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CpG island hypermethylation-associated silencing of small nucleolar RNAs in human cancer

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Much effort in cancer research has focused on the tiny part of our genome that codes for mRNA. However, it has recently been recognized that microRNAs also contribute decisively to tumorigenesis. Studies have also shown that epigenetic silencing by CpG island hypermethylation of microRNAs with tumor suppressor activities is a common feature of human cancer. The importance of other classes of non-coding RNAs, such as long intergenicncRNAs (lincRNAs) and transcribed ultraconserved regions (T-UCRs) as altered elements in neoplasia, is also gaining recognition. Thus, we wondered whether there were other ncRNAs undergoing CpG island hypermethylation-associated inactivation in cancer cells. We focused on the small nucleolar RNAs (snoRNAs), a subset of ncRNA with a wide variety of cellular functions, such as chemical modification of RNA, pre-RNA processing and control of alternative splicing. By data mining snoRNA databases and the scientific literature, we selected 49 snoRNAs that had a CpG island within ≤ 2 Kb or that were processed from a host gene with a 5′-CpG island. Bisulfite genomic sequencing of multiple clones in normal colon mucosa and the colorectal cancer cell line HCT-116 showed that 46 snoRNAs were equally methylated in the two samples: completely unmethylated (n = 26) or fully methylated (n = 20). Most interestingly, the host gene-associated 5′-CpG islands of the snoRNAs SNORD123, U70C and ACA59B were hypermethylated in the cancer cells but not in the corresponding normal tissue. CpG island hypermethylation was associated with the transcriptional silencing of the respective snoRNAs. Results of a DNA methylation microarray platform in a comprehensive collection of normal tissues, cancer cell lines and primary malignancies demonstrated that the observed hypermethylation of snoRNAs was a common feature of various tumor types, particularly in leukemias. Overall, our findings indicate the existence of a new subclass of ncRNAs, snoRNAs, that are targeted by epigenetic inactivation in human cancer.

Introduction

Coding exons account for only 1.5% of the genome,[1] despite being the focus of most of the current biomedical research. A large proportion of the genome is made up of non-protein coding regions that might have critical biological important, containing gene regulatory regions (transcriptional and splicing types), matrix attachment sites, origins of replication, other functional elements and non-coding RNAs (ncRNAs).[2,3] The physiological and pathological importance of this functional part of the non-protein-coding genome is particularly apparent in a large class of small non-coding RNAs (ncRNAs) known as microRNAs.[4] These are about 22 nucleotides long and repress gene expression in a variety of eukaryotic organisms.[4] In human cancer, miRNA expression profiles differ between normal tissues and derived tumors and between tumor types.[5] miRNAs can act as oncogenes or tumor suppressors, with a key role in tumorigenesis.[6-8] Defects in miRNA function have been associated with a failure of miRNA post-transcriptional regulation,[9] miRNA transcriptional repression by oncogenic factors,[10] loss-of-function genetic alterations in the genes involved in miRNA-processing pathways[11-13] and transcriptional silencing associated with hypermethylation of CpG island promoters.[14] Thus, as occurs with miRNAs, it is likely that other types of ncRNAs are also involved in human tumorigenesis and are characterized by epigenetic and genetic defects in this disease.[3] In this context, Ultraconserved Regions (UCRs), a subset of conserved sequences that are located in intragenic and intergenic regions,[15,16] are altered at the transcriptional level in human tumorigenesis.[17] Interestingly, transcribed UCRs (T-UCRs) undergo DNA methylation-associated silencing in cancer cells.[18]

Another important class of ncRNAs that are potentially altered in human cancer are the small nucleolar RNAs (snoRNAs), which are localized in the nucleolus.[19] They are responsible for methylation[20,21] and pseudouridylation[22,23] of rRNA (rRNA) at about 50–100 sites per eukaryotic ribosome. However there

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is increasing evidence of the targeting of other classes of RNAs, such as mRNAs.[24] snoRNAs are divided into two main classes: box C/D and box H/ACA,[25] on the basis of their conserved secondary structural characteristics and associated modification reactions.[19,26] The C/D box snoRNAs guide the position-specific 2'-O-methylation and are associated with a core of four proteins: fibrillarin (the methyltransferase), NOP56, NOP5/NOP58 and NHPX. The H/ACA snoRNAs direct RNA pseudouridylation of rRNA and are associated with dyskerin (the pseudouridine synthase), GAR1, NHP2 and NOP10.[24,26,27] Mutations in the human dyskerin gene, NHP215 and NOP1016 gene are associated with the X-linked genetic disorder, dyskeratosiscongenita (DC), where malfunction of rRNA and shortening of telomeres have been observed.[28-30] As mutations in the dyskerin gene have also been associated with cancer susceptibility,[28-30] it was suggested that snoRNAs were involved in the onset and progression of cancer. One of the first studies to address this possibility reported a snoRNA to be highly expressed in normal brain, but significantly reduced in meningioma.[31] Other studies showed that GAS5, a snoRNA-host gene, controls cell survival by inducing or sensitizing cells to apoptosis.[32,33] A substantial decrease of GAS5 in breast cancer samples compared with adjacent unaffected normal breast epithelial tissues also suggests that it has a role as a tumor suppressor gene.[33]

The association between snoRNAs and cancer was underlined by other studies showing that a homozygous 2-bp (TT) deletion of the snoRNA U50 is strongly associated with prostate cancer[34] and that U50 undergoes frequent genomic heterozygous deletion and transcriptional downregulation in breast cancer.[35] U50 overexpression reduces colony-forming ability in prostate and breast cancer cells.[34,35] Taken together, these studies suggest that noncoding snoRNA U50 is important in the development and progression of breast and prostate cancers.[34,35] More recently, it was shown that a diversity of snoRNAs are differentially expressed in non-small-cell lung cancer with respect to the corresponding matched tissue,[36] encouraging investigation into the possible role of snoRNAs in oncogenesis. Another study has linked at least one snoRNA to the post-transcriptional processing of a protein-coding gene.[37] There is also evidence that other snoRNAs might be involved in the regulation of gene expression by giving rise to other regulatory RNA species, such as miRNAs, linking snoRNAs to RNA silencing.[38] It would therefore be very interesting to identify the function and mechanisms of the orphan snoRNAs.

The downregulation of tumor suppressor protein-coding genes (e.g., hMLH1, BRCA1, VHL and $p16^{INK4a}$),[39,40] and ncRNAs with growth inhibitory functions, such as miRNAs[14] has been closely linked to the presence of CpG island promoter hypermethylation. Thus, we wondered whether the same mechanism could play a role in the loss of adequate snoRNA expression in tumors. Usually, snoRNAs are genomically found in the introns of protein-coding or non-protein-coding host genes, with each intron carrying only one single snoRNA and their transcription being synchronized with that of the host gene. After splicing they are generally processed by debranching and exonucleolytic trimming of the 5'- and 3'-ends,[24,41-43] and assembled with specific core proteins that are essential for their localization and correct enzymatic activity, and for preventing their degradation.[27] However, intergenicsnoRNAs are independently transcribed by RNA polII as independent units,[24] and some human intron-encoded snoRNAs may have their own independent promoters.[23] Herein, we present a double candidate and genomic approach to unmask snoRNA-associated CpG islands that undergo cancer-specific hypermethylation-associated transcriptional silencing, such as SNORD123, U70C and ACA59B. These findings support a model in which epigenetic disruption of emerging new classes of ncRNAs, such as snoRNAs, is a common feature of human tumorigenesis.

Results and Discussion

snoRNA CpGisland DNA methylation analyses. To identify snoRNAs with putative DNA methylation-related inactivation in human tumors, we data-mined the scientific literature on snoRNAs published during 2000–2011, as made available by PubMed.gov, the human genome browser at UCSC[44] and the snoRNA-LBME-db database.[24] The CpG Island Searcher Program[45] was used to determine which snoRNAs were located within ≤2 Kb of a CpG island, since it has been estimated that more than 90% of the human promoters of another type of ncRNA, the microRNAs, are located 1 Kb upstream of the mature transcript.[46,47] The DNA methylation status of CpG islands within 2 Kb are also important for regulating the expression of a second type of ncRNA, the T-UCRs.[18] We also included snoRNAs that were processed from a host gene RNA containing a 5'-CpG island. **Figure 1** illustrates both categories of snoRNA-related CpG islands. Using the described conditions, we selected 49 snoRNAs that had a CpG island within a distance of ≤ 2 Kb (15 intergenic independent snoRNAs and 24 within an intron of a host gene) or that were processed from the transcript of a host gene with a 5'-CpG island (n = 10). The characteristics of the 49 selected snoRNAs and the summarized results are shown in **Table 1**.

We performed bisulfite genomic sequencing of multiple clones using primers encompassing the 49 snoRNA-associated CpG islands to determine the DNA methylation patterns in normal colon mucosa and the colorectal cancer cell line HCT-116. We observed a completely unmethylated status for 23 snoRNA-related CpG islands in normal tissue and colon cancer cells (**Table 1**). Examples of the bisulfite sequencing analyses are shown in **Figure 2**. We also found a dense DNA methylation profile for 20 snoRNA-associated CpG islands in normal colon mucosa and HCT-116 colorectal cancer cells (**Table 1**). Examples of the bisulfite sequencing analyses are shown in **Figure 3**. Most importantly, we found a cancer-specific hypermethylation event for the snoRNAs SNORD123, U70C and ACA59B. Their associated CpG islands were completely unmethylated in normal colon mucosa and heavily hypermethylated in HCT-116 colorectal cancer cells (**Table 1 and Fig. 4**). For all three cases, the CpG islands studied were in the 5'-region of the host gene where the snoRNA is located: the long non-coding gene LOC100505806 (SNORD123), Astrotactin 2 (U70C) and Solute Carrier Family 47 Member 1 (ACA59B). The DNA methylation results were also confirmed using methylation-specific PCR (**Fig. S1**).

Hypermethylation of snoRNA-related CpG islands is associated with transcriptional silencing. To demonstrate transcriptional silencing of these snoRNAs in cancer cells in association with the presence of CpG island hypermethylation, we measured transcript levels by quantitative RT-PCR. For the SNORD123, we analyzed the expression of the snoRNA itself, the long non-coding RNA LOC100505806 from which the snoRNA is processed and the mRNA of the Semaphorin 5A (SEMA5A) that it is transcribed in the opposite direction from the same CpG island (**Fig. 4**). No expression of the SNORD123, LOC100505806 and SEMA5A transcripts could be detected in HCT-116 cells in which the shared CpG island was methylated (**Fig. 5A**). Normal colon mucosa expressed the three transcripts (**Fig. 5A**). Methylation-specific PCR analyses of two additional colon cancer cell lines identified a hypermethylated and unmethylatedCpG island in SW48 and DLD1 cells, respectively (**Fig. S1**). Loss of the SNORD123, LOC100505806 and SEMA5A transcripts was observed in the hypermethylated SW48 cells and expression of the three transcripts was found in the unmethylated DLD1 cells (**Fig. 5A**). The absence of the SNORD123 transcript in HCT-116 and SW48 cells and its presence in DLD1 cells was also validated by northern-blot analyses (**Fig. 5B**). For the snoRNAs U70C and ACA59B, no expression of the snoRNA U70C, its host gene ASTN2 and the host gene of ACA59B (SLC47A1) could be detected in HCT-116 cells in which the corresponding CpG island was methylated (**Fig. S2**). Normal colon mucosa expressed the three transcripts (**Fig. S2**). Most importantly, the expression for ACA59B (SLC47A1) was restored upon treatment with the DNA demethylating agent 5-aza-2'-deoxycytidine in the HCT-116 cell line (**Fig. S2**). These results were confirmed using an alternative model of an isogenic HCT-116 cell line in which the two major DNA methyltransferases, DNMT1 and DNMT3b, had been genetically disrupted (HCT116 DKO).[48] The CpG island for ACA59B (SLC47A1) was hypomethylated in DKO cells (**Fig. S2**), but hypermethylated in the HCT116 parental cell line. ACA59B (SLC47A1) expression was restored in DKO cells (**Fig. S2**), reinforcing the link between CpG island hypermethylation and snoRNA silencing.

Profile of snoRNA hypermethylation in different tumor types. To address the cancer-specific hypermethylation event in snoRNAs, we ruled out the possible presence of SNORD123, U70C and ACA59B CpG island methylation in a panel of normal tissues from the colon (n = 5), breast (n = 7) and lung (n = 22), in addition to four blood samples from healthy volunteers, using a DNA methylation microarray approach (**Fig. 6**).[49] The observed high-throughput DNA methylation platform included five, two and four CpG sites corresponding to the bisulfite genomic sequenced CpG islands for SNORD123, U70C and ACA59B, respectively (**Fig. 6**). The presence of SNORD123, U70C and ACA59B cancer-specific CpG island hypermethylation and transcriptional silencing was not a unique feature of the colorectal cancer cell line HCT-116; analyzing the NCI60 panel of human cancer cell lines (n = 60) from nine tumor types, we also found them in other colon cancer cell line and lung, breast, prostate, ovarian, renal, melanoma, lymphoma and leukemia cells (**Fig. 7**).

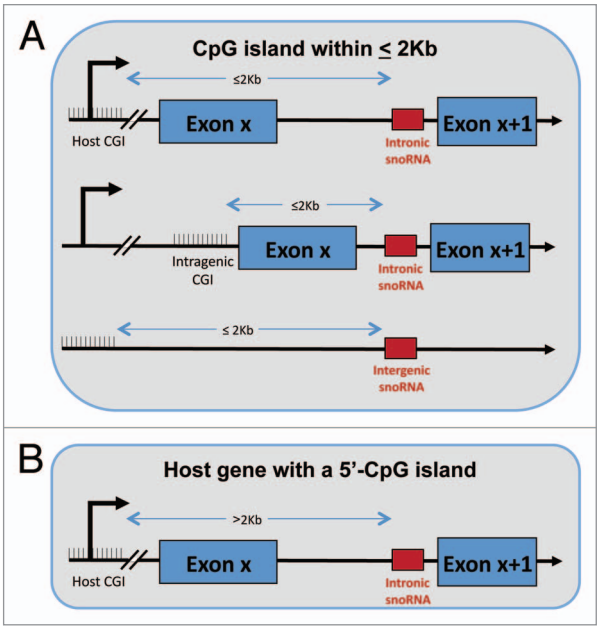

Figure 1. Types of CpG islands associated with snoRNAs in this study. (A) Upstream CpG islands of a snoRNA located within 2 Kb. It includes intergenic independent snoRNAs and snoRNAs within an intron of a host gene. (B) 5'-CpG islands of host genes for which RNA processing generates the expression of an intronic resident snoRNA.

Most importantly, the CpG island hypermethylation of SNORD123, U70C and ACA59B was not an in vitro phenomenon. Having noted the high frequency of CpG island hypermethylation for the three snoRNAs in leukemia cell lines (**Fig. 7**), we examined the presence of these epigenetic events in 48 primary samples from acute lymphoblastoid leukemia (ALL) patients, 43 had a B-cell phenotype while five were T-ALLs (**Fig. 8**). Using the described DNA methylation platform, we observed SNORD123, U70C and ACA59B hypermethylation in 27% (13 of 48), 39% (19 of 48) and 29% (14 of 48) of acute lymphoblastoid leukemia cases, respectively (**Fig. 8**). We did not observe any association between CpG Island hypermethylation of the three studied snoRNAs and disease-free survival or overall survival in these patients (Log rank Mantel-Cox test $p > 0.05$). We also extended the analyses of snoRNA DNA methylation to primary acute myelogenous leukemia (AML) samples. Among 16 primary acute myelogenous leukemia cases, we observed SNORD123 CpG island hypermethylation in 25% (4 of 16) of samples, while ACA59B and U70C were unmethylated in all cases. Finally, among 20 primary multiple myeloma cases, we observed ACA59B CpG island hypermethylation in 10% (2 of 20) of samples, while SNORD123 and U70C were unmethylated in all cases.

Overall, our results reveal the existence of cancer-specific hypermethylation events in CpG islands associated with snoRNAs that lead to their transcriptional inactivation in transformed cells. Despite our increasing knowledge about the biological roles of snoRNAs, one of the main challenges in cancer research into ncRNAs is the identification of a particular function that is relevant for cellular transformation. As coding genes,[39,40]

Table 1. DNA methylation profile of CpG islands associated with snoRNAs

Name	Class	Chr.	Strand	Length (bp)	Target RNA	Host gene	Analyzed CpG Island	Normal Colon	HCT116
ACA24	HAcaBox	4	+	131	18S rRNA U863 and 18S rRNA U609	SNHG8	CpG island within ≤ 2Kb		
U50	CDBox	6	-	74	28S rRNA C2848 and 28S rRNA G2863	SNHG5	CpG island within ≤ 2Kb		
U50B	CDBox	6	-	71	Unknown	SNHG5	CpG island within ≤ 2Kb		
ACA9	HAcaBox	7	-	133	28S rRNA U1670 and 28S rRNA U1769	C7orf40	CpG island within ≤ 2Kb		
HBII-336	CDBox	7	+	74	18S rRNA A576	Independent	CpG island within ≤ 2Kb		
hTR	scaRna	3	-	548	Unknown	Independent	CpG island within ≤ 2Kb		
U91	scaRna	18	+	83	U4 snRNA C8	Independent	CpG island within ≤ 2Kb		
mgU12-22/U4-8	scaRna	18	+	421	U4 snRNA C8 and U12 snRNA G22	Independent	CpG island within ≤ 2Kb		
HBII-382	scaRna	1	+	82	U2 snRNA C61 and U2 snRNA G11	Independent	CpG island within ≤ 2Kb		
mgU2-25/61	scaRna	1	+	420	U2 snRNA G25 and U2 snRNA C61	Independent	CpG island within ≤ 2Kb		
U13	CDBox	8	+	104	Unknown	Independent	CpG island within ≤ 2Kb		
ACA62	HAcaBox	17	+	133	18S rRNA U34 and 18S rRNA U105	Independent	CpG island within ≤ 2Kb		
U104	CDBox	17	+	80	28S rRNA C1327	Independent	CpG island within ≤ 2Kb		
U60	CDBox	16	-	83	28S rRNA G4340	Independent	CpG island within ≤ 2Kb		
U3	CDBox	17	+	217	Unknown	Independent	CpG island within ≤ 2Kb		
U3-2	CDBox	17	+	217	Unknown	Independent	CpG island within ≤ 2Kb		
U3-2B	CDBox	17	-	217	Unknown	Independent	CpG island within ≤ 2Kb		
U3-3	CDBox	17	-	217	Unknown	Independent	CpG island within ≤ 2Kb		
U3-4	CDBox	17	-	217	Unknown	Independent	CpG island within ≤ 2Kb		
U98b	HAcaBox	1	+	133	Unknown	PPP2R5A	Host gene with a 5'-CpG island		
ACA20	HAcaBox	6	-	132	18S rRNA U651	TCP1	Host gene with a 5'-CpG island		
ACA29	HAcaBox	6	-	140	Unknown	TCP1	Host gene with a 5'-CpG island		
U32A	CDBox	19	+	77	18S rRNA G1328 and 28S rRNA A1511	RPL13A	Host gene with a 5'-CpG island		
U33	CDBox	19	+	83	18S rRNA U1326	RPL13A	Host gene with a 5'-CpG island		
U34	CDBox	19	+	66	28S rRNA U2824	RPL13A	Host gene with a 5'-CpG island		
U35A	CDBox	19	+	86	28S rRNA C4506	RPL13A	Host gene with a 5'-CpG island		
SNORD123	CDBox	5	+	70	Unknown	LOC100505806	Host gene with a 5'-CpG island		
U70C	HAcaBox	9	-	136	18S rRNA U1692	ASTN2	Host gene with a 5'-CpG island		
ACA59B	HAcaBox	17	+	152	Unknown	SLC47A1	Host gene with a 5'-CpG island		
U38A	CDBox	1	+	71	28S rRNA A1858	RPS8	CpG island within ≤ 2Kb		
U38B	CDBox	1	+	69	28S rRNA A1858	RPS8	CpG island within ≤ 2Kb		
HBII-52-1	CDBox	15	+	82	serotonin receptor 5HT-2C mRNA?	SNURF-SNRNP-UBE3A antisense	CpG island within ≤ 2Kb		
HBII-85-3	CDBox	15	+	97	Unknown	SNURF-SNRNP-UBE3A antisense	CpG island within ≤ 2Kb		
SNORD116.1	CDBox	1	-	92	Unknown	USH2A	CpG island within ≤ 2Kb		
ACA55	HAcaBox	1	-	137	18S U36	PABPC4	CpG island within ≤ 2Kb		
U88	scaRna	2	+	266	U5 snRNA U41	ATG16L1	CpG island within ≤ 2Kb		
HBII-316	CDBox	2	+	89	28S rRNA A3846	WDR43	CpG island within ≤ 2Kb		
ACA58	HAcaBox	3	-	137	28S rRNA U3823	MRPL3	CpG island within ≤ 2Kb		
U19-2	HAcaBox	5	+	204	28S rRNA U3741 and 28S rRNA U3743	ATP6V0E	CpG island within ≤ 2Kb		
U63	CDBox	5	-	68	28S rRNA A4541	HSPA9	CpG island within ≤ 2Kb		
ACA54	HAcaBox	11	-	123	28S rRNA U3801 and 28S rRNA U4539	NAP1L4	CpG island within ≤ 2Kb		
ACA49	HAcaBox	12	+	136	Unknown	EP400	CpG island within ≤ 2Kb		
ACA2b	HAcaBox	12	-	137	28S rRNA U4263 and 28S rRNA U4282	C12orf41	CpG island within ≤ 2Kb		
ACA28	HAcaBox	14	+	127	18S rRNA U815 and 18S rRNA U866	EIF5	CpG island within ≤ 2Kb		
HBII-82	CDBox	16	+	94	28S rRNA G3923	SF3B3	CpG island within ≤ 2Kb		
U105B	CDBox	19	+	79	18S rRNA U799	PPAN	CpG island within ≤ 2Kb		
U37	CDBox	19	-	66	28S rRNA A3697	EEF2	CpG island within ≤ 2Kb		
HBII-180A	CDBox	19	-	97	28S rRNA C3680	C19orf48	CpG island within ≤ 2Kb		
HBII-180B	CDBox	19	-	97	28S rRNA C3680	C19orf48	CpG island within ≤ 2Kb		

Green and red rectangles represent unmethylated and methylated CpG islands, respectively.

microRNAs[14] and T-UCRs[18] undergoing cancer-specific CpG island hypermethylation-associated silencing are known to have tumor suppressor roles, it is possible that snoRNAs act in a similar manner. This additional level of complexity is really true for the epigenetic silencing of two of the identified snoRNAs, SNORD123,[50] and ACA59B (also known as SNORA59B),[51] because their target RNAs are unknown. ACA59B resides in an intron of the Solute Carrier Family 47 Member 1 (SLC47A1) gene, while SNORD123 is a C/D box snoRNA that resides within a long ncRNA transcript (LOC100505806) while in opposite direction is transcribed from the same CpG island the coding gene SEMA5A, adding another level of complexity in this case. For U70C (also known as SNORA70 or ACA70), the task might be a little easier. U70C was originally cloned from a cervical cancer cell line and belongs to the H/ACA box class of snoRNAs, having the predicted hairpin-hinge-hairpin-tail structure, conserved H/ACA-box motifs, and an association with the GAR1 protein.[50,52,53] The snoRNA U70C resides in an intron of the Astrotactin 2 (ASTN2) gene in the sense orientation and it serves as a guide for the pseudouridylation of selected bases of rRNA by forming short duplexes with the 18S rRNA U1692, the target for this snoRNA.[50,52,53] A role for 18S rRNA in tumorigenesis is starting to emerge,[54-56] and our findings provide additional information about this role.

The enormous task of understanding the mechanisms by which snoRNA epigenetic silencing contributes to the origin and progression of human tumors still lies ahead. In the meantime, our observation that epigenetic inactivation by CpG island hypermethylation of a subset of snoRNAs, such as SNORD123, U70C and ACA59B, occurs across a wide spectrum of human cancer cell lines and primary tumors of diverse cellular and tissue origin provides clear support for the concept that major disruption of ncRNA programming is a common feature of cancer cells.

Materials and Methods

Cell lines, culture conditions and primary study samples. The human cancer cell lines examined in this study were obtained from the American Type Culture Collection (ATCC). HCT-116 and DKO cells were a generous gift from Dr. Bert Vogelstein (Johns Hopkins Kimmel Comprehensive Cancer Center). Cell were maintained in appropriate media and treated with 1 μM 5-aza-2'-deoxycytidine (Sigma) for 48 h to achieve demethylation.[18] DNA samples from normal tissues and primary leukemias were

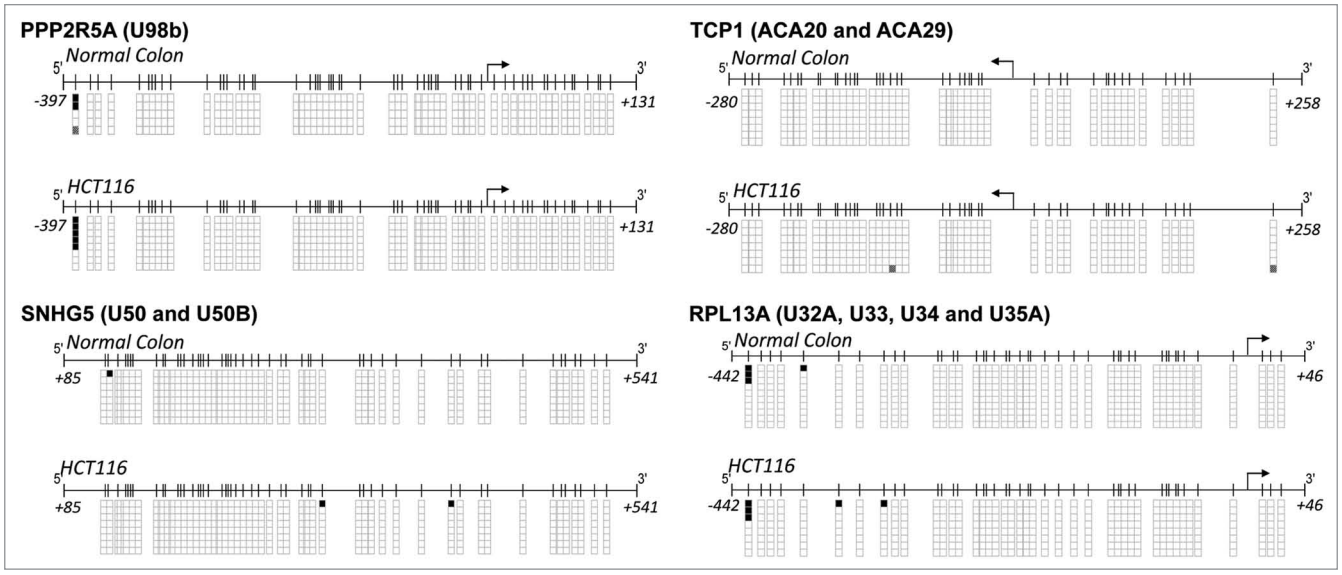

Figure 2. Bisulfite genomic sequencing of the CpG islands associated with the snoRNAs U98b (host gene PPP2R5A), ACA20/ACA29 (host gene TCP1), U50/U50B (host gene SNHG5) and U32A/U33/U34/U35A (host gene RPL13A) in normal colon and the colorectal cancer cell line HCT-116. CpG dinucleotides are represented as short vertical lines. Eight single clones are represented for each sample. Presence of a methylated or unmethylated cytosine is indicated by a black or white square, respectively. Transcription start sites are represented by vertical black arrows.

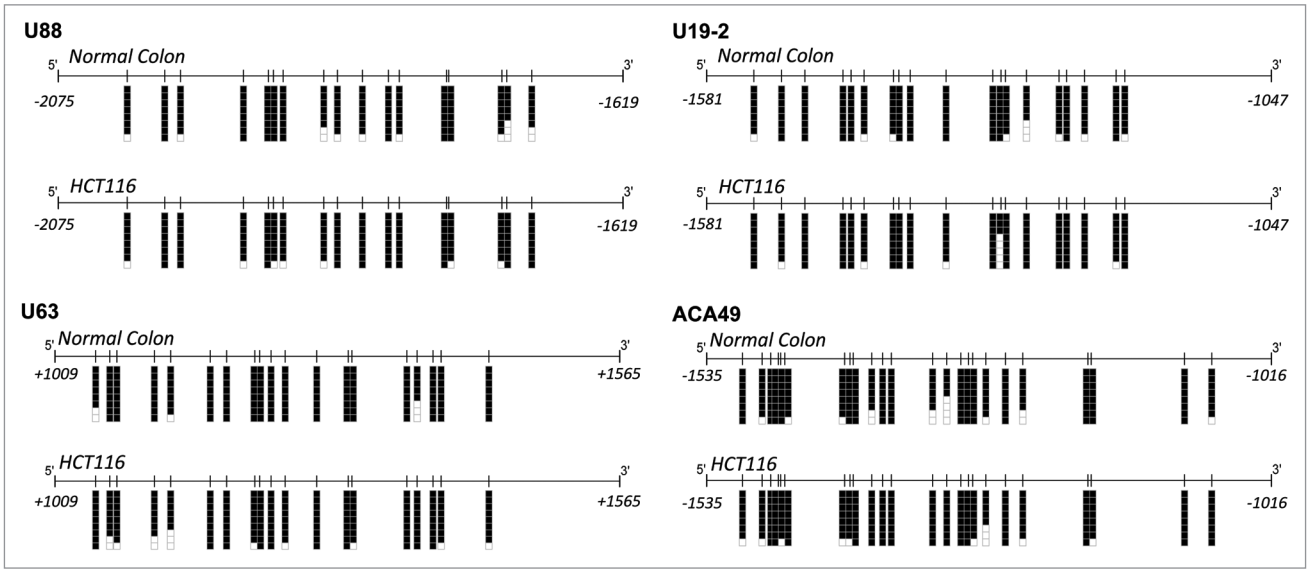

Figure 3. Bisulfite genomic sequencing of the CpG islands associated with the snoRNAs U88, U19-2, U63 and ACA49 in normal colon and the colorectal cancer cell line HCT-116. CpG dinucleotides are represented as short vertical lines. Eight single clones are represented for each sample. Presence of a methylated or unmethylated cytosine is indicated by a black or white square, respectively. Transcription start sites are represented by vertical black arrows.

obtained at the time of the clinically indicated surgical procedures. All patients gave written consent to participate in the study and the Ethics Committee of each hospital approved the study protocol.

DNA methylation analyses. The CpG Island Searcher Program[45] was used to determine which snoRNAs were located within 2 Kb of a CpG island. DNA methylation status was established by PCR analysis of bisulfite-modified genomic DNA, which induces chemical conversion of unmethylated, but not methylated, cytosine to uracil. Two procedures were used. First, methylation status was established by bisulfite genomic sequencing of the corresponding CpG islands. Eight independent clones were analyzed. The second analysis used methylation-specific PCR with primers specific for either the methylated or modified unmethylated DNA. The primers used are described in **Table S1**.

Quantification of snoRNAs with real-time PCR. Quantitative real-time PCR was performed to quantify the level

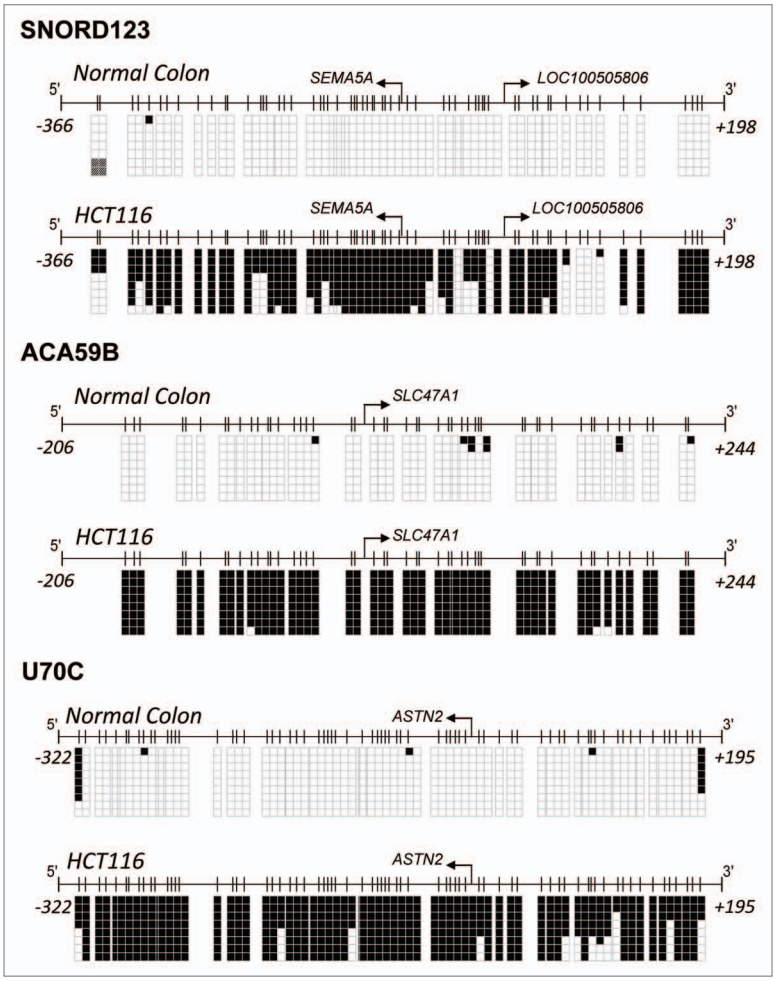

Figure 4. Bisulfite genomic sequencing of the CpG islands associated with the snoRNAs SNORD123 (host gene is the lncRNA LOC100505806), ACA59B (host gene SLC47A1) and U70C (host gene ASTN2) in normal colon and the colorectal cancer cell line HCT-116. CpG dinucleotidesare represented as short vertical lines. Eight single clones are represented for each sample. Presence of a methylated or unmethylated cytosine is indicated by a black or white square, respectively. Transcription start sites are represented by vertical black arrows.

of snoRNAs, as described previously in reference 18. Briefly, to quantify SEMA5A and the snoRNA-host genes 1 μg of purified and DNase-treated (TURBO DNA-free, Ambion) total RNA was reverse-transcribed using Thermoscript RT and random primer hexamers. cDNA was amplified by real-time PCR using SYBR (Applied Biosystems) green detection. Reverse transcription using a custom-designed TaqMan microRNA Reverse Transcription Kit (Applied Biosystems) was used to quantify the SNORD123 and U70C, providing specificity for the mature RNA target. Reactions were performed in an Applied Biosystems 7900HT Fast Real-Time PCR system in 384-well plates. Expression values of ASTN2, LOC100505806, SLC47A1 and SEMA5A were normalized to HPRT1 and expression values of U70C and SNORD123 to RNU19, respectively. Total RNA was extracted from three independent experiments and real-time PCR reactions were performed in triplicate. The primers used are described in **Table S1**.

Northern-blot. Fifteen micrograms of total RNA were loaded in a 15% denaturating polyacrylamide gel containing 8 M Urea in 0.5x TBE buffer system. Decade Marker (Ambion) was prepared according to manufacturer's protocol, using [γ-^{32}P] ATP (PerkinElmer) and simultaneously loaded into the gel. Both RNA and marker were resolved by gel electrophoresis and transferred onto Hybond-N^{+} membrane (Amersham) in 0.5x TBE, followed by UV-cross linking (1,200 Jules). Both SNORD123 and 5.8S rRNA probes were radiolabeled with 25 μCie using T4 kinase (Invitrogen) and purified with Nucaway Spin columns (Ambion). The membrane was prehybridized in hybridization buffer for 1 h and hybridized overnight in the same solution at 45°C containing the SNORD123 probe previously heated at 95°C for 2 min. The membrane was washed at low stringency followed by film exposure. The membrane was then hybridized with the 5.8S rRNA probe using the same conditions followed by quantification using phosphorimager technology. All the probes used are described in **Table S1**.

Infinium 450K DNA methylation array. The DNA methylation levels at 10 CpG sites encompassing the SNORD123-associated CpG island were determined using the Infinium 450K DNA methylation microarray, as previously described in reference 49. Briefly, DNA was quantified by Quant-iT™

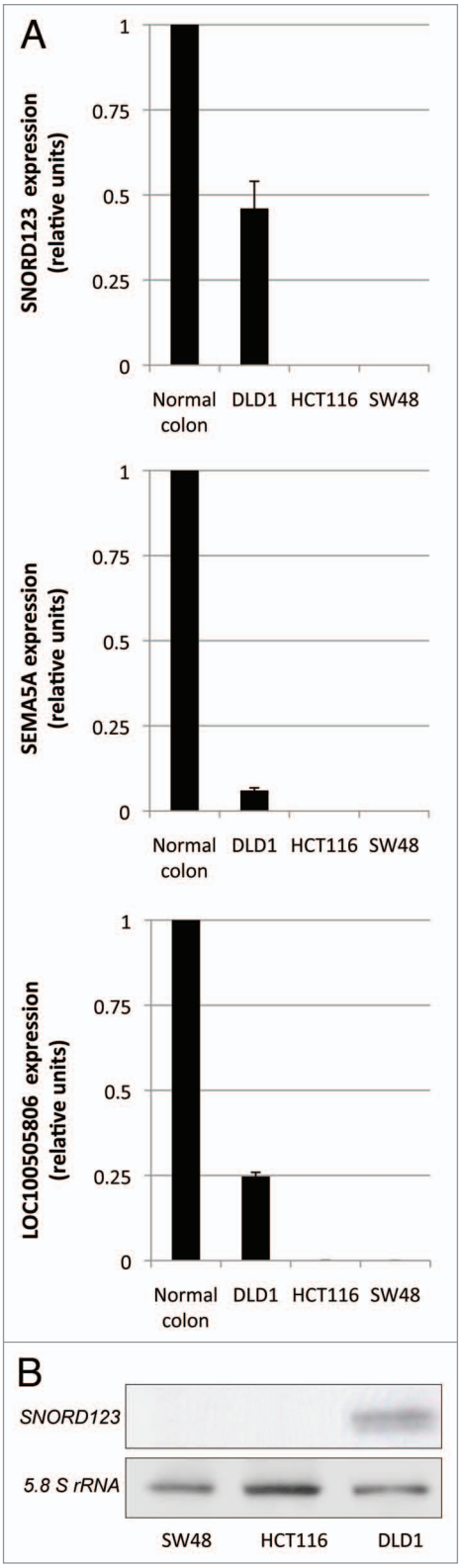

Figure 5. Expression analyses of of the transcripts derived from the SNORD123/LOC100505806/SEMA5A CpG island. (A) Quantitative RT-PCR of SNORD123, LOC10050580 and SEMA5A showed loss of expression in the CpG island hypermethylated HCT-116 and SW48 cells. SNORD123 ACA59B and U70C are expressed in the unmethylated DLD1 cancer cells and in normal colon mucosa. (B) Northern-blot analysis shows the absence of the SNORD1 transcript in the hypermethylated HCT-116 and SW48 cells and its presence in the unmethylated DLD1 cells.

PicoGreendsDNA Reagent (Invitrogen) and the integrity was analyzed in a 1.3% agarose gel. Bisulfite conversion of 600 ng of each sample was done according to the manufacturer's recommendations for the IlluminaInfinium Assay. Effective bisulfite conversion was checked for three controls that were converted simultaneously with the samples. Four μl of bisulfite-converted DNA were used to hybridize on an InfiniumHumanMethylation 450 BeadChip, following the IlluminaInfinium HD Methylation protocol. The chip was analyzed using an IlluminaHiScan SQ fluorescent scanner. The intensities of the images were measured using GenomeStudio (2010.3) Methylation module (1.8.5) software. The methylation score of each CpG is represented as a β value.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental Material can be found at: www.landesbioscience.com/journals/rnabiology/article/19353

References

1. International Human Genome Sequencing Consortium. Finishing the euchromatic sequence of the human genome. Nature 2004; 431:931-45; PMID:15496913; http://dx.doi.org/10.1038/nature03001.
2. Mattick JS. Challenging the dogma: the hidden layer of non-protein-coding RNAs in complex organisms. Bioessays 2003; 25:930-9; PMID:14505360; http://dx.doi.org/10.1002/bies.10332.
3. Esteller M. Non-coding RNAs in human disease. Nat Rev Genet 2011; 12:861-74; PMID:22094949; http://dx.doi.org/10.1038/nrg3074.
4. He L, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet 2004; 5:522-31; PMID:15211354; http://dx.doi.org/10.1038/nrg1379.
5. Calin GA, Croce CM. MicroRNA signatures in human cancers. Nat Rev Cancer 2006; 6:857-66; PMID:17060945; http://dx.doi.org/10.1038/nrc1997.
6. Esquela-Kerscher A, Slack FJ. Oncomirs—microRNAs with a role in cancer. Nat Rev Cancer 2006; 6:259-69; PMID:16557279; http://dx.doi.org/10.1038/nrc1840.

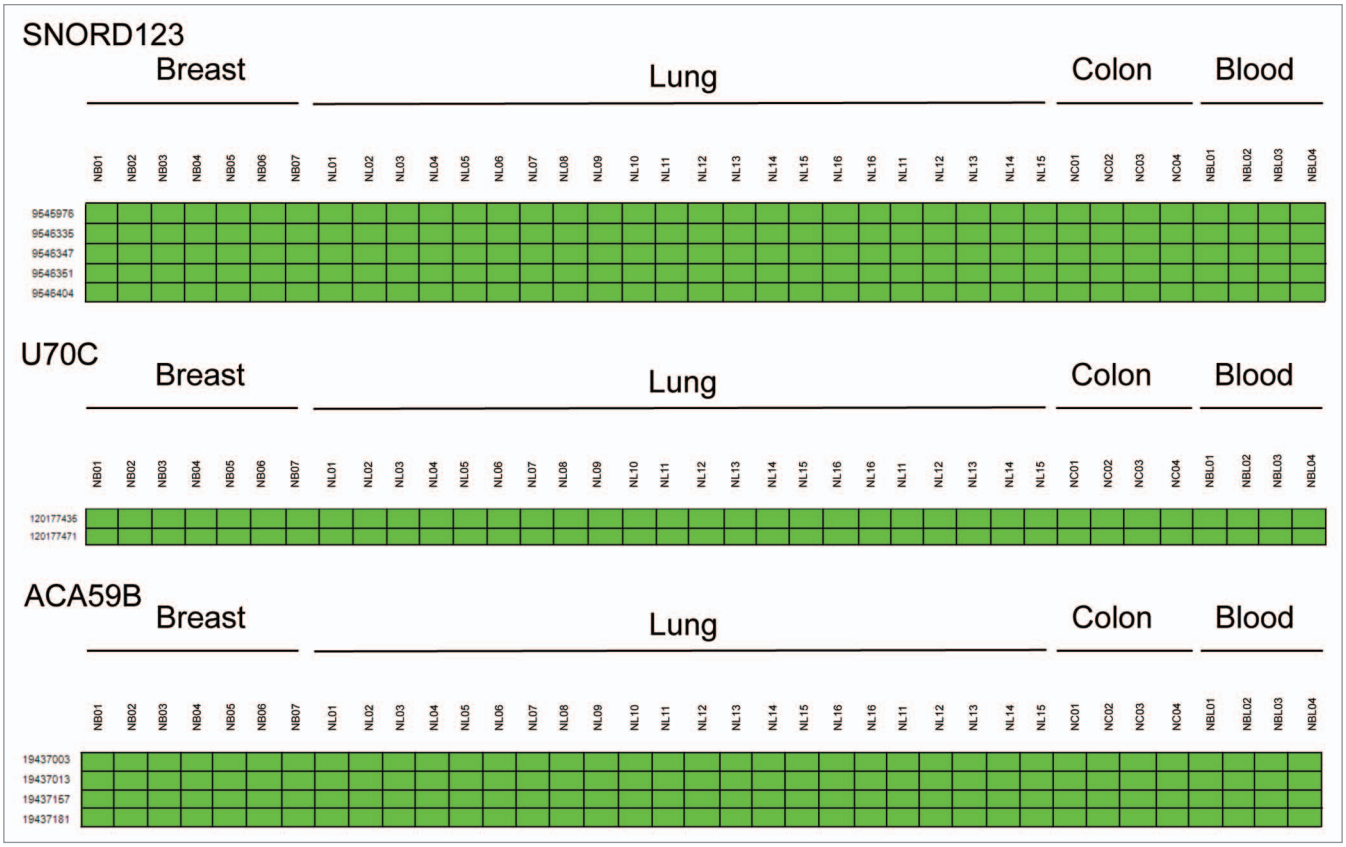

Figure 6. SNORD123, U70C and ACA59B CpG island methylation in normal tissues. DNA methylation microarray analyses of five, two and four CpG sites corresponding to the bisulfite genomic sequenced CpG islands for SNORD123, U70C and ACA59B in normal breast, lung and colon tissues and blood samples. Each square represents a single CpG: green square, unmethylated CpG; red square, methylated CpG. The three snoRNA-associated CpG islands were unmethylated in all the normal tissues tested.

7. Hammond SM. MicroRNAs as tumor suppressors. Nat Genet 2007; 39:582-3; PMID:17460676; http://dx.doi.org/10.1038/ng0507-582.
8. Melo SA, Esteller M. Dysregulation of microRNAs in cancer: playing with fire. FEBS Lett 2011; 585:2087-99; PMID:20708002; http://dx.doi.org/10.1016/j.febslet.2010.08.009.
9. Thomson JM, Newman M, Parker JS, Morin-Kensicki EM, Wright T, Hammond SM. Extensive post-transcriptional regulation of microRNAs and its implications for cancer. Genes Dev 2006; 20:2202-7; PMID:16882971; http://dx.doi.org/10.1101/gad.1444406.
10. Chang TC, Yu D, Lee YS, Wentzel EA, Arking DE, West KM, et al. Widespread microRNA repression by Myc contributes to tumorigenesis. Nat Genet 2008; 40:43-50; PMID:18066065; http://dx.doi.org/10.1038/ng.2007.30.
11. Melo SA, Ropero S, Moutinho C, Aaltonen LA, Yamamoto H, Calin GA, et al. A TARBP2 mutation in human cancer impairs microRNA processing and DICER1 function. Nat Genet 2009; 41:365-70; PMID:19219043; http://dx.doi.org/10.1038/ng.317.
12. Hill DA, Ivanovich J, Priest JR, Gurnett CA, Dehner LP, Desruisseau D, et al. DICER1 mutations in familial pleuropulmonaryblastoma. Science 2009; 325:965; PMID:19556464; http://dx.doi.org/10.1126/science.1174334.
13. Melo SA, Moutinho C, Ropero S, Calin GA, Rossi S, Spizzo R, et al. A genetic defect in exportin-5 traps precursor microRNAs in the nucleus of cancer cells. Cancer Cell 2010; 18:303-15; PMID:20951941; http://dx.doi.org/10.1016/j.ccr.2010.09.007.
14. Lopez-Serra P, Esteller M. DNA methylation-associated silencing of tumor-suppressor microRNAs in cancer. Oncogene 2011; 354:1-14; PMID:21860412.
15. Bejerano G, Pheasant M, Makunin I, Stephen S, Kent WJ, Mattick JS, et al. Ultraconserved elements in the human genome. Science 2004; 304:1321-5; PMID:15131266; http://dx.doi.org/10.1126/science.1098119.
16. Katzman S, Kern AD, Bejerano G, Fewell G, Fulton L, Wilson RK, et al. Human genome ultraconserved elements are ultraselected. Science 2007; 317:915; PMID:17702936; http://dx.doi.org/10.1126/science.1142430.
17. Calin GA, Liu CG, Ferracin M, Hyslop T, Spizzo R, Sevignani C, et al. Ultraconserved regions encoding ncRNAs are altered in human leukemias and carcinomas. Cancer Cell 2007; 12:215-29; PMID:17785203; http://dx.doi.org/10.1016/j.ccr.2007.07.027.
18. Lujambio A, Portela A, Liz J, Melo SA, Rossi S, Spizzo R, et al. CpG island hypermethylation-associated silencing of non-coding RNAs transcribed from ultraconserved regions in human cancer. Oncogene 2010; 29:6390-401; PMID:20802525; http://dx.doi.org/10.1038/onc.2010.361.
19. Gerbi SA. Small nucleolar RNA. Biochem Cell Biol 1995; 73:845-58; PMID:8722000; http://dx.doi.org/10.1139/o95-092.
20. Cavaillé J, Nicoloso M, Bachellerie JP. Targeted ribose methylation of RNA in vivo directed by tailored antisense RNA guides. Nature 1996; 383:732-5; PMID:8878486; http://dx.doi.org/10.1038/383732a0.
21. Kiss-László Z, Henry Y, Bachellerie JP, Caizergues-Ferrer M, Kiss T. Site-specific ribose methylation of preribosomal RNA: a novel function for small nucleolar RNAs. Cell 1996; 85:1077-88; PMID:8674114; http://dx.doi.org/10.1016/S0092-8674(00)81308-2.
22. Ganot P, Bortolin ML, Kiss T. Site-specific pseudouridine formation in preribosomal RNA is guided by small nucleolar RNAs. Cell 1997; 89:799-809; PMID:9182768; http://dx.doi.org/10.1016/S0092-8674(00)80263-9.
23. Li T, Zhou X, Wang X, Zhu D, Zhang Y. Identification and characterization of human snoRNA core promoters. Genomics 2010; 96:50-6; PMID:20353816; http://dx.doi.org/10.1016/j.ygeno.2010.03.010.
24. Lestrade L, Weber MJ. snoRNA-LBME-db, a comprehensive database of human H/ACA and C/D box snoRNAs. Nucleic Acids Res 2006; 34:158-62; PMID:16381836; http://dx.doi.org/10.1093/nar/gkj002.
25. Balakin AG, Smith L, Fournier MJ. The RNA world of the nucleolus: two major families of small RNAs defined by different box elements with related functions. Cell 1996; 86:823-34; PMID:8797828; http://dx.doi.org/10.1016/S0092-8674(00)80156-7.
26. Bachellerie JP, Cavaillé J, Hüttenhofer A. The expanding snoRNA world. Biochimie 2002; 84:775-90; PMID:12457565; http://dx.doi.org/10.1016/S0300-9084(02)01402-5.
27. Reichow SL, Hamma T, Ferré-D'Amaré AR, Varani G. The structure and function of small nucleolarribonucleoproteins. Nucleic Acids Res 2007; 35:1452-64; PMID:17284456; http://dx.doi.org/10.1093/nar/gkl1172.

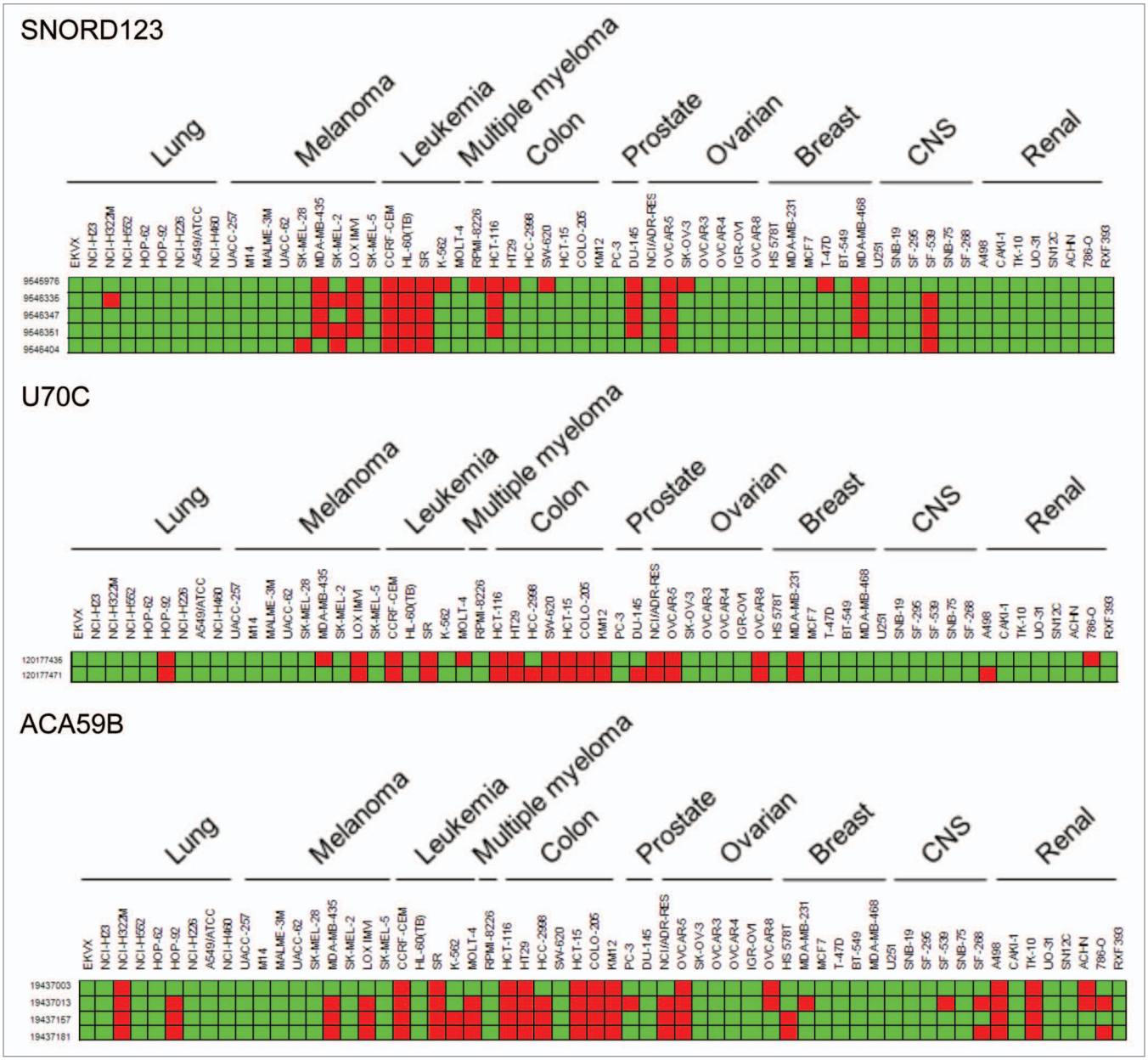

Figure 7. SNORD123, U70C and ACA59B CpG island methylation in human cancer cell lines. DNA methylation microarray analyses of five, two and four CpG sites corresponding to the bisulfite genomic sequenced CpG islands for SNORD123, U70C and ACA59B in the NCI60 panel of seven different tumor types. Each square represents a single CpG: green square, unmethylated CpG; red square, methylated CpG. The three snoRNA-associated CpG islands were methylated in the originally studied HCT-116 cells, but hypermethylation events were also observed in other classes of malignancies, such as leukemias.

28. Heiss NS, Knight SW, Vulliamy TJ, Klauck SM, Wiemann S, Mason PJ, et al. X-linked dyskeratosiscongenita is caused by mutations in a highly conserved gene with putative nucleolar functions. Nat Genet 1998; 19:32-8; PMID:9590285; http://dx.doi.org/10.1038/ng0598-32.
29. Walne AJ, Vulliamy T, Marrone A, Beswick R, Kirwan M, Masunari Y, et al. Genetic heterogeneity in autosomal recessive dyskeratosiscongenita with one subtype due to mutations in the telomerase-associated protein NOP10. Hum Mol Genet 2007; 16:1619-29; PMID:17507419; http://dx.doi.org/10.1093/hmg/ddm111.
30. Vulliamy T, Beswick R, Kirwan M, Marrone A, Digweed M, Walne A, et al. Mutations in the telomerase component NHP2 cause the premature ageing syndrome dyskeratosiscongenita. Proc Natl Acad Sci USA 2008; 105:8073-8; PMID:18523010; http://dx.doi.org/10.1073/pnas.0800042105.
31. Chang LS, Lin SY, Lieu AS, Wu TL. Differential expression of human 5S snoRNA genes. Biochem Biophys Res Commun 2002; 299:196-200; PMID:12437969; http://dx.doi.org/10.1016/S0006-291X(02)02623-2.
32. Mourtada-Maarabouni M, Hedge VL, Kirkham L, Farzaneh F, Williams GT. Growth arrest in human T-cells is controlled by the non-coding RNA growth-arrest-specific transcript 5 (GAS5). J Cell Sci 2008; 121:939-46; PMID:18354083; http://dx.doi.org/10.1242/jcs.024646.
33. Mourtada-Maarabouni M, Pickard MR, Hedge VL, Farzaneh F, Williams GT. GAS5, a non-protein-coding RNA, controls apoptosis and is downregulated in breast cancer. Oncogene 2009; 28:195-208; PMID:18836484; http://dx.doi.org/10.1038/onc.2008.373.
34. Dong XY, Guo P, Boyd J, Sun X, Li Q, Zhou W, et al. Implication of snoRNA U50 in human breast cancer. J Genet Genomics 2009; 36:447-54; PMID:19683667; http://dx.doi.org/10.1016/S1673-8527(08)60134-4.
35. Dong XY, Rodriguez C, Guo P, Sun X, Talbot JT, Zhou W, et al. SnoRNA U50 is a candidate tumor-suppressor gene at 6q14.3 with a mutation associated with clinically significant prostate cancer. Hum Mol Genet 2008; 17:1031-42; PMID:18202102; http://dx.doi.org/10.1093/hmg/ddm375.

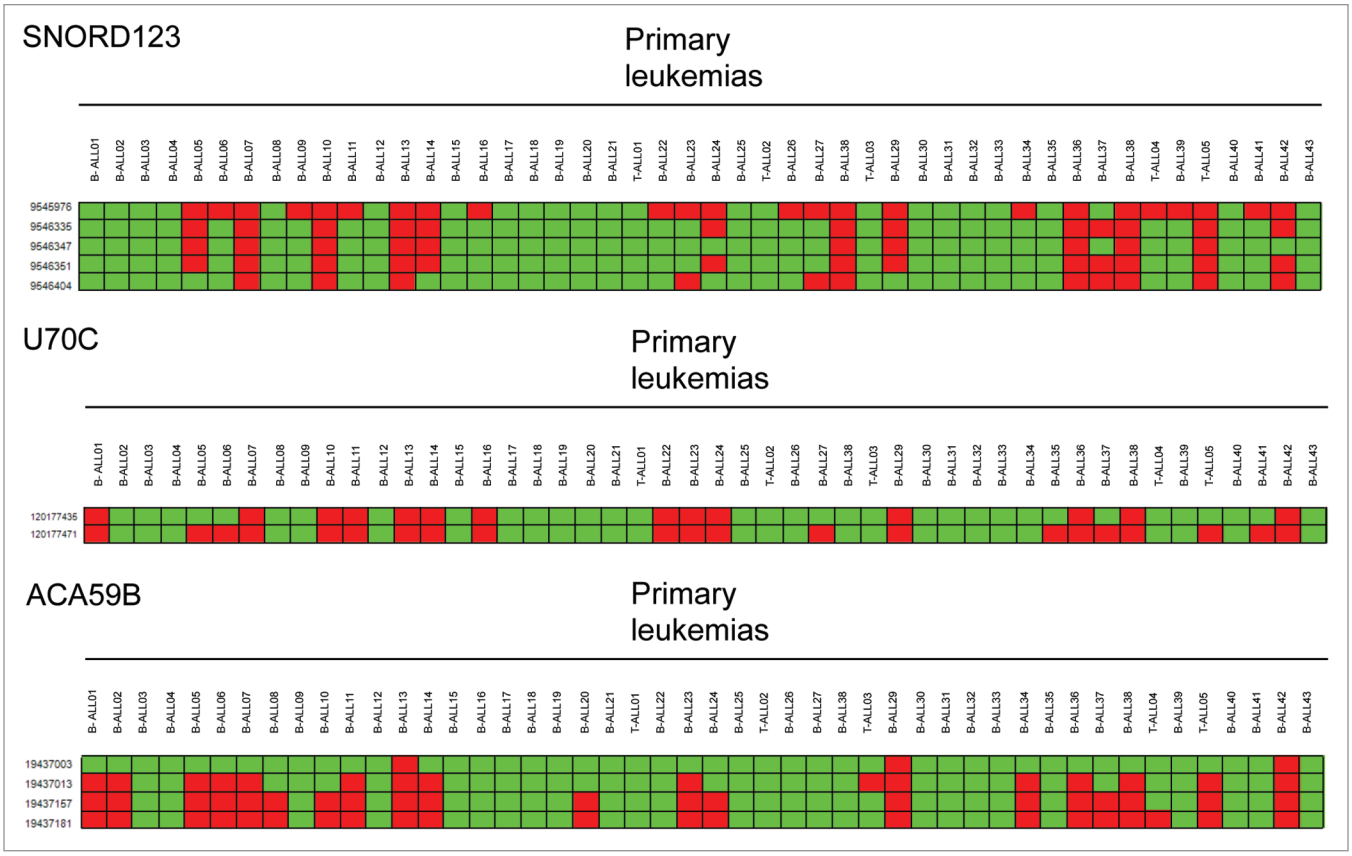

Figure 8. SNORD123, U70C and ACA59B CpG island methylation in primary acute lymphoblastoidleukemias. DNA methylation microarray analyses of five, two and four CpG sites corresponding to the bisulfite genomic sequenced CpG islands for SNORD123, U70C and ACA59B in leukemia patients demonstrated hypermethylation. Each square represents a single CpG: green square, unmethylated CpG; red square, methylated CpG. The B-cell or T-cell phenotype of the studied ALLs is indicated.

36. Horsthemke B, Wagstaff J. Mechanisms of imprinting of the Prader-Willi/Angelman region. Am J Med Genet A 2008; 146:2041-52; PMID:18627066; http://dx.doi.org/10.1002/ajmg.a.32364.
37. Kishore S, Stamm S. The snoRNA HBII-52 regulates alternative splicing of the serotonin receptor 2C. Science 2006; 311:230-2; PMID:16357227; http://dx.doi.org/10.1126/science.1118265.
38. Bratkovic T, Rogelj B. Biology and applications of small nucleolar RNAs. Cell Mol Life Sci 2011; 68:3843-51; PMID:21748470; http://dx.doi.org/10.1007/s00018-011-0762-y.
39. Jones PA, Baylin SB. The epigenomics of cancer. Cell 2007; 128:683-92; PMID:17320506; http://dx.doi.org/10.1016/j.cell.2007.01.029.
40. Rodríguez-Paredes M, Esteller M. Cancer epigenetics reaches mainstream oncology. Nat Med 2011; 17:330-9; PMID:21386836; http://dx.doi.org/10.1038/nm.2305.
41. Kiss T, Filipowicz W. Exonucleolytic processing of small nucleolar RNAs from pre-mRNA introns. Genes Dev 1995; 9:1411-24; PMID:7797080; http://dx.doi.org/10.1101/gad.9.11.1411.
42. Tycowski KT, Shu MD, Steitz JA. A mammalian gene with introns instead of exons generating stable RNA products. Nature 1996; 379:464-6; PMID:8559254; http://dx.doi.org/10.1038/379464a0.
43. Hoeppner MP, White S, Jeffares DC, Poole AM. Evolutionarily stable association of intronicsnoRNAs and microRNAs with their host genes. Genome Biol Evol 2009; 1:420-8; PMID:20333211; http://dx.doi.org/10.1093/gbe/evp045.
44. Kent WJ, Sugnet CW, Furey TS, Roskin KM, Pringle TH, Zahler AM, et al. The human genome browser at UCSC. Genome Res 2002; 12:996-1006; PMID:12045153.
45. Takai D, Jones PA. The CpG island searcher: a new WWW resource. In Silico Biol 2003; 3:235-40; PMID:12954087.
46. Saini HK, Griffiths-Jones S, Enright AJ. Genomic analysis of human microRNA transcripts. Proc Natl Acad Sci USA 2007; 104:17719-24; PMID:17965236; http://dx.doi.org/10.1073/pnas.0703890104.
47. Zhou X, Ruan J, Wang G, Zhang W. Characterization and identification of microRNA core promoters in four model species. PLoS Comput Biol 2007; 3:37; PMID:17352530; http://dx.doi.org/10.1371/journal.pcbi.0030037.
48. Rhee I, Bachman KE, Park BH, Jair KW, Yen RW, Schuebel KE, et al. DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. Nature 2002; 416:552-6; PMID:11932749; http://dx.doi.org/10.1038/416552a.
49. Sandoval J, Heyn HA, Moran S, Serra-Musach J, Pujana MA, Bibikova M, et al. Validation of a DNA methylation microarray for 450,000 CpG sites in the human genome. Epigenetics 2011; 6:692-702; PMID:21593595; http://dx.doi.org/10.4161/epi.6.6.16196.
50. Yang JH, Zhang XC, Huang ZP, Zhou H, Huang MB, Zhang S, et al. snoSeeker: an advanced computational package for screening of guide and orphan snoRNA genes in the human genome. Nucleic Acids Res 2006; 34:5112-23; PMID:16990247; http://dx.doi.org/10.1093/nar/gkl672.
51. Kiss AM, Jády BE, Bertrand E, Kiss T. Human box H/ACA pseudouridylation guide RNA machinery. Mol Cell Biol 2004; 24:5797-807; PMID:15199136; http://dx.doi.org/10.1128/MCB.24.13.5797-807.2004.
52. Luo Y, Li S. Genome-wide analyses of retrogenes derived from the human box H/ACA snoRNAs. Nucleic Acids Res 2007; 35:559-71; PMID:17175533.
53. Weber MJ. Mammalian small nucleolar RNAs are mobile genetic elements. PLoS Genet 2006; 2:205; PMID:17154719; http://dx.doi.org/10.1371/journal.pgen.0020205.
54. McMahon M, Ayllón V, Panov KI, O'Connor R. Ribosomal 18 S RNA processing by the IGF-I-responsive WDR3 protein is integrated with p53 function in cancer cell proliferation. J Biol Chem 2010; 285:18309-18; PMID:20392698; http://dx.doi.org/10.1074/jbc.M110.108555.
55. Peng Q, Wu J, Zhang Y, Liu Y, Kong R, Hu L, et al. 1A6/DRIM, a novel t-UTP, activates RNA polymerase I transcription and promotes cell proliferation. PLoS One 2010; 5:14244; PMID:21151873; http://dx.doi.org/10.1371/journal.pone.0014244.
56. Uemura M, Zheng Q, Koh CM, Nelson WG, Yegnasubramanian S, De Marzo AM. Overexpression of ribosomal RNA in prostate cancer is common but not linked to rDNA promoter hypomethylation. Oncogene 2011; 319:1-10; PMID:21822302.