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 directing 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-

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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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
were added to the SALSA MLPA P300-
tom-made MS-MLPA probes (Table 2).

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
and representative CpG dinucleotides that
were part of the restriction site for the methylation-sensitive enzyme HhaI (GGCG)
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 bisulfite sequencing (Figure 1A),
and the ligated probes can be amplified
by the polymerase chain reaction. 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 ex-
traction 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 din-
ucleotides that were part of the restriction site for the methylation-sensitive enzyme HhaI (GGCG) 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 con-
sists of two oligonucleotides: the left
probe oligonucleotide (LPO) and the
right probe oligonucleotide (RPO). Cus-
tom-made MS-MLPA probes (Table 2)
were added to the SALSA MLPA P300-
A1 Reference-2 kit (MRC-Holland, Am-
erstdam, the Netherlands), to make a
complete MS-MLPA assay.

All MS-MLPA reactions were per-
formed 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 geno-
typing software (Applied Biosystems).

The methylation dosage ratio was ob-
tained by the following calculation:

\[
D_m = \frac{P_x}{P_{ctrl}} \frac{D_{dig}}{D_{undig}}
\]

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 \(\geq 0.20\) were therefore
considered to reliably indicate methyla-
tion by the present MS-MLPA assays.

Thresholds for hypermethylation or hy-
pomethylation in tumor DNA, com-
pared with normal DNA, were deter-
mined 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 pres-
ent miRNAs was derived from a global
investigation of miRNA expression pat-
terns in cancer cell lines that will be pub-
lished separately (S. Kaur et al., unpub-
lished data). We used Human miRNA
Microarray G4470B (Agilent Technolo-
gies, Santa Clara, CA, USA), which con-
tains 723 human and 76 human viral
miRNAs, sourced from the Sanger miR-
Base (version 10.1). Signals obtained by

Alterations of CpG Island Methylation at miRNA Loci in Cancers

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 ap-
proach 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 in-
tergenic 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 epi-
genetic machinery (miR-1-1 targeting
HDAC4 [26], miR-148a targeting
DNMT3B [27] and miR-152 targeting
DNMT1 [28]) and three additional miR-
NAs 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).
Table 2. Sequences for MS-MLPA probes used to construct two multiplex assays (kits 1 and 2).

<table>
<thead>
<tr>
<th>miRNA</th>
<th>MLP probes (5′→3′)</th>
<th>Product size (bp)</th>
<th>Distance to miRNA (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Custom kit 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18b-I</td>
<td>LPO \textbf{GGGTCCCTAAGGGTTGGA}</td>
<td>97</td>
<td>1,590</td>
</tr>
<tr>
<td>&amp; RPO \textbf{CCGACAGGCCGCGGCTTCAAGGGGCA}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&amp; 200a</td>
<td>LPO \textbf{GGGTCCCTAAGGGTTGGA}</td>
<td>102</td>
<td>508</td>
</tr>
<tr>
<td>&amp; RPO \textbf{CCGACAGGCCGCGGCTTCAAGGGGCA}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>124a-3</td>
<td>LPO \textbf{GGGTCCCTAAGGGTTGGA}</td>
<td>114</td>
<td>595</td>
</tr>
<tr>
<td>&amp; RPO \textbf{CCGACAGGCCGCGGCTTCAAGGGGCA}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>124a-2</td>
<td>LPO \textbf{GGGTCCCTAAGGGTTGGA}</td>
<td>119</td>
<td>1,367</td>
</tr>
<tr>
<td>&amp; RPO \textbf{CCGACAGGCCGCGGCTTCAAGGGGCA}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18b-II</td>
<td>LPO \textbf{GGGTCCCTAAGGGTTGGA}</td>
<td>133</td>
<td>1,900</td>
</tr>
<tr>
<td>&amp; RPO \textbf{CCGACAGGCCGCGGCTTCAAGGGGCA}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>208a</td>
<td>LPO \textbf{GGGTCCCTAAGGGTTGGA}</td>
<td>139</td>
<td>1,616</td>
</tr>
<tr>
<td>&amp; RPO \textbf{CCGACAGGCCGCGGCTTCAAGGGGCA}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>152</td>
<td>LPO \textbf{GGGTCCCTAAGGGTTGGA}</td>
<td>143</td>
<td>59</td>
</tr>
<tr>
<td>&amp; RPO \textbf{CCGACAGGCCGCGGCTTCAAGGGGCA}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>124a-1</td>
<td>LPO \textbf{GGGTCCCTAAGGGTTGGA}</td>
<td>152</td>
<td>1,750</td>
</tr>
<tr>
<td>&amp; RPO \textbf{CCGACAGGCCGCGGCTTCAAGGGGCA}</td>
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<td></td>
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<tr>
<td>Custom kit 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-1</td>
<td>LPO \textbf{GGGTCCCTAAGGGTTGGA}</td>
<td>121</td>
<td>2,884</td>
</tr>
<tr>
<td>&amp; RPO \textbf{CCGACAGGCCGCGGCTTCAAGGGGCA}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>148a</td>
<td>LPO \textbf{GGGTCCCTAAGGGTTGGA}</td>
<td>138</td>
<td>413</td>
</tr>
<tr>
<td>&amp; RPO \textbf{CCGACAGGCCGCGGCTTCAAGGGGCA}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>let-7a-3</td>
<td>LPO \textbf{GGGTCCCTAAGGGTTGGA}</td>
<td>152</td>
<td>150</td>
</tr>
<tr>
<td>&amp; RPO \textbf{CCGACAGGCCGCGGCTTCAAGGGGCA}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>373</td>
<td>LPO \textbf{GGGTCCCTAAGGGTTGGA}</td>
<td>163</td>
<td>510</td>
</tr>
<tr>
<td>&amp; RPO \textbf{CCGACAGGCCGCGGCTTCAAGGGGCA}</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a}LPO, left probe oligonucleotide; RPO, right probe oligonucleotide. Bold, PCR primer recognition sequence; underlined, sequence homologous to miRNA; italic, stuffer; in gray, HhaI site.

\textsuperscript{b}Distance between the closest HhaI site in a MS-MLPA probe and the mature miRNA.
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, Dm) (Figure 3). The first group consists of loci with absent (Dm <0.20) or low-level methylation (Dm <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 (Dm around 0.5; Figure 3B). The third group consists of miRNAs 208a, 373, and let-7a-3 with high methylation (Dm 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,
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
ALTERED CPG ISLAND METHYLATION AT miRNA LOCI IN CANCERS

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-

Figure 3. Average methylation dosage ratio ± 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.
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 normal tissues; Table 3 shows the percentage of tumors with hypermethylation and hypomethylation.

### Table 3. Percentage of tumors with hypermethylation and hypomethylation.

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

P values:

- **I**
  - (e) versus (j): 0.026 ns, 0.026 ns, 0.026 ns, 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, ns
  - (f) versus (g): 0.012 ns, 0.032 ns, 0.012, <0.01, 0.015 ns, ns, <0.01, <0.01
  - (b) versus (c): ns, ns, <0.01 ns, ns, ns, <0.01 ns, ns, <0.01, <0.01
  - (g) versus (h): ns, ns, <0.01 ns, ns, ns, <0.01 ns, ns, 0.023 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.
- ns, Nonsignificant.
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 might be perceived to influence the results and discussion reported in this paper.

REFERENCES


