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 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 disclosed 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. 12 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 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...
from 40 of these patients, and additionally 40 blood samples of donors 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 the PCR reactions for all assays were performed using 1X PCR buffer, 1.5 mM MgCl₂, 200 µM dNTPs, 5 pmol of each primer, and 2 U of Platinum Taq DNA polymerase.

**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 were included in PCR reactions. All assays for methylated and unmethylated DNA included an initial denaturation at 94°C for 5 min and 37 cycles of 94°C for 30 s, 56.4°C for 30 s, and 72°C for 30 s, 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′-GCAACCTCTTTAGACCGCC-3′ and 5′-GACACACCTTCCACCCGC-3′ for methylated DNA and 5′-CTATGGTGTGTTTTGAGAT-3′ and 5′-ACACACCTTCCACCCCG-3′ for unmethylated DNA. PCR conditions for methylated and unmethylated DNA were similar; 35 cycles of 94°C for 30 s, 70.2°C for 30 s, and 72°C for 30 s, 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 AgNO₃. 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 3100, 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 1** Characteristics of CRC patients and reference group

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

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

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′-GACCGGAGATTGTTAGC-3′ and 5′-TTATGTGTTTCGTCTTTTCAGA-3′ and 5′-TATGG AGTGGAGATTGTTAGT-3′ and 5′-ATTGTTTTGTGGTTTTGAAATT-3′, respectively. PCR steps for methylated DNA included an initial denaturation at 94°C for 5 min and 37 cycles of 94°C for 30 s, 56.4°C for 30 s, and 72°C for 30 s, 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′-GGCTTGTGGTTTCGAGTTGC-3′ and 5′-GACACCTTTCCCCGACCCG-3′ for methylated DNA and 5′-GTGTGGTTGTTGTAGGT-3′ and 5′-ACACACCTTTCCACCCCA-3′ for unmethylated DNA. PCR conditions for methylated and unmethylated DNA were similar; 35 cycles of 94°C for 30 s, 70.2°C for 30 s, and 72°C for 30 s, except for the annealing temperature of 60°C used for unmethylated DNA.

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

| MIR200B/MIR200A/MIR429 cluster | Tumor tissue* n=50 (%) | Adjacent normal tissue* n=50 (%) | Peripheral blood* n=40 (%) | Reference group Peripheral blood* n=40 (%) |
|--------------------------------|------------------------|-------------------------------|-----------------------------|--------------------------------------|
| 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.
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. Different studies have focused on methylation pattern of cancer-associated genes to identify tumor-specific biomarkers effective for developing targeted therapies. 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. Davalos 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. 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. 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. Hypomethylation of MIR200B/ MIR200A/MIR429 cluster has been reported in various liver and pancreatic cancer cell lines, whereas hypermethylation has been detected in transformed cells and breast cancer cells with mesenchymal phenotype.

Furthermore, in prostate and breast cancer cell lines, the MIR200C/MIR141 promoter methylation is correlated with expression and presumably with EMT. 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. 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

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

| MIR200C/MIR141 cluster | CRC patients | Reference group |
|------------------------|--------------|-----------------|
|                        | Tumor tissue | Adjacent normal | Peripheral blood |
|                        | n=51 (%)**† | n=51 (%)*       | n=40 (%)†        | 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.

**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 H3K27me3.23 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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