

Altered Methylation at MicroRNA-Associated CpG Islands in Hereditary and Sporadic Carcinomas: A Methylation-Specific Multiplex Ligation-Dependent Probe Amplification (MS-MLPA)-Based Approach

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MicroRNAs (miRNAs) are small noncoding RNAs that contribute to tumorigenesis by acting as oncogenes or tumor suppressor genes and may be important in the diagnosis, prognosis and treatment of cancer. Many miRNA genes have associated CpG islands, suggesting epigenetic regulation of their expression. Compared with sporadic cancers, the role of miRNAs in hereditary or familial cancer is poorly understood. We investigated 96 colorectal carcinomas, 58 gastric carcinomas and 41 endometrial carcinomas, occurring as part of inherited DNA mismatch repair (MMR) deficiency (Lynch syndrome), familial colorectal carcinoma without MMR gene mutations or sporadically. Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) assays were developed for 11 miRNA loci that were chosen because all could be epigenetically regulated through the associated CpG islands and some could additionally modulate the epigenome by putatively targeting the DNA methyltransferases or their antagonist retinoblastoma-like 2 (RBL2). Compared with the respective normal tissues, the predominant alteration in tumor tissues was increased methylation for the miRNAs 1-1, 124a-1, 124a-2, 124a-3, 148a, 152 and 18b; decreased methylation for 200a and 208a; and no major change for 373 and let-7a-3. The frequencies with which the individual miRNA loci were affected in tumors showed statistically significant differences relative to the tissue of origin (colorectal versus gastric versus endometrial), MMR proficiency versus deficiency and sporadic versus hereditary disease. In particular, hypermethylation at miR-148a and miR-152 was associated with microsatellite-unstable (as opposed to stable) tumors and hypermethylation at miR-18b with sporadic disease (as opposed to Lynch syndrome). Hypermethylation at miRNA loci correlated with hypermethylation at classic tumor suppressor promoters in the same tumors. Our results highlight the importance of epigenetic events in hereditary and sporadic cancers and suggest that MS-MLPA is an excellent choice for quantitative analysis of methylation in archival formalin-fixed, paraffin-embedded samples, which pose challenges to many other techniques commonly used for methylation studies.

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INTRODUCTION

In addition to covalent modifications of DNA and histone proteins, microRNAs (miRNAs) have emerged as important epigenetic regulators that control gene expression without altering the DNA sequence itself. MicroRNAs are small noncoding RNAs that exert their regulatory effect by repressing translation or direct-

ing degradation of mRNA, after binding to a complementary sequence usually located in the 3'-untranslated region of target genes. The human genome encodes nearly 1,000 miRNAs that may regulate one-third of all human transcripts (1). MicroRNAs are important for normal development, differentiation and cellular growth and their aberrant function may

give rise to human disease (2). In cancer, miRNAs may act as tumor suppressors when they have proto-oncogene transcripts as targets (for example, miR-124a, which targets CDK6) (3) and as oncogenes when they target tumor suppressor gene (TSG) transcripts (for example, miR-372 and miR-373, which target LATS2) (4). Different cancers display both shared and unique signatures of miRNA alterations, reflecting broader patterns of genetic and epigenetic instability characteristic of such cancers (5–7).

Epigenetic changes can promote tumorigenesis in two main ways, by altering the activity of specific genes and by inducing genomic instability. DNA hyper-

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Table 1. Basic molecular and clinicopathological characteristics of the study series.

	CRC				GC			EC
	Sporadic MSS	Sporadic MSI	Lynch	FCCX	Sporadic MSS	Sporadic MSI	Lynch	Lynch
Total number of tumors	30	14	34	18	34	11	13	41
Mean age of diagnosis (years)	71.9	76.6	45.6	56.4	71.1	76.5	57.5	49.7
Germline mutation present in MMR genes (total)	N/A	N/A ^a	34	0	N/A	N/A ^a	13	41
<i>MLH1</i>			32				11	35
<i>MSH2</i>			2				2	3
<i>MSH6</i>			0				0	3
High MSI	0	14	33	1	0	11	13	26 ^b
Tumor stage (Dukes/WHO/FIGO) ^c								
A/I/I	4/29	2/14	8/25	3/11	11/34	1/11	2/13	13/26
B/II/II	13/29	9/14	12/25	6/11	6/34	5/11	5/13	8/26
C/III/III	10/29	1/14	5/25	2/11	11/34	3/11	5/13	1/26
D/IV/IV	2/29	2/14	0/25	0/11	6/34	2/11	1/13	4/26

^aDue to the *MLH1* promoter methylation (present in 12 of 14 (86%) of sporadic MSI CRCs and 8 of 11 (73%) of sporadic MSI GCs). N/A, not applicable.

^bIn the same series, MMR protein corresponding to the germline mutation was lost in 29 of 29 (100%) cases available for immunohistochemical analysis.

^cAccording to the Dukes (A–D), World Health Organization (WHO) (I–IV) and International Federation of Gynecology and Obstetrics (FIGO) (I–IV) staging system for CRC, GC and EC, respectively. The denominator indicates the number of tumors for which the data were available.

methylation is important in silencing critical TSGs. Cancer cells often show manifestations of the CpG island methylator phenotype (CIMP), the basic mechanisms of which remain elusive (8,9). Furthermore, promoter methylation of the DNA mismatch repair (MMR) gene *MLH1* underlies microsatellite instability (MSI) and explains the majority of sporadic MMR-deficient colorectal carcinomas (CRCs) (10). DNA hypomethylation activates oncogenes, and global hypomethylation of DNA induces chromosomal instability (11). One-third of all human miRNAs has a CpG island in the upstream region and may be regulated by DNA methylation (12). Some microRNAs (called “epi-miRNAs”) may even target the epigenetic machinery itself, such as the DNA methyltransferases (DNMTs) or their antagonist retinoblastoma-like 2 (RBL2), suggesting that the epigenome and miRNome are closely connected (13). A better understanding of the epigenetic processes that contribute to cancer development provides tools for anticancer therapy (14).

Current knowledge of cancer associations of miRNAs is mainly based on cell

lines and sporadic forms of cancer. We have previously shown that, in hereditary or familial CRC or endometrial carcinoma (EC) syndromes, TSG promoter methylation reflects tumor type and family category (15). We now wanted to find out how DNA methylation profiles apply to miRNA loci, by focusing on miRNAs that could be targets of epigenetic regulation on the one hand and target the epigenome on the other hand, theoretically allowing for self-amplified loops. Our series consisted of archival formal-fixed paraffin-embedded samples, which prompted us to test novel methods (custom-made methylation-specific multiplex ligation-dependent probe amplification [MS-MLPA]) for the present purposes.

MATERIALS AND METHODS

Patients and Samples

This study was on the basis of 195 tumor and paired normal tissue samples, including 96 CRCs, 58 gastric carcinomas (GCs) (41 of which were intestinal and 17 were diffuse) and 41 ECs (Table 1). The series was further stratified according to

germline mutation status into Lynch syndrome (having germline mutations in *MSH2*, *MLH1* or *MSH6*); familial colorectal carcinoma, type X (FCCX; familial nonpolypotic CRC without MMR gene mutations); and sporadic cases. Among the latter, microsatellite-stable (MSS) and MSI subgroups were distinguished (15,16), ultimately resulting in eight patient categories. DNA was extracted from paraffin-embedded tumors from selected areas with high tumor percentages and matching normal tissue by a method modified from Isola *et al.* (17). Additionally, commercial cell lines representing colon cancer (HCT116, HCT15, HCA7, RKO, LIM1215, KM12, SW48, LoVo) or EC (HEC59) were used for methodological optimization. The appropriate institutional review boards of the Helsinki University Central Hospital approved this study.

Data Mining

The following repositories and computer tools were used: miRBase (<http://www.mirbase.org>) (18) for basic information of miRNAs, miRanda

(<http://www.microrna.org>) (19) and TargetScan (<http://www.targetscan.org>) (20) for miRNA target gene identification, CpG Island Searcher (<http://www.uscnorris.com/cpgislands2>) (21) for CpG island delineation, miRGator (<http://genome.ewha.ac.kr/miRGator/miRGator.html>) (22) for functional information of miRNAs and Ensembl (<http://www.ensembl.org>) for genomic sequences flanking the miRNA loci.

Bisulfite Modification and Sequencing

DNA was modified by using the CpGenome DNA Modification Kit (Chemicon, Temecula, CA, USA). Bisulfite-converted DNA was amplified with methylation-unbiased primers specified in Supplementary Table S4 and used for sequencing directly or after cloning. For the latter purpose, amplification products were cloned into a pCR2.1-TOPO vector by using the TOPO TA Cloning System (Invitrogen, Carlsbad, CA, USA), and all resulting white colonies were used for DNA extraction and sequencing.

Custom-Made MS-MLPA

The methylation statuses of all CpG sites in a miRNA-associated CpG island were determined in normal tissues and cancer cell lines by bisulfite sequencing (see above) and representative CpG dinucleotides that were part of the restriction site for the methylation-sensitive enzyme *HhaI* (GCGC) chosen for the design of probes for custom-made MS-MLPA. In MS-MLPA (23), a signal peak is generated if the sample DNA is methylated, which protects the DNA probe hybrids against *HhaI* digestion, and the ligated probes can be amplified by the polymerase chain reaction. For the synthetic MS-MLPA probe design, the manufacturer's instructions (<http://www.mrc-holland.com>) were followed. An MS-MLPA probe pair consists of two oligonucleotides: the left probe oligonucleotide (LPO) and the right probe oligonucleotide (RPO). Custom-made MS-MLPA probes (Table 2) were added to the SALSA MLPA P300-

A1 Reference-2 kit (MRC-Holland, Amsterdam, the Netherlands), to make a complete MS-MLPA assay.

All MS-MLPA reactions were performed according to the manufacturer's instructions (<http://www.mrc-holland.com>) by using 100–150 ng DNA. The MS-MLPA products were separated by capillary electrophoresis (on an ABI 3730 Automatic DNA Sequencer [Applied Biosystems, Carlsbad, CA, USA]) and analyzed by using GeneMapper4.0 genotyping software (Applied Biosystems). The methylation dosage ratio was obtained by the following calculation:

$$D_m = (P_x/P_{ctrl})_{Dig} / (P_x/P_{ctrl})_{Undig}$$

where D_m is the methylation dosage ratio, P_x is the peak area of a given probe, P_{ctrl} is the sum of the peak areas of all control probes, Dig stands for *HhaI* digested sample and Undig stands for undigested sample. D_m can vary between 0 and 1.0 (corresponding to 0–100% of methylated DNA). The D_m value of 0.20 divided the test specimens into two distinct categories (methylation present versus absent) when evaluated against bisulfite sequencing (Figure 1A), and D_m values of ≥ 0.20 were therefore considered to reliably indicate methylation by the present MS-MLPA assays. Thresholds for hypermethylation or hypomethylation in tumor DNA, compared with normal DNA, were determined on the basis of methylation levels in normal DNAs of the same tissue type, as described below in Results.

miRNA Expression Analysis by Microarrays

Information of expression of the present miRNAs was derived from a global investigation of miRNA expression patterns in cancer cell lines that will be published separately (S. Kaur *et al.*, unpublished data). We used Human miRNA Microarray G4470B (Agilent Technologies, Santa Clara, CA, USA), which contains 723 human and 76 human viral miRNAs, sourced from the Sanger miR-Base (version 10.1). Signals obtained by

using Agilent's Feature Extraction software (version 10.7.3.1) were processed according to standard procedures (24) by GeneSpring GX11.0.2 and compared with methylation data.

Statistical Analysis

Statistical analyses were performed by using SPSS Statistics (IBM SPSS, Chicago, IL, USA). Statistical significance for the differences between frequencies or distributions was evaluated with Fisher exact probability test or *t* test (or Wilcoxon signed ranks test for series that were not normally distributed), respectively. For correlations, the Pearson product-moment correlation coefficient (*r*) for linear correlation was determined. *P* values < 0.05 (two-tailed) were considered significant.

All supplementary materials are available online at www.molmed.org.

RESULTS

Study Design

We investigated 195 carcinomas equally from many patients representing familial/hereditary or sporadic cases (Table 1) by using a candidate gene approach described in Figure 2. The final selection included 11 miRNA loci that could all be regulated by methylation by virtue of CpG islands associated with these loci. All but one (208a [25]) were intergenic according to available databases. A significant proportion could in theory additionally contribute to aberrant methylation by targeting the DNMTs or their antagonist RBL2. The latter, so-called "epi-miRNAs," included three miRNAs with verified targets in the epigenetic machinery (miR-1-1 targeting HDAC4 [26], miR-148a targeting DNMT3B [27] and miR-152 targeting DNMT1 [28]) and three additional miRNAs with putative epigenetic targets (18b and 200a predicted to target RBL2, and 373 predicted to target DNMT1, DNMT3A and RBL2; additionally, miR-1-1 and miR-148a were predicted to target DNMT1 besides their verified targets).

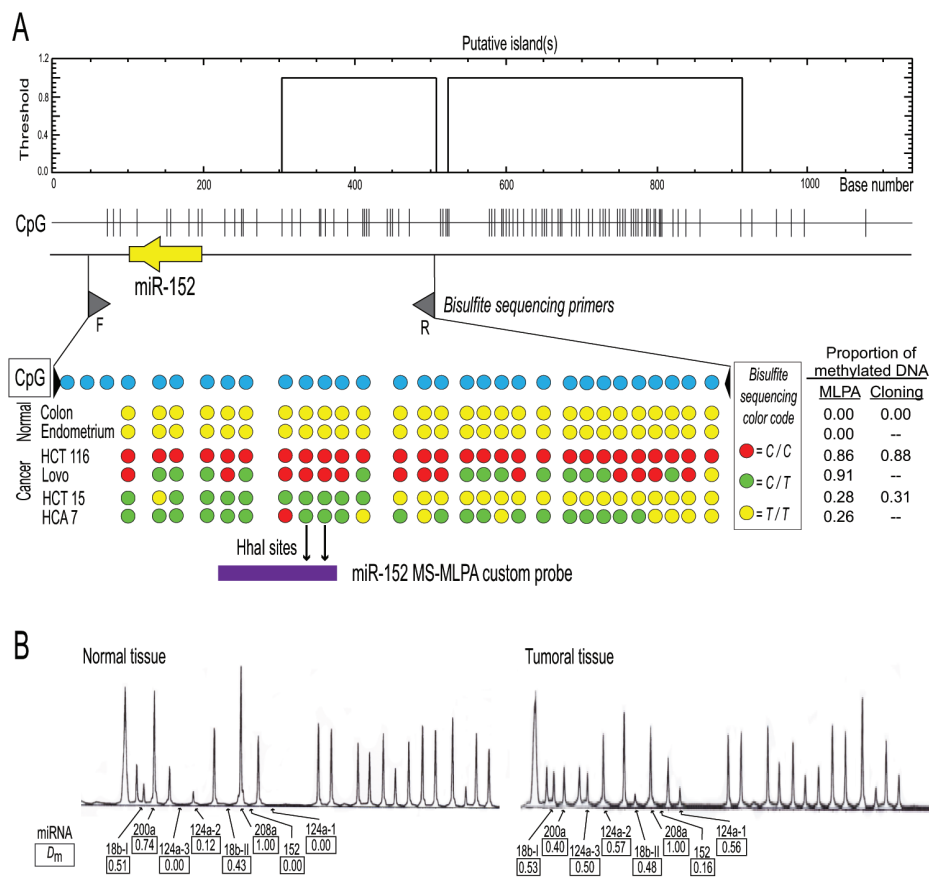


Figure 1. Design and validation of custom MS-MLPA using miR-152 as an example (see Supplementary Figure S4 for the remaining miRNAs). Borders of the CpG island(s) relative to the mature miRNA (yellow arrow) were first defined by CpG Island Searcher (A, top) followed by bisulfite sequencing of normal tissues and cell lines to select a representative region for MS-MLPA probe design (A, bottom). Results from direct bisulfite sequencing are displayed on the left. The relative proportions of DNA with the particular two *HhaI* sites methylated were quantified by MS-MLPA and by choosing one sample of each type for cloning after bisulfite conversion (shown on the right). D_m values by MS-MLPA were well in agreement with results from the cloning analysis. The probes for multiple miRNAs that performed well together were combined to make a custom assay (B). Methylation dosage ratios (D_m) were concordant with bisulfite sequencing in the test samples (values for miR-152 are shown in A, right); in a Lynch syndrome patient with EC (B), a typical methylation pattern was evident in normal tissue (compare to Figure 3) combined with some altered methylation in tumor tissue (for miRNAs 200a, 124a-1, 124a-2 and 124a-3). Peaks without a label correspond to control probes used for normalization.

Optimization and Validation of Custom-Made MS-MLPA

The boundaries of CpG islands associated with the miRNA loci in question were defined by appropriate computer programs, and all CpG sites within an island were analyzed for methylation by bisulfite sequencing. Regions that showed stable patterns of methylation

over long stretches of DNA (and contained one or several *HhaI* sites, which was a prerequisite for the method used) were chosen for MS-MLPA probe design (Figure 1A, Supplementary Figure S4, Table 2). Custom MS-MLPA assays were validated against bisulfite sequencing by using normal tissues and cell lines. On the basis of comparison of MS-MLPA

data with bisulfite sequencing results with and without cloning, optimization was considered successful when there were no false-positive results (methylation dosage ratio was <0.20 by MS-MLPA against T/T by direct bisulfite sequencing) and no false-negative results (methylation dosage ratio was ≥ 0.20 against C/T or C/C by bisulfite sequencing; see the Figure 1A legend). The present 11 miRNA loci were examined in two multiplex assays, kit 1 and kit 2 (kit 1 included two probe pairs for 18b, which gave highly concordant results, and the values obtained with 18b-I are presented in this report). An example of the outcome of the validated kit 1 using normal and tumor tissue from a patient included in the present study is shown in Figure 1B.

Microarray-based expression analysis of cell lines and normal tissues against methylation at the miRNA loci of interest showed that increasing methylation dosage ratios by MS-MLPA significantly correlated with reduced expression of the mature miRNAs 148a, 152 and 200a (the remaining miRNAs either were uninformative because of similar patterns in all samples or displayed no correlation) (Supplementary Figure S5).

DNA Methylation Patterns at miRNA Loci in Normal versus Tumor Tissues

MS-MLPA analysis of the individual miRNAs revealed three groups in normal tissues on the basis of the degree of methylation (expressed as the methylation dosage ratio, D_m) (Figure 3). The first group consists of loci with absent ($D_m < 0.20$) or low-level methylation ($D_m < 0.30$ on average) and includes miRNAs 124a-1, 124a-2, 124a-3, 1-1, 148a, and 152 (Figure 3A). The second group comprises miRNAs 18b and 200a with intermediate methylation (D_m around 0.5; Figure 3B). The third group consists of miRNAs 208a, 373, and let-7a-3 with high methylation (D_m close to 1.0; Figure 3B). In paired tumor tissues, the predominant change was increased methylation for all miRNAs from the first group as well as for miR-18b,

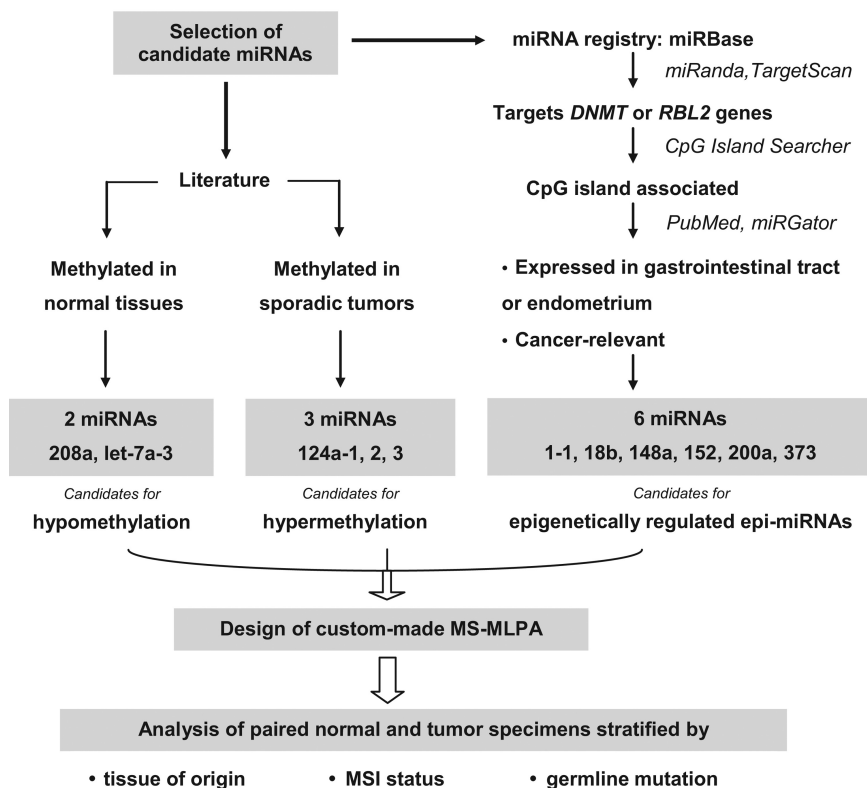


Figure 2. Flowchart of this investigation.

whereas miR-200a and miR-208a showed decreased methylation. Finally, miR-373 and let-7a-3 displayed mainly minor alterations and no consistent pattern.

Among the eight different patient categories examined (please see key in Figure 3B), those that showed statistically significant differences between normal and tumor tissues are indicated by asterisks in Figure 3. Whereas some miRNA loci (notably, miR-124a-1, miR-124a-2 and miR-124a-3) invariably showed a statistically significant difference in tumor versus normal tissue regardless of colorectal, gastric or endometrial origin, or the patient category in question, other miRNA loci were involved in a more selective manner. The number of miRNA loci with a statistically significant methylation difference between tumor and normal tissue varied from 3 to 8 (out of 11) per patient category, where 8 was attributable to Lynch CRC and FCCX CRC.

Methylation Alterations at miRNA Loci in Tumors: Relationship to Tissue of Origin, MSI Status and Sporadic versus Hereditary Disease

The nine miRNA loci, each of which showed a predominant pattern of alterations in Figure 3, were used to assign frequencies of hypermethylation or hypomethylation to the individual groups of tumors (Table 3). Methylation dosage ratios (D_m) in normal tissues (Supplementary Table S6) were first used to determine the thresholds for hypermethylation (for the seven hypermethylation-prone markers) and hypomethylation (for the two hypomethylation-prone markers) in a tissue-specific manner. Specifically, a tumor sample was considered to be hypermethylated at a given miRNA locus if the D_m value in that particular specimen was equal to or higher than the mean D_m plus 1 standard deviation calculated for all normal specimens of the same tissue type (colorectal, gastric or endometrial). (If D_m plus 1 standard deviation was

<0.20, the value of 0.20 was used instead, because it was our threshold for a reliable detection of methylation by the present method [see Materials and Methods].) Similarly, a tumor sample was considered to be hypomethylated if the D_m value in the specimen was equal to or lower than the mean D_m minus 1 standard deviation calculated for all normal specimens of that tissue type.

The miRNAs 124a-1, 124a-2, 124a-3 and 18b were associated with the highest frequencies of hypermethylation in tumors (41–100% for the 124a family and 18–86% for 18b; Table 3). When assessed against the tissue of origin (set I of comparisons in Table 3), the miRNAs 124a-1, 124a-3 and 18b were significantly more often hypermethylated in CRC (and to a lesser extent, GC) compared with EC. In contrast, hypermethylation at miR-152 was typical of EC, occurring in 54% of such tumors, as opposed to 24% of CRC ($P < 0.01$) and 17% of GC ($P < 0.01$).

Sporadic CRCs and GCs with both MSS and MSI subgroups available were used to evaluate the role of the MSI status (set II in Table 3). The miRNAs 148a and 152 provided the best discrimination between MSS and MSI tumors among both CRCs and GCs, with hypermethylation being significantly associated with MSI tumors. A similar association was seen for miR-1-1 in CRC and for miR-124a-1, miR-124a-3 and miR-18b in GC.

MSI cancers were used to assess the possible impact of the disease setting (sporadic versus Lynch syndrome). In this regard, the most informative miRNA was 18b, which showed a hypermethylation frequency of 86% among sporadic CRCs with MSI and 73% among sporadic GCs with MSI, compared with only 18% ($P < 0.01$) and 23% ($P = 0.015$), respectively, among the corresponding tumors from Lynch syndrome patients.

For the two miRNAs with consistent hypomethylation in tumors, miR-200a and miR-208a, hypomethylation frequencies were significantly higher for CRC (and to a lesser extent, GC) compared with EC (Table 3). Evaluation of hypomethylation against MSI status did not

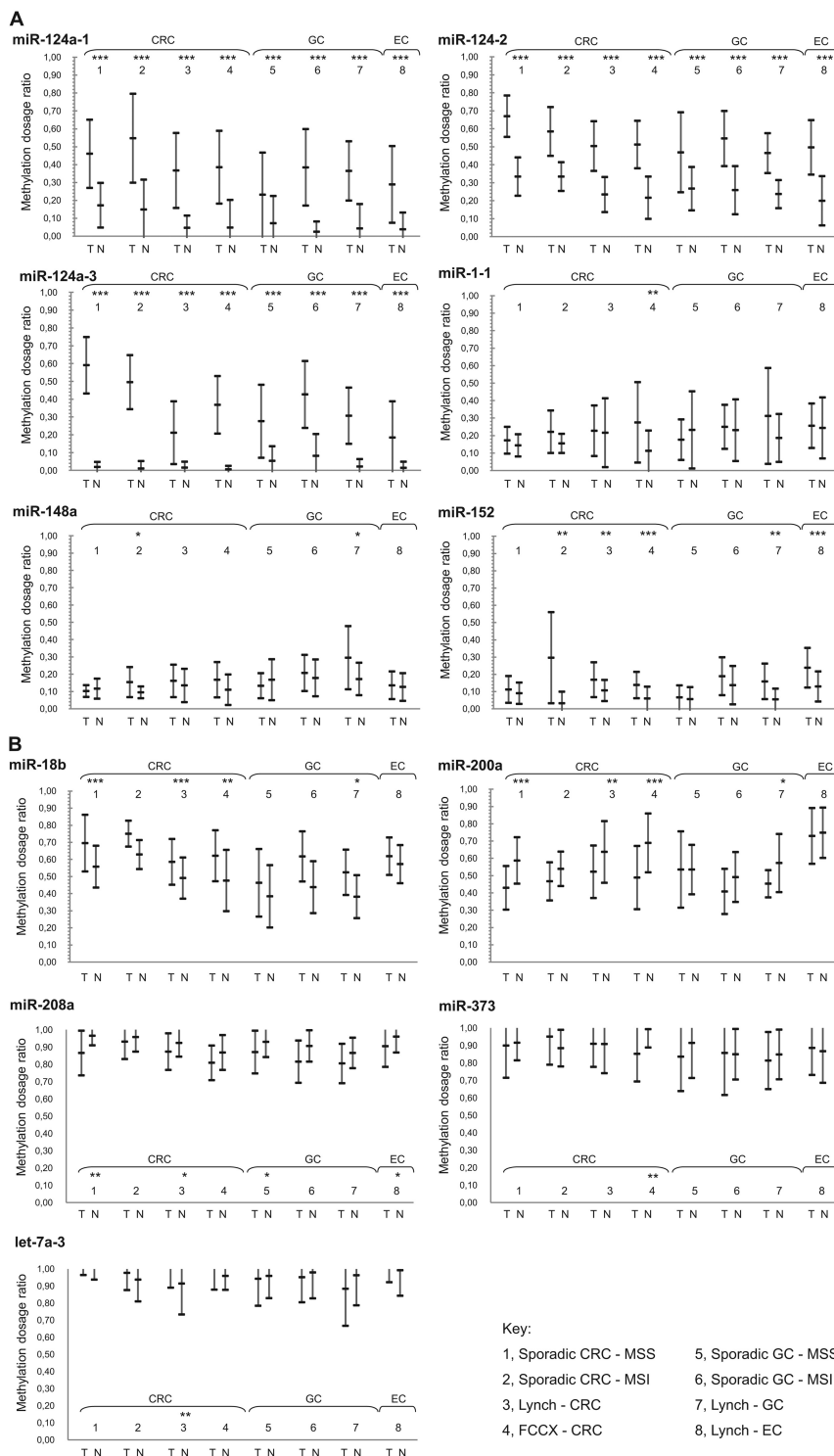


Figure 3. Average methylation dosage ratio \pm 1 standard deviation in tumor (T) versus normal (N) tissues for miRNA loci that displayed low (A) or intermediate/high methylation (B) in normal tissues. The results are given separately for each patient category out of eight listed in B, and the exact numerical values are available in Supplementary Table S5. Asterisks indicate statistical significance for the difference between T and N on the level of * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ on the basis of a t test or Wilcoxon test.

reveal any statistically significant differences. Hypomethylation at miR-200a distinguished sporadic microsatellite-unstable GC from the Lynch counterpart (55% versus 8%, $P = 0.023$).

Hypermethylation at miRNA-Associated versus TSG-Associated CpG Islands

Among the seven miRNAs with consistent hypermethylation in tumors (124a-1, 124a-2, 124a-3, 1-1, 148a, 152 and 18b), the average number of miRNAs showing hypermethylation per tumor was calculated for each tumor category (Table 3, right). This value showed statistically significant differences ($P < 0.01$) relative to the tissue of origin (higher in CRC [3.6] than in GC [3.0] or EC [2.9]), MSI status (higher in MSI versus MSS tumors) and sporadic versus hereditary disease (higher among sporadic cases). We previously determined the occurrence of hypermethylation at 24 traditional TSG promoters in the same tumors (15,16). Hypermethylation at miRNA-associated CpG islands was tightly correlated with the number of hypermethylated TSGs in the same tumors ($r = 0.481$, $P < 0.001$, calculated for the entire tumor series).

DISCUSSION

Concerted methylation at multiple CpG islands (CIMP) is common in various types of cancers and is measured by using combinations of CpG island-containing sequences, some of which reside in promoter regions of TSGs, whereas others are not associated with any known genes (15,29,30). Available information of the role of miRNA loci in CIMP is limited as far as primary human malignancies are concerned and even more so for familial and hereditary cancers. We examined a large series of sporadic and hereditary carcinomas, stratified into clinically relevant subcategories, for methylation at 11 miRNA loci that could all be targets of epigenetic regulation through associated CpG islands. Some even target the epigenome by themselves, on the basis of computer predictions and existing func-

Table 3. Percentage of tumors with hypermethylation and hypomethylation.

Tumor category	Number	Percentage of tumors with methylation change ^a									Average number of hypermethylated	
		Hypermethylation						Hypomethylation			miRNA out of 7	TSG out of 24 ^b
		124a-1	124a-2	124a-3	1-1	148a	152	18b	200a	208a		
(a) Sporadic CRC MSS	30	90	97	100	7	0	7	70	53	43	3.7	3.4
(b) Sporadic CRC MSI	14	93	93	100	29	29	50	86	50	14	4.7	7.3
(c) Lynch CRC	34	74	82	44	32	35	32	18	38	35	3.2	3.4
(d) FCCX CRC	18	72	78	83	22	28	17	28	56	67	3.3	3.9
(e) CRC total	96	81	88	77	22	22	24	46	48	41	3.6	4.1
(f) Sporadic GC MSS	34	44	85	53	0	0	3	29	32	35	2.2	2.0
(g) Sporadic GC MSI	11	91	100	91	9	27	45	73	55	55	4.4	6.1
(h) Lynch GC	13	92	92	85	23	46	31	23	8	54	3.9	4.1
(i) GC total	58	64	90	67	7	16	17	36	31	43	3.0	3.2
(j) Lynch EC	41	66	83	41	10 ^c	18	54	22 ^c	20	20	2.9	3.1

P value^d

I ^e												
(e) versus (i)	0.026	ns ^f	ns	0.026	ns	ns	ns	ns	ns	ns	<0.01	0.049
(e) versus (j)	0.048	ns	<0.01	ns	ns	<0.01	0.019	<0.01	0.028	<0.01	0.019	
(i) versus (j)	ns	ns	0.019	ns	ns	<0.01	ns	ns	0.025	ns	ns	
II												
(a) versus (b)	ns	ns	ns	0.048	<0.01	<0.01	ns	ns	ns	ns	<0.01	<0.01
(f) versus (g)	0.012	ns	0.032	ns	0.012	<0.01	0.015	ns	ns	ns	<0.01	<0.01
III												
(b) versus (c)	ns	ns	<0.01	ns	ns	ns	<0.01	ns	ns	ns	<0.01	<0.01
(g) versus (h)	ns	ns	ns	ns	ns	ns	0.015	0.023	ns	ns	ns	0.027

Gray shading indicates values corresponding to the total cases in each group of tissue of origin.

^aUsing cutoffs determined by methylation in the respective normal tissues (Supplementary Table S6).

^bData from Joensuu *et al.* (15) and Gylling *et al.* (16).

^cPercentage based on 40 tumors.

^dDetermined by Fisher exact test except for average number of hypermethylated miRNA out of 7, where *t* test was used.

^eComparison groups: I, tissue of origin; II, MSS versus MSI; III, sporadic versus hereditary disease.

^fns, Nonsignificant.

tional evidence. The miR-124a family was selected because it was known to be frequently hypermethylated in sporadic cancers (3,31), but no prior expectations existed regarding the other miRNA loci. That in fact most miRNA loci were involved in methylation changes is consistent with observations that miRNA gene methylation in cancer may be even higher than that for protein-coding genes, perhaps because miRNA genes are subject to a more stringent expression control (32). Whatever the basic mechanism of CIMP, frequent hypermethylation at miRNA loci and its correlation with hypermethylation at traditional TSG loci shows that miRNA loci are no less efficiently targeted by this process than other types of genes. Co-occurrence of hypermethylation at miRNA and TSG loci is

concordant with the findings by Lehmann *et al.* (33) from sporadic carcinomas of the breast. Besides miR-124a, miR-1-1 (26,34,35) and miR-148a (33,36) have also been reported to show reduced expression and/or hypermethylation in colorectal and other cancers. The miRNA 18b stood out as a novel marker of hypermethylation in our investigation. The biological function of miR-18b is largely unknown, except that it may regulate tumor growth by targeting ESR1 (37).

Current evidence of a possible association between certain miRNAs and MSI status is derived from expression profiling of CRCs (35,38,39). In our investigation, hypermethylation at a total of six miRNA loci (124a-1, 124a-3, 1-1, 148a, 152 and 18b) was associated with MSI in CRC or GC. The association observed for

miR-1-1 in CRC is supported by expression and/or methylation results from colorectal (35) and hepatocellular carcinomas (26); we were unable to find any published MSI-related data for the remaining loci. The MSI connections of miRNA gene methylation may be clinically important, since the presence versus absence of CIMP combined with the mode of genomic instability is a prognostic indicator in colorectal cancers (40).

In comparison to the silencing of multiple TSGs in CIMP, there are reports of coordinated activation of multiple proto-oncogenes by promoter hypomethylation (41,42). The mechanism(s) leading to coordinated hypomethylation as well as the relationship to CIMP, if any, are unknown. Our marker panel included three miRNAs with almost complete methylation in nor-

mal tissues (208a, 373 and let-7a-3) and another two with approximately 50% methylation (18b and 200a), and these loci could *a priori* be hypomethylated in tumors. Whereas miR-18b was prone to hypermethylation instead (see above), miR-200a and miR-208a were associated with frequent hypomethylation in tumors (Table 3). While the level at which hypomethylation becomes functionally significant is unknown, our observation of hypomethylation at miR-200a is in agreement with reported overexpression of this miRNA in CRC (43). Furthermore, the number of hypomethylated miRNA loci was significantly associated with advanced stage (Dukes C or D) among the present CRCs (data not shown). Finally, the miRNAs 373 and let-7a-3 revealed variable patterns with minor alterations in our tumor series. Hypomethylation at the let-7a-3-associated CpG island may depend on the cancer type, since a functionally significant hypomethylation (in the same region that we studied) was observed in lung cancer (44) and epithelial ovarian cancer (45).

Methylation changes at miRNA loci were able to distinguish sporadic from hereditary disease. Among tumors with MSI, hypermethylation at miR-18b was significantly more frequent in sporadic CRC and GC compared with the same cancers arising in the Lynch syndrome (Table 3). Among MSS tumors, CRCs from FCCX patients showed a statistically significant difference for eight miRNA loci relative to the paired normal mucosae (Figure 3), including increased methylation (miR-124a-1, miR-124a-2, miR-124a-3, miR-1-1, miR-152 and miR-18b) and decreased methylation (miR-200a and miR-373). The etiology of FCCX is likely to be heterogeneous, since among FCCX tumors reported by Goel *et al.* (46), hypermethylation at *CACNAG1*, *SOCS1*, *RUNX3*, *NEUROG1* and *MLH1* loci was infrequent, but a distinguishing feature was a low degree of *LINE-1* methylation (surrogate marker for global hypomethylation). In our FCCX tumors, the occurrence of global hypomethylation is unknown.

Archival formalin-fixed, paraffin-embedded samples provide a valuable source of biological specimens for epigenetic studies; however, both the quality and quantity of DNA may limit subsequent analyses. We could overcome such common limitations by using the MS-MLPA approach, where custom-made assays were designed for the analysis of 11 miRNA loci in two multiplex polymerase chain reaction (PCR)-based reactions without the need of bisulfite conversion. Similar assays can be developed for any miRNA loci of interest. The observed methylation changes are likely to be functionally important on the basis of correlation between methylation and expression of miRNAs (Supplementary Figure S5), literature evidence of expression consequences of the miRNAs and ensuing effects on target mRNAs (see Introduction and Discussion) and connections to broader patterns of CIMP and clinicopathological parameters (see Results). Further studies are needed to investigate in depth the functional consequences of methylation changes at individual miRNA loci. Because a single miRNA is able to target multiple genes that may be involved in several pathways, methylation alterations that we observed at miRNA-associated CpG islands may have widespread biological and clinical significance.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that

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