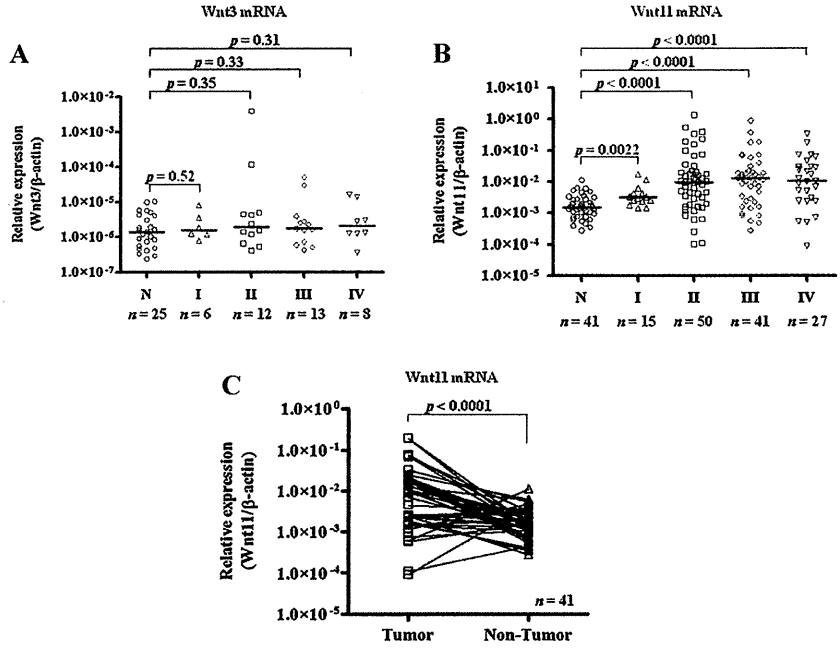

Figure 5. Wnt3 (A) and Wnt11 (B) mRNA levels in primary colorectal tumor tissues and non-tumor tissues. I–IV indicates the pathological stage of colorectal cancer. The horizontal lines represent the median level in each group. (C) The mRNA expression levels of Wnt11 were compared in 41 paired primary colorectal tumor and non-tumor tissues. All values were normalized for β-actin.

significantly higher in patients with higher FZD7 mRNA levels than in those with the lower FZD7 mRNA levels ($P = 0.00013$ by Mann–Whitney *U*-test; Figure 7A) (FZD7 high or low indicates patients with the FZD7 mRNA levels $\geq$ or $<$ the median value of all tumors tested). Their expression levels of Wnt11 mRNA were statistically correlated with those of FZD7 mRNA ($r = 0.481$, $P < 0.0001$ by two tailed Spearman's test; Figure 7B). In addition, we divide all of the patients to the combination of Wnt11 and FZD7 expression levels into four groups according to the median to evaluate the impact of these molecular profiles on the recurrence rates. (FZD7-high and Wnt11-high group ($n = 40$), FZD7-high and Wnt11-low group ($n = 25$), FZD7-low and Wnt11-high group ($n = 26$), FZD7-low and Wnt11-low group ($n = 42$)). FZD7-high and Wnt11-high group was significantly associated with shorter disease free survival compared to FZD7-low and Wnt11-low group ($P = 0.016$ by Kaplan–Meier analysis; Figure 7C).

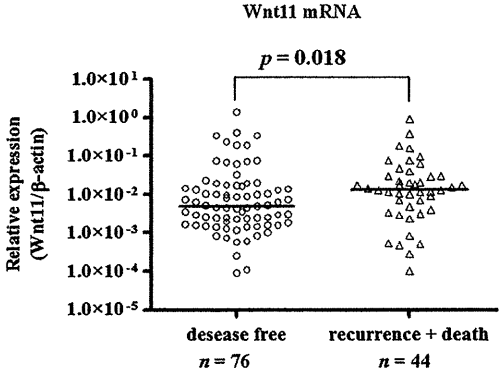

Figure 6. Comparison of Wnt11 mRNA expression levels between patients with disease free after surgery and those with recurrence or death after surgery. Disease free indicated a patient group with no recurrence after surgery. Recurrence + death indicated a patient group with recurrence or death after surgery. The horizontal lines represent the median level in each group. All values were normalized for β-actin.

DISCUSSION

Our present findings show, for the first time, that Wnt11 mRNA expression was increased in primary CRC tissues compared to adjacent non-tumor tissues, and that it was of prognostic significance and correlated with FZD7 mRNA expression. In contrast, Wnt3 mRNA expression was markedly low in both cell lines and primary CRC tissues and there was no significant difference in the

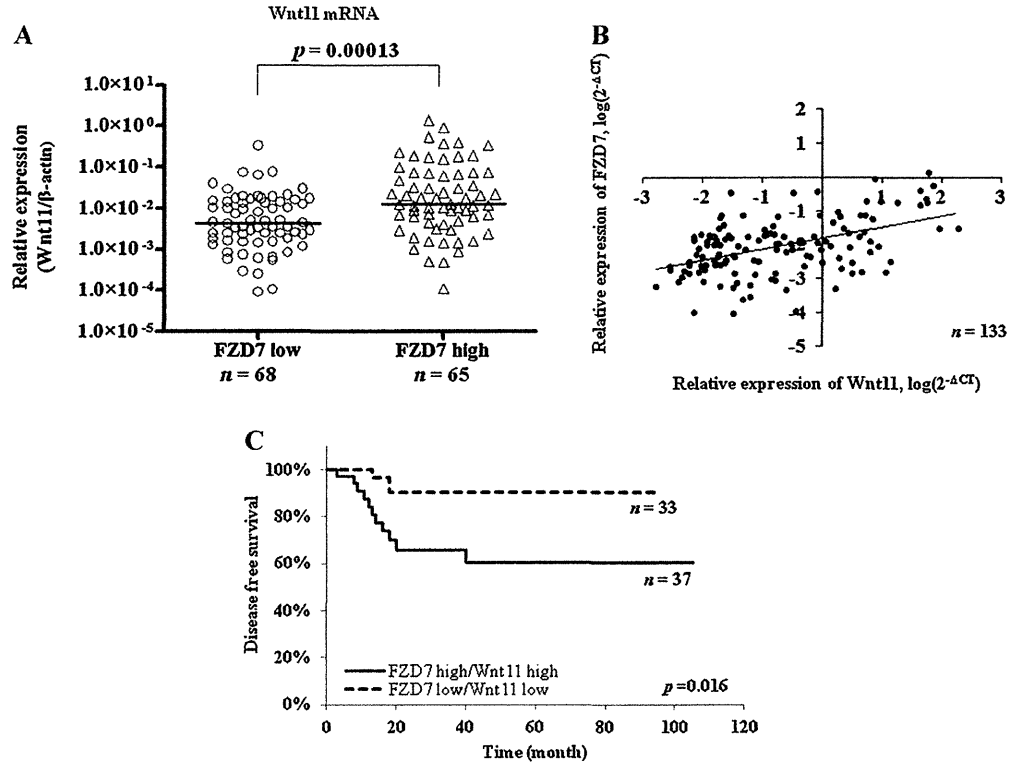

Figure 7. Relationship of the expression level of Wnt11 mRNA with that of FZD7 mRNA in primary colorectal tumor tissues. (A) FZD7 high or low indicates the patients with the FZD7 mRNA levels ≥ or < the median value of all tumors tested. The horizontal lines represent the median level in each group. (B) Correlation between Wnt11 and FZD7 mRNA expression level in CRC tissues. (C) Kaplan–Meier analysis for recurrence of patients. FZD7 high/Wnt11 high indicates the patients with high levels of FZD7 and Wnt11 mRNAs. FZD7 low/Wnt11 low indicates the patients with the low levels of FZD7 and Wnt11mRNAs. All values were normalized for β-actin.

expression level between tumor and non-tumor primary CRC tissues. These findings are consistent with a previous report showing no detectable Wnt3 mRNA in the murine colon by in situ hybridization [22].

Wnt11 is a non-canonical Wnt protein and represses the canonical Wnt signaling [23]. It is expressed in a wide variety of mouse tissues including small intestine and colon [24]. A role for Wnt11 in tumorigenesis has been implicated as it is expressed in prostate and breast cancer cell lines [19,25] and primary prostate cancer tissues [19]. It should be noted that Wnt11 is expressed in androgen-independent prostate cancer cell lines and high-grade prostatic tumors, and it has been reported that stable transfection of the Wnt11 gene inhibits androgen receptor transcriptional activity and cell growth in androgen-dependent prostate cancer cells, but not in androgen-independent cells [19]. Wnt11 has also been shown to induce transformation of non-transformed mouse C57MG mammary epithelial cells [26] and rat IEC6 intestinal epithelial cells [24] via autocrine and/or paracrine signaling mechanisms. On the other hand, Wnt11 did not promote cell growth in MCF-7 (breast cancer), CHO-K1, and LNCap/PC-3 (prostate cancer) cells [20,21,25] but increased cell viability by reducing apoptosis [20,21,25]. Those findings suggest that the tumor-promoting activity of Wnt11 may vary depending on the type of cell. In this study, Wnt11 transfectants of colon cancer HCT-116 cells showed the increased proliferative activity compared to mock cells as was found in mouse C57MG mammary epithelial cells and rat IEC6 intestinal epithelial cells [24,26]. It was also found that migration and invasion activities were increased in Wnt11 transfectans of HCT-116 cells. In previous reports, Wnt11 decreased the migratory activity of CHO-K1 cells [20] whereas it increased that of prostate and breast cancer cells [21,27].

Our previous study demonstrated that down-regulation of FZD7 with siRNA in HCT-116 cells resulted in decreased phosphorylation of JNK and

c-*jun*, suggesting the involvement of non-canonical Wnt/JNK signaling pathway in the tumor-promoting activity of FZD7 [14]. We therefore examined the expression and phosphorylation of JNK and c-*jun* in this study, and revealed that their phosphorylation levels were increased in Wnt11 transfectants compared to mock cells. As described above, CHO-K1 cells were shown to decrease their migratory activity after Wnt11 transfection [20]. In that report, phosphorylation levels of JNK and c-*jun* were reduced in the transfectants compared with control cells. This suggests that Wnt11 might regulate cell migration activity at least partly through activation or inactivation of Wnt/JNK signaling pathway depending on the type of cell.

Accumulating evidence suggests that Wnt11 interacts with FZD7 [15–17,28]. However, these studies have been restricted to cell or developmental biology, and there have thus far been no study with cancer cells. Wnt11 stimulates proliferation, migration, cytoskeletal rearrangement, and contact-independent growth of rat intestinal epithelial IEC6 cells with increased PKC and Ca^{2+}/calmodulin-dependent protein kinase II activity [24]. Although it is unknown whether IEC6 cells express FZD7, Wnt11 is expressed in normal murine intestine and colon [24] where FZD7 mRNA expression has been shown by in situ hybridization [22]. In this study, we found that FZD7 siRNA transfer into Wnt11 transfectants decreased their migration activity, whereas it did not affect the invasion ability (Figure 4). These data suggested that FZD7 could be involved in Wnt11-induced migration activity of HCT-116 cells, but the Wnt11-induced invasion ability might occur mainly through some receptor other than FZD7. There is accumulating evidence that Wnt11 may interact with FZD4, FZD5, or LRP5/6 [29]. Furthermore, our present and previous data on Wnt11 and FZD7 expressions [13,14] revealed that both Wnt11 and FZD7 mRNAs are expressed in both human colon cancer cell lines and primary CRC tissues, and we observed a significant correlation between these levels of mRNA. An important finding of this report is the prognostic significance of Wnt11 and FZD7 expressions in primary CRC tissues. Wnt11 expression was higher in the recurrence + death group than in the disease free group (Figure 6), and FZD7-high and Wnt11-high group had worse disease free survival than FZD7-low and Wnt11-low group (Figure 7C). Taken together, these findings support the idea that the interaction between Wnt11 and FZD7 may play an important role in CRC progression.

In preparation of this manuscript, it was reported that Wnt11 expression was decreased in hepatocellular carcinoma (HCC) tissues compared to adjacent non-tumor tissues, and that Wnt11 inhibited HCC cell proliferation and migration [30]. This is reminiscent of the opposing roles of Wnt5a, a non-canonical Wnt, in various cancers [31]. Wnt5a functions as tumor suppressor in a number of cancers including colorectal, breast, hepatocellular and thyroid cancers, whereas it is oncogenic in gastric, pancreatic, prostatic cancers and melanoma [27]. Further studies are warranted to disclose whether Wnt11 has similar roles in various cancers.

In conclusion, this is the first report to show that Wnt11 may play an important role in the regulation of proliferation and migration/invasion activities of CRC cells at least partly through Wnt/JNK signaling pathway, and that the expression level of Wnt11 mRNA was increased and correlated with that of FZD7 in primary CRCs. These results suggest that Wnt11-FZD7 interaction might be involved in CRC progression.

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REVIEW

Emerging links between epigenetic alterations and dysregulation of noncoding RNAs in cancer

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Abstract Epigenetic changes, including DNA methylation and histone modification, play key roles in the dysregulation of tumor-related genes, thereby affecting numerous cellular processes, including cell proliferation, cell adhesion, apoptosis, and metastasis. In recent years, numerous studies have shown that noncoding RNAs (ncRNAs) are key players in the initiation and progression of cancer and epigenetic mechanisms are deeply involved in their dysregulation. Indeed, the growing list of microRNA (miRNA) genes aberrantly methylated in cancer suggests that a large number of miRNAs exert tumor-suppressive or oncogenic effects. In addition, it now appears that long ncRNAs may be causally related to epigenetic dysregulation of critical genes in cancer. Dissection of the relationships between ncRNAs and epigenetic alterations may lead to the development of novel approaches to the diagnosis and treatment of cancer.

Keywords Noncoding RNA · MicroRNA · lincRNA · DNA methylation · Histone modification

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Introduction

Epigenetics are inherited factors that influence gene activity but do not alter primary DNA sequences; among them, DNA methylation and histone modification are key events that silence gene expression. Cancer is thought to arise through the accumulation of multiple genetic alterations that lead to activation of oncogenes and loss of function of tumor suppressor genes. However, a growing body of evidence now suggests that, in addition to genetic alterations, epigenetic changes such as DNA methylation and histone modification also play crucial roles in the development and progression of human malignancies [1].

The first identified cancer-related change in DNA methylation was genome-wide hypomethylation [2]. Subsequently, however, it became apparent that hypermethylation of 5′ CpG islands is crucial for silencing tumor suppressor genes. Although the classical two-hit theory posits that tumor suppressor genes are inactivated by gene mutation or deletion, it is now recognized that DNA hypermethylation is a third mechanism by which inactivation of tumor suppressor genes occurs and that it plays a significant role in tumorigenesis. The specific mechanisms by which DNA methylation is altered in cancer remain unclear, however.

To date, approximately half of the classical tumor suppressor genes known to be mutated in familial cancer syndromes have been shown to be inactivated by promoter hypermethylation. These include *RB*, *VHL*, *CDKN2A* (*p16INK4A*), *CDH1* (*E-cadherin*), and *BRCA1* [1, 2]. In addition to these classical tumor suppressors, increasing numbers of genes related to cell-cycle control, DNA repair, tumor invasiveness, and the response to growth factors are being identified as inactivated by hypermethylation in malignancies [3, 4]. In recent years, moreover, noncoding RNAs (ncRNAs) have been attracting the interest of many

researchers, and the accumulated evidence suggests that they too are strongly involved in cancer. For example, altered expression of microRNAs (miRNAs) is a common feature of malignancies [5–7], and epigenetic mechanisms are strongly involved in the dysregulation of miRNAs in cancer [8]. Moreover, recent studies have shown that certain ncRNAs are causally related to epigenetic alterations in cancer cells. In this review, we will highlight the contribution made by epigenetic alteration of ncRNA genes in cancer and discuss their clinical application as biomarkers and therapeutic targets.

Epigenetic dysregulation of miRNAs in cancer

miRNAs are a class of small ncRNAs that regulate gene expression by inducing translational inhibition or direct degradation of target mRNAs through base pairing to partially complementary sites [9]. miRNA genes are transcribed as large precursors, 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 Patha to produce ~70-nt hairpin-structured second precursors, called pre-miRNAs. The pre-miRNAs are transported to the cytoplasm and processed by another RNase III enzyme, Dicer, to generate mature miRNA products. miRNAs are highly conserved among species and play critical roles in a variety of biological processes, including cell proliferation, development, differentiation, and apoptosis. In addition, subsets of miRNAs are thought to act as tumor suppressor genes or oncogenes, and their dysregulation is a common feature of human cancers [6, 7]. More specifically, expression of miRNAs is generally downregulated in tumor tissues, as compared to corresponding healthy tissues, which suggests that some miRNAs may behave as tumor suppressors in some tumors. Although the mechanism underlying the alteration of miRNA expression in cancer is still not fully understood, recent studies have shown that cancer affects multiple mechanisms involved in regulating miRNA levels. For example, genetic mutations that affect proteins involved in the processing and maturation of miRNA, such as *TARBP2* and *XPO5*, can lead to overall reductions in miRNA expression [10, 11]. In addition, epigenetic alterations also appear to be a major mechanism by which the normal patterns of miRNA expression are disrupted in cancer (Table 1).

Pharmacological or genetic unmasking through DNA demethylation and/or histone deacetylase (HDAC) inhibition is a common method of identifying epigenetically silenced genes in cancer, and the link between DNA methylation and miRNA expression in cancer was first identified using this technique. Microarray-based screening of miRNAs in T24 human bladder cancer cells and normal

Table 1 Epigenetically silenced miRNA genes in cancer

Name	Tumor type	Target genes	Reference
miR-1-1	Colon, liver	*FOXP1*, *MET*, *HDAC4*, *ANXA2*	[52, 53]
miR-9 family	Multiple types	*FGFR1*, *CDK6*, *CDX2*, *E-cadherin*	[18, 24, 29, 38, 39, 51, 70, 71]
miR-34 family	Multiple types	*MET*, *CDK4*, *CCNE2*, *C-MYC*	[14, 15, 24, 51, 72–74]
miR-124 family	Multiple types	*CDK6*	[13, 20, 21, 51, 71, 75, 76]
miR-125b	Breast	*ETS1*	[77]
miR-127	Prostate, bladder, colon	*BCL6*	[12]
miR-129-2	Endometrium, colon	*SOX4*	[29, 78]
miR-137	Head and neck, colon, stomach	*CDK6*, *LSD1*, *CDC42*	[15, 28–32]
miR-148a	Colon, pancreas	*TGIF2*	[24, 79]
miR-152	Endometrium, bladder, lung	*DNMT1*, *E2F3*, *MET*, *Rictor*	[18, 71, 80]
miR-181c	Stomach	*NOTCH4*, *KRAS*	[17]
miR-196b	Stomach		[81]
miR-200 family	Bladder, lung, colon	*ZEB1*, *ZEB2*	[44, 45, 82]
miR-203	Leukemia, liver, MALToma	*ABL1*, *ABCE1*, *CDK6*	[26, 27, 51]
miR-205	Bladder, lung	*ZEB1*, *ZEB2*	[44, 45]
miR-218	Head and neck	*Rictor*	[83]
miR-941	Colon		[19]
miR-1224	Bladder		[18]
miR-1237	Colon		[19]
miR-1247	Colon		[19]

fibroblasts (LD419) followed by treatment with 5-aza-2′-deoxycytidine, a DNA methyltransferase inhibitor, and 4-phenylbutyric acid, a histone deacetylase inhibitor, revealed cancer-specific upregulation of miR-127 by the drugs [12]. Similar upregulation of miR-127 by epigenetic drug treatment has been observed in variety of other human cancer cells. On the other hand, miR-127 was also downregulated by the drugs in primary tumors of the prostate, bladder, and colon. Experimental evidence confirmed that the proto-oncogene *BCL6* is a target of miR-127, suggesting that it can act as a tumor suppressor [12]. Consistent with that idea, the miR-127 gene is embedded in a typical CpG island, which was hypermethylated in cancer cells, suggesting that the expression of miR-127 is epigenetically silenced in cancer. Importantly, acetylation of histone H3 and trimethylation of histone H3 lysine 4 (H3K4) restore miR-127 expression, confirming that DNA methylation and histone modification are linked in the regulation of miRNA gene expression in cancer.

Several groups have carried out expression-based screening for epigenetically silenced miRNAs in various cancers, including colorectal, oral, gastric, and bladder cancers [13–19]. Methylation of the miR-124 family (miR-124-1, miR-124-2, and miR-124-3) was first discovered in colorectal cancer by analyzing HCT116 colorectal cancer cells, in which both *DNMT1* and *DNMT3B* were knocked out [13]. Since then, methylation of miR-124 family genes has been reported in gastric cancer, acute lymphoblastic leukemia, and hepatocellular carcinoma [20–22]. miR-124 is thought to exert tumor suppressor effects by targeting *CDK6* and inducing phosphorylation of Rb protein [13, 21].

Members of the miR-34 family (miR-34a, miR-34b, and miR-34c) are direct targets of p53, and their ectopic expression induces cell-cycle arrest and apoptosis in cancer cells [23]. Pharmacological or genetic unmasking of epigenetically silenced miRNAs in oral, colorectal, and gastric cancer cells revealed that downregulation of miR-34b/c is associated with hypermethylation of the neighboring CpG island [14–16]. The CpG island of miR-34b/c is frequently methylated, silencing the gene, in primary colorectal cancers, but exogenous expression of miR-34b/c in the cells inhibits cell growth and induces dramatic changes in the gene expression profile [14]. Notably, methylation of miR-34b/c has also been linked to cancer metastasis [24]. These findings, as well as its contribution to the p53 network, imply that miR-34b/c acts as a tumor suppressor in cancer. Consistent with that idea, introduction of miR-34b/c into cancer cells downregulates candidate target genes, including *MET*, *CDK4*, *CCNE2*, and *C-MYC* [14, 24]. Likewise, restoration of endogenous miRNA expression through demethylation also downregulates target genes, suggesting that miRNAs could be important targets for epigenetic cancer therapy [14]. In addition, we recently reported that methylation of a panel of genes, including miR-34b/c, in mucosal wash fluid collected during colonoscopy could be a useful biomarker for predicting the invasiveness of colorectal cancers [25].

miR-203 is a candidate tumor suppressor targeted for epigenetic silencing in oral cancer, hematopoietic malignancies, and hepatocellular carcinoma [15, 22, 26]. miR-203 directly controls *ABL1* expression, and it also targets *BCR-ABL1* translocation protein induced by Philadelphia chromosome in chronic myelogenous leukemia and B cell acute lymphoblastic leukemia in children. The CpG island of miR-203 is specifically methylated in Philadelphia-positive tumors, as compared to other hematopoietic malignancies, suggesting that epigenetic downregulation of miR-203 enhances the expression of *BCR-ABL1* oncogene. Epigenetic silencing of miR-203 has also been shown to activate *ABL1* in *Helicobacter*-associated gastric mucosa-associated lymphoid tissue lymphoma [27]. Conversely, expression of miR-203 suppresses cellular growth and downregulate other possible target genes, including *ABCE1* and *CDK6*, in hepatocellular carcinoma [22].

Methylation of the CpG island of miR-137 was first discovered in oral cancer [15] and was subsequently reported in colon [28, 29] and gastric cancers [30]. miR-137 methylation is associated with a poorer survival rate among patients with head and neck squamous cell carcinoma (HNSCC) [31] and was detected in oral rinses collected from HNSCC patients, suggesting its utility as a cancer biomarker [32]. Within cancer cells, miR-137 targets *CDK6*, *LSD1*, and *CDC42*, indicating it to be a tumor suppressor [15, 28, 33], whereas in normal cells, miR-137 regulates neuronal differentiation by targeting *EZH2* and *MIB1* [34, 35].

Methylation of several miRNA genes has been identified using genome-wide DNA methylation analysis. Methylated CpG island amplification (MCA) was first developed by Toyota et al. [36], and the combination of MCA and CpG island microarray (MCAM) analysis is a high-throughput method for determining methylation status with high specificity and sensitivity [37]. Global gene promoter methylation analysis using MCAM identified miR-9-1 methylation in pancreatic cancer cells [38]. Methylation of miR-9 family genes (miR-9-1, miR-9-2, and miR-9-3) was also identified in metastatic cancer cell lines [24] and is associated with metastatic recurrence of renal cell carcinoma [39]. miR-9 has been shown to target *FGFR1* and *CDK6* in acute lymphoblastic leukemia [40] and *CDX2* in gastric cancer cells [41], suggesting it to function as a tumor suppressor. In contrast to these results, however, one recent study showed that miR-9 is activated by MYC and MYCN in breast cancer and that it promotes metastasis through downregulation of *E-cadherin* [42]. These results are indicative of the functional complexity of miRNAs in cancer and suggest that miRNAs may exert opposite effects in different tissues or settings.

The miR-200 family (miR-200a, miR-200b, miR-200c, miR-141, and miR-429) and miR-205 act as key regulators of epithelial–mesenchymal transition (EMT) by directly targeting *ZEB1* and *ZEB2*, which are transcriptional repressors that downregulate *E-cadherin* [43]. Downregulation of miR-200 family and miR-205 in bladder cancer cells is associated with DNA methylation and repressive histone marks at their promoters [44]. Epigenetic silencing of the miR-200 family and miR-205 was also observed in carcinogen-treated lung epithelial cells, suggesting that induction of EMT through miRNA dysregulation occurs early during lung carcinogenesis [45].

Most studies on the epigenetic silencing of miRNA genes in cancer have focused on CpG island hypermethylation. However, one recent study showed that in bladder cancer, CpG island shore methylation is frequently associated with miRNA downregulation. CpG island shores are regions located within 2 kb of CpG islands, and their methylation strongly affects gene expression [46]. Microarray analysis of miRNA expression coupled with demethylating treatment in bladder cancer cells revealed a number of epigenetically silenced miRNA genes [18]. Interestingly, for several miRNAs, the hypermethylation was more frequent in the CpG island shore region than in the CpG island (miR-9, miR-149, miR-210, miR-212, miR-328, miR-503, miR-1224, miR-1227, and miR-1229). Methylation of these miRNA genes is associated with tumor grade, stage, and prognosis, and the expression levels of the silenced miRNAs are also downregulated in urine specimens from bladder cancer patients, suggesting their utility as diagnostic biomarkers. So far, the regulatory role of the CpG island shore methylation on miRNA gene silencing has not been reported in other cancer types, and it might be specific to bladder cancer. Further study will be needed to clarify the association of CpG island shore methylation with miRNA expression in cancer.

Although epigenetic dysregulation leads to the silencing of a number of miRNAs in cancer, several are upregulated through epigenetic mechanisms. The CpG island of let-7a-3 is heavily methylated in normal cells but is hypomethylated in lung adenocarcinoma, leading to its elevated expression [47]. In lung cancer cells, let-7a-3 exerts oncogenic effects through action on a number of genes involved in cell proliferation, adhesion, and differentiation. Another study showed that elevated expression of miR-375 in estrogen receptor α (ERα)-positive breast cancer cells promotes tumor cell proliferation [48]. Overexpression of miR-375 is caused by loss of repressive histone marks and DNA methylation, which leads to dissociation of the transcriptional repressor CTCF from the miR-375 promoter and the binding of ERα to its regulatory region. In addition, whereas miR-200a and miR-200b are downregulated in many cancer types (see above), they are overexpressed in pancreatic cancer due to hypomethylation [49]. Their elevation in the serum of pancreatic cancer patients means that miR-200a and miR-200b could potentially serve as diagnostic biomarkers.

Chromatin signatures of miRNA genes in cancer

As with protein-coding genes, epigenetic silencing of miRNA genes is strongly linked to histone modification, including loss of trimethylated histone H3 lysine 4 (H3K4me3) and increases in di- and trimethylated H3 lysine 9 (H3K9me2 and H3K9me3) and trimethylated H3 lysine 27 (H3K27me3). Several studies have utilized histone modifications to identify dysregulated miRNAs in cancer. The combination of chromatin immunoprecipitation (ChIP)-on-chip and miRNA microarray analyses in prostate cancer cells revealed that miRNA expression is positively correlated with H3K4me3 and inversely correlated with H3K27me3 in the miRNA promoter regions [50]. Analysis of histone modification using ChIP-on-chip analysis in acute lymphoblastic leukemia revealed that the CpG islands of 13 miRNA genes are associated with high H3K9me2 and low H3K4me, suggesting that these miRNAs are epigenetically silenced in acute lymphoblastic leukemia [51]. Subsequent methylation analysis confirmed the hypermethylation of the selected miRNAs, including miR-9 family, miR-34 family, and miR-124 family genes. Notably, acute lymphoblastic leukemia patients with miR gene methylation showed significantly poorer disease-free survival and overall survival, suggesting that epigenetic silencing of miRNAs is an important prognostic factor.

We recently used ChIP-seq analysis to carry out genome-wide chromatin signature analysis in colorectal cancer cells [52]. Using H3K4me3 as a hallmark of the active promoter region, we identified presumed promoter regions of 233 miRNA genes. By comparing the expression and H3K4me3 status before and after DNA demethylation, we found that 47 miRNAs encoded in 37 primary transcripts are targets of epigenetic silencing in colorectal cancer. Among them, CpG island methylation was observed in 22 miRNA genes, including miR-1-1. Methylation of miR-1-1 was found in approximately 70% of colorectal adenomas and 80% of colorectal cancers, indicating that this methylation is an early event in colorectal tumorigenesis. Downregulation of miR-1 is also involved in hepatocellular carcinoma and lung cancer, and its restoration inhibited cellular growth and suppressed potential target genes, including *MET*, *HDAC4*, and *FOXP1* [53, 54]. Similarly, expression of miR-1 in lung cancer and colorectal cancer cells inhibited cellular migration and invasion [52, 54] and targeted *ANXA2* and *BDNF*, two genes reportedly overexpressed in cancer and implicated in invasion and metastasis [52].

General features and functions of long ncRNAs

Long ncRNAs are generally defined as transcribed RNA molecules greater than 200 nt in length [55]. Although small ncRNAs, including miRNAs, small interfering RNAs, and piwi-interacting RNAs, have been intensively studied over the last decade, only a small number of long ncRNAs have been functionally characterized, and it remains uncertain whether these molecules have specific functions or just represent by-products of RNA polymerase infidelity. This fundamental question was addressed in several recent studies using high-throughput sequencing technology and bioinformatics. Here we briefly outline what is known about long intergenic ncRNAs (lincRNAs).

Using powerful bioinformatics analysis, Cabili et al. generated a reference catalog of 8,195 human lincRNAs based on integrated RNA-seq data from 24 tissues and cell types [56]. Their analysis and the resultant lincRNA dataset are comprehensive and useful. There are several differences and similarities between lincRNAs and protein-coding mRNAs. Like protein-coding transcripts, lincRNAs are transcribed by RNA polymerase II, spliced, and polyadenylated, but the maximum expression level of lincRNAs is ten times lower than that of protein-coding transcripts. lincRNAs are also smaller in size than protein-coding RNAs, and they have fewer exons; on average, lincRNAs are ~1 kb in length with 2.9 exons, whereas protein-coding transcripts are ~2.9 kb in length with 10.7 exons. In addition, lincRNAs are alternatively spliced more frequently than protein-coding mRNAs (2.3 isoforms per lincRNA locus, on average). It is noteworthy that the vast majority of lincRNAs exhibit tissue-specific expression patterns: 78% of examined lincRNAs are tissue-specific, but only 19% of protein-coding transcripts are so. Tissue (cell or context)-specific expression of lincRNAs was also reported elsewhere [57, 58], suggesting that their highly specific expression patterns is an important feature of lincRNAs.

The results summarized above support the notion that lincRNAs are actively regulated rather than just by-products and that they likely have specific biological functions. To test that idea, Guttman and colleagues performed an unbiased loss-of-function analysis of 147 lincRNAs expressed in murine embryonic stem cells and showed that lincRNAs primarily act in *trans* to affect global gene expression [59]. They identified 26 lincRNAs that have major effects on endogenous Nanog levels, leading to the knockdown of known protein-coding regulatory genes involved in pluripotency (e.g., Oct4), which suggests that lincRNAs may be involved in maintaining the pluripotent state. They also found that lincRNAs are direct transcriptional targets of pluripotency-associated transcription factors and are dynamically expressed throughout differentiation. Thus, at least some lincRNAs are clearly functional and may be integral components of mechanisms involved in determination of lineage specificity and stem cell biology. Collectively, these studies suggest that lincRNAs are important regulatory components functioning at the same level as protein-coding genes and that some lincRNAs may act as transcription factors.

Role of lincRNA as a scaffold for chromatin-modifying complexes

lincRNAs appear to be involved in virtually all steps of gene regulation, including transcription, mRNA splicing, and translation. Among their functions, one of the most important with regard to epigenetic regulation is their role as a scaffold for chromatin-modifying complexes. The concept originated with the discovery of *HOTAIR* by Rinn and colleagues [60]. The lincRNA *HOTAIR* is encoded within the *HOXC* gene cluster and acts in *trans* to regulate *HOXD* genes through the recruitment of Polycomb repressive complex 2 (PRC2) to induce trimethylation of H3K27 (H3K27me3). Remarkably, pull-down experiments with PRC2 components demonstrated a direct and specific interaction with *HOTAIR*. The observation that *HOTAIR* binds PRC2 and induces epigenetic silencing of another *HOX* cluster on a different chromosome was an unexpected and novel finding. Following this discovery, several other long ncRNAs were found to associate with chromatin-modifying complexes, including *XIST*, which recruits PRC2 to the inactive X chromosome [61]; *AIR*, which is associated with the H3K9me2 methyltransferase G9a [62]; *Kcnq1ot1*, which binds both G9a and PRC2 [63]; and *ANRIL*, which interacts with components from both PRC1 and PRC2 [64].

A later study revealed that *HOTAIR* interacts with two chromatin-modifying complexes, the PRC2 complex ("writer" of K27 repressive marks) and the LSD1/CoREST H3K4 demethylase complex ("eraser" of activating marks) [65]. Using a series of deletion mutants, the PRC2 binding domain was mapped to the 5′ end (the first 300 nt) of *HOTAIR*, whereas the LSD1 binding site corresponds to the 3′ end of *HOTAIR*. This observation suggests that *HOTAIR* acts as a scaffold that bridges between the PRC2 and LSD1 complexes and that the *HOTAIR*/PRC2/LSD1 complex can suppress gene expression via multiple mechanisms. This finding does not apply only to *HOTAIR*, as many other lincRNAs also appear to interact with both the PRC2 and LSD1 complexes. For example, Khalil and colleagues performed RIP-chip assays (RNA coimmunoprecipitation combined with high-throughput lincRNA microarray) using antibodies directed against several proteins involved in chromatin-modifying complexes (PRC2, CoREST, and SMCX) [66]. They found that as many as 38% of lincRNAs expressed in the cell types studied are reproducibly

associated with one of these complexes. In mouse embryonic stem cells, moreover, a number of lincRNAs were found to be strongly associated with multiple chromatin complexes [59]. For example, eight lincRNAs bind to the Prc2 H3K27 and Eset H3K9 methyltransferase complexes and the Jarid1c H3K4 demethylase complex. Similarly, 17 lincRNAs were found to bind to the Prc2, Prc1, and Jarid1b complexes. Taken together, these results suggest the attractive hypothesis that lincRNAs bind to ubiquitously expressed chromatin-modifying complexes in order to guide them to specific genomic regions.

The findings summarized above suggest that differentially expressed lincRNAs help to establish cell type-specific epigenetic states. However, the mechanism by which lincRNAs specifically regulate their target genes remains unclear, and their binding sites throughout the genome are largely unknown. In a recent study, Chu and colleagues addressed this question using a novel assay they named chromatin isolation by RNA purification and sequencing, which is a method for mapping genome-wide long ncRNA binding sites in vivo [67]. This analysis enabled them to obtain a high-resolution map of ncRNA occupancy throughout the genome and to identify a set of 832 *HOTAIR* binding sites in human breast cancer cells. Interestingly, binding sites for *HOTAIR* are focal (<500 bp) and located in the midst of a broad Polycomb binding domain. This suggests that *HOTAIR* may serve as a pioneering factor that recruits Polycomb, which then spreads out bilaterally. They also discovered an underlying DNA sequence motif enriched in *HOTAIR* binding sites, indicating the existence of a new class of regulatory element: long ncRNA target sites. We now think that long ncRNAs function like sequence-specific transcription factors.

Dysregulation of long ncRNAs in human cancer

In breast cancer, increased expression of *HOTAIR* reportedly correlates with a poor prognosis and tumor metastasis [68]. It is noteworthy that expression of a single lincRNA in primary tumors can be a powerful predictor of eventual metastasis and death. Enforced expression of *HOTAIR* induces genome-wide re-targeting of PRC2, leading to altered H3K27me3 and gene expression and increased cancer invasiveness and metastasis. The link between *HOTAIR* and metastatic disease depends on the direct interaction between the ncRNA and its protein partner and the association between the ncRNA and its target DNA sequence. This suggests that lincRNAs may play active roles in modulating the cancer epigenome and could be novel targets for cancer diagnosis and therapy.

More recently, Prensner and colleagues used high-throughput RNA-Seq with a large panel of clinical samples to comprehensively assess the long ncRNAs dysregulated in prostate cancer [69]. They identified approximately 1,800 long ncRNAs in prostate tissue, of which 121 were transcriptionally dysregulated in prostate cancer. Among those, prostate cancer associated transcript 1 (*PCAT-1*) showed tissue-specific expression and was selectively upregulated only in prostate cancer. Like *HOTAIR*, *PCAT-1* functions predominantly as a transcriptional repressor by facilitating *trans*-regulation of genes preferentially involved in mitosis and cell division, including known tumor suppressor genes such as *BRCA2*. The discovery of *PCAT-1* highlights the usefulness of unbiased transcriptome analysis to investigate the actions of long ncRNAs in cancer.

Concluding remarks

In this review, we highlighted the relationship between epigenetic alteration and dysregulation of ncRNAs in cancer. Aberrant DNA methylation and histone modification are the major mechanisms underlying miRNA dysregulation in cancer, and methylation of a subset of miRNA genes may be useful biomarkers for detecting cancer or predicting clinical outcome. Replacement of silenced tumor-suppressive miRNAs in cancer cells could be a promising strategy for cancer treatment. Moreover, emerging evidence suggests that long ncRNAs also play critical roles in cancer. It has been speculated that the human genome may harbor as many functional long ncRNAs as protein-coding genes, and it is plausible that dysregulation of long ncRNAs can drive the development and progression of tumors as effectively as protein-coding genes and miRNAs. We anticipate that further studies of small and long ncRNAs will lead to the discovery of novel biomarkers and therapeutic targets in cancer.

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Conflicts of interest None

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Tumorigenesis and Neoplastic Progression

Molecular Dissection of Premalignant Colorectal Lesions Reveals Early Onset of the CpG Island Methylator Phenotype

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The concept of the CpG island methylator phenotype (CIMP) in colorectal cancer (CRC) is widely accepted, although the timing of its occurrence and its interaction with other genetic defects are not fully understood. Our aim in this study was to unravel the molecular development of CIMP cancers by dissecting their genetic and epigenetic signatures in precancerous and malignant colorectal lesions. We characterized the methylation profile and *BRAF/KRAS* mutation status in 368 colorectal tissue samples, including precancerous and malignant lesions. In addition, genome-wide copy number aberrations, methylation profiles, and mutations of *BRAF*, *KRAS*, *TP53*, and *PIK3CA* pathway genes were examined in 84 colorectal lesions. Genome-wide methylation analysis of CpG islands and selected marker genes revealed that CRC precursor lesions are in three methylation subgroups: CIMP-high, CIMP-low, and CIMP-negative. Interestingly, a subset of CIMP-positive malignant lesions exhibited frequent copy number gains on chromosomes 7 and 19 and genetic defects in the *AKT/PIK3CA* pathway genes. Analysis of mixed lesions containing both precancerous and malignant components revealed that most aberrant methylation is acquired at the precursor stage, whereas copy number aberrations are acquired during the progression from precursor to malignant lesion. Our integrative genomic and epigenetic analysis suggests early onset of CIMP during CRC development and indicates a previously unknown CRC development pathway in which epigenetic instability associates with genomic alterations. ***(Am J Pathol 2012, 181:1847–1861; http://dx.doi.org/10.1016/j.ajpath.2012.08.007)***

Colorectal cancer (CRC) is a leading cause of cancer mortality worldwide, but the incidence of CRC can be reduced through detection and removal of colorectal adenomas. Notably, however, most small colorectal polyps do not progress to malignancy; thus, the identification of precursor lesions likely to become cancerous is also extremely important for reducing CRC mortality.

CRCs are thought to arise, in part, through the accumulation of genetic changes, including mutations of oncogenes and tumor suppressor genes.[1] It is also generally accepted that CRCs can exhibit either of two genetic instabilities: chromosomal instability (CIN) or microsatellite instability (MSI).[2] In addition to these genetic changes, epigenetic alterations, including DNA methylation and histone

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Table 1. Primer Sequences Used in this Study

Gene	Primer/Target	Forward	Reverse	Product size (bp)
Methylation Analysis				
CDKN2A	Pyroseq PCR	5′-GGTTGTTTTGGTTGGTGTTTT-3′	5′-Bio-ACCCTATCCCTCAAATCCTCTAAAA-3′	169
	Sequence primer	5′-TTTTTTTGTTTGGAAAGAT-3′		
	Target	5′-ATYGYG-3′		
DFNA5	Pyroseq PCR	5′-GGYGGAGAGAGGGTTYGTT-3′	5′-Bio-RAACCCCTCCCRCAACCT-3′	91
	Sequence primer	5′-YGGGYGTTTTAGAGT-3′		
	Target	5′-YGYGGGATTGGTYGTYG-3′		
DKK2	Pyroseq PCR	5′-GGGTTTTTTGATTAATTAAGAGGAGA-3′	5′-Bio-TCTACAATAACTAAAAACAATCAAATAC-3′	179
	Sequence primer	5′-TAATTAAGAGGAGAGTTAAA-3′		
	Target	5′-TYGTYGAGATTTYGGYG-3′		
DLX4	Pyroseq PCR	5′-GGTTTYGGTTTAGTTTTGGATTTAGTT-3′	5′-Bio-CAATTCTACTCCCAAAAAACTCCCA-3′	182
	Sequence primer	5′-TGTTTYGTTTTATTTTAAGT-3′		
	Target	5′-TGGYGTTATYGTTYG-3′		
FZD10	Pyroseq PCR	5′-GGGATTTATTATAAAAGGAAGAGAAGAT-3′	5′-Bio-AATAATCCCCRACACCCCRAAAAC-3′	129
	Sequence primer	5′-AAAGGAAGAGAAGATGTATT-3′		
	Target	5′-TYGYG-3′		
GALNT14	Pyroseq PCR	5′-GAGYGGGAAAGTTTTTTTAGGTATAG-3′	5′-Bio-CCTAAACRCAACTCCCAAACCATC-3′	153
	Sequence primer	5′-GAAAGTTTTTTTAGGTATAG-3′		
	Target	5′-YGTYGTTTGGYG-3′		
IGF2BP1	Pyroseq PCR	5′-GAAGGGGTTTGTAGAGTTTTAGGGA-3′	5′-Bio-CCCACCCACCCTACAAAAAAAAACC-3′	157
	Sequence primer	5′-TTGAGTTTTTTATTTTTAGG-3′		
	Target	5′-YGGGAGATTATYG-3′		
IGFBP7	Pyroseq PCR	5′-AGGGTTYGGGGTAGGGGATTGGGGAT-3′	5′-Bio-AAAACCACACCCCRAAACRATAAAAACAC-3′	208
	Sequence primer	5′-YGGGTGTTYGTTTATTTT-3′		
	Target	5′-TYGAYGTTAGTAGGAGYGYGYGYG-3′		
KCNV1	Pyroseq PCR	5′-TAAGGAGAGGTAATTTTTTYGGGAGTT-3′	5′-Bio-CGCTAAAAAACATCTCTAACCCAATC-3′	150
	Sequence primer	5′-GGAGTTYGGGGAATTT-3′		
	Target	5′-YGGTYG-3′		
LRP1B	Pyroseq PCR	5′-GATGTAAGATTAGAYGTATTTTGTATTG-3′	5′-Bio-AACCAATCAACCTTCTCCTACCTAA-3′	148
	Sequence primer	5′-TATTGAAAAGTTAAGATATA-3′		
	Target	5′-YGGGYGTTTYGTTYGYG-3′		
MEOX2	Pyroseq PCR	5′-TAGAGTTTGGAGGGTAGAGTTGTTGT-3′	5′-Bio-ATTCCACTTCCTATCTCCTACTAAAC-3′	137
	Sequence primer	5′-GGGTAGAGTTGTTGTTTTT-3′		
	Target	5′-TYGGGYG-3′		
MINT1	Pyroseq PCR	5′-GGTTTTTTGTTAGYGTTTGTATTT-3′	5′-Bio-ATTAATCCCTCTCCCCTCTAAACTT-3′	133
	Sequence primer	5′-TTTAGTAAAAATTTTTTGGG-3′		
	Target	5′-GYGTTTGTTGTG-3′		
MINT2	Pyroseq PCR	5′-YGTTATGATTTTTTTGTTTAGTTAAT-3′	5′-Bio-TACACCAACTACCCAACTACCTC-3′	203
	Sequence primer	5′-TTTTGTTTAGTTAATTGAATTT-3′		
	Target	5′-GTYGTYGTTTYGAGTTTTAGG-3′		
MINT12	Pyroseq PCR	5′-YGGGTTATGTTTTATTTTTTGTGTTT-3′	5′-Bio-CTCAAAAAAATCAAACAACCAACCAA-3′	190

(*table continues*)

Y equals C or T, and R equals A or G.
Pyroseq, pyrosequencing.

Table 1. *Continued*

Gene	Primer/Target	Forward	Reverse	Product size (bp)
	Sequence primer	5′-TAATTYGGATTTTAAATTAAATA-3′		
	Target	5′-AAAYGTTTTTATTTT-3′		
MINT31	Pyroseq PCR	5′-GAYGGYGTAGTAGTTATTTTGTT-3′	5′-Bio-CATCACCACCCCTCACTTTAC-3′	184
	Sequence primer	5′-TGTAGTTTTAGGAGAGTGAATA-3′		
	Target	5′-AYGTTTAGGGGTGATGGTTTTAGTAAA-3′		
MIR34B	Pyroseq PCR	5′-GGTYGAGTGATTGTGGYGGGGG-3′	5′-Bio-CCTCCATCTTCTAAACRTCTCCCTTA-3′	176
	Sequence primer	5′-TAATYGTTTTTGGAATTT-3′		
	Target	5′-YGYGGGTYGAGGGGYGGGGYGGGYGYG-3′		
MLH1	Pyroseq PCR	5′-TTGGTATTTAAGTTGTTTAATTAATAGTTG-3′	5′-Bio-AAAATACCTTCAACCAATCACCTC-3′	119
	Sequence primer	5′-AGTTATAGTTGAAGGAAGAA-3′		
	Target	5′-YGTGAGTAYG-3′		
RASSF2	Pyroseq PCR	5′-GGTAGGGGTTGAAAAAGGTTAA-3′	5′-Bio-CRCRACTAAAAAACTACTTCAACT-3′	177
	Sequence primer	5′-GGYGTTYGGTTTTTA-3′		
	Target	5′-GTYGYGYGGTTATYG-3′		
RASSF5	Pyroseq PCR	5′-TYGTTATTAGTYGGGTATGGTTATGG-3′	5′-Bio-CRAAACCRCTCAAACTCTATAAATAAC-3′	110
	Sequence primer	5′-TATTYGTTATTATTGGATTT-3′		
	Target	5′-YGAGTYGTYGYG-3′		
SFRP1	Pyroseq PCR	5′-GTTTTGTTTTTTAAGGGGTGTTGAG-3′	5′-Bio-CTCCRAAAACTACAAAACTAAAATAC-3′	202
	Sequence primer	5′-GYGTTTGGTTTTAGTAAAT-3′		
	Target	5′-TTGYGYGGGGYGGTTTYGAGGGTTYG-3′		
SFRP2	Pyroseq PCR	5′-AATTTYGGATTGGGGTAAAATAAGTT-3′	5′-Bio-TTAAACAACAAACAAAAAAACCTAACC-3′	182
	Sequence primer	5′-YGTTTTYGTTAGTATTTGG-3′		
	Target	5′-TYGYGAGGTYGTTYGYG-3′		
SOX5	Pyroseq PCR	5′-GATTTGGAGGGAGYGGGAGTTTT-3′	5′-Bio-CAAAAACAAACAACACAATACRAATACA-3′	184
	Sequence primer	5′-GTYGTATTTTTYGGGG-3′		
	Target	5′-YGGGYGTYG-3′		
WIF1	Pyroseq PCR	5′-GTTTTYGTAGGTTTTTTGGTATTTAGG-3′	5′-Bio-GAACCATACTACTCAAAACCTCCTC-3′	174
	Sequence primer	5′-AGGTTTTTTGGTATTTAGG-3′		
	Target	5′-TYGGGAGGYGAYGYGTTTAGTYGTTTAAAYG-3′		
WNT5A	Pyroseq PCR	5′-ATATTTGGGGTTGGAAAGTTTTAATTAT-3′	5′-Bio-AACCRACAACAAAAACAAAACCTAATC-3′	149
	Sequence primer	5′-GGTTGGAAAGTTTTAATTAT-3′		
	Target	5′-YGTYGTYG-3′		
ZNF569	Pyroseq PCR	5′-TAGTYGATTGTAAGAAGGAAGTGTTT-3′	5′-Bio-CRCAAAAAAACTCAACCTAAATTTTACA-3′	199
	Sequence primer	5′-GGTTTTTGGGAAATGTA-3′		
	Target	5′-GTTYGGYG-3′		
Mutation Analysis				
AKT1	Pyroseq PCR	5′-Bio-AGTGTGCGTGGCTCTCACC-3′	5′-CATTCTTGAGGAGGAAGTAGCG-3′	83
	Sequence primer	5′-GCCAGGTCTTGATGTACT-3′		
	Target	5′-YCCCTA-3′		

modification, play critical roles in the development of CRC; in addition, an increasing number of genes involved in cell cycle control, DNA repair, tumor invasiveness, and the response to growth factors have been identified as targets of hypermethylation in CRC.[3–6] These epigenetic alterations are thought to be the main driving force in a subset of CRCs exhibiting concurrent hypermethylation of multiple loci, which is termed the CpG island methylator phenotype (CIMP).[7] CIMP-positive CRCs show characteristic clinicopathological and molecular features, including proximal tumor location, female sex, older age, high tumor grade, wild-type *TP53*, frequent *BRAF* and *KRAS* mutations, and MSI. In addition, several studies support the hypothesis that CRCs can be categorized into three subclasses based on aberrant CpG island methylation: CIMP-high (CIMP-H; also known as CIMP1), CIMP-low (CIMP-L; also known as CIMP2), and CIMP-negative (CIMP-N). CIMP-H CRCs are significantly associated with a *BRAF* mutation, *MLH1* methylation, and subsequent MSI.[7–11] A link between CIMP-L CRCs and *KRAS* mutations was first reported by Ogino et al,[12] and it was subsequently confirmed by other groups,[10,13] but much remains unknown about their respective molecular and clinicopathological features.

Reducing the incidence of cancers, such as CRC, will require a better understanding of the mechanisms underlying carcinogenesis and the molecular alterations occurring in premalignant lesions. For example, although experimental evidence has confirmed the presence of CIMP in CRCs, the role of CIMP in the progression of precancerous lesions toward cancer is not yet fully understood. In recent years, sessile serrated adenomas (SSAs) have been the origin of MSI-positive/CIMP-H cancers, which account for approximately 10% to 15% of sporadic CRCs.[14,15] In addition, studies have also shown that CIMP is frequently observed among MSI-negative CRCs.[10,16] From these and other studies of many tumors, a model was suggested in which CIN and epigenetic instability (CIMP) represent the two major pathways of CRC development, with up to 50% of CRCs being characterized as CIMP.[16,17] This means that the origin of a large fraction of CIMP cancers remains unclear.

High-resolution magnifying colonoscopy is a powerful diagnostic tool for detecting premalignant lesions. According to Kudo's classification, the pit patterns of nonneoplastic lesions are classified as type I (normal colon) or type II [hyperplastic polyp (HP)], whereas the pit patterns of neoplastic lesions are classified as types III, IV, and V.[18,19] Recently, we performed an integrative analysis of the morphological, pathological, and molecular signatures in colorectal precancerous lesions and identified a novel pit pattern (type II, open pits) that was specific to SSAs.[20] Those results depict an important relationship between morphological characteristics and molecular alterations that will significantly improve our ability to detect premalignant lesions. In the present study, our aim was to uncover the molecular evolution of CIMP cancers through an integrative analysis of many precursor and malignant colorectal lesions. Based on their genetic and epigenetic signatures, we propose a model in which CRCs develop via four distinct pathways. We also provide evidence that CIMP and CIN are not completely mutually exclusive, so that chromosomal aberrations may play important roles in a subset of CIMP cancers. These findings will improve our understanding of the pathogenesis of CRCs and could potentially contribute to better clinical management of premalignant lesions.

Materials and Methods

Study Population and Tissue Specimens

Colorectal tumor tissues were collected from Japanese patients who underwent endoscopic or surgical resection of a colorectal tumor at Akita Red Cross Hospital (Akita, Japan). A total of 368 specimens from 192 precursor lesions, 38 noninvasive carcinomas [carcinoma *in situ* (CIS)], 100 CRCs, and 38 samples of adjacent normal tissue were analyzed in this study. Informed consent was obtained from all patients before collection of the specimens. Approval of this study was obtained from the Institutional Review Board of Akita Red Cross Hospital and Sapporo Medical University (Sapporo, Japan). By using the standard phenol/chloroform procedure, genomic DNA was extracted from biopsy specimens obtained before endoscopic or surgical resection. CRC cell lines were maintained and treated with 5-aza-2′-deoxycytidine, as previously described.[21]

Endoscopic and Histological Analysis

High-resolution magnifying endoscopes (CF260AZI; Olympus, Tokyo, Japan) were used for all colonoscopic analyses. Colorectal subsite locations were defined as right side colon proximal to the splenic flexure (cecum, ascending colon, hepatic flexure, and transverse colon), left side colon distal to the splenic flexure (splenic flexure, descending colon, and sigmoid colon), and rectum (rectosigmoid and rectum). All detected colorectal tumors were observed at high magnification after staining with indigo carmine dye and 0.05% crystal violet. Surface microstructures were classified according to Kudo's pit pattern classification system.[18,19] Most often, one biopsy specimen was collected from each lesion for the extraction of genomic DNA. However, when two or more pit patterns were found in a single lesion (eg, adenoma to carcinoma transition), biopsy specimens were obtained for each respective pit pattern (see Supplemental Figure S1 at *http://ajp.amjpathol.org*). Thereafter, the lesions underwent endoscopic mucosal resection, endoscopic submucosal dissection, or surgical resection, after which histological analyses were performed (see Supplemental Figure S1 at *http://ajp.amjpathol.org*). Conventional adenomas, such as tubular adenoma and tubulovillous adenoma, were diagnosed using standard criteria. Serrated lesions, including HP, traditional serrated adenoma (TSA), and SSA, were classified based on criteria previously described by Torlakovic et al.[22] Serrated lesions that did not satisfy the criteria for SSA or TSA were defined as HP. Tumors were classified into three categories: precursor lesions (HP, tubular adenoma, tubulovillous adenoma, TSA, and SSA), CIS, and CRCs.

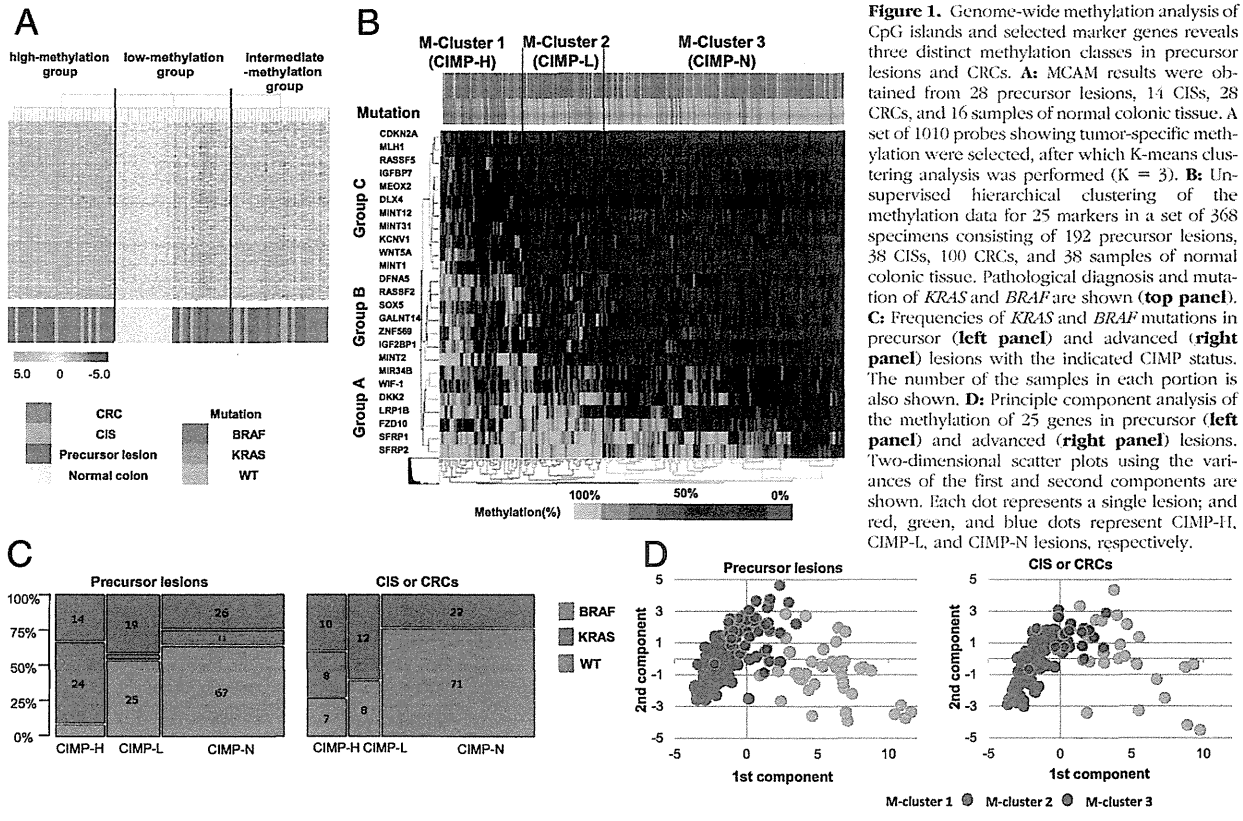

Figure 1. Genome-wide methylation analysis of CpG islands and selected marker genes reveals three distinct methylation classes in precursor lesions and CRCs. **A:** MCAM results were obtained from 28 precursor lesions, 14 CISs, 28 CRCs, and 16 samples of normal colonic tissue. A set of 1010 probes showing tumor-specific methylation were selected, after which K-means clustering analysis was performed (K = 3). **B:** Unsupervised hierarchical clustering of the methylation data for 25 markers in a set of 368 specimens consisting of 192 precursor lesions, 38 CISs, 100 CRCs, and 38 samples of normal colonic tissue. Pathological diagnosis and mutation of *KRAS* and *BRAF* are shown (**top panel**). **C:** Frequencies of *KRAS* and *BRAF* mutations in precursor (**left panel**) and advanced (**right panel**) lesions with the indicated CIMP status. The number of the samples in each portion is also shown. **D:** Principle component analysis of the methylation of 25 genes in precursor (**left panel**) and advanced (**right panel**) lesions. Two-dimensional scatter plots using the variances of the first and second components are shown. Each dot represents a single lesion; and red, green, and blue dots represent CIMP-H, CIMP-L, and CIMP-N lesions, respectively.

MCAM Data

Methylated CpG island amplification microarray (MCAM) analysis was performed as previously described.[23] A BioPrime Plus Array CGH Genomic Labeling System (Life Technologies, Carlsbad, CA) was used to label MCA amplicons from tumor samples with Alexa Fluor 647 (Life Technologies, Carlsbad, CA), and those from a pooled mixture of normal colonic tissue were labeled with Alexa Fluor 555 (Life Technologies). Labeled MCA amplicons were then hybridized to a custom human CpG island microarray (G4497A; Agilent Technologies, Santa Clara, CA), which included 15,134 probes covering 6157 unique genes. After washing, the array was scanned using an Agilent DNA Microarray Scanner (Agilent Technologies), and the data were processed using Feature Extraction software version 10.7 (Agilent Technologies), and analyzed using GeneSpring GX version 11 (Agilent Technologies). Unsupervised hierarchical clustering and statistical analyses were then performed.

Methylation Analysis by Bisulfite Pyrosequencing

Bisulfite pyrosequencing was performed as previously described.[24] Briefly, genomic DNA (1 μg) was modified with sodium bisulfite using an EpiTect Bisulfite Kit (Qiagen, Hilden, Germany). Pyrosequencing was then performed using a PSQ96 system with a PyroGold reagent Kit (Qiagen), after which the results were analyzed using Q-CpG software version 1.0.9 (Qiagen). Unsupervised hierarchical clustering, principal component analysis, and correspondence analysis of validation data were performed using JMP version 8 (SAS Institute Inc., Cary, NC). For the statistical analysis, quantitative methylation levels of each gene were converted to *z* scores, which were defined as follows: (Methylation Level in Each Sample - Mean Methylation Level in All Samples)/(SD of Methylation Levels in All Samples). Primer sequences are listed in Table 1.

Mutation and MSI Analysis

Mutations in codon 600 of *BRAF* and codons 12 and 13 of *KRAS* were examined by pyrosequencing using *BRAF* and *KRAS* pyrokits (Qiagen), respectively, according to the manufacturer's instructions. Mutation of *PIK3CA*, *AKT2*, and *PDK1* was analyzed by direct sequencing, as previously described.[25] Mutation of *TP53* was initially detected by PCR–single-stranded conformational polymorphism analysis, followed by direct sequencing, as previously described.[26] Mutation of *AKT1* was analyzed by pyrosequencing using the primer sequences listed in Table 1. MSI was assessed as previously described,[27] using primers proposed by the National Cancer Institute Workshop on Microsatellite Instability (*BAT25*, *BAT26*, *D5S346*, *D2S123*, and *D17S250*).[28] MSI was defined by the presence, in the tumor sample, of bands that were abnormal in size, compared with a corresponding normal

Table 2. Clinicopathological Features of the Colorectal Tumors Used in this Study

Feature	Total (N = 192)	CIMP-H (n = 42)	CIMP-L (n = 46)	CIMP-N (n = 104)	P value
Precursor Lesion					
Age (years)	69.61 ± 9.68	72.14 ± 8.01	72.43 ± 9.82	67.34 ± 9.73	<0.05
Sex					
F	73 (38.02)	21 (50)	26 (56.52)	26 (25)	<0.001
M	119 (61.98)	21 (50)	20 (43.48)	78 (75)	
Location					
Right	92 (47.92)	32 (76.19)	27 (58.7)	33 (31.73)	<0.001
Left	40 (20.83)	5 (11.9)	7 (15.22)	28 (26.92)	
Rectum	60 (31.25)	5 (11.9)	12 (26.09)	43 (41.35)	
Size (mm)	12.98 ± 9.61	13.73 ± 8.44	16.67 ± 12.55	8.38 ± 6.48	<0.001
Morphological characteristics					
Protruding type	82 (42.71)	17 (40.48)	21 (45.65)	44 (42.31)	
Flat type	110 (57.29)	25 (59.52)	25 (54.35)	60 (57.69)	
Depressed type	0	0	0	0	
Histological features					
HP	28 (14.58)	2 (4.76)	5 (10.87)	21 (20.19)	<0.001
SSA	29 (15.1)	26 (61.90)	1 (2.17)	2 (1.92)	
TSA	25 (13.02)	6 (14.29)	5 (10.87)	14 (13.46)	
Tubular adenoma	53 (17.6)	2 (4.76)	7 (15.22)	44 (42.31)	
Tubulovillous adenoma	57 (29.69)	6 (14.29)	28 (60.87)	23 (22.12)	
CIS					
	(N = 38)	(n = 8)	(n = 5)	(n = 25)	
Age (years)	66.71 ± 13.77	75.75 ± 7.78	73.6 ± 5.5	62.44 ± 14.61	<0.05
Sex					
F	13 (34.21)	3 (37.5)	2 (40)	8 (32)	
M	25 (65.79)	5 (62.5)	3 (60)	17 (68)	
Location					
Right	18 (47.37)	6 (75)	4 (80)	8 (32)	<0.05
Left	9 (23.68)	0	1 (20)	8 (32)	
Rectum	11 (28.95)	2 (25)	0	9 (36)	
Size (mm)	17.66 ± 9.75	20.63 ± 12.96	19.6 ± 10.21	16.32 ± 8.64	
Morphological features					
Protruding type	20 (52.63)	6 (75)	1 (20)	13 (52)	
Flat type	16 (42.11)	2 (25)	4 (80)	10 (40)	
Depressed type	2 (5.26)	0	0	2 (8)	
CRC					
	(N = 100)	(n = 17)	(n = 15)	(n = 68)	
Age (years)	67.81 ± 12.48	71 ± 9.54	72.13 ± 12.78	66.06 ± 12.82	
Sex					
F	43 (43)	14 (82.35)	8 (53.33)	21 (30.88)	<0.01
M	57 (57)	3 (17.65)	7 (46.67)	47 (69.12)	
Location					
Right	48 (48)	16 (94.12)	6 (40)	26 (38.24)	<0.001
Left	22 (22)	1 (5.88)	3 (20)	18 (26.47)	
Rectum	30 (30)	0 (0)	6 (40)	24 (35.29)	
Stage (UICC)					
I	38 (38)	8 (47.06)	3 (20)	27 (39.71)	
II	31 (31)	4 (23.53)	5 (33.33)	22 (32.35)	
III	23 (23)	5 (29.41)	7 (46.67)	11 (16.18)	
IV	8 (8)	0	0	8 (11.76)	

F, female; M, male; UICC, Union for International Cancer Control.

sample. A tumor sample was defined as MSI positive when two or more markers showed instability.

Array-Based Comparative Genomic Hybridization

Array-based comparative genome hybridization (array CGH) analysis was performed as previously described.[29] Briefly, 500 ng of genomic DNA from colorectal tumor specimens and sex-matched reference DNA from noncancerous colonic mucosa were labeled with Cy5 and Cy3, respectively, using a Genomic DNA Enzymatic Labeling Kit (Agilent Technologies), and were then hybridized to Human Genome CGH Microarray Kit 180A (G4449A; Agilent Technologies). DNA copy number aberrations (CNAs) were identified using the ADM-2 algorithm included in the Agilent Genomic Workbench software version 5 (Agilent Technologies). A copy number loss was defined as a log 2 ratio of <-0.5, and a copy number gain was defined as a log 2 ratio of >0.5. Unsupervised hierarchical clustering of CNAs was performed using JMP, version 8. The microarray data in this study

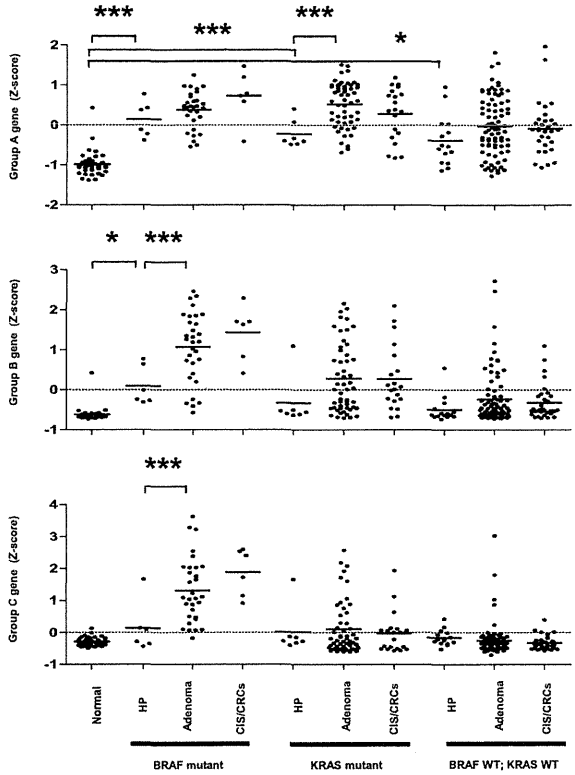

Figure 2. Association between methylation and *BRAF*/*KRAS* mutations. The levels of methylation of groups A to C genes in normal colon, precursor, and malignant lesions are shown as z scores. Colorectal lesions are divided into three groups, according to their *BRAF* or *KRAS* mutation status. Among the *BRAF* mutants, levels of methylation of group B and C genes are significantly higher in adenomas than HPs but are not further up-regulated in advanced lesions. $^{*}P < 0.05$, $^{***}P < 0.001$.

have been submitted to the Gene Expression Omnibus, and the accession number is GSE35534.

Statistical Analysis

To compare differences in continuous variables between groups, *t*-tests or an analysis of variance with a post hoc Tukey's test was performed. A Fisher's exact test or a χ^2 test was used for analysis of categorical data. $P < 0.05$ (two sided) was considered statistically significant. Statistical analyses were performed using JMP, version 8, and SPSS statistics 18 (IBM Corporation, Somers, NY).

Results

Three Methylation Subclasses in Precancerous and Malignant Colorectal Tumors

To clarify the epigenetic changes occurring early during colorectal tumorigenesis, we first performed global methylation analysis using MCAM in a series of normal colonic tissues (n = 16), precursor lesions (HP, n = 3; SSA, n = 5; TSA, n = 3; tubular adenoma, n = 6; tubulovillous adenoma, n = 10), CISs (n = 14), and CRCs (n = 28). Hierarchical clustering analysis using the MCAM results identified 1010 probe sets that detected frequent hypermethylation in tumor tissues (see Supplemental Figure S2 at *http://ajp.amjpathol.org*). Subsequent K-means clustering analysis using these probe sets revealed that the samples could be clearly categorized into three subclasses based on the level of their methylation (Figure 1A); subclasses with high and intermediate methylation were presumed to reflect CIMP-H and CIMP-L tumors, respectively. Among the 1010 probe sets, 538 unique genes were in the high-methylation group, whereas 259 genes were in the intermediate-methylation group (see Supplemental Figure S2C and Supplemental Tables S1 and S2 at *http://ajp.amjpathol.org*). A subset of the precursor lesions could also be categorized into these subclasses, indicating that CIMP-like methylation patterns were already established early during carcinogenesis.

To further characterize the genes that acquired methylation progressively during CIMP pathway tumorigenesis, we next performed MCAM analysis with a series of precursor lesions in which CIMP-N flat components were present, along with CIMP-positive protruding components within the same lesions (see Supplemental Figure S2D at *http://ajp.amjpathol.org*). Because both components were presumed to arise from the same origin, these lesions could represent an ideal model for analyzing the molecular progression of CIMP cancers. CIMP status was defined using classic CIMP markers (*MINT1*, *MINT2*, *MINT12*, *MINT31*, and *CDKN2A*), and tumors with methylation of three or more markers were defined as CIMP. When we analyzed three pairs of precursor lesions using MCAM, we identified 36 unique genes that were differentially methylated between CIMP-positive and CIMP-N precursor lesions (see Supplemental Table S3 at *http://ajp.amjpathol.org*). These genes were potentially the earliest targets of aberrant methylation during CIMP pathway tumorigenesis, and most of them overlapped with the genes identified in the initial MCAM analysis (see Supplemental Figure S2E at *http://ajp.amjpathol.org*).

Methylation Profiling Identified CIMP in Precancerous Lesions

Based on the results previously summarized, we selected a series of marker genes to characterize the methylation profile of precursor and malignant lesions. We initially selected 14 genes (*LRP1B*, *CDKN2A*, *WNT5A*, *MEOX2*, *ZNF569*, *GALNT14*, *SOX5*, *DFNA5*, *DLX4*, *SFRP2*, *WIF1*, *FZD10*, *KCNV1*, and *IGF2BP1*) identified in the MCAM analysis (see Supplemental Figure S2 at *http://ajp.amjpathol.org*). Among them, *IGF2BP1*, *KCNV1*, *DLX4*, *GALNT14*, and *ZNF569* were not previously reported to be methylated in CRCs, but RT-PCR analysis using multiple CRC cell lines confirmed that they were frequent targets of epigenetic silencing in CRC (see Supplemental Figure S3 at *http://ajp.amjpathol.org*). In addition, we selected 11 well-characterized markers (*MLH1*, *SFRP1*, *IGFBP7*, *DKK2*, *MIR34B*, *MINT1*, *MINT2*, *MINT12*, *MINT31*, *RASSF2*, and *RASSF5*) used for methylation analysis.[4,7,21,24,30–32]