Genome-wide profiling of chromatin signatures reveals epigenetic regulation of microRNA genes in colorectal cancer

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Abstract

Altered expression of microRNAs (miRNAs) occurs commonly in human cancer but the mechanisms are generally poorly understood. In this study, we studied the contribution of epigenetic mechanisms to miRNA dysregulation in colorectal cancer (CRC) by performing high-resolution ChIP-seq. Specifically, we conducted genome-wide profiling of trimethylated histone H3 lysine 4 (H3K4me3), trimethylated histone H3 lysine 27 (H3K27me3) and dimethylated histone H3 lysine 79 (H3K79me2) in CRC cell lines. Combining miRNA expression profiles with chromatin signatures enabled us to predict the active promoters of 233 miRNAs encoded in 174 putative primary transcription units. By then comparing miRNA expression and histone modification before and after DNA demethylation, we identified 47 miRNAs encoded in 37 primary transcription units as potential targets of epigenetic silencing. The promoters of 22 transcription units were associated with CpG islands (CGIs), all of which were hypermethylated in CRC cells. DNA demethylation led to increased H3K4me3 marking at silenced miRNA genes, whereas no restoration of H3K79me2 was detected in CGI methylated miRNA genes. DNA demethylation also led to upregulation of H3K4me3 and H3K27me3 in a number of CGI-methylated miRNA genes. Among the miRNAs we found to be dysregulated, many of which are implicated in human cancer, miR-1-1 was methylated frequently in early and advanced CRC where it may act as a tumor suppressor. Our findings offer insight into the association between chromatin signatures and miRNA dysregulation in cancer, and they also suggest that miRNA re-expression may contribute to the effects of epigenetic therapy.
Introduction

MicroRNAs (miRNAs) are a class of small non-coding RNAs that regulate gene expression by inducing translational inhibition or direct degradation of target mRNAs through base pairing to partially complementary sites (1). MicroRNA genes are transcribed as large precursor RNAs, called pri-miRNAs, which may encode multiple miRNAs in a polycistronic arrangement. Pri-miRNAs are then processed by the RNase III enzyme Drosha and its cofactor Pasha to produce ~70 nt hairpin structured second precursors (pre-miRNAs). The pre-miRNAs are then transported to the cytoplasm and processed by another RNase III enzyme, Dicer, to generate mature miRNA products. MicroRNAs are highly conserved among species, and play critical roles in a variety of biological processes, including development, differentiation, cell proliferation and apoptosis. Subsets of miRNAs are thought to act as tumor suppressor genes (TSGs) or oncogenes, and their dysregulation is a common feature of human cancers (2). More specifically, expression of miRNAs is generally downregulated in tumor tissues, as compared to the corresponding normal tissues, which suggests some miRNAs may behave as TSGs in some tumors. Although the mechanism underlying the alteration of miRNA expression in cancer is still not fully understood, recent studies have shown that multiple mechanisms involved in regulating miRNA levels are affected in cancer. For example, genetic mutations that affect proteins involved in the processing and maturation of
miRNA can lead to overall reductions in miRNA expression levels (3, 4). In addition, genetic and epigenetic alterations can disrupt expression of specific miRNAs in cancer.

Epigenetic gene silencing due to promoter CpG island (CGI) hypermethylation is one of the most common mechanisms by which TSGs are inactivated during tumorigenesis. In recent years, it has become evident that some miRNA genes are also targets of epigenetic silencing in cancer. We and others have previously shown that pharmacological or genetic disruption of DNA methylation in cancer cell lines induces upregulation of substantial numbers of miRNAs (5, 6). These analyses led to identification of candidate tumor-suppressive miRNAs whose silencing was associated with CGI methylation. For example, miR-127 is embedded in a typical CGI, and treatment of human bladder cancer cells with inhibitors of histone deacetylase (HDAC) and DNA methyltransferase (DNMT) induced CGI demethylation and re-expression of the miRNA (7). In addition, methylation of miR-124 family members (miR-124-1, -124-2 and -124-3) was identified in colorectal cancer (CRC), and was subsequently reported in tumors of other origins (5). Similarly, we found frequent methylation and downregulation of miR-34b/c in both CRC and gastric cancer (6, 8).
Epigenetic regulation of miRNA genes is tightly linked to chromatin signatures. For instance, transcriptionally active miRNA genes are characterized by active chromatin marks, such as trimethylated histone H3 lysine 4 (H3K4me3) (9). We previously showed that restoring H3K4me3 through DNA demethylation could be a useful marker for predicting the promoter region of a silenced miRNA gene (6). However, the chromatin signatures, including both active and repressive histone marks on miRNA genes, within the cancer genome are still largely unknown. In the present study, we carried out genome-wide profiling of chromatin signatures in CRC cells, and identified the active promoter regions of miRNA genes. We also demonstrate that changes in chromatin signatures before and after removal of DNA methylation lead to robust identification of miRNA genes that are epigenetically regulated in cancer.
Materials and methods

Cell lines and tissue specimens

Colorectal cancer cell lines and HCT116 cells harboring genetic disruptions within the \textit{DNMT1} and \textit{DNMT3B} loci (DKO) have been described previously (6). Treatment of cells with 5-aza-2’-deoxycytidine (DAC; Sigma-Aldrich, St Louis, MO, USA) and 4-phenylbutyrate (PBA; Sigma-Aldrich) were carried out as described (8). A total of 90 primary CRC specimens were obtained as described (6, 10). Samples of adjacent normal colorectal mucosa were also collected from 20 patients. A total of 78 colorectal adenoma specimens were obtained through endoscopic biopsy. Informed consent was obtained from all patients before collection of the specimens. Total RNA from normal colonic mucosa from healthy individuals was purchased from Ambion (Austin, TX, USA). Total RNA was extracted using a mirVana miRNA isolation kit (Ambion) or TRIZOL reagent (Invitrogen, Carlsbad, CA). Genomic DNA was extracted using the standard phenol-chloroform procedure.

miRNA expression profiling

Expression of 470 miRNAs was analyzed using Human miRNA Microarray V1 (G4470A; Agilent technologies, Santa Clara, CA, USA) as described previously (8). In addition, expression of 664 miRNAs was analyzed using a TaqMan MicroRNA Array v2.0 (Applied
Biosystems, Foster City, CA, USA). Briefly, 1 μg of total RNA was reverse transcribed using Megaplex Pools kit (Applied Biosystems), after which they were amplified and detected using PCR with specific primers and TaqMan probes. The PCR was run in a 7900HT Fast Real-Time PCR System (Applied Biosystems), and SDS2.2.2 software (Applied Biosystems) was used for comparative delta Ct analysis. U6 snRNA (RNU6B; Applied Biosystems) served as an endogenous control. Microarray data and TaqMan Array data (delta Ct values) were further analyzed using GeneSpring GX ver. 11 (Agilent technologies). The Gene Expression Omnibus accession number for the microarray data is GSE29900.

Real-time RT-PCR of miRNA

Expression of selected miRNAs was analyzed using TaqMan microRNA Assays (Applied Biosystems). Briefly, 5 ng of total RNA were reverse transcribed using specific stem-loop RT primers, after which they were amplified and detected using PCR with specific primers and TaqMan probes as describe above. U6 snRNA (RNU6B; Applied Biosystems) served as an endogenous control. Expression of the primary miR-1-1 transcript was analyzed using a TaqMan Pri-miRNA assay (assay ID, Hs03303345_pri; Applied Biosystems). GAPDH (assay ID, Hs99999905_m1; Applied Biosystems) served as an endogenous control.
ChIP-on-chip analysis

Chromatin immunoprecipitation (ChIP)-on-chip was performed according to Agilent Mammalian ChIP-on-chip Protocol version 10.0 (Agilent Technologies). Briefly, 1 x 10^8 cells were treated with 1% formaldehyde for 10 min to crosslink histones with the DNA. After washing with PBS, the cell pellets were resuspended in 3 ml of lysis buffer and sonicated. Chromatin was immunoprecipitated for 16 h at 4°C using 10 μl of anti-trimethyl-histone H3K4 (clone MC315; Upstate, Lake Placid, NY), anti-trimethyl-histone (clone H3K27, Upstate) or anti-dimethyl-histone H3K79 (clone NL59, Upstate) antibody. Before adding antibodies, 50 μl of the each cell lysate was saved as an internal control for the input DNA. After washing, elution and reversal of the cross-links, input DNA and the immunoprecipitate were ligated to linkers and PCR amplified. Input DNA and the immunoprecipitate were then labeled with Cy3 and Cy5 using an Agilent Genomic DNA Enzymatic Labeling Kit (Agilent Technologies) and hybridized to the 244K Human Promoter ChIP-on-chip microarray (G4489A; Agilent technologies). After washing, the array was scanned using an Agilent DNA Microarray Scanner (Agilent technologies), and the data was processed using Feature Extraction software (Agilent Technologies).

ChIP-seq analysis
ChIP experiments were performed as described above, after which massively parallel sequencing was performed using a SOLiD3 Plus system (Applied Biosystems) according to the manufacturer’s instructions. Briefly, 100 ng of input DNA or the immunoprecipitate were ligated to adapters and PCR amplified using a SOLiD Fragment Library Construction Kit (Applied Biosystems). Template bead preparation was carried out using a SOLiD ePCR Kit V2 (Applied Biosystems) and a SOLiD Bead Enrichment Kit (Applied Biosystems). Approximately 40 to 50 million beads per sample were sequenced using SOLiD Opti Fragment Library Sequencing Master Mix 50 (Applied Biosystems) and a SOLiD3 Plus sequencer (Applied Biosystems). Sequence reads which were of poor quality or those that were not uniquely mapped were excluded from the study. Peaks were identified using the Model-based Analysis for ChIP-seq (MACS) software (11), and visualized using the USCS genome browser.

Reference sequence

Genomic locations are based on the UCSC hg18 (NCBI Build 36.1, March 2006), which was produced by the International Human Genome Sequencing Consortium. We also obtained locations of CpG islands, ReSeq genes and UCSC genes from the UCSC hg18 data sets.

Methylation analysis


Genomic DNA (2 μg) was modified with sodium bisulfite using an EpiTect Bisulfite Kit (QIAGEN). Methylation-specific PCR (MSP), bisulfite sequencing and bisulfite pyrosequencing were carried out as described (6). For bisulfite sequencing analysis, amplified PCR products were cloned into pCR2.1-TOPO vector (Invitrogen), and 10 to 12 clones from each sample were sequenced using an ABI3130x automated sequencer (Applied Biosystems). Primer sequences and PCR product sizes are listed in Supplementary Table S1.

**Transfection of miRNA precursor molecules**

CRC cells (1x10^6 cells) were transfected with 100 pmol of Pre-miR miRNA Precursor Molecules (Ambion) or Pre-miR miRNA Molecules Negative Control #1 (Ambion) using a Cell Line Nucleofector kit V (Lonza, Basel, Switzerland) with a Nucleofector I electroporation device (Lonza) according to the manufacturer’s instructions. Total RNA or cell lysate was extracted 48 h after transfection. Cell viability assays, Western blotting, wound-healing assays and Matrigel invasion assays are described in the Supplementary Methods.

**Gene expression profiling**

Total RNA (700 ng) was amplified and labeled using a Quick Amp Labeling Kit one-color (Agilent Technologies), after which the synthesized cRNA was hybridized to the Whole Human
Genome Oligo DNA microarray (G4112F; Agilent technologies). Data analysis was performed using GeneSpring GX ver. 11 (Agilent technologies). The Gene Expression Omnibus accession number for the microarray data is GSE29760.

miRNA target predictions and luciferase reporter assays

The predicted targets of miR-1 and their downstream target sites were analyzed using TargetScan and miRanda. Construction of luciferase reporter vectors containing the predicted target sites and dual luciferase reporter assays were carried out as described in the Supplementary Methods.
Results

miRNA profiling in colorectal cancer cell lines

In order to screen for epigenetically silenced miRNAs, we first carried out miRNA microarray analysis in a series of CRC cell lines (HCT116, DLD1 and RKO) and normal colonic tissue. Hierarchical clustering analysis revealed that expression of a majority of miRNAs was downregulated in all three CRC cell lines tested, as compared to normal colonic mucosa (Supplementary Fig. S1A). DAC treatment upregulated expression of a large number of miRNAs in all three CRC cell lines (Supplementary Fig. S1B), and combination treatment with DAC plus PBA induced even greater numbers of miRNAs in CRC cells (Supplementary Fig. S1C,D). But the most profound effect on the miRNA expression profile was induced by genetic disruption of \textit{DNMT1} and \textit{DNMT3B} in HCT116 cells (double knockout; DKO cells) (Supplementary Fig. S1C). We also noted a novel overlap between miRNAs upregulated by pharmacological or genetic disruption of DNA methylation and those downregulated in CRC cells, as compared to normal colonic mucosa (Supplementary Fig. S1E-G). To test the tumor suppressive potentials of the downregulated miRNAs, we constructed expression vectors encoding selected miRNAs and carried out colony formation assays. We found that a majority of the miRNAs exerted growth suppressive effects when they were ectopically expressed in CRC cells (Supplementary Fig. S2). These results suggest that an epigenetic mechanism plays
an essential role in the downregulation of a number of miRNAs in cancer, and that such
downregulation of numerous miRNAs may contribute to tumorigenesis.

Chromatin signatures of active and silenced miRNA genes

We next examined the chromatin signatures of miRNA genes in HCT116 CRC cells, with
and without genetic disruption of DNMT1 and DNMT3B (DKO cells). We performed ChIP
analysis using antibodies against trimethylated histone H3 lysine 4 (H3K4me3), which marks
active promoters; dimethylated histone H3 lysine 79 (H3K79me2), which is associated with
transcriptional elongation; and trimethylated histone H3 lysine 27 (H3K27me3), which is a
repressive mark. We started our analysis using the Agilent 244K Promoter array, which covers
approximately 370 human miRNA genes, and we subsequently migrated to ChIP-seq analysis to
increase our scope within the genome. We observed a good correlation between the results of
the ChIP-on-chip and ChIP-seq analyses (Supplementary Fig. S3). We also validated the
reliability of our ChIP-seq data by checking representative protein-coding genes that were
transcriptionally active or silenced in HCT116 cells (Supplementary Fig. S4).

Representative chromatin signatures of miRNA genes are shown in Figure 1A. We found
enrichment of the H3K4me3 mark around the proximal upstream CGI regions of two
abundantly expressed miRNA clusters, miR-200b and miR-17, in both wild-type HCT116 and DKO cells (Fig. 1A). Gene bodies were marked by H3K79me2, which indicates active transcriptional elongation, whereas they almost completely lacked the repressive H3K27me3 mark. With respect to the H3K4me3 mark in the miR-17 cluster, we observed a sharp dip at the transcription start site (TSS) of the host gene and another dip downstream, which is consistent with a previous report that miR-17 has its own TSS within the intron of the host gene (Fig. 1A) (12).

By contrast, miRNAs whose silencing was associated with promoter CGI hypermethylation completely lacked both of the active histone marks. The CGIs of miR-34b/c, miR-124-1 and miR-9-3 were densely methylated in HCT116 cells (5, 6, 13), and were completely devoid of H3K4me3 and H3K79me2 marks (Fig. 1B). miR-124-1 and miR-9-3 showed moderate enrichment of H3K27me3, whereas miR-34b/c was almost H3K27me3-free, which corresponds to previous reports that DNA methylation and H3K27me3 are sometimes observed independently in cancer (14). In DKO cells, where DNA methylation was significantly diminished and gene expression was restored, increased H3K4me3 marks were found at the upstream CGI, though restoration of H3K79me2 was quite limited. Upregulation of H3K27me3 was also seen around miR-124-1 and miR-9-3, which is consistent with previous
observations that genes with methylated CGIs adopt a bivalent chromatin pattern after DNA demethylation (15, 16).

Identification of putative miRNA promoter regions

Identification of epigenetically silenced miRNAs is sometimes hampered by a lack of knowledge of the transcription initiation region of the primary miRNA transcripts. Previous studies have shown that H3K4me3 is a useful marker for identifying active miRNA gene promoters (9, 12), and we employed that approach with CRC cells. Using miRNA microarrays and TaqMan low density arrays, we detected expression of 339 and 429 distinct mature miRNAs in HCT116 and DKO cells, respectively. We then searched for the putative promoter regions of these miRNAs using H3K4me3 as a marker.

More than half of miRNAs are located in the introns of protein-coding or long non-coding RNA genes, and it is generally believed that intragenic miRNAs share common promoters with their host genes (17). We identified the putative promoters of 166 intragenic miRNAs located in RefSeq genes and/or UCSC genes, and a majority of the H3K4me3 marks were observed at the TSS of the host genes, many of which were located more than 10 kb upstream of the pre-miRNA coding regions (Fig. 2A,C, Supplementary Fig. S5A, Supplementary Table S2).
By contrast, intragenic H3K4me3 marks were identified in the proximal upstream of 22 pre-miRNAs, indicating these miRNAs have their own promoters and are transcribed independently of their host genes (Supplementary Fig. S6, Supplementary Table S3). To identify promoters of intergenic miRNAs, we first searched 10 kb upstream for H3K4me3 marks, and also explored the initiation sites of overlapping 5' ESTs. We identified the putative promoters of 66 intergenic miRNAs, the majority of which (47 of 66) were identified in the proximal upstream (< 2 kb) of the pre-mRNA coding region (Fig. 2B,D, Supplementary Fig. S5B, Supplementary Table S2). In total, we identified the putative promoters of 174 transcript units encoding 233 distinct pre-miRNAs, whereas promoters of 135 miRNAs remain unidentified, despite their positive expression in CRC cells.

We validated our promoter search by comparing our results with previously reported transcription initiation regions. Promoters of 177 pre-miRNAs that we identified overlapped with those identified in human embryonic stem (ES) cells by Marson et al. (9), whereas the promoters of only 38 pre-miRNAs did not match. Similarly, the TSS of 65 miRNAs identified in human melanoma and breast cancer cell lines by Ozsolak et al. overlapped with the promoters we identified (12). For example, we found H3K4me3 marks overlapping with known TSS of the miR-17 cluster, let-7a-a/let7f-1/let-7d and miR-200c/141 (Fig. 2B, Supplementary Fig. S5).
We also identified an H3K4me3 mark at the intronic transcription initiating region of miR-21 (Supplementary Fig. S6C). The high degree of consistency between our results and those of earlier studies attests the accuracy of our promoter prediction.

Identification of epigenetically silenced miRNAs

We next endeavored to identify epigenetically silenced miRNA genes by taking advantage of the observation that DNA demethylation can induce increases in H3K4me3 in the promoters of the epigenetically silenced genes (6). We searched for miRNA genes showing reduction or loss of both expression and H3K4me3 marks in HCT116 and DKO cells. We identified 47 pre-miRNA genes encoded in 37 primary transcription units as potential targets of epigenetic silencing in HCT116 cells. Promoters of 22 transcription units were associated with CGIs, and MSP analysis revealed that all of the CGIs were methylated (Fig. 3A,B, Table 1). In most cases, DNA demethylation led to increases in H3K4me3 and H3K27me3 marking of the methylated CGIs of miRNA genes, whereas H3K79me2 marks were not restored by demethylation (Fig. 3C). By contrast, the chromatin signatures of miRNAs without promoter CGIs were more variable among genes. We 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 RT-PCR but not by microarray, and robust upregulation after DNA demethylation indicates 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 towards 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 CRC (Fig. 3E).
We further analyzed CGI methylation in a series of CRC 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 CRC 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 CRC tumors (n = 90) and normal colonic tissue obtained from CRC 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, S10). Elevated levels of miRNA gene methylation (>15.0%) were frequently detected in primary CRC 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%; miR-548b, 47.8%), while 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 CRC
Among the epigenetically silenced miRNAs, we next focused on miR-1-1 because it has received relatively little attention in CRC, despite its frequent hypermethylation in that disease. Using bisulfite-pyrosequencing, we detected elevated levels (>15.0%) of miR-1-1 methylation in both primary CRC tumors and colorectal adenomas (54 of 78, 69.2%), suggesting that its methylation is an early event in colorectal tumorigenesis (Fig. 5A). By contrast, levels of miR-1-1 methylation were relatively low (<15.0%) in the normal colonic tissues tested (Fig. 5A). We performed bisulfite sequencing analysis to confirm the methylation results in selected tissue specimens and CRC cell lines (Fig. 5B, Supplementary Fig. S11A,B). We also confirmed that DNA demethylation could restore expression of the primary transcript of miR-1-1 (pri-miR-1-1) in CRC cells (Supplementary Fig. S11C).

To determine whether miR-1-1 serves as a tumor suppressor in CRC, we transfected CRC 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 three cell lines (Fig. 5C). Colony formation assays also revealed reduced colony formation by CRC cells transfected with a miR-1-1 expression vector (Fig. 5D).
To further clarify the effect of the miRNA, we next performed a gene expression microarray analysis of HCT116 cells transfected with a miR-1 precursor molecule or a negative control. We found that 2769 probe sets were downregulated (> 1.5-fold) by ectopic miR-1 expression, and gene ontology analysis revealed that “extracellular regions”, “membrane” and “response to wound healing” 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 two 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 CRC cells was confirmed by Western blotting and real-time 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,G, Supplementary Fig. S12B,C). Finally, we carried out wound-healing and Matrigel invasion assays to test the effect of miR-1 expression on CRC cell migration and invasion. We found that wound closure by HCT116 cells transfected with the negative control was complete within 28 h, 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. 5f). These results strongly suggest that miR-1 acts as a tumor suppressor in CRC.
Discussion

In the present study, we provide a comprehensive view of the epigenetic regulation of miRNA genes in CRC 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 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, we carried out high resolution ChIP-seq analyses in an effort to detect the chromatin signatures of miRNA genes in CRC.

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 CRC cells. The majority of such miRNAs (103 of 135) are located in the intergenic regions, and if we increase our searching 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 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 demonstrated that the Alu repetitive sequences within which C19MC is embedded exhibits 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 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 suggests 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 CRC cells revealed that hypermethylated genes adopt a bivalent chromatin pattern upon DNA demethylation (16). More recently, Jacinto et al. 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 re-expression (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 CRC 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 CRC 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 CRC tumors, as compared to 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 that survived for only a short period, suggesting 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 CRC 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 CRC, we conducted a number of functional analyses, and our findings indicate that ectopic expression of miR-1 in CRC cells suppresses
cell growth, colony formation, cell motility and invasion. In addition, our gene expression analysis revealed that miR-1 was able to induce global changes in gene expression in CRC cells, especially genes related to the extracellular region, cell membrane and wound healing. We identified two novel miR-1 target genes, \textit{ANXA2} and \textit{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 CRC.

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 CRC. 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 CRC tumors are lower than in normal colonic tissue, which is in agreement with its possible oncogenic properties in CRC.
Finally, our chromatin signature analysis revealed that a number of miRNAs without promoter CGIs are also potential targets of epigenetic silencing in CRC. 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). Their abundant expression in normal colon and downregulation in multiple CRC cell lines indicates their tumor suppressive properties in CRC (data not shown), though further study is need to define their functions in colorectal tumorigenesis.
With this study, we provide compelling evidence that both CGI-positive and -negative miRNAs are targets of epigenetic silencing in CRC. Our data suggest that DNA demethylation can alter the chromatin signatures of numerous miRNAs in cancer, and that re-expression of these miRNAs has important relevance to the effects of epigenetic cancer therapy.
Acknowledgments

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References


Figure legends

Figure 1
Chromatin signatures of transcriptionally active and epigenetically silenced miRNA genes in CRC.  A, ChIP-seq results for H3K4me3, H3K79me2 and H3K27me3 in transcriptionally active miRNA genes in HCT116 and DKO cells.  Chromosomal locations are indicated on the top.  Locations of host genes, pre-miRNA genes and CpG islands are shown below.  B, ChIP-seq results for epigenetically silenced miRNAs with associated CGI hypermethylation.  CGI methylation is lost and miRNAs are re-expressed in DKO cells.  Note that H3K4me3 marking is upregulated in the putative promoter regions in DKO cells, whereas H3K79me2 shows only a minimal increase.

Figure 2
Identification of miRNA gene promoter regions using chromatin signatures.  A, Examples of H3K4me3 marks in intragenic miRNAs.  Let-7g is located within the intron of the protein-coding gene WDR82, and miR-34a is located within the exon of a non-coding host gene.  H3K4me3 marks are observed in the TSS regions of the host genes, suggesting that these miRNAs share common promoters with their host genes.  B, Examples of H3K4me3 marking of intergenic miRNA genes.  C, Summarized distances between intragenic pre-miRNA coding regions and their putative promoter regions (n = 166).  D, Summarized distances between intergenic pre-miRNA coding regions and their putative promoter regions (n = 67).

Figure 3
Identification of epigenetically silenced miRNA genes.  A, Flowchart for the selection of epigenetically silenced miRNA genes in CRC.  B, Graph showing the number of epigenetically
silenced miRNAs associated with CGI methylation and those without CGI methylation. C, Chromatin signatures of two representative miRNA genes, with and without promoter CGI methylation. miR-1-1 (upper) was silenced in association with CGI methylation in HCT116 cells. In DKO cells, H3K4me3 marking was observed around the transcription start site of the host gene C20orf166. miR-146a (lower) is another candidate target for epigenetic silencing in HCT116, though its promoter is not associated with CGI. Both H4K4me3 and H3K79me2 were restored in DKO cells. D, Expression levels of epigenetically silenced miRNAs and their host genes in HCT116 and DKO cells. TaqMan RT-PCR data for 57 mature miRNAs encoded by 47 pre-miRNA genes were imported into Gene Spring GX, after which the data were normalized and shown in box plots (left). Expression data of 13 host genes of epigenetically silenced miRNAs were obtained using an Agilent Whole Human Genome microarray (right). E, miRNAs targeted by the PcG group in ES cells are more likely to be silenced by CGI hypermethylation in CRC cells. CGI methylated miRNAs (n = 22) (left) or transcriptionally active miRNAs (n = 146) (right) were selected, and their SUZ12 binding and H3K27me3 enrichment in human ES cells were assessed. Of 22 CGI methylated miRNAs, 13 (59%) were positive for SUZ12, and 16 (73%) for H3K27me3. ND, not determined.

**Figure 4**

DNA methylation and expression analysis of miRNAs in CRC cells. A, Representative results of MSP analysis of a series of CRC 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 CRC. Bisulfite pyrosequencing results for miRNA promoter CGIs (black bars) and TaqMan
RT-PCR results for mature miRNAs (gray bars) in a series of CRC cell lines and normal colonic tissue are shown. RT-PCR results were normalized to internal U6 snRNA expression.

**Figure 5**

Methylation and functional analysis of miR-1-1 in CRC. A, Summarized bisulfite pyrosequencing results for the miR-1-1 promoter CGI in normal colonic tissue (n = 20), colorectal adenomas (n = 78) and primary CRC tumors (n = 90). B, Representative bisulfite sequencing results for the miR-1-1 promoter in a sample of normal colonic tissue and a primary CRC tumor. Open and filled circles represent unmethylated and methylated CpG sites, respectively. C, MTT assays with CRC cell lines transfected with a miR-1 precursor molecule or a negative control. Cell viabilities were determined 48 h after transfection. Values were normalized to cells transfected with the negative control. Shown are the means of eight replications; error bars represent standard deviations. 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 three replications; error bars represent standard deviations. 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’ 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, or a miR-17 precursor. Shown are the means of four replications; error bars represent standard deviations. H, Wound healing assay using HCT116 cells transfected with a
miR-1 precursor or a negative control. The wound was made 24 h after transfection, and pictures were taken at the indicated time points. 1, 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 arrows. Shown on the right are the means of three random microscopic fields per membrane; error bars represent the standard deviations.
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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.
Figure 1

A

HCT116

H3K4me3
H3K79me2
H3K27me3
H3K4me3
H3K79me2
H3K27me3

DKO

H3K4me3
H3K79me2
H3K27me3
H3K4me3
H3K79me2
H3K27me3

hsa-miR-200b
hsa-miR-200a
hsa-miR-429
CpG island

chr1:1,085,300 1,099,500

chr13:90,794,600 90,807,000

MIR-17-HG
MIR-17-HG
hsa-miR-17
hsa-miR-18a
hsa-miR-19a
hsa-miR-20a
hsa-miR-19b-1
hsa-miR-92a-1
CpG island

B

HCT116

H3K4me3
H3K79me2
H3K27me3
H3K4me3
H3K79me2
H3K27me3

DKO

H3K4me3
H3K79me2
H3K27me3
H3K4me3
H3K79me2
H3K27me3

BTG4
BC021736
BC021736
hsa-miR-34b
hsa-miR-34c

chr11:110,885,000 110,893,100

chr8:9,795,800 9,804,300

chr15:87,707,800 87,715,800

hsa-miR-9-3
AK054931
hsa-miR-124-1
CpG island

5 kb

2 kb

2 kb
Figure 2

A. chr3:52,273,600 - 52,290,500

H3K4me3

hsa-let-7g

CpG island

chr1:9,124,600 - 9,173,500

H3K4me3

hsa-miR-34a

CpG island

B. chr19:13,843,000 - 13,848,500

H3K4me3

hsa-miR-181c

hsa-miR-181d

CpG island

chr12:6,939,000 - 6,946,000

H3K4me3

PTPN6

PHB2

hsa-miR-200c

hsa-miR-141

CpG island

C. Pie chart showing distribution of distances:

- <2 kb
- 2-5 kb
- 5-10 kb
- >10 kb

D. Pie chart showing distribution of distances:

- <2 kb
- 2-5 kb
- 5-10 kb
- >10 kb
Expression analysis

miRNA microarray
miRNA TaqMan array

Chromatin signature analysis

Presumed promoter regions of 174 transcription units encoding 233 pre-miRNAs are identified

Comparison between HCT116 and DKO

37 transcription units encoding 47 pre-miRNAs are potential targets of epigenetic silencing

22 of the presumed promoters are associated with CpG islands (CGIs)

DNA methylation analysis

22 CGIs are hypermethylated in HCT116

B

No CGI methylation (22 pre-miRNAs)
CGI methylation (25 pre-miRNAs)

D

Normalized miRNA expression levels (log2)

HCT116
DKO

Normalized host gene expression levels (log2)

HCT116
DKO

E

CGI methylated miRNAs

Transcriptionally active miRNAs

SUZ12
K27

SUZ12
K27

(%) 100
80
60
40
20
0

(%) 100
80
60
40
20
0

ND negative positive

H3K4me3
H3K79me2
H3K27me3

H3K4me3
H3K79me2
H3K27me3

C20orf200
C20orf166

hsa-miR-1-1

hsa-miR-146a

chr20:60,556,000 60,564,000

2 kb

chr5:159,825,000 159,848,500

5 kb

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Figure 4

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B

Graphs showing methylation levels and miR/U6 ratios for different miRNAs in various cell lines and samples.
Figure 5

A

Methylation (%)

Normal  Cancer

B

Normal

Cancer

0 50 100 150 200 250 bp

C

HCT116  DLD1  RKO

Cell viability (%)

Control  Pre-miR-1  Control  Pre-miR-1  Control  Pre-miR-1

D

Control  miR-1-1

Colony formation (%)

E

Annexin A2

β-actin

Pre-miR-1  Pre-miR-17

F

ANXA2 3' UTR 485 bp

210 bp

Position 197-203 of ANXA2 3' UTR

ANXA2 5'...AAGCCAAAGAAAUGAACAUUCCA...

miR-1 3' GGUGUGUGAAGGAAGUAAGGU

G

Relative luc activity (%)

Vector  ANXA2

H

Control  0 h  8 h  18 h  28 h

Pre-miR-1

I

Control  Pre-miR-1

Cell invasion (%)

control  pre-miR-1  pre-miR-17
Genome-wide profiling of chromatin signatures reveals epigenetic regulation of microRNA genes in colorectal cancer

Hiromu Suzuki, Shintaro Takatsuka, Hirofumi Akashi, et al.

Cancer Res  Published OnlineFirst July 6, 2011.

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