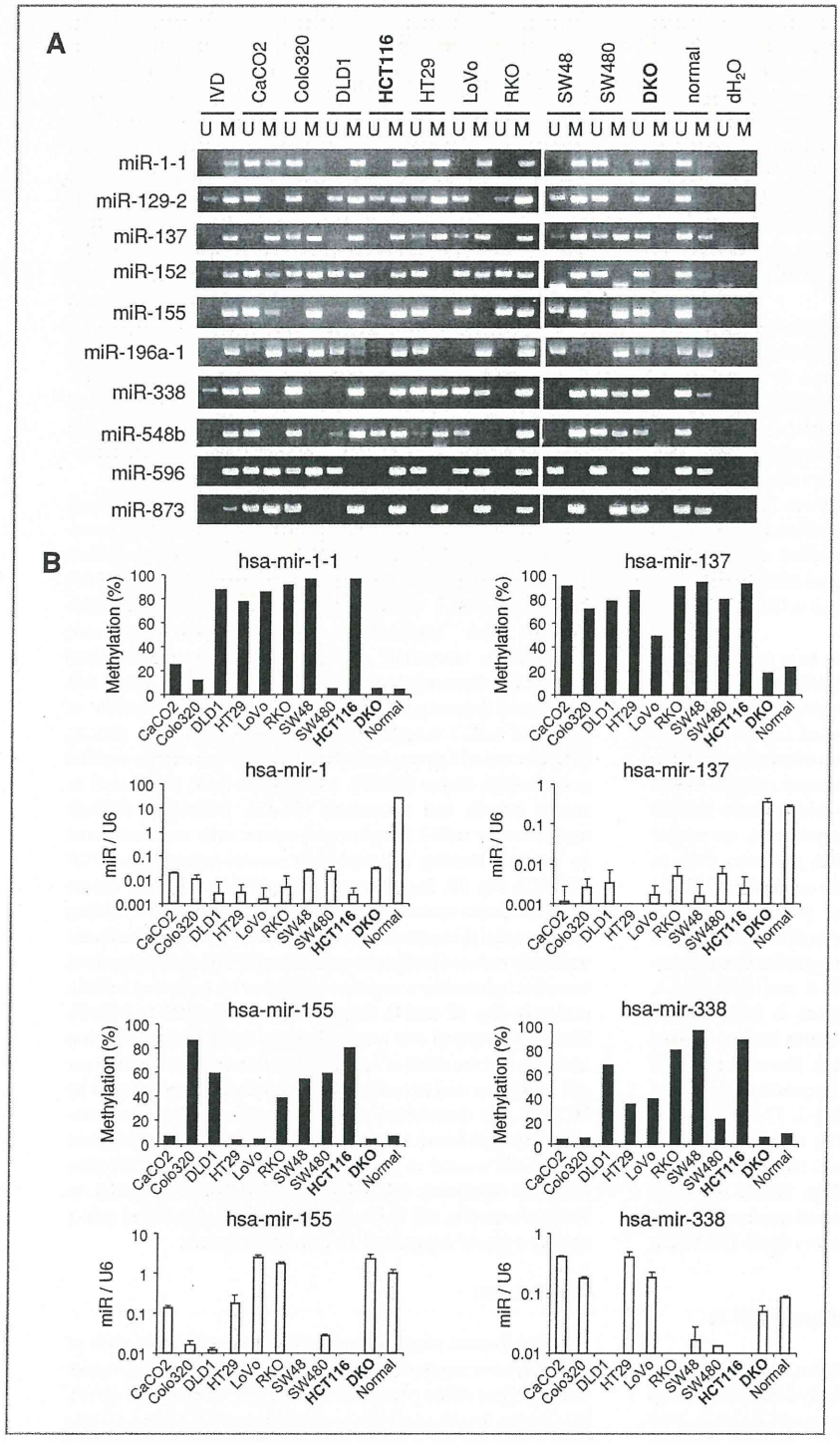

Figure 4. DNA methylation and expression analysis of miRNAs in colorectal cancer cells. A, representative results of MSP analysis of a series of colorectal cancer cell lines and normal colonic tissue. Bands in the "M" lanes are PCR products obtained with methylation-specific primers; those in the "U" lanes are products obtained with unmethylated-specific primers. *In vitro* methylated DNA (IVD) serves as a positive control. B, relationship between DNA methylation and expression of miRNAs in colorectal cancer. Bisulfite pyrosequencing results for miRNA promoter CGIs (black bars) and TaqMan real-time PCR results for mature miRNAs (gray bars) in a series of colorectal cancer cell lines and normal colonic tissue are shown. RT-PCR results were normalized to internal U6 snRNA expression.

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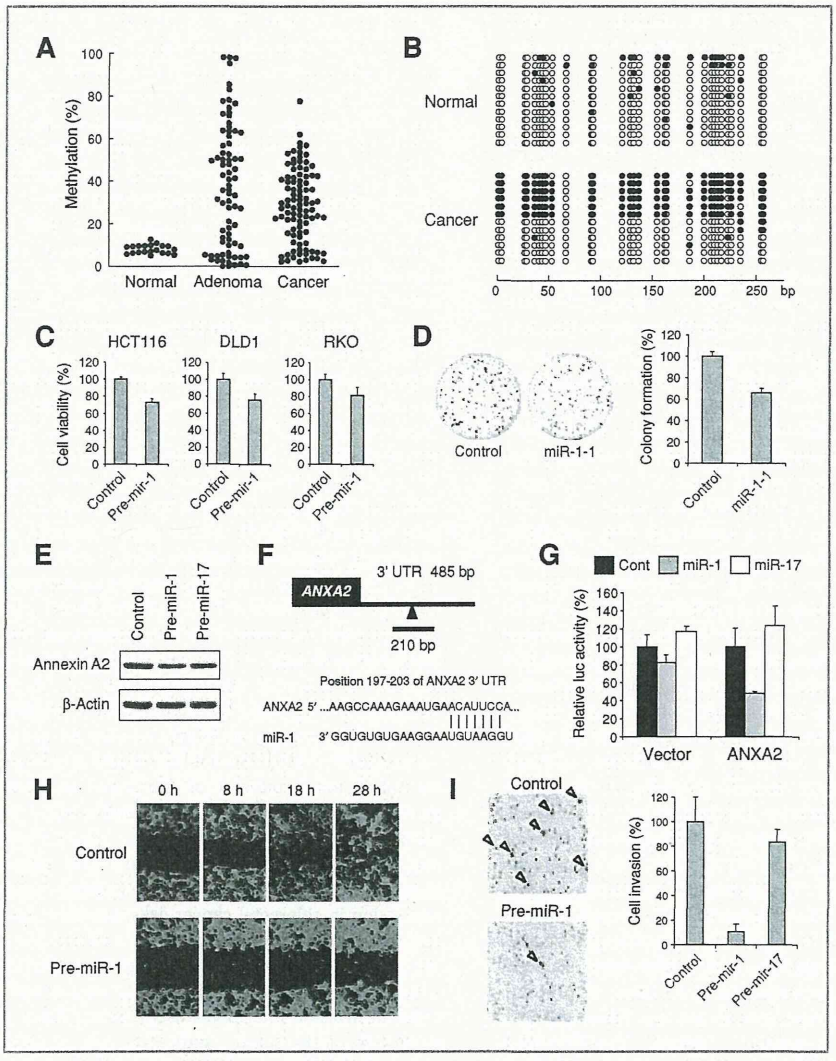

Figure 5. Methylation and functional analysis of miR-1-1 in colorectal cancer. A, summarized bisulfite pyrosequencing results for the miR-1-1 promoter CGI in normal colonic tissue ($n = 20$), colorectal adenomas ($n = 78$), and primary colorectal cancer tumors ($n = 90$). B, representative bisulfite sequencing results for the miR-1-1 promoter in a sample of normal colonic tissue and a primary colorectal cancer tumor. Open and filled circles represent unmethylated and methylated CpG sites, respectively. C, MTT assays with colorectal cancer cell lines transfected with a miR-1 precursor molecule or a negative control. Cell viabilities were determined 48 hours after transfection. Values were normalized to cells transfected with the negative control. Shown are the means of 8 replications; error bars represent SDs. D, colony formation assays using HCT116 cells transfected with a miR-1-1 expression vector or a control vector. Representative results are shown on the left, and relative colony formation efficiencies are on the right. Shown are means of 3 replications; error bars represent SDs. E, Western blot analysis of Annexin A2 in HCT116 cells transfected with a miR-1 precursor molecule or a negative control. Precursor of miR-17, which is abundantly expressed in HCT116 cells and is irrelevant to miR-1, served as another negative control. F, putative miR-1 binding site in the 3′ untranslated region (UTR) of *ANXA2*. A fragment that included the binding site was PCR amplified and cloned into pMIR-REPORT vector. G, reporter assay results using the luciferase vector with the 3′ UTR of *ANXA2* or an empty vector in HCT116 cells cotransfected with a miR-1 precursor, a negative control (Cont), or a miR-17 precursor. Shown are the means of 4 replications; error bars represent SDs. H, wound-healing assay using HCT116 cells transfected with a miR-1 precursor or a negative control. The wound was made 24 hours after transfection, and photographs were taken at the indicated time points. I, Matrigel invasion assay using HCT116 cells transfected with a miR-1 precursor, a negative control, or a miR-17 precursor. Invading cells are indicated by arrowheads. Shown on the right are the means of 3 random microscopic fields per membrane; error bars represent the SDs.

polymerase (pol) II binding patterns (23, 24), evolutionally conserved regions (25), EST mapping (26), and computationally predicted promoters (27, 28). Active promoters are reportedly marked by H3K4me3 (29), and recent studies that have applied such histone marks have successfully identified miRNA gene promoters or TSSs (9, 12). In the present study,

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we carried out high-resolution ChIP-seq analyses in an effort to detect the chromatin signatures of miRNA genes in colorectal cancer.

Although we were able to identify the putative promoters of a number of miRNAs, the present study has several limitations. First, our strategy to identify miRNA promoters can be applied only to transcriptionally active genes. Second, promoters of 135 miRNAs remain unidentified, although their expression was detected in colorectal cancer cells. The majority of such miRNAs (103 of 135) are located in the intergenic regions, and if we increase our search scope, we may identify putative promoter regions in the further upstream, although the accuracy may be decreased. For example, in DKO cells, we detected abundant expression of placenta-specific miRNAs transcribed from a miRNA cluster on chromosome 19 (C19MC), suggesting these miRNAs are epigenetically silenced in normal adult tissues. We found an H3K4me3 mark around a CGI located approximately 18 kb upstream of the cluster, suggesting that this region may be a putative promoter of C19MC (Supplementary Fig. S13), which is consistent with a recent report that hypermethylation of this CGI is associated with epigenetic silencing of C19MC in human cancer cell lines (30). However, other studies have shown that the Alu repetitive sequences within which C19MC is embedded exhibit RNA pol II or pol III promoter activities (31, 32), but we failed to detect obvious active histone marks in these Alu repeats. These results suggest that C19MC may have multiple promoter regions and point to a limitation of the strategy we employed in the current study.

Despite this limitation, chromatin signatures provided important clues to the identity of epigenetically silenced miRNAs in cancer. In HCT116 cells, for instance, the miR-9-1 promoter showed significant enrichment of active histone marks and mature miR-9 was abundantly expressed (data not shown). On the other hand, lack of H3K4me3 in the same cells and its restoration after DNA demethylation clearly suggest that miR-9-2 and miR-9-3 are epigenetically silenced in these cells, which is indicative of the utility of our strategy. We also noted that chromatin signatures of epileptically silenced miRNA genes exhibit patterns similar to those of protein-coding genes. Recent studies have shown that TSGs with CGI methylation retain repressive histone modifications (H3K9me3 and H3K27me3) even after demethylation (15). A genome-wide analysis of the chromatin signature using ChIP-on-chip in colorectal cancer cells revealed that hypermethylated genes adopt a bivalent chromatin pattern upon DNA demethylation (16). More recently, Jacinto and colleagues found that DNA demethylation never results in restoration of the H3K79me2 mark in TSGs with methylated CGIs, suggesting that such incomplete chromatin reactivation leads to relatively low levels of reexpression (33). In the present study, we found that miRNA genes with methylated CGIs never return to a full euchromatin status after DNA demethylation. In addition, we observed significant overlap between PcG marked miRNAs in ES cells and miRNAs with CGI methylation in cancer cells, suggesting a strong predisposition of these miRNAs toward aberrant DNA methylation in cancer.

Many of the epigenetically silenced miRNA genes we identified have been implicated in human malignancies. miR-124 family, miR-9 family, miR-34b/c, and miR-129-2 were identified by screening for epigenetically silenced miRNAs in colorectal cancer cell lines (5, 6, 13), and their methylation was subsequently found in various cancers (8, 34–36). Methylation-associated silencing of miR-137 was first reported in oral cancer (37), and a recent study revealed its frequent methylation in the early stages of colorectal tumorigenesis (38). The high frequency of CGI hypermethylation in these miRNAs in primary colorectal cancer is suggestive of their tumor suppressor function. It was also recently shown that the muscle-specific miRNAs miR-1 and miR-133a are downregulated in primary colorectal cancer tumors as compared with normal colonic tissues (39). Reduced expression of miR-1 is also found in lung cancer (40), and CGI methylation–mediated silencing of miR-1-1 has been reported in hepatocellular carcinoma (41). In addition, levels of miR-1 expression were diminished in the serum of non–small-cell lung cancer (NSCLC) patients who survived for only a short period, suggesting that it is predictive of prognosis in NSCLC patients (42). Ectopic expression of miR-1 in lung cancer, liver cancer, and rhabdomyosarcoma cells reportedly inhibits cellular growth through suppression of its target genes, which include *MET*, *FOXP1*, and *HDAC4* (40, 41, 43). In the present study, we found frequent methylation of the miR-1-1 promoter CGI in both colorectal adenoma and primary colorectal cancer tissues, suggesting that aberrant methylation of miR-1-1 is an early event in colorectal tumorigenesis. The strong tumor specificity of the methylation indicates that it could be a novel tumor marker for early detection of colorectal neoplasia. Because the tumor suppressor potential of miR-1 has not been tested in colorectal cancer, we conducted a number of functional analyses, and our findings indicate that ectopic expression of miR-1 in colorectal cancer cells suppresses cell growth, colony formation, cell motility, and invasion. In addition, our gene expression analysis revealed that miR-1 could induce global changes in gene expression in colorectal cancer cells, especially genes related to the extracellular region, cell membrane, and wound healing. We identified 2 novel miR-1 target genes, *ANXA2* and *BDNF*, which are frequently overexpressed in cancer and are implicated in invasion and metastasis (20–22). These results are suggestive of the tumor suppressor role of miR-1 and its potential therapeutic application in colorectal cancer.

On the other hand, we unexpectedly detected silencing of several miRNAs with known oncogenic properties. For example, miR-155 is a well-characterized oncogenic miRNA that is overexpressed in various human malignancies (44). Although we found miR-155 to be silenced with CGI methylation in HCT116 cells, its methylation was rarely observed in primary tumors, suggesting that epigenetic silencing of miR-155 may not be functionally important in colorectal cancer. Similarly, miR-196a-1 is reportedly overexpressed in several human malignancies, including esophageal adenocarcinoma and glioblastoma (45, 46). Methylation levels of miR-196a-1 in primary colorectal cancer tumors are lower than in normal colonic tissue, which is in agreement with its possible oncogenic properties in colorectal cancer.

Finally, our chromatin signature analysis revealed that a number of miRNAs without promoter CGIs are also potential

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targets of epigenetic silencing in colorectal cancer. These miRNAs were identified through restoration of both their expression and H3K4me3 marking after DNA demethylation, whereas the signatures of H3K79me2 and H3K27me3 varied among genes. This category may thus include miRNAs induced by secondary effects of DNA demethylation, such as upregulation of transcription factors. It is noteworthy, however, that some functionally important miRNAs showed chromatin signatures that were distinct from CGI-methylated miRNAs. Upon DNA demethylation, miR-142 and miR-146a exhibited more active chromatin states, which were characterized by enrichment of both H3K4me3 and H3K79me2 marks. Earlier studies implicated their tumor suppressor roles in cancers of various origins. For instance, miR-142 was found to be downregulated in murine and human lung cancer and its expression suppressed cancer cell growth (47). Loss of miR-146a was reported in hormone-refractory prostate cancer (48), and expression of miR-146a suppressed NF-κB activity and metastatic potential in breast and pancreatic cancer cells (49, 50). The abundant expression of miRNAs in normal colon and downregulation in multiple colorectal cancer cell lines indicates their tumor-suppressive properties in colorectal cancer (data not shown), though further study is need to define the functions of miRNAs in colorectal tumorigenesis.

With this study, we provide compelling evidence that both CGI-positive and -negative miRNAs are targets of epigenetic silencing in colorectal cancer. Our data suggest that DNA demethylation can alter the chromatin signatures of numerous miRNAs in cancer and that reexpression of these miRNAs has important relevance to the effects of epigenetic cancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Research Article

Cancer Prevention Research

Epigenetic Alteration of DNA in Mucosal Wash Fluid Predicts Invasiveness of Colorectal Tumors

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Abstract

Although conventional colonoscopy is considered the gold standard for detecting colorectal tumors, accurate staging is often difficult because advanced histology may be present in small colorectal lesions. We collected DNA present in mucosal wash fluid from patients undergoing colonoscopy and then assessed the methylation levels of four genes frequently methylated in colorectal cancers to detect invasive tumors. We found that methylation levels in wash fluid were significantly higher in patients with invasive than those with noninvasive tumors. Cytologic and *K-ras* mutation analyses suggested that mucosal wash fluid from invasive tumors contained greater numbers of tumor cells than wash fluid from noninvasive tumors. Among the four genes, levels of *mir-34b/c* methylation had the greatest correlation with the invasion and showed the largest area under the receiver operating characteristic curve (AUC = 0.796). Using cutoff points of *mir-34b/c* methylation determined by efficiency considerations, the sensitivity/specificity were 0.861/0.657 for the 13.0% (high sensitivity) and 0.765/0.833 for the 17.8% (well-balanced) cutoffs. In the validation test set, the AUC was also very high (0.915), the sensitivity/specificity were 0.870/0.875 for 13.0% and 0.565/0.958 for 17.8%. Using the diagnostic tree constructed by an objective algorithm, the diagnostic accuracy of the invasiveness of colorectal cancer was 91.3% for the training set and 85.1% for the test set. Our results suggest that analysis of the methylation of DNA in mucosal wash fluid may be a good molecular marker for predicting the invasiveness of colorectal tumors. *Cancer Prev Res; 4(5); 674–83. ©2011 AACR.*

Introduction

Colorectal cancer is one of the most common neoplasias worldwide (1), and its early detection and accurate preoperative staging are essential for reducing the incidences of invasion and metastasis. The fecal occult blood test is widely used to screen for colorectal tumors, though its sensitivity and specificity are not high (2, 3). Conventional colonoscopy is considered the gold standard for detecting colorectal cancers and adenomas, whereas several other methods, including computed tomography, ultrasonography, and 3D magnetic resonance, have been used for staging (4, 5). Generally, tumor size is used as the marker for invasion and lymph node metastasis; however, accurate staging is often difficult because advanced histology may be present in as much as 10% of small (5–10 mm) colorectal adenomas (6–8).

Magnified endoscopy is a highly useful method for diagnosing invasive colorectal cancer (9, 10). Although conventional endoscopic examination with indigo carmine dye is not sufficient to determine whether or not a colorectal cancer is invasive, pit pattern analysis, using high-magnification observation with crystal violet, reportedly enables the diagnosis of invasive colorectal cancers. Recently, narrow-band imaging magnification endoscopy has also been used to predict the invasiveness of colorectal tumors (11). However, it has been suggested that the skills required for pit pattern analysis will limit the number of endoscopists who use the technique.

DNA methylation plays a critical role in the tumorigenesis of colorectal cancer (12, 13). For example, promoter hypermethylation is associated with the silencing of various cancer-related genes (14, 15), and aberrant methylation of the CpG islands of genes in stool and serum/plasma can be used as a molecular marker for detection of colorectal tumors (16–20). On the contrary, because DNA

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methylation is an early event (21–23), it does not enable one to distinguish between premalignant lesions and invasive tumors. As yet, there is no study describing a molecular test for predicting the invasiveness of colorectal tumors.

In this study, we examined the methylation levels of 4 genes frequently methylated in colorectal cancers by using DNA obtained from mucosal wash fluid. We found that the methylation level of DNA in the wash fluid was significantly higher in patients with invasive than those with noninvasive tumors. Our results suggest that methylation of DNA in mucosal wash fluid could be a good molecular marker for predicting the invasiveness of colorectal tumors.

Materials and Methods

Specimens and sample preparation

Colorectal tumor tissues and washing fluid were collected from Japanese patients who underwent endoscopic mucosal resection (EMR) or surgical resection of colorectal tumors at Akita Red Cross Hospital. Informed consent was obtained from all patients before collection of the specimens. Approval for this study was obtained from the Institutional Review Board of Akita Red Cross Hospital and Sapporo Medical University.

We used 2 methods to obtain DNA from mucosal washing fluid. When a colorectal tumor was detected during colonoscopy, the tumor's surface mucus was either washed away by using 20 mL of water, which was aspirated through the suction channel of the endoscope, and suspended in ThinPrep PreservCyt solution (Hologic, Inc.; method 1) or washed away with 20 mL of normal saline by using an NT tube and collected (method 2). Each sample of collected washing fluid was placed in a Non-GYN PreservCyt vial (Hologic, Inc.) for a minimum of 15 minutes, after which the solution was transferred to a disposable centrifuge tube and centrifuged at 500 × *g* for 20 minutes. The resultant supernatant was discarded, and the cell pellet was suspended in ThinPrep solution until DNA extraction. After collection of the washing fluid and endoscopic observation, biopsies of the colorectal tumor and corresponding normal colonic mucosa were carried out by using biopsy forceps under endoscopic guidance. For cytology analysis, a ThinPrep slide was prepared by a T2000 ThinPrep processor (Hologic, Inc.) and a nongynecologic ThinPrep Filter with 5-μm pores. The slide was then Papanicolaou stained and examined by GLab cytotechnologists and a pathologist (GeneticLab Co. Ltd.), and atypical cells were identified. Nuclei were stained by 4′,6-diamidino-2-phenylindole (DAPI) and visualized under a fluorescence microscope (Olympus) as described previously (24).

A total of 337 biopsy specimens including 150 colorectal tumors and 187 normal colonic mucosa specimens were examined. We also examined 88 samples of washing fluid from 70 colorectal tumor patients and 18 healthy patients. For testing the clinical usefulness of the study, we examined an additional 47 colorectal tumor biopsy samples as the test set, which were independently obtained several months after the collection of prior samples. On the basis of histologic examination after any type of resection, biopsy specimens and wash fluid samples from a colorectal tumor were divided into 2 groups: invasive tumors and noninvasive tumors. Invasive tumors were defined as submucosal invasive tumors.

Bisulfite pyrosequencing

DNA was extracted from biopsy specimens and washing fluid by using the standard phenol-chloroform procedure, after which 1-μg samples of genomic DNA were modified with sodium bisulfite by an EpiTect Bisulfite Kit (Qiagen). Bisulfite pyrosequencing was then carried out as described previously (25). Primers for pyrosequencing were designed by PSQ Assay Design software (Qiagen). Following PCR, the biotinylated PCR product was purified, made single-stranded, and then used as a template in a pyrosequencing reaction run according to the manufacturer's instructions. The PCR products were bound to streptavidin Sepharose beads HP (Amersham Biosciences), after which beads with the immobilized PCR product were purified, washed, and denatured by 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 by a PSQ96MA system (Qiagen) and Pyro Q-CpG software (Qiagen). The primer sequences are listed in Supplementary Table S1.

K-ras mutation analysis

Mutation of codons 12 and 13 of *K-ras* was examined by using direct sequencing and pyrosequencing, as described previously (26). Pyrosequencing was done by a *K-ras* mutation detection kit (Qiagen) as suggested by the supplier.

Statistical analysis

All statistical analyses were carried out by SPSSJ 15.0 (SPSS Japan Inc.). To compare differences in methylation levels or other continuous values between groups, *t* tests or ANOVA with a post hoc Tukey test were carried out. Fisher's exact test was used for analysis of categorical data. To evaluate correlations between continuous values, Pearson's correlation coefficients were calculated. Receiver operating characteristic (ROC) curves for the diagnosis of invasive tumors were constructed on the basis of methylation levels, followed by area under the curve (AUC) calculation. A diagnostic tree to discriminate invasive tumors was constructed by using the training set based on the following objective algorithm. Step 1: classify the samples based on the most efficient cutoff of tumor size. Step 2: classify the samples based on the most efficient cutoffs of methylation levels in 4 sequences under the classification of the previous step. Step 3: repeat step 2 until no additional efficacy is observed. $P < 0.05$ (2-sided) were considered significant.

Results

Preparation of specimens

We first compared the 2 methods used to obtain tumor cells from colonoscopy wash fluid. When tumors were

Table 1. Amounts of DNA and quality of cytology obtained by using the 2 collection methods tested

	Biopsy (n = 11)	Wash fluid (method 1)			Biopsy (n = 37)	Wash fluid (method 2)		
		Total (n = 11)	IT (n = 8)	NI (n = 3)		Total (n = 37)	IT (n = 18)	NI (n = 19)
DNA, μg	21.28 ± 15.25	15.00 ± 11.12			17.08 ± 13.00	16.53 ± 16.88		
Cytology								
Diagnosable		2	1	1		17	11	6
Not diagnosable		9	7	2		20	7	13
		18.2%	12.5%	33.3%		45.9%	61.1%	31.6%

Abbreviations: IT: invasive tumors, NI: noninvasive tumors.

found during colonoscopy, the tumor surfaces were washed with water (method 1) or saline (method 2; Supplementary Fig. S1A and B). Both methods enabled us to obtain enough DNA for molecular analysis (Table 1), but comparison of the cytology revealed that wash fluid obtained with saline (method 2) enabled more accurate detection of anaplastic cells (Supplementary Fig. S1B; Supplementary Table 1). We therefore used method 2 for subsequent analyses.

DNA methylation in biopsy specimens

The clinical features of the patients examined in this study are summarized in Table 2. There was no statistically significant difference in age or gender between patients with invasive and noninvasive tumors. We selected 4 genes for analysis, *mir-34b/c*, *SFRP1*, *SFRP2*, and *DKK2*, which are frequently methylated in colorectal cancer (25, 27, 28). Methylation analysis was carried out by using bisulfite pyrosequencing with DNA from 337 specimens, including 52 invasive tumors, 98 noninvasive tumors, and 187 specimens of normal colon tissue. We found that the methylation levels of the 4 genes were significantly higher in cancerous tissue than in normal colorectal mucosa (Fig. 1); however, we found no difference in tissue methylation levels between invasive and noninvasive tumors.

DNA methylation in mucosal washing fluid

We next examined gene methylation in 76 samples of mucosal wash fluid from 36 patients with invasive tumors and 34 with noninvasive tumors, and from 18 patients without colorectal lesions (Fig. 1). When we compared the invasive and noninvasive tumors, we found no differences for *SFRP2* and *DKK2*. However, *mir-34b/c* and *SFRP1* showed higher levels of methylation in wash fluid from patients with invasive than with noninvasive tumors.

We then used ROC analysis to further compare mucosal wash fluid from invasive and noninvasive tumors (Fig. 3A and B). The odds ratios (OR) for the risk of invasion associated with the methylation levels of the 4 genes tested are shown in Table 3. High levels of methylation were significantly associated with an increased risk of invasion. Among the 4 genes, levels of *mir-34b/c* methylation had the greatest impact on the risk of invasion and showed the largest AUC (0.796). Using various cutoff points of *mir-34b/c* methylation determined on the basis of efficiency considerations for clinical use, the sensitivity/specificity were 0.861/0.657 for the 13.0% (high sensitivity) cutoff point and 0.765/0.833 for the 17.8% (well balanced) cutoff. We also subdivided the tumors on the basis of whether they were ≥25 mm or <25 mm in size. In tumors 25 mm or larger, *mir-34b/c* showed the highest AUC (0.816); its sensitivity/specificity was 0.862/0.667 and its OR was 12.5. In tumors smaller than 25 mm, *SFRP1* showed the highest AUC (0.810), with a sensitivity/specificity of 0.821/0.833 and an OR of 23.0.

We further verified the utility of DNA methylation in mucosal washing, using an independent set of specimens, which was established as the test set (Figs. 2, 3C and D; Table 4). All of these ROC analyses were considered the training set. In the test set, the methylation levels of *mir-34b/c* showed very high AUC again (0.915), sensitivity/specificity was 0.870/0.875 for the 13.0% cutoff, and 0.565/0.958 for the 17.8% cutoff. In tumors 25 mm or larger, the AUC of the methylation levels of *mir-34b/c* was 0.778. In tumors smaller than 25 mm, the AUC of the methylation levels of *SFRP1* was 0.695.

To make a more efficient diagnostic method suitable for clinical situations, we then constructed a diagnostic tree to classify invasive and noninvasive tumors on the basis of the combination of methylation levels detected in wash fluid (Fig. 4A). First, because most endoscopists make an endoscopic diagnosis according to the size of lesions, we defined the most efficient cutoff of tumor size as the first node of the diagnostic tree. As the next nodes, we used the most efficient cutoffs of the methylation levels of the 4 genes (*mir-34*, *SFRP1*, *SFRP2*, and *DKK2*). For example, as shown in Figure 4A, if the tumor size is more than 25 mm and the methylation level of *mir-34b/c* is more than 15%, this lesion is diagnosed as an invasive tumor. In the training set, the sensitivity and specificity were 0.943 (33/35) and 0.882 (30/34), respectively. The total accuracy of the diagnosis was 91.3% (63/69). For use in clinical situations, we validated this diagnostic tree by using an independent test set (n = 47). The application of the diagnostic tree to the test set is shown in Figure 4B. Although a slight reduction in