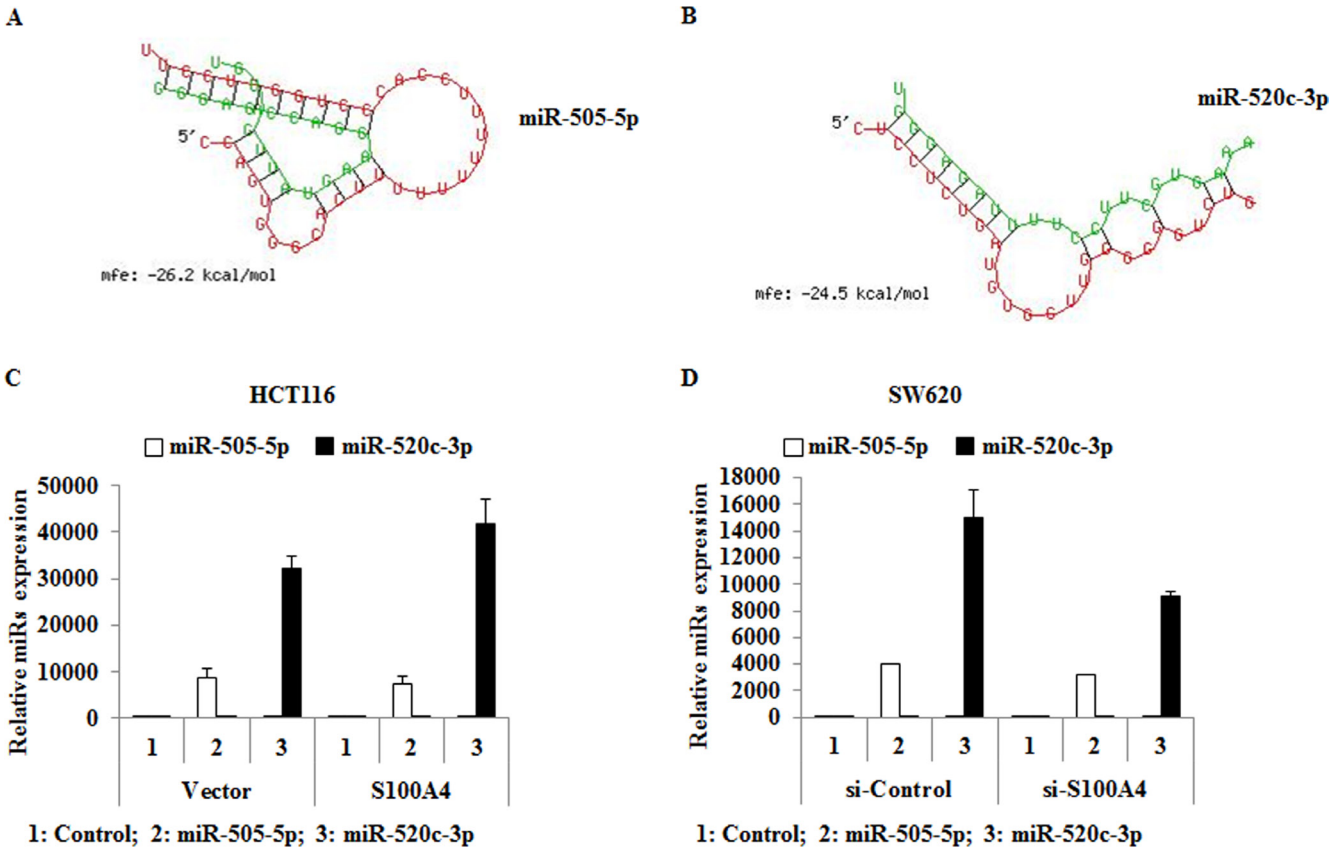
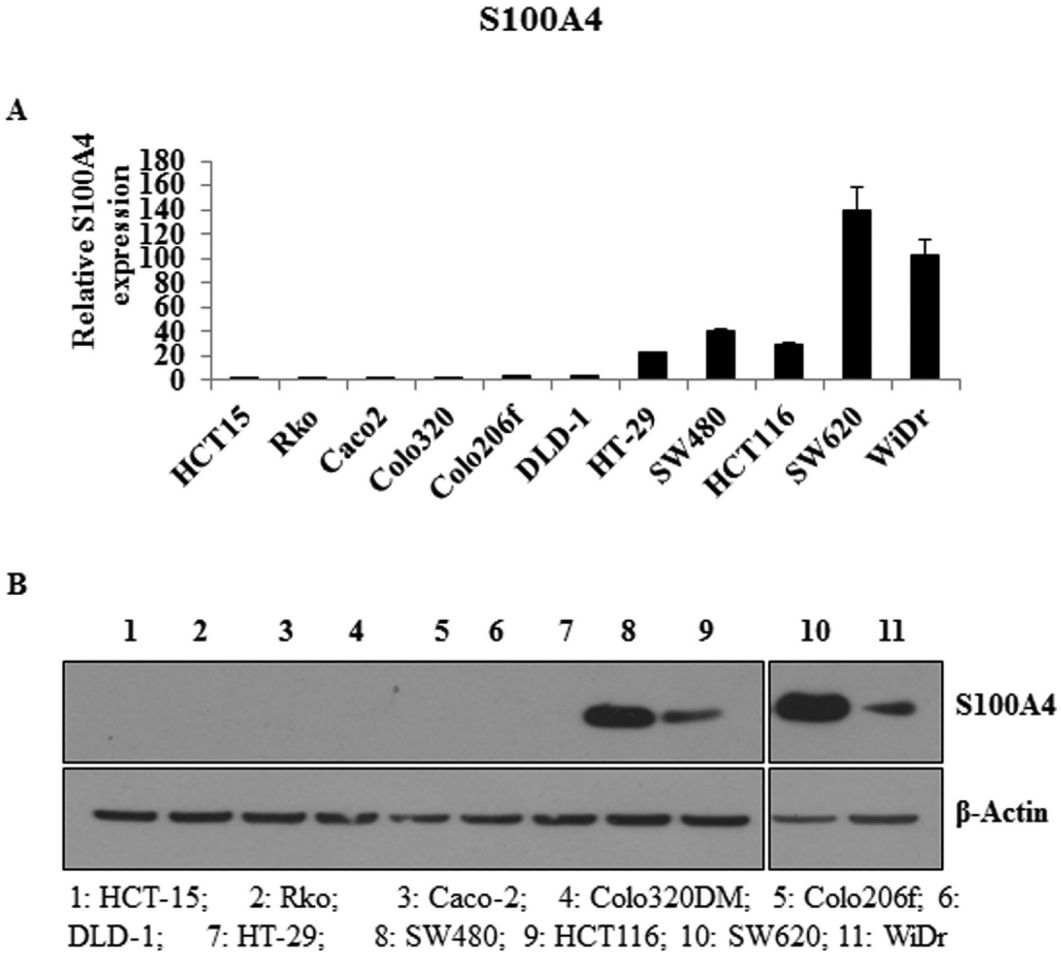
# Epigenetic silencing of miR-520c leads to induced S100A4 expression and its mediated colorectal cancer progression

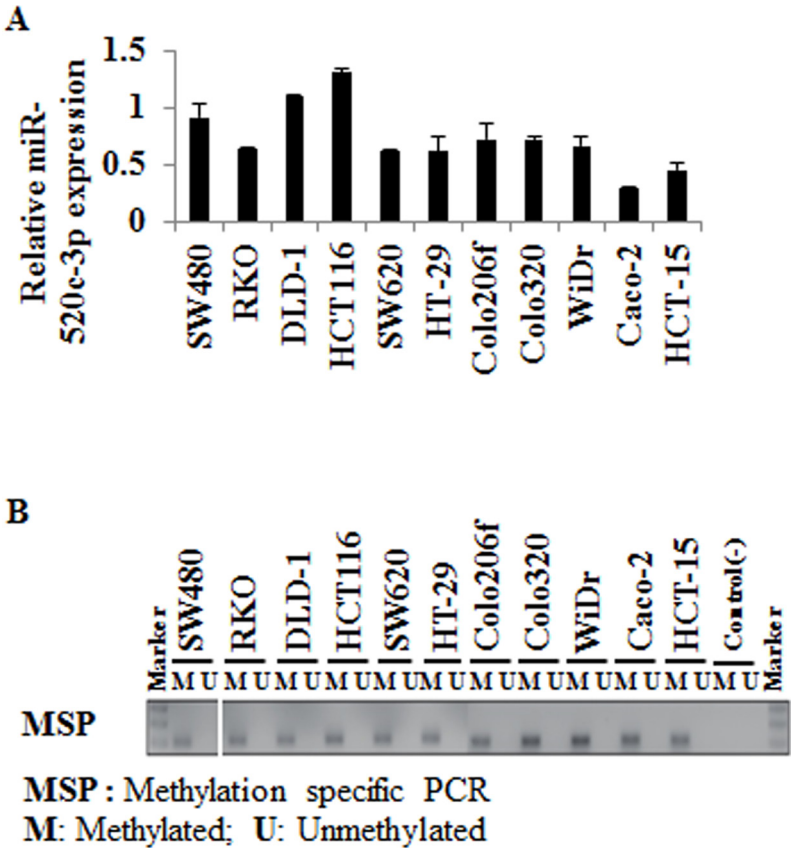
## Supplementary Materials



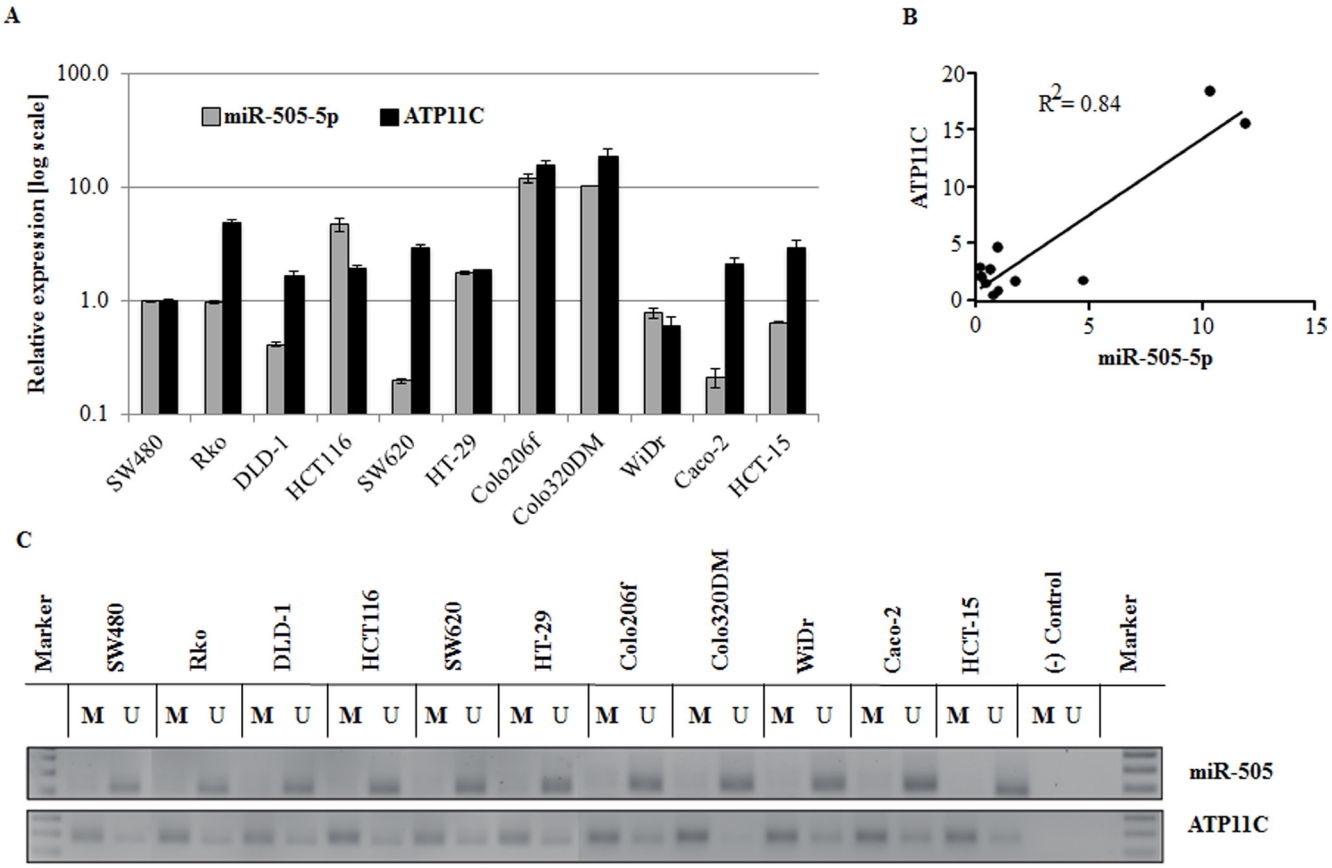

**Supplementary Figure 1: Secondary structure prediction (RNAhybrid) of the binding between the seed sequences of miR-505-5p and miR-520c-3p within the S100A4-3′-UTR and ectopic overexpression of miR-505-5p and miR-520c-3p.** (**A**, **B**) The S100A4-3′-UTR contains seed sequences for miR-505-5p (green-highlighted sequence) at position 77 to 85 nt and miR-520c-3p (green-highlighted sequence) position 59 to 65 nt and showed a strong hybridization energy ΔG of -26.2 kcal/mol and -24.5 kcal/mol, respectively. (**C**, **D**) HCT116 or SW620 cells were transfected with control-miR, miR-505-5p, miR-520c-3p along with either vector-control or -S100A4 expression (HCT116) or si-Control and si–S100A4 (SW620). Transfection efficiency of miRs was measured using qRT-PCR, whereas RNUB6 served as internal control.



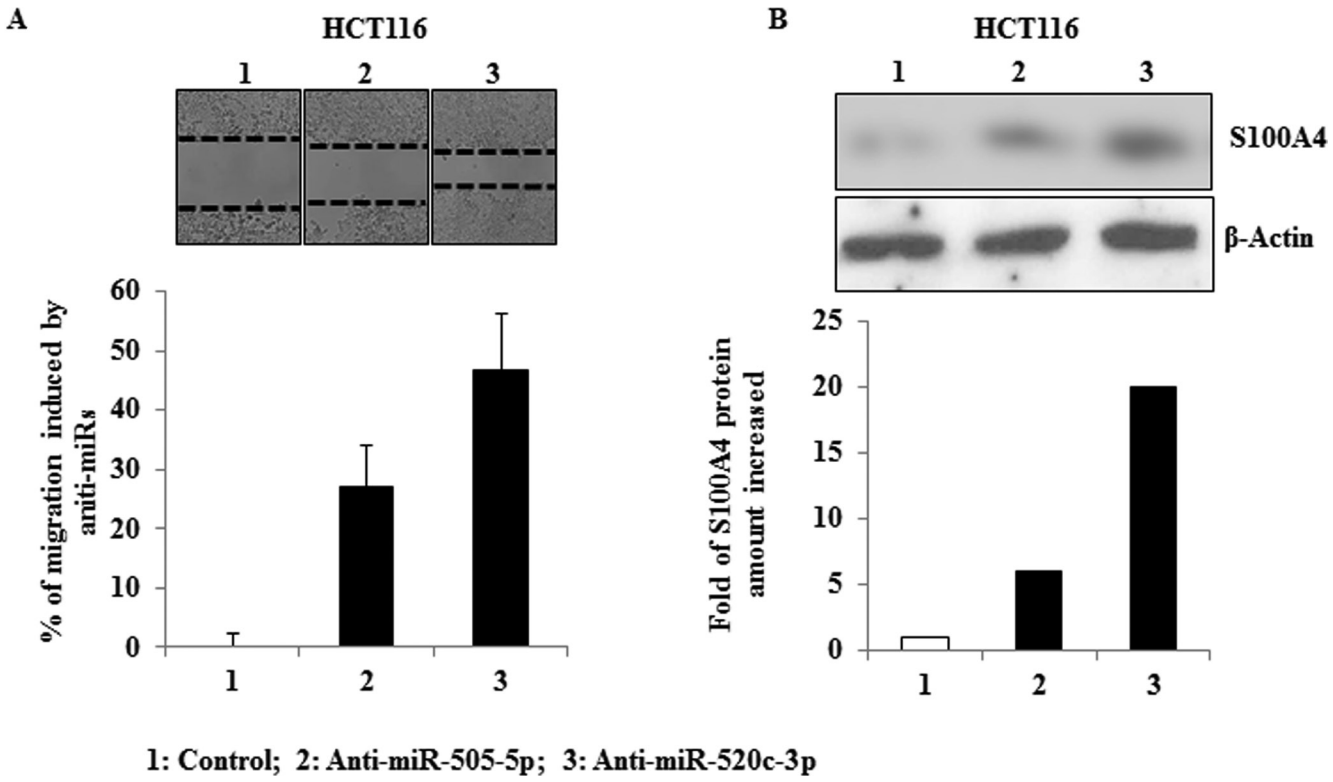

**Supplementary Figure 2: S100A4 endogenous mRNA expression and protein amount in CRC cell lines.** (**A**) S100A4 expression in a panel of CRC cell lines was analyzed by qRT-PCR, whereas RPII served as internal control. (**B**) S100A4 protein amounts were screened in the same panel of CRC cell lines, whereas β-actin served as internal control (Samples were analyzed using two gels, whereas sample preparation, gel electrophoresis, blotting and development were simultaneously performed).



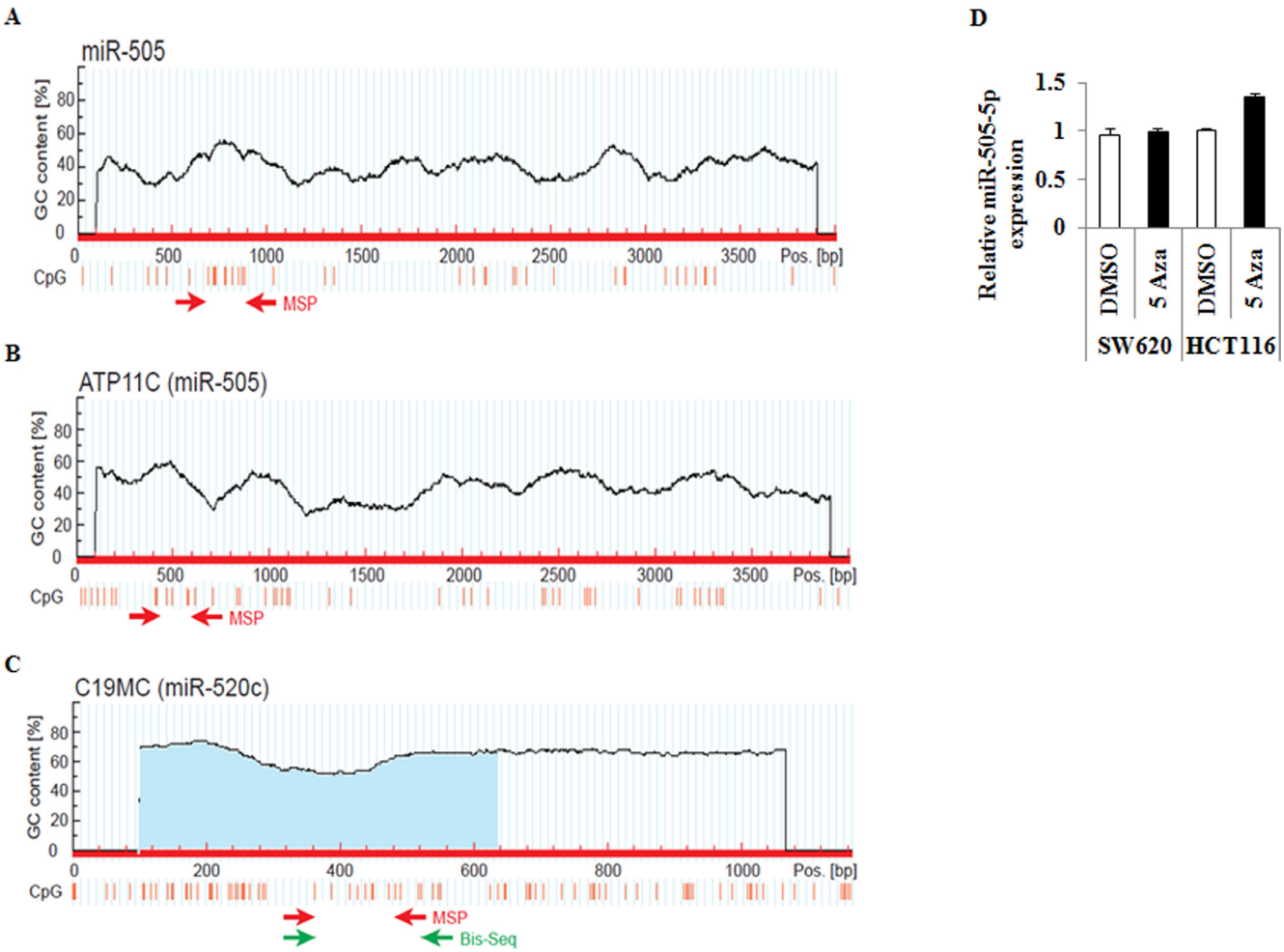

**Supplementary Figure 3: miR-520c expression and methylation status in a panel of CRC cell lines.** (**A**) Endogenous miR-520c-3p expression was quantified using qRT-PCR. RNUB6 served as internal control. (**B**) Results of the methylation-specific PCR of the CRC cell lines were analyzed using gel electrophoresis of the PCR products.




**Supplementary Figure 4:miR-505-5p and its host gene ATP11C expression, and methylation status in a panel of CRC cell lines.** (**A**, **B**) Relative miR-505-5p and ATP11C expression levels in a panel of CRC cell lines were analyzed by qRT-PCR, whereas RNUB6 and RPII served as internal controls. miR-505-5p and ATP11C expression levels positively correlated in CRC cell lines ($R^2 = 0.84$). (**C**) PCR products of CRC cell lines obtained from methylation-specific PCR for the possible regulatory region of miR-505 and its host gene ATP11C were analyzed by agarose gel electrophoresis.




**Supplementary Figure 5: Inhibition of miR-520c-3p significantly induces wound healing and S100A4 protein expression in HCT116 cells.** (**A**) HCT116 cells were transfected with anti-miR-505-5p and anti-miR-520-3p. After 48 h cells were plated in wound healing culture plates (#80241, Ibidi, Germany) and incubated for 24 h to allow the cells to attach. The cell separating plastic covers were removed. After 24 h of incubation the wound was photographed under a brightfield microscope. Wound healing was measured comparative to control and the bar graph shows the relative migration (wound healing) induced by anti-miR-505-5p and anti-miR-520c-3p. (**B**) After 48 h, protein was isolated from anti-miR transfected cells and S100A4 protein expression was analyzed using Western blot (presented here is the shortest exposure to show the clear differences), whereas β-actin served as internal control. The bar graph shows the densitometric analysis of S100A4 protein in comparison to the control.




**Supplementary Figure 6: Diagrammatic representation of CpG-islands in the upstream regions of miR-520c, miR-505 and the promoter region of its host gene ATP11C.** (**A**–**C**) The Methprimer tool was used to predict CpG islands in upstream regions of miR-505, ATP11C and miR-520c. For miR-520c a reported region was analyzed, whereas for miR-505 and ATP11C the 4 kb upstream region was used for methylation analysis. Primer locations for methylation specific PCR and bisulfite sequencing are represented by arrows in red (MSP) and green (Bis-Seq). (**D**) The expression of miR-505-5p and ATP11C after 5-Aza (2 μM) treatment for 3 days was analyzed in SW620 and HCT116 cells using qRT-PCR.

**Supplementary Table 1: Oligos used for S100A4-3′-UTR cloning**

| No | Oligo Name | Sequence (5′ --- 3′) |
|---|---|---|
| 1 | S100A4_UTR_For | 5′ CCCTCGAGCTTCCCAGATAAGCAGCCCA 3′ |
| 2 | S100A4_UTR_Rev | 5′ CCGTCGACCTTCCAAGAATCTTTATTGAAC 3′ |

**Supplementary Table 2: Oligos used to mutate miR-505 and miR-520c seed sequences at S100A4-3′-UTR**

| No | Oligo Name | Sequence (5′ --- 3′) |
|---|---|---|
| 1 | S100A4_m505_For | AGCCAGGGTGGAAAAAAAATTCAGCCCACTGGCGACAGGGAG |
| 2 | S100A4_m505_Rev | CTCCCTGTCGCCAGTGGGCTGAATTTTTTTTCCACCCTGGCT |
| 3 | S100A4_m520c_For | CAGCATCAAGCACGTGTCTGAAAAGGCCAGGGTG GAAAAAAAAAAGT |
| 4 | S100A4_m520c_Rev | ACTTTTTTTTTTCCACCCTGGCCTTTTCAGACACGTGCTTGATGCTG |

**Supplementary Table 3: Oligos used for measuring the Human microsatellite DNA (HMD) in mice liver tissue**

| No | Gene Name | Sequence (5′ --- 3′) |
|---|---|---|
| 1 | HMD_F | GGGATAATTTCAGCTGACTAAACAG |
| 2 | HMD_R | AAACGTCCACTTGCAGATTCTAG |

**Supplementary Table 4: Patients information**

| | | Total | Percentage |
|---|---|---|---|
| **Sex** | M | 36 | 61% |
| | F | 23 | 39% |
| **Age median (min to max)** | 63.0 (37.9 to 87.8) | | |
| **UICC (7th Edition)** | I | 17 | 29% |
| | II | 22 | 37% |
| | III | 20 | 34% |
| **pT** | 1 | 6 | 10% |
| | 2 | 16 | 27% |
| | 3 | 27 | 46% |
| | 4 | 10 | 17% |
| **pN** | 0 | 39 | 66% |
| | 1 | 14 | 24% |
| | 2 | 6 | 10% |
| **Metachronous metastases** | no | 35 | 59% |
| | yes | 24 | 41% |

Clinico-pathological features of the 59 CRC patients.
pT = Pathologic tumor classification. pN = Pathologic lymph node status.

**Supplementary Table 5: Oligos used for S100A4 and RPII screening by qRT-PCR**

| No | Gene Name | Sequence (5′ --- 3′) |
|---|---|---|
| 1 | S100A4_For | TGTGATGGTGTCCACCTTCC |
| 2 | S100A4_Rev | CCTGTTGCTGTCCAAGTTGC |
| 3 | RPII _For | GCACCACGTCCAATGACAT |
| 4 | RPII _Rev | GTGCGGCTGCTTCCATAA |

**Supplementary Table 6: Oligos used for methylation-specific PCR (MSP) of miR-505 and miR-520c**

| No | Gene Name | Sequence (5′ --- 3′) |
|---|---|---|
| 1 | miR-505_M_F | ATTTAGGTTGGAGTGTAATGGTACG |
| 2 | miR-505_M_R | TTAAAAAACCGAAATAAAAAAATCG |
| 3 | miR-505_U_F | TTTAGGTTGGAGTGTAATGGTATGA |
| 4 | miR-505_U_R | AAAAAACCAAAATAAAAAAATCACC |
| 5 | miR-520c_M_F | CGGTAAAATTTTGAATTTTTTTGTC |
| 6 | miR-520c_M_R | AAAATACATACTAACCTACCGCGTT |
| 7 | miR-520c_U_R | TGGTAAAATTTTGAATTTTTTTGTTG |
| 8 | miR-520c_U_R | AAAATACATACTAACCTACCACATT |

M: Methylated; U: Unmethylated.

**Supplementary Table 7: Oligos used for Bisulfite sequencing and COBRA analysis of miR-520c**

| No | Gene Name | Sequence (5′ --- 3′) |
|---|---|---|
| 1 | miR-505_Bis_F | GGTAAAATTTTGAATTTTTTTGT |
| 2 | miR-505_Bis_R | AAAAAAACCTATTAACTAAAAACCC |