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Methylation analysis of MIR200 family in Mexican patients with colorectal cancer

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ABSTRACT

The present study aimed to analyze the methylation pattern of the *MIR200* family in the colorectal tissues and peripheral blood of colorectal cancer (CRC) patients. Previous informed consent, 102 samples of colorectal tissues (tumor and adjacent normal tissues) and 40 peripheral blood samples were collected from CRC patients. Additionally, we included a reference group of 40 blood samples. DNA extraction was done for colorectal tissues and peripheral blood. For methylation-specific PCR, we used bisulfite-treated DNA and controls for methylated and unmethylated DNA were included to each assay. PCR fragments were separated by 6% polyacrylamide gel electrophoresis. Methylation-positive and methylation-negative results were confirmed by bisulfite genomic sequencing technique. We analyzed 102 colorectal tissues and 40 blood samples from 51 CRC patients. *MIR200B/MIR200A/MIR429* methylation analysis discloses no differences among tissues ($p>0.05$). However, *MIR200C/MIR141* methylation showed differences between colorectal tissues and peripheral blood of CRC patients ($p<0.0001$) and mainly methylated alleles were observed in peripheral blood. These findings suggest a tissue-specific methylation pattern for the *MIR200C/MIR141* promoter.

INTRODUCTION

The addition of a methyl group in position 5 of the cytosines occurring in CpG islands and nearby shores leads to “the fifth base” five methyl-cytosine (5-mC). The presence of 5-mC is related to gene expression regulation, genomic imprinting, X inactivation, and tissue-specific regulation.^{1,2} In colorectal cancer (CRC), hypermethylation-mediated gene silencing has been described in protein-coding genes such as *RUNX3*, *CACNA1G*, *IGF2*, and *MLH1*.³ Moreover, this epigenetic modification has been reported in microRNAs (miRNAs).⁴ The miRNAs are typically 23 nucleotides long and influence gene regulation.⁵ Among the miRNAs, *MIR200* family has been related

Significance of this study

What is already known about this subject?

- ▶ miRNAs influence gene expression.
- ▶ Dysregulation of *MIR200* family has been related to metastasis in colorectal cancer (CRC).

What are the new findings?

- ▶ Methylation status of *MIR200* family in Mexican patients with CRC.
- ▶ High proportion of methylated alleles of the *MIR200C/MIR141* cluster in peripheral blood of CRC patients and reference group.

How might these results change the focus of research or clinical practice?

- ▶ The results found in peripheral blood suggest that different tissues and several CpG sites must be included during the design of methylation assays to find the best biomarker in multifactorial diseases.

to epithelial–mesenchymal transition (EMT) found in CRC.⁶ The *MIR200* family consists of the following five members clustered in two loci: *MIR200B*, *MIR200A*, and *MIR429* are located on 1p36.33, whereas *MIR200C* and *MIR141* are on 12p13.3. The respective CpG island is located on –378 to +2568 for *MIR200B/MIR200A/MIR429* and between –343 and –115 nucleotides for *MIR200C/MIR141*.^{7,8} The present study aimed to analyze the methylation pattern of the *MIR200* family in the colorectal tissues and peripheral blood of CRC patients.

MATERIALS AND METHODS

Patients and samples

Post histopathological confirmation of CRC, tumor and adjacent normal tissues were collected from surgically removed biopsy samples of 51 Mexican patients. These 51 patients had not received any prior treatment. The peripheral blood samples could be obtained



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Table 1 Characteristics of CRC patients and reference group

	Mean age	Age range (%)	Gender (%)	Tumor localization (%)	Stage (%)
CRC patients n=51	61	30–50: 22 51–70: 53 +71: 25	F: 33 M: 67	Colon: 59 Rectum: 41	0-II: 28 III-IV: 68 NA: 4
Reference n=40	51	30–50: 50 51–70: 50 +71: 0	F: 42 M: 58	-----	-----

CRC, colorectal cancer; F, female; M, male; NA, not available.

from 40 of these patients, and additionally 40 blood samples of donors were included as a reference group. All subjects were admitted for treatment at Hospital Civil “Juan I. Menchaca” during the period 2016–2017 and signed an informed consent in accordance with the Declaration of Helsinki. The project was approved by the Local committee in Bioethics (CUA/CEI/081/2016).

DNA extraction

After histopathological classification as normal mucosa or tumor tissue, the DNA was extracted with the High Pure PCR Template preparation kit (Roche Diagnostics #11796828001). The tissues were maintained in RNA later (Ambion, #AM7020) at -20°C until the DNA isolation. DNA extraction from blood samples was carried out according to Miller *et al* and DTAB/CTAB protocols.^{9,10} DNA concentration and purity were determined with a spectrophotometer at 260 and 280 nm. DNA was storage at -80°C until the time of methylation-specific PCR (MS-PCR).

DNA bisulfite conversion

Prior to amplification, all DNA samples were converted using EZ DNA methylation-gold kit at a concentration of 100 ng/ μL (Zymo Research #D5008), according to the manufacturer’s instructions. DNA conversion process transformed unmethylated cytosines to uracil, while methylated cytosines were not modified. Additionally, human methylated and unmethylated DNA controls from HCT116 DKO cell line were used during DNA conversion to assess the reaction efficiency (Zymo Research # D5014).

Methylation-specific PCR

Converted DNA was subjected to MS-PCR with primers using the protocol described by Davalos *et al*.⁷ Briefly, the PCR reactions for all assays were performed using 100 ng/ μL of DNA in a volume of 25 μL mixed with 1X PCR buffer, 1.5 mM MgCl_2 , 200 μM dNTPs, 5 pmol of each primer, and 2 U of Platinum Taq DNA polymerase.

Previously converted controls for methylated and unmethylated DNA were included in PCR reactions. All assays were performed in duplicate. For the *MIR200B/MIR200A/MIR429* cluster, the primers for methylated and unmethylated DNA were 5'-GAGCGGAGATTGGTTAGC-3' and 5'-TTATTGTTTCGTCGTTTTTCGA-3' and 5'-TAGGAGTGGAGATTGGTTAGT-3' and 5'-ATTGTTTTGTTGTTTTGAAATT-3', respectively. PCR steps for methylated DNA included an initial denaturation at 94°C for 5 min and 37 cycles of 94°C for 30s, 56.4°C for 30s, and 72°C for 30s, whereas the cycling conditions for unmethylated DNA were similar except for the annealing temperature of 49.2°C . The primers for *MIR200C/MIR141* cluster were 5'-GCGTTGGTTGTTTCGGTAGGC-3' and 5'-GACAACCTTTCCCGACCCG-3' for methylated DNA and 5'-GTGTTGGTTGTTTGGTAGGT-3' and 5'-AACAACCTTTCCCAACCCA-3' for unmethylated DNA. PCR conditions for methylated and unmethylated DNA were similar; 35 cycles of 94°C for 30s, 70.2°C for 30s, and 72°C for 30s, except for the annealing temperature of 60°C used for unmethylated DNA.

Electrophoresis

PCR amplicons were detected in a 6% polyacrylamide gel electrophoresis stained with AgNO_3 . A PCR fragment of size 141 and 149 base pairs (bp) was considered methylated and unmethylated, respectively, for the *MIR200B/MIR200A/MIR429* cluster, while a 240bp PCR fragment size was considered both methylated and unmethylated for the *MIR200C/MIR141* cluster.

Bisulfite genomic sequencing

Bisulfite genomic DNA sequencing was performed on samples positive for methylated and unmethylated DNA (ABI PRISM 3110, ThermoFisher Scientific). In all 12 CpG sites were sequenced. CpGviewer software was used for reading and aligning the DNA sequences generated by Sanger sequencing.¹¹

Statistical analysis

The Fisher’s Exact test was used to compare methylation frequencies and $p < 0.05$ was considered as significant.

RESULTS

We analyzed 102 colorectal tissue samples (tumor and adjacent normal tissues) from 51 CRC patients (67% men) with an average age of 61 years. Additionally, 40 peripheral blood samples from the same patients and 40 from healthy individuals (reference group) were included in the methylation assay. Table 1 shows the characteristics of both groups.

Table 2 Methylation analysis of *MIR200B/MIR200A/MIR429* cluster in CRC patients and reference group

	CRC patients			Reference group
	Tumor tissue* n=50 (%)	Adjacent normal tissue* n=50 (%)	Peripheral blood* n=40 (%)	Peripheral blood* n=40 (%)
<i>MIR200B/MIR200A/MIR429</i> cluster				
Methylated and unmethylated alleles	50 (100)	48 (96)	40 (100)	40 (100)
Only methylated alleles	0	2 (4)	0	0
Only unmethylated alleles	0	0	0	0

*Comparisons among the colorectal tissues and peripheral blood of CRC patients and between the groups were not significant ($p > 0.05$). CRC, colorectal cancer.

Table 3 Methylation analysis of *MIR200C/MIR141* cluster in CRC patients and reference group

<i>MIR200C/MIR141</i> cluster	CRC patients			Reference group
	Tumor tissue n=51 (%) ^{*†}	Adjacent normal tissue n=51 (%) [*]	Peripheral blood n=40 (%) [†]	Peripheral blood n=40 (%)
Methylated and unmethylated alleles	48 (94)	49 (96)	8 (20)	5 (12.5)
Only methylated alleles	2 (4)	2 (4)	32 (80)	35 (87.5)
Only unmethylated alleles	1 (2)	0 (0)	0	0

^{*}Comparisons between colorectal tissues of CRC patient were not significant (p>0.05).

[†]Comparisons between colorectal tissues versus peripheral blood of CRC patients or versus reference group were significant (p<0.0001). CRC, colorectal cancer.

The methylation analysis of *MIR200B/MIR200A/MIR429* and *MIR200C/MIR141* clusters did not reveal significant differences between tumor and adjacent normal tissues (p>0.05) (tables 2 and 3). Both of these clusters mostly exhibited a mix of methylated and unmethylated alleles.

However, in the *MIR200C/MIR141* cluster, we observed mainly methylated alleles in the peripheral blood compared with colorectal tissues from CRC patients (p<0.0001). The methylation pattern in the reference group was similar to that observed in the peripheral blood of patients with CRC (table 3). Figure 1 depicts the methylation pattern of the *MIR200C/MIR141* cluster in the peripheral blood and colorectal tissues of CRC patients. Samples positive for methylated and unmethylated DNA were confirmed by bisulfite genomic DNA sequencing (figure 2).

DISCUSSION

DNA methylation-mediated silencing of gene expression in cancer cells promotes tumor development.^{12 13} Different studies have focused on methylation pattern of cancer-associated genes to identify tumor-specific biomarkers effective for developing targeted therapies.¹³ In the current study, we analyzed the methylation status of *MIR200* family in samples of the colorectal tissues (adjacent normal and tumor tissues) and peripheral blood from CRC patients as well as in blood samples of a reference group of healthy individuals. The absence of significant differences between the tumor and adjacent normal tissues observed in the present study is consistent with the previous findings for both the *MIR200* family clusters.^{7 14 15} Dávalos *et al* analyzed the *MIR200* family methylation pattern in the colorectal tissues of 25 CRC patients and RKO and HCT116 cell lines. They detected only methylated sites in RKO cells but

unmethylated ones in HCT116 cells. In colorectal tissues, they reported a predominant mix of unmethylated and methylated alleles with only the *MIR200C/MIR141* cluster exhibiting hypermethylation in 40% of tumor samples.⁷ Wiklund *et al* analyzed both clusters and found more hypermethylated DNA in invasive bladder cancer than in superficial tumors and normal urothelium (n=5); moreover, the same pattern was observed in undifferentiated cell lines but not in differentiated cells.¹⁶ Li *et al* reported hypermethylation of *MIR200C* in tumor tissue and not in adjacent non-tumor tissues in 39 gastric cancer samples (63% vs 54.6%, respectively, p<0.01).¹⁴ Hypomethylation of *MIR200B/MIR200A/MIR429* cluster has been reported in various liver and pancreatic cancer cell lines,^{17 18} whereas hypermethylation has been detected in transformed cells and breast cancer cells with mesenchymal phenotype.¹⁹

Furthermore, in prostate and breast cancer cell lines, the *MIR200C/MIR141* promoter methylation is correlated with expression and presumably with EMT.^{7 20} In 14 prostate cancer biopsies, a common methylation pattern of the *MIR200C/MIR141* cluster was observed in both normal and tumor tissues; however, when the authors divided the promoter region in three sections, they found variable results with higher percentage of methylation in regions 2 and 3 than in region 1.¹⁵ In our methylation analysis that included a part of region 1, only 4% of CRC patients had methylated alleles in the cluster. Since these patients showed mainly methylated alleles for *MIR200C/MIR141* in blood, a tissue-specific methylation pattern can be inferred. In samples of the reference group, we observed the same pattern of methylated alleles. Although methylation regulates tissue-specific gene expression, it is possible that the methylated *MIR200C/MIR141* alleles modulate the expression of these miRNAs in the peripheral blood. Evidence supporting the addition of the region analyzed in this study to the list of tissue-specific differentially methylated regions, which are contiguous genomic segments with different methylation patterns across various biological samples and located around the promoter or within the gene, even in intergenic

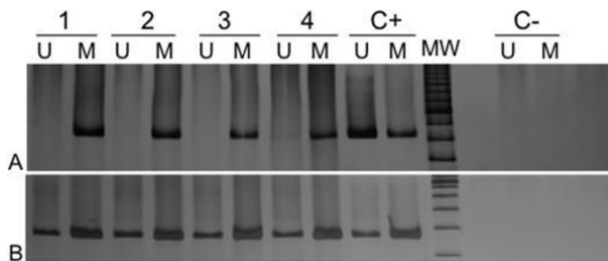


Figure 1 Methylation pattern of *MIR200C/MIR141* cluster in CRC patients; The figure shows mainly methylated alleles in the peripheral blood (1A), and methylated and unmethylated alleles in adjacent normal tissues (1B) (MW: Molecular weight marker of 50 bp, C+: positive control and C-: negative control, M (methylated), U (unmethylated)). CRC, colorectal cancer.

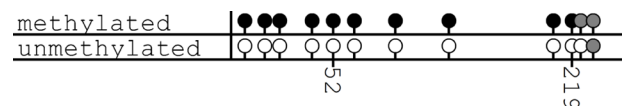


Figure 2 Lollipop plot shows 12 CpG sites of *MIR200C/MIR141* cluster in methylated and unmethylated DNA; black and white circles represent methylated and unmethylated cytosines respectively; the CpG sites not aligned with respect to reference sequence are shown in gray circles.

regulatory regions, is accumulating.²¹ This is supported by Vrba *et al*, who analyzed the methylation and expression of *MIR200C/MIR141* in human mammary epithelial cells and human mammary fibroblasts and found a cell type-specific repression by DNA methylation of this cluster in HMF.²² They also included the *MIR200B/MIR200A/MIR429* cluster and a similar pattern was seen; however, the repression was induced by DNA methylation and H3K27me3.²² Additionally, the expression levels of *MIR200C/MIR141* cluster in blood samples of control individuals have been analyzed and lower expression of *MIR141* has been reported compared with *MIR200C*.^{23, 24} Thus, results from our study and previous literature seem to indicate that the region analyzed here could be involved in the regulation of *MIR141* gene.

The lack of significant differences between the tumor and adjacent normal tissues could be related to the small sample size, and this is the main limitation of the current study. Moreover, MS-PCR technique could be considered as another limitation because it is related with a high frequency of false-positive results; however, to overcome this limitation, bisulfite genomic sequencing was performed for confirming a few positive and negative results. The findings of this study highlight the importance to include several tissues in the analysis to identify the best biomarker for CRC.

In conclusion, Mexican patients with CRC exhibited a methylation pattern similar to the *MIR200* family in tumor and adjacent normal tissues; however, in the *MIR200C/MIR141* cluster, methylated alleles were more frequently detected than unmethylated ones in the peripheral blood of such patients. These findings could be considered as tissue-specific methylation for the *MIR141* gene.

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