

## SHORT REPORT

# Epigenetic regulation of microRNA expression in colorectal cancer

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**ABSTRACT**

In the last years, microRNAs (miRNA) have emerged as new molecular players involved in carcinogenesis. Deregulation of miRNAs expression has been shown in different human cancer but the molecular mechanism underlying the alteration of miRNA expression is unknown. To identify tumor-suppressor miRNAs silenced through aberrant epigenetic events in colorectal cancer (CRC), we used a sequential approach. We first identified 5 miRNAs down-regulated in colorectal cancer patient samples and located around/on a CpG island. Treatment with a DNA methyltransferase inhibitor and a HDAC inhibitor restored expression of 3 of the 5 microRNAs (*hsa-miR-9*, *hsa-miR-129* and *hsa-miR-137*) in 3 CRC cell lines. Expression of *hsa-miR-9* was inversely correlated with methylation of their promoter regions as measure by MSP and bisulphate sequencing. Further, methylation of the *hsa-miR-9-1*, *hsa-miR-129-2* and *hsa-miR-137* CpG islands were frequently observed in CRC cell lines and in primary CRC tumors, but not in normal colonic mucosa. Finally, methylation of *hsa-miR-9-1* was associated with the presence of lymph node metastasis. In summary, our results aid in the understanding of miRNA gene regulation showing that aberrant DNA methylation and histone modifications work together to induce silencing of miRNAs in CRC.

## INTRODUCTION

MicroRNAs (miRNAs) are 19- to 25-nt non coding RNAs that are cleaved from 70- to 100-nt hairpin-shaped precursors <sup>1</sup>. They can regulate gene expression inducing direct mRNA degradation or translational inhibition <sup>2</sup>. miRNAs are expressed in a tissue-specific manner and represent crucial factors in the regulation of different pathways involved in development, cell differentiation, proliferation and apoptosis <sup>3-6</sup>. Moreover, abnormal regulation of miRNA expression may contribute to the development of human cancer <sup>7-10</sup>. Interestingly, down-regulation of miRNAs has been demonstrated in tumors in comparison with normal tissue <sup>11</sup>, suggesting that some miRNAs may behave as putative tumor suppressor genes <sup>12, 13</sup>. However, although the biological importance of miRNA is becoming increasingly apparent, the molecular mechanism underlying regulation of miRNA expression in cancer is not completely understood. Specific transcriptional regulation, epigenetic mechanisms including methylation and histone deacetylation, gene mutations affecting proteins involved in the processing and maturation of microRNAs, or regulation of miRNA stability include some of the mechanism involved in the regulation of microRNA expression <sup>14-16</sup>.

While methylation of CpG dinucleotides in the promoter region of genes is an important mechanism of gene regulation, aberrant hypermethylation of tumor suppressor genes plays a significant role in tumor development <sup>17</sup>. In fact down-regulation of tumor suppressor genes in human cancer has been tightly linked to the presence of CpG island promoter hypermethylation <sup>18, 19</sup> and establishes epigenetic regulation as a key mechanism in tumorigenesis. Interestingly, recent studies indicate that microRNA expression may be regulated by different epigenetic mechanism including abnormal methylation of the promoter regions or histone modifications <sup>20, 21</sup>.

We and others have recently demonstrated that expression of microRNAs is down-regulated in CRC <sup>22, 9</sup> and two different studies <sup>23, 24</sup>, comparing the miRNA expression profile of the parental HTC116 cell line and the DNA methyltransferase 1 and 3b (DNMT1 and DNMT3b) double knockout cell line suggest that DNA hypermethylation contributes to the transcriptional down-regulation of microRNAs in CRC.

To explore the role of epigenetic mechanisms in the down-regulation of miRNAs in CRC we examined the presence of DNA methylation and histone modifications-associated silencing of miRNAs and observed that abnormal hypermethylation of the promoters of *hsa-miR-9*, *hsa-miR-129* and *hsa-miR-137* is associated with reduced expression in CRC tissues but rare in normal tissue. Our results suggest that DNA hypermethylation and histone modifications contribute to the transcriptional down-regulation of miRNAs and may participate in human colorectal tumorigenesis.

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## **MATERIAL AND METHODS**

### **Human cancer cell lines and primary tumor samples**

CRC derived cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA) and were grown according to the supplier's instructions. Matched sets of primary colon tumors (n=50) and adjacent normal tissues (n=50) were obtained at the time of clinically indicated procedures. Total DNA and RNA were isolated from CRC cells and primary tissues by Trizol (Invitrogen, San Diego, CA, USA) extraction according to the manufacturer's instructions.

### **Drug treatment**

CRC derived cell lines were seeded at a concentration of  $5 \times 10^5$  cells per 100 mm dish 24 hr prior to treatment with 5-Aza-2'-deoxycytidine (AZA: 1 and 3  $\mu$ M; Sigma, St Louis, MO, USA) and/or 4-phenylbutyric acid (PBA; 1 and 3 mM; Sigma). For 5-Aza-2'-deoxycytidine treatment, cells were treated for 4 days and for histone acetylase inhibition, PBA was continuously administered by replacing the medium containing PBA every 24 hr for 5 days. 5-Aza-2'-deoxycytidine with PBA treatment, 5-Aza-2'-deoxycytidine was removed after 48 hrs, while PBA was continuously administered every 24 hr for 5 days.

### **miRNA and mRNA expression analysis**

miRNA expression analysis was performed as previously described<sup>25</sup>. In brief, cDNA was synthesized from total RNA using gene-specific primers according to the TaqMan MicroRNA Assay protocol (Applied Biosystems, Inc., Foster City, CA, USA). Real-time PCR was performed using an Applied Biosystems 7300 Sequence Detection system. The 10  $\mu$ l PCR included 0.67  $\mu$ l RT product, 1x TaqMan Universal PCR master mix and 1  $\mu$ l of primers and probe mix of the TaqMan MicroRNA Assay protocol (Applied Biosystems, Inc.). Expression was normalized using TaqMan microRNA endogenous control assay RNAU6B.

### **DNA methylation analyses**

We searched the human genome database (<http://genome.ucsc.edu/>) (University of California Santa Cruz, Genome Bioinformatics) for the existence of miRNAs embedded in a CpG island.

Genomic DNA samples were modified by sodium bisulfite using the CpGenome DNA modification kit (Chemicon, Temecula, CA, USA) following the manufacturer's instructions.

The DNA methylation status was analyzed by methylation specific PCR (MSP) after sodium bisulfite modification of DNA. Human male genomic DNA universally methylated for all genes (Intergen Company, Purchase, NY) was used as a positive control for methylated alleles. Water blanks were included with each assay. Primer sequences of MSP of each miRNA are described in Supplementary Table S1. Following amplification, PCR products were subjected to gel electrophoresis through a 2.5% agarose gel and were visualized by ethidium bromide staining and UV transillumination. The second analysis was realized by bisulfite genomic sequencing (BSP) of both strands of the corresponding CpG islands. Following sodium bisulfite treatment, the 5' region of miRNAs was PCR amplified. PCR products were subcloned using the One Shot® TOP10 kit (Invitrogen) into pCR® 4-TOPO® plasmid and candidate plasmid clones were sequenced. Primer sequences are described in Supplementary Table S1.

#### **Q-CHIP assay**

Colon carcinoma cell lines CACO2 and SW620 before and after treatment with the histone deacetylase inhibitor 4-phenylbutyric acid (PBA) were subjected to chromatin immunoprecipitation. The ChIP assays were performed as previously described<sup>23</sup> and the ChIP fractions were used for quantitative-PCR assay. Quantitative-PCR assay was performed in order to assess the acetylated Histone 3 (AcH3: Upstate Biotechnologies, Lake Placid, NY) modification. Immunoprecipitated DNA fractions (10 ng) from antibody-bound and input chromatin were analyzed by real-time PCR using SYBR Green detection and the LightCycler platform. The amplification of the immunoprecipitation fraction (IP) was used as a target sequence and input DNA (In) as reference sequence. Both fractions were amplified in the same run and following the same procedure of Q-PCR-Chip. The specific primers used for the Q-CHIP are described in Supplementary Table S1. The following program conditions were applied for Q-PCR-Chip running: denaturation program, consisting in one cycle at 95°C for 10 minutes; amplification program, consisting in 45 cycles at 95°C for 10 s, 60°C for 10 s and 72°C for 10 s; melting program, one cycle at 95°C for 0 s, 40°C for 60 s and 90°C for 0 s; and cooling program,

one cycle at 40°C for 60s. The temperature transition rate was 20°C/s, except for the melting program, which was 0.2°C/s between 40°C and 90°C. A procedure based on the relative quantification of target sequence (IP) vs. their controls/calibrators in relation to the reference sequence (In) was used to assess the level of Ach3. Calculations were automatic performed by LightCycler software (RealQuant, version 1.0, Roche). The normalized ratio was obtained from the next equation and expressed as percentage of the control/calibrator:

$$\text{Normalised ratio (N)} = (E_{\text{target}})^{\Delta C_p \text{ target (control - sample)}} \div (E_{\text{ref}})^{\Delta C_p \text{ ref (control - sample)}}$$

Efficiencies (E) of each amplifications were calculated from the slopes of crossover points (Cp) vs DNA concentration plot, according to the formula  $E = 10^{(-1/\text{slope})}$ .  $\Delta C_p$  corresponded to the difference between control/calibrator Cp and sample Cp, either for the target or for the reference sequences. The level of Ach3 was determined relative to input chromatin of each sample.

### Statistical Analysis

Correlations between methylation state and clinico-pathological features were assessed using  $\chi^2$  or Fisher's Exact probability tests as appropriate. All p-values presented are two-sided. A p-value < 0.05 was regarded as statistically significant. All statistical tests were performed using the SPSS software package (version 15, SPSS, Chicago, IL).

## RESULTS

### Identification of epigenetically regulated miRNAs in human colorectal cancer

We have previously identified 23 miRNAs down-regulated in primary CRC in comparison with matched normal colorectal epithelial tissues<sup>22</sup>. In order to determine whether these microRNAs could be epigenetically regulated we first examined their location in the genome. Out of the 23 microRNAs down-regulated in CRC, 5 of them (*hsa-miR-9*, *hsa-miR-124*, *hsa-miR-129*, *hsa-miR-137*, *hsa-miR-149*) were included within 1000bp of a CpG island. Since it has been predicted that >90% of the human miRNAs promoter are located 1000 bp upstream of the mature miRNA we decided to analyze the methylation status of these microRNAs<sup>26</sup>. In order to do so, 3 CRC cell lines were treated with 5-Aza-2'-deoxycytidine and/or PBA followed by the analysis of miRNAs expression. While expression of *hsa-miR-9* and *hsa-miR-137* was up-regulated after treatment with 5-Aza-2'-deoxycytidine or PBA, the effect was more pronounced when both 5-Aza-2'-deoxycytidine and PBA were combined. Furthermore, expression of *hsa-miR-129* was mostly up-regulated when both a demethylating agent and an inhibitor of histone deacetylases were used. Expression changes showed some variation according to the cell line employed (Figure 1A, 1B and 1C). Changes in the expression of the cell-cycle inhibitor p16/CDKN2A gene (data not shown) and the *hsa-miR-127* were used as positive control of genes regulated by epigenetic inactivation as both genes have been previously demonstrated to be regulated epigenetically (Figure 1A, 1B and 1C)<sup>20</sup>. These results indicate that *hsa-miR-9*, *hsa-miR-129* and *hsa-miR-137* could be regulated by aberrant DNA methylation and histone modification in CRC.

### Analysis of miRNAs promoter hypermethylation in CRC

We next examined the methylation of the promoter regions of *hsa-miR-9*, *hsa-miR-129* and *hsa-miR-137* by MSP to determine whether aberrant hypermethylation is responsible for silencing of miRNAs expression. *Hsa-miR-9* is represented by three genomic loci *hsa-miR-9-1* (1q22), *hsa-miR-9-2* (5q14.3), *hsa-miR-9-3* (15q26.1). While *hsa-miR-9-1* is located at 183bp of a CpG island and *hsa-miR-9-3* is embedded in a CpG island, *hsa-miR-9-2* is located more than 1000pb of a CpG island. *Hsa-miR-129* is represented by two genomic loci but only one of them, *hsa-miR-129-2* at region 11p11.2, is embedded in a CpG island. On the contrary, *hsa-miR-137* is



represented by one single locus located in 1p21.3 chromosome region which is inside on a CpG island. As shown in figure 2, all 4 microRNAs (*hsa-miR-9-1*, *hsa-miR-9-3*, *hsa-miR-129-2* and *hsa-miR-137*) were found to be hypermethylated in Caco2 and LS513 CRC cell lines and methylation was partially reverted by treatment with 5-Aza-2'-deoxycytidine (Figure 2A). MSP analysis of *hsa-miR-9-1*, *hsa-miR-9-3*, *hsa-miR-129-2* and *hsa-miR-137* loci in a panel of 7 CRC cell lines revealed that the *hsa-miR-129-2* and *hsa-miR-137* were un-methylated in the LS174 cell line (Figure 2B) while the rest of cell lines showed methylation of at least one allele. Expression of the mature *hsa-miR-129* and *hsa-miR-137* were not detected in either of methylated CRC cell lines (data not shown). Interestingly, MSP analysis of *hsa-miR-137* revealed a dramatic size difference in the PCR products among the different cell lines consistent with the presence of heterozygous VNTR alleles in RKO and LS174 cell lines (Figure 2B). A variable number of tandem repeats (VNTR) 5' to the pre-miR-137 has been previously identified<sup>27</sup>. The design of the primers for the MSP analysis used a reference DNA sequence that included three repeats (VNTR) of 15 bases (GenBank no. AK094607). While the Caco2 cell lines contained 6 copies of the 15 base-repetition VNTR (TAGTAGCGGTAGCGG) only 3 copies were detected in the LS513 cell line. Sequencing analysis of this region showed that the VNTR copy number affect the number of CpG dinucleotides that can modify the chromatin structure (Supplementary Figure S1).

Methylation of *hsa-miR-9-1* and *hsa-miR-9-3* loci and expression of *hsa-miR-9* showed a heterogenous behavior (Figure 2B). Low-expression of the mature miRNA (*hsa-miR-9*) was correlated mainly with methylation of *hsa-miR-9-1*, but not with methylation of the *hsa-miR-9-3* as indicated by the fact that the *hsa-miR-9-3* locus was found methylated on HCT116 and SW620 cell lines that showed high *hsa-miR-9* expression (Figure 2B and Figure 2C).

MSP results obtained for *hsa-miR-9-1* and *hsa-miR-9-3* were confirmed by bisulphite sequencing (Figure 3A and 3B).

### **Regulation of microRNA expression by histone 3 acetylation**

The fact that expression of microRNAs was upregulated by the combined treatment with 5-Aza-2'-deoxycytidine and PBA suggested that histone acetylation could also be involved in the

regulation of microRNA expression. CRC cell lines were subjected to chromatin immunoprecipitation with an antibody against acetylated H3 (a mark of transcriptional active chromatin) followed by Q-PCR for *hsa-miR-9-1*, *hsa-miR-9-3*, *hsa-miR-129* and *hsa-miR-137* before and after treatment with PBA. Treatment of Caco2 and SW620 cells with PBA induced an increase in the Ach3 in the case of *hsa-miR-9-3* and *hsa-miR-127*. However, we could only detected slight changes of Ach3 by quantitative CHIP assay when we examined *hsa-miR-129-2*, *hsa-miR-9-1* and *hsa-miR-137*. These results in addition to the expression analysis shown in figure 1 indicate that expression of the mature *hsa-miR-9* could be regulated by hypermethylation of the *hsa-miR-9-1* locus and H3 de-acetylation of the *hsa-miR-9-3* locus at least in Caco2 cells (Figure 4).

#### **Methylation analysis of *hsa-miR-9-1*, *hsa-miR-129* and *hsa-miR-137* in primary CRC tissues**

Finally, we analyzed the methylation status of *hsa-miR-9-1*, *hsa-miR-129-2* and *hsa-miR-137* in a group of matched samples of primary CRC samples and adjacent normal tissue. Tumor-specific DNA methylation of *hsa-miR-9-1*, *hsa-miR-129-2* and *hsa-miR-137* was detected in 56% (20 of 36), 91% (31 of 34) and 100% (31 of 31) of primary CRC cases analyzed, respectively. Representative examples are shown in Figure 5. *Hsa-miR-9-1* was completely unmethylated in samples of normal colonic tissue. Interestingly, we found methylation of *hsa-miR-129-2* and *hsa-miR-137* in 12% (4 of 34) and 23% (7 of 31) in histologically normal colorectal mucosa from individuals with colorectal cancer. These results suggest the possibility of a colorectal mucosal 'field defect' related to aberrant microRNA-methylation.

Similar to CRC cell lines, *hsa-miR-129-2* and *hsa-miR-137* hypermethylation was found in almost every sample analyzed and these alterations did not correlate any of the clinico-pathological features of primary CRC. However, when we evaluated the correlation between *hsa-miR-9-1* methylation and clinico-pathological features of primary CRC we observed that methylation was more frequent in advanced stages of CRC and was associated with regional nodal invasion ( $p=0.008$ ), vascular invasion ( $p=0.004$ ) and metastasis ( $p=0.016$ ) (Table 1).

These results suggest that *hsa-miR-9-1* silencing could have a role in the malignant progression of colorectal cancer.

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## DISCUSSION

In the current study we have shown the first demonstration that suppression of *hsa-miR-9*, *hsa-miR-129* and *hsa-miR-137* genes in colorectal cancer is mediated at least in part by epigenetic mechanisms including DNA hypermethylation and histone acetylation. Moreover, the frequent hypermethylation of these miRNA genes in CRC and the correlation between methylation and clinico-pathological supports the concept that epigenetic instability is an important event in human tumorigenesis specifically in colorectal carcinoma.

Epigenetic regulation of miRNAs mediated by inadequate methylation has been previously described. Saito et al <sup>14</sup> provided the first evidence for the involvement of DNA methylation in the regulation of miRNA expression in carcinoma cells identifying 17 miRNA up-regulated in bladder cancer cells after treatment with 5-Aza and PBA. However, no changes in miRNA expression were detected after treatment with 5-Aza or PBA alone suggesting that epigenetic control of miRNA expression includes DNA methylation and histone modifications at least in their model. Other report <sup>21</sup> suggested that treatment of a breast cancer cell line with a proapoptotic dose of the HDAC inhibitor hydroxamic acid LAQ824 resulted in both up-regulation and down regulation of several miRNA transcripts supporting a role of epigenetic mechanism in the regulation of microRNA expression. On the other hand, Diederichs et al <sup>28</sup> showed that miRNA expression are not induced by demethylating agents or HDAC inhibitors in the lung cancer cell line A549. This discrepancy may reflect different responses in the cell lines tested as well as dose-dependent effects of the different HDAC inhibitors. Our results confirm previous studies, suggesting that aberrant alteration in DNA methylation and histone modification suppress miRNA expression in colorectal cancer.

The differences between methylation of *hsa-miR-9-1*, *hsa-miR-129* and *hsa-miR-137* in primary CRC tumors and normal mucosa suggest that similar to classic tumor-suppressor genes, DNA methylation could be an important molecular mechanism for the down-regulation of miRNA expression. Moreover, the association between *hsa-miR-9-1* methylation and more advanced stages and nodal involvement supports a role for *hsa-miR-9-1* methylation as a potential tumor

marker of poor outcome in colorectal cancer. However, further investigation would be required to study the involvement of *hsa-miR-9-1* in distant metastasis and patient survival.

Other miRNAs has been shown to be hypermethylated in CRC. Methylation of the *hsa-miR-34b/c* CpG island was frequently observed in CRC cell lines and in primary CRC tumors, but not in normal colonic mucosa suggesting a tumor-specific methylation<sup>29</sup>. Moreover, Grady et al<sup>30</sup> showed that the *EVL/hsa-hsa-miR-342* locus was methylated in 86% of colorectal adenocarcinomas and in 67% of adenomas, indicating that it is an early event in colorectal carcinogenesis. In addition, similar to our results with *hsa-miR-137* they observed a high frequency of methylation (56%) in histologically normal colorectal mucosa from individuals with concurrent cancer, suggesting the existence of a 'field defect' involving miRNAs.

The precise function of *hsa-miR-9*, *hsa-miR-129*, and *hsa-miR-137* have not been characterized although abnormal expression has been described in tumor samples. *Hsa-miR-9* has been described as a putative tumor suppressor gene in recurrent ovarian cancer<sup>31</sup> and genome-wide profiling of methylated promoters identified *hsa-miR-9-1* as a miRNA frequently hypermethylated in pancreatic adenocarcinoma<sup>32</sup>. Lehmann et al<sup>33</sup> showed differential methylation of *hsa-miR-9-1* in a large series of primary human breast cancer specimens. In agreement with our results a good correlation between hypermethylation of the *hsa-miR-9-1* gene and down-regulation of expression in tumour tissue was found. On the other hand, Kozaki et al<sup>34</sup> identified *hsa-miR-137* as a tumor suppressor miRNA frequently silenced by DNA methylation in oral squamous cell carcinoma which was implicated in the regulation of cell growth mediated by regulation of cyclin-dependent kinase 6 (CDK6). Expression of *hsa-miR-137* was also significantly down-regulated in glioblastoma multiforme cells and over-expression of microRNA-137 induced differentiation of brain tumor stem cells<sup>35</sup>. The presence of a 15-bp VNTR in the primary miRNA of *hsa-miR-137* detected in CRC cell lines may affect directly gene methylation since the number of CpGs differs according to the VNTR copy number. Bemis et al<sup>27</sup> have already described the presence of this VNTR in melanoma cell lines and showed that alterations in the number of tandem repeats in this area interfere with mature *hsa-miR-137* processing and function. In this sense, it has been postulated that local sequence features can

contribute to the susceptibility of a CpG island to become methylated or to be protected from methylation<sup>36</sup>

In summary, our study shows that miRNA-specific hypermethylation in CRC and histone-deacetylation could be an important molecular mechanism causing the global down-regulation of miRNAs. Considering the high frequency of miRNA hypermethylation found in CRC cancer, miRNA gene methylation could be considered as useful tumor marker.

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**FIGURE LEGENDS**

**Figure 1: Expression of *hsa-miR-9*, *hsa-miR-129*, *hsa-miR-137* and *hsa-miR-127* is regulated epigenetically in CRC**

**A)** qRT-PCR analysis of the *hsa-miR-9*, *hsa-miR-129*, *hsa-miR-137* and *hsa-miR-127* in CRC cell lines before and after treatment with 5-Aza-2'-deoxycytidine (AZA). **B)** qRT-PCR analysis of the *hsa-miR-9*, *hsa-miR-129*, *hsa-miR-137* and *hsa-miR-127* in CRC cell lines before and after treatment with 4-phenylbutyric acid (PBA) **C)** qRT-PCR analysis of the *hsa-miR-9*, *hsa-miR-129*, *hsa-miR-137* and *hsa-miR-127* in CRC cell lines before and after treatment with 5-Aza-2'-deoxycytidine and histone deacetylase inhibitor 4-phenylbutyric acid (PBA). ddCT: is the value of the normalized expression of the miRNA (Ct miRNA-Ct RNU6B) in treated cell line minus normalized expression value of the miRNA in non-treated cell line.

**Figure 2: Methylation of *hsa-miR-9-1*, *hsa-miR-9-3*, *hsa-miR129-2* and *hsa-miR137* in CRC cell lines**

**A)** MSP analysis of the *hsa-miR-9-1*, *hsa-miR9-3*, *hsa-miR129-2* and *hsa-miR137* CpG island regions in Caco2 and LS513 CRC cells lines control and treated with 5-Aza-2'-deoxycytidine. **B)** MSP analysis of the *hsa-miR-9-1*, *hsa-miR9-3*, *hsa-miR129-2* and *hsa-miR137* CpG island regions in a panel of CRC cell lines. U: un-methylated, M: methylated. C+: methylated control. **C)** Expression analysis of mature *hsa-miR-9* in CRC cell lines measured by qRT-PCR. Data are expressed as  $2^{-\Delta Ct}$  values obtained by normalization using RNU6B as endogenous control.

**Figure 3: Bisulphite sequencing of *hsa-miR-9-1* and *hsa-miR-9-3*.**

**a)** Bisulphite sequencing of the *hsa-miR-9-1* CpG island region in positive methylated control (+ control) and CRC derived cell lines (control and treated with 5-Aza-2'-deoxycytidine). Each circle indicates a CpG dinucleotide (black circle: methylated CpG; open circle: unmethylated CpG). Eight clones were sequenced for each cell line.

**b)** Bisulphite sequencing of the *hsa-miR-9-3* CpG island region in positive methylated control (+ control) and CRC derived cell lines (control and treated with 5-Aza-2'-deoxycytidine). Each box indicates a CpG dinucleotide (black circle: methylated CpG; open circle: unmethylated CpG). Eight clones were sequenced for each cell line.

**Figure 4: Histone 3 acetylation analysis in promoter region of the *hsa-miR-127*, *hsa-miR-9-1*, *hsa-miR-9-3*, *hsa-miR-129* and *hsa-miR-137* genes in CRC cell lines before and after treatment with AZA and PBA.**

Quantitative-ChIP-PCR analysis for the acetylation of histone 3 in the *hsa-miR-127*, *hsa-miR-9-1*, *hsa-miR-9-3*, *hsa-miR-129* and *hsa-miR-137* CpG islands. Values are expressed as the percentage of the acetylation of histone 3 mark in CRC derived CACO and SW620 cell lines after treatment with AZA+PBA in comparison with expression in these cell lines before treatment (100%). AZA: 5-Aza-2'-deoxycytidine; PBA: 4-phenylbutyric acid.

**Figure 5: Methylation analyses for *hsa-miR-9-1*, *hsa-miR-129-2* and *hsa-miR-137* CpG islands in primary colorectal tumors.**

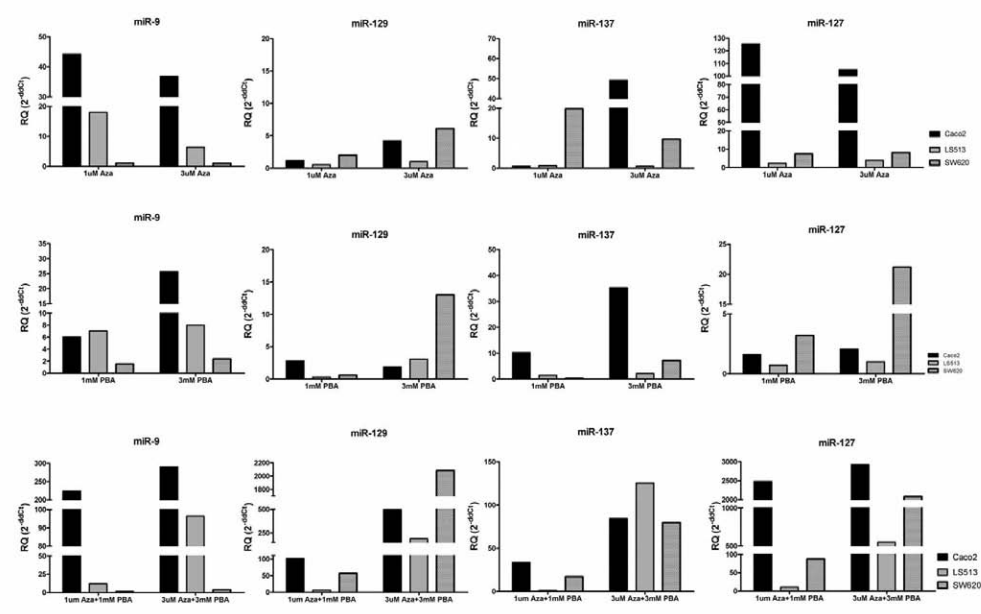
MSP analysis for *-miR-9-1*, *hsa-miR-129-2* and *hsa-miR-137* in surgically resected primary colorectal tumors (*T*) and corresponding noncancerous colon mucosa (*N*). Unmethylated (*U*) or methylated (*M*) sequences. *In vitro* methylated DNA (*IVD*) is shown as methylated control sequences.

**Supplementary Figure 1:** Sequencing analysis of the VNTR repeat region (TAGCAGCGGCAGCGG) in *hsa-miR-137* from cell lines Caco2 and LS513. Six repeats are found in Caco2 and 3 repeats in LS513.

**Table 1: Clinico-pathological characteristics of CRC patients according to *hsa-miR-9-1* methylation status.**

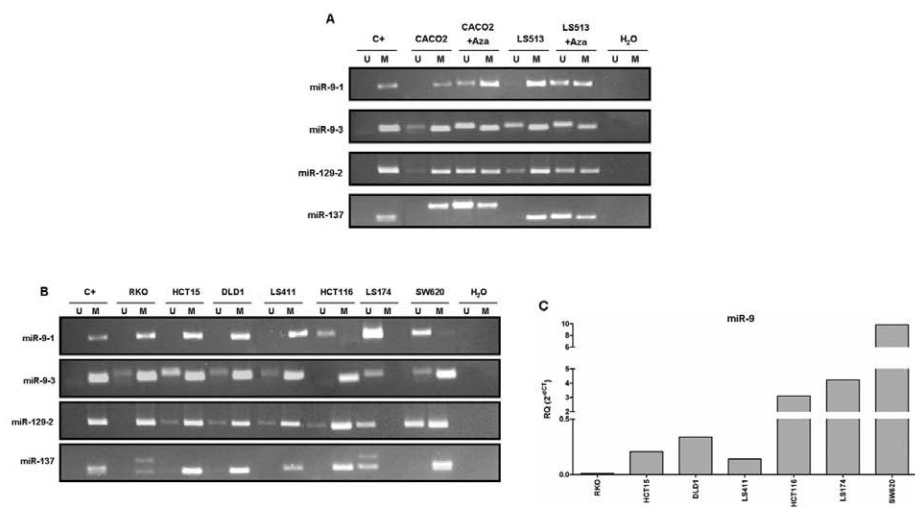
Feature	miR-9-1 Non Methylated (n=16)	miR-9-1 Methylated (n=20)	p-value
<b>Age</b>			
<65 years	5	5	ns
>65 years	11	15	
<b>Sex</b>			
M	11	12	ns
F	5	8	
<b>Localization</b>			
proximal	4	6	ns
distal	12	16	
<b>Hystological grade</b>			
well-differentiated	8	8	ns
poor-differentiated	8	12	
<b>Stage</b>			
I+II	14	9	<b>0.008</b>
III+IV	2	11	
<b>Vascular invasion</b>			
non	16	12	<b>0.004</b>
yes	0	8	
<b>lymph-node involvement</b>			
non	14	9	<b>0.008</b>
yes	2	11	
<b>Metastasis</b>			
non	16	14	<b>0.016</b>
yes	0	6	

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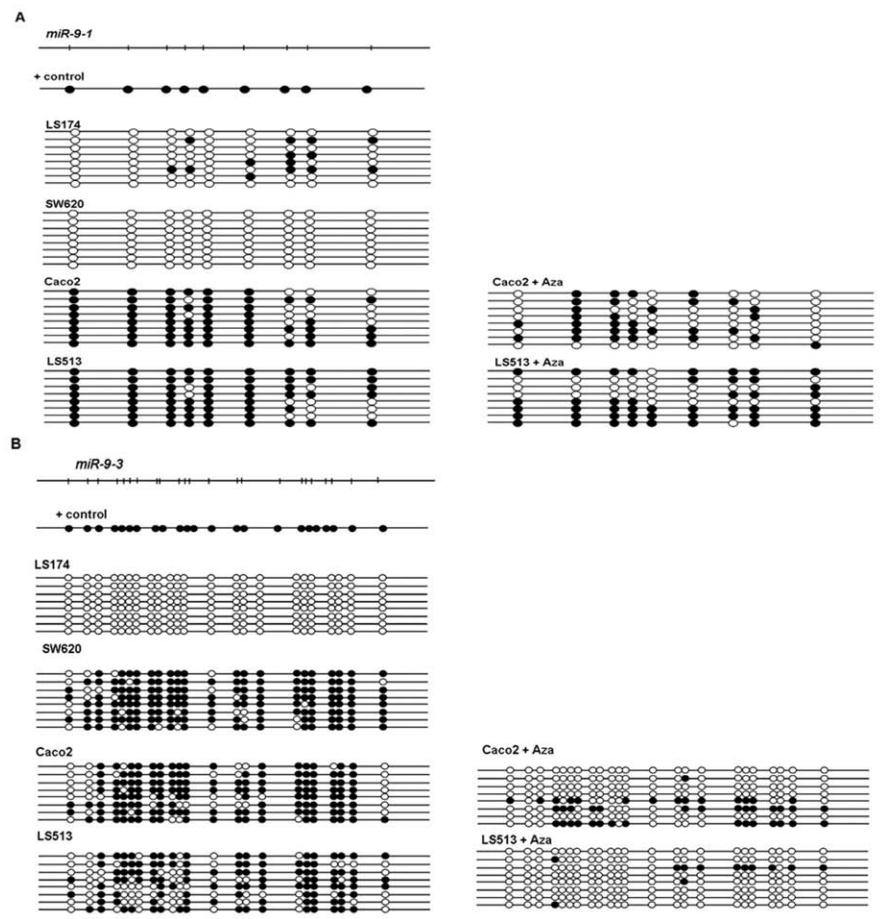
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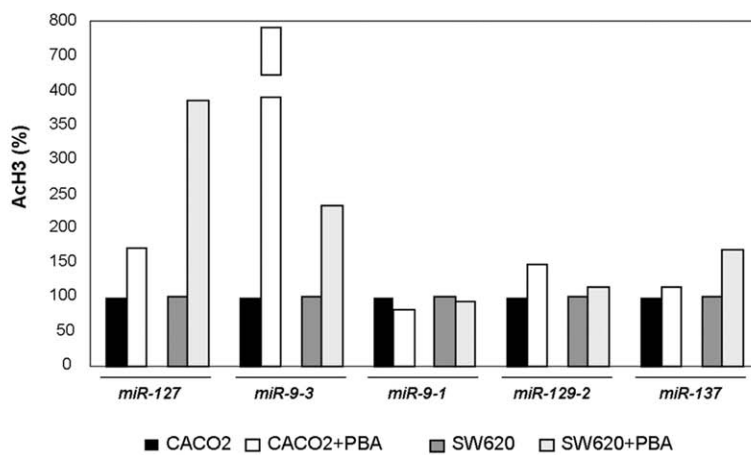


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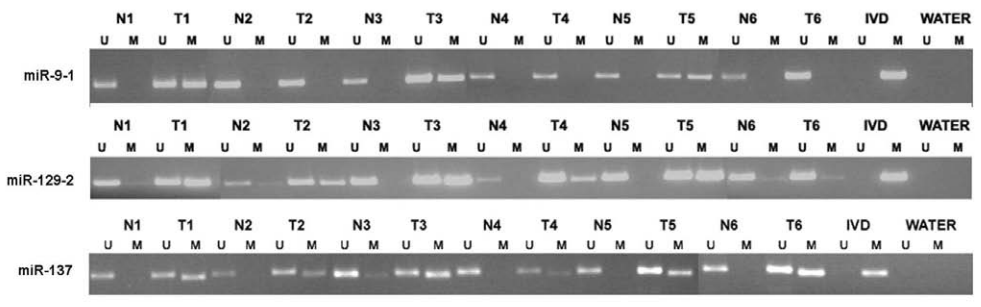


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