miR-185 and *miR-133b* deregulation is associated with overall survival and metastasis in colorectal cancer

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Received March 15, 2011; Accepted April 20, 2011

DOI: 10.3892/ijo.2011.1043

Abstract. Colorectal cancer (CRC) is one of the most common and deadly forms of cancer. Despite improved treatment modalities, post-operative recurrence and metastasis remain the major problems for extending patient survival after surgery. This highlights the need to search for biomarkers for prognostication and treatment stratification of colorectal cancer patients. In this study, we applied the SYBR-green quantitative PCR-based array approach to screen for differentially expressed miRNAs between patients with short (<50 months, range 10-33 months) and long survival (≥50 months, range 50-152 months). The selected candidate prognostic miRNAs were validated in a cohort of 50 CRC patients by TaqMan quantitative PCR. We found that high expression of *miR-185* and low expression of *miR-133b* were correlated with poor survival (p=0.001 and 0.028, respectively) and metastasis (p=0.007 and 0.036, respectively) in colorectal cancer. Our findings suggest the potential prognostic values of these miRNAs for predicting clinical outcome after surgery.

Introduction

Colorectal cancer (CRC) is the second most common cancer in women and the third in men worldwide (1). It ranks the second most common cause of cancer death in the Western world (2). Currently, there are several treatment modalities for CRC, including surgery, radiotherapy, chemotherapy and targeted therapy (e.g., cetuximab). However, the long-term survival remains low in metastatic disease (3).

Given that CRC usually follows a stepwise progression from benign to malignant lesion and distant metastasis, there is a possibility for early diagnosis in order to reduce morbidity and mortality. The commonly used method to characterize CRC tumor in clinic is T1-T3 staging system. However, the staging system reflects only morphological characteristics of the tumor and does not consider tumor molecular biology, thus, it is inaccurate in predicting the future outcome for each particular case. Therefore, the development of screening tools and new biomarkers to facilitate early diagnosis is particularly warranted. Further investigations in the search for new prognostic biomarkers may help to improve post-operative treatment approaches for CRC patients.

Several studies have documented a link between the aberrant expression of a class of small non-coding RNAs, termed microRNAs (miRNAs), and the pathogenesis/prognosis of several cancer types, including colorectal cancer (4,5). These molecules provide a potentially valuable diagnostic/prognostic tool for CRC pathology, because mature miRNA species are relatively more stable than mRNAs and well preserved in formalin-fixed, paraffin-embedded samples which are commonly used in clinical routine (6). Furthermore, only a small number of miRNAs are required to distinguish cancerous tissues from non-cancerous tissues compared with mRNA profiles (7), which makes them more feasible candidate biomarkers.

miRNAs are endogenous single-stranded non-coding RNAs of ~22-nucleotides in length, which are generated by an RNase III enzyme Dicer from endogenous hairpin-shaped transcripts (8). miRNAs regulate gene expression at post-transcriptional level through mRNA degradation and/or translational repression, by forming hybrids with the 3'-untranslated region sequences of their target mRNAs (9). They are involved in the regulation of many cellular functions, including proliferation, differentiation and apoptosis (9-11).

Alteration of specific miRNA expressions has been described for many tumor types (12-21). In colorectal cancer, *miR-143* and *miR-145* were first reported to have reduced expression in both benign and malignant lesions compared to corresponding normal tissues (12). Importantly, these molecules appear to have tumor suppressive roles in CRC (22,23). Besides their potential roles in CRC tumorigenesis, several recent studies have pioneered the use of miRNA expression profiles in tumor classification and prognostication. Among the important findings, deregulation of specific miRNAs was shown to be associated

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Key words: miRNA, colorectal cancer, prognosis, survival, metastasis

with tumor classification (24), microsatellite status [e.g., *miR-25* and *miR-92* (25)], recurrence [e.g., *miR-498* and *miR-320*, (26)], survival and therapeutic outcome [e.g., *miR-21* (24)] of CRC. Despite the studies mentioned above, only limited information is available on the impact of deregulated miRNA expression on CRC patient survival.

In this study, we evaluated the impact of deregulated miRNA expressions on patient survival and metastasis in colorectal cancer. We identified two miRNAs that were significantly associated with shorter CRC survival and metastasis after surgery.

Materials and methods

Clinical samples. A total of 50 snap-frozen primary sporadic colorectal tumors from patients who had undergone surgery between 1993 and 1998 at Karolinska University Hospital were included in this study. The clinical, histopathological and follow-up details of all cases are detailed in Table I, and have been partly published for subsets of the cases in previous studies (27). All tumor specimens were histopathologically verified. The patients were followed-up until August 2010 or the time of death. All samples were obtained with informed consent and the study of the tissue materials was approved by the local ethics committee.

Total RNA extraction. Total RNA isolation was performed using mirVana miRNA Isolation Kit (Applied Biosystems/ Ambion, Austin, TX) according to the manufacturer's protocol. RNA concentrations were measured using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

SYBR Green-based miRNA profiling. The SYBR Green-based qRT-PCR (quantitative real-time PCR) miRNA array platform (human miRNome microRNA Profilers QuantiMir™; cat. no. RA660A-1; System Biosciences, Mountain View, CA) was used for profiling of 10 patients with distinct survival (5 patients <50 months, 5 patients ≥50 months). The experimental procedures were done according to the protocol recommended by the manufacturer. In brief, 800 ng of total RNA was tailed with poly(A) polymerase, and reverse transcribed by oligo(dT)-adaptor primer at 42˚C for 1 h. Quantitative real-time PCR was performed using the Power SYBR Green Master Mix (cat. no. 4367659; Applied Biosystems, Foster City, CA) on the 7900HT Real-time PCR System (Applied Biosystems) employing QuantiMir universal reverse primers and miRNA-specific forward primers. All data values were normalized by geometric mean (i.e., a measure of central tendency similar to median or arithmetic mean) of three different reference genes (Human U6, RNU43 and U1), and relative quantification was calculated as $2^{-\Delta CT}$. Normalized miRNAs with <20% missing values were included in subsequent analyses for hierarchical clustering based on non-centered correlation and complete linkage using Cluster 3.0 (28) and visualized using Java TreeView (29), and significance analysis of microarrays (SAM) (30).

TaqMan qRT-PCR assays of individual miRNAs. The expression of selected mature miRNAs was quantified using commercially available TaqMan qRT-PCR assays (Applied Biosystems) and a 7900HT Real-Time PCR System (Applied Biosystems). cDNA was synthesized from 100 ng total RNA using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) and used for quantification of *miR-133b* (ID 002247), *miR-185* (ID 002271), *miR-320b* (ID 002844), *miR-21* (ID 000397), *miR-663b* (ID 002857), *miR-892b* (ID 002214) and *miR-615-5p* (ID 002353), *RNU6B* (ID 001093) and *miR-16* (ID 000391). Due to the variability of *RNU6B* expression across the samples analyzed, the expression of miRNAs was normalized to *miR-16*. All reactions were performed in triplicate, and relative expression levels were determined with the ΔC_T method and reported as $2^{-\Delta CT}$.

Statistical analysis. SYBR Green-based miRNA profiling data were evaluated by using SAM analysis, in order to identify the most significant miRNAs associated with survival. p-values were obtained for the Cox score statistics using the χ^2 distribution.

Selected miRNAs potentially associated with the outcome were analyzed by TaqMan qRT-PCR and tumor samples were classified into two different groups with high or low expression of each miRNA according to median level. The interrelationship of miRNAs with survival was studied using Kaplan-Meier plots, while significant differences between curves were evaluated using log-rank test. The significance of individual miRNA expression in correlation with metastasis and other clinical characteristics, including age, gender, stage and recurrence, was studied using Fisher's exact test. All p-values obtained in this study were 2-tailed, and p-values <0.05 were considered as significant. All statistical tests were performed in Statistica 8.0 (StatSoft, Inc., Tulsa, OK), unless otherwise stated.

Results

Distinct miRNA expression patterns between the long and short survival groups of colorectal cancer patients. In order to determine whether miRNA expression could distinguish colorectal cancer patients with distinct survival outcomes after surgery, we characterized miRNA expression patterns in colorectal tumors of 10 chosen patients with distinct survival using SYBR-green qRT-PCR array-based platform. Among the samples analyzed in the screening experiment, five patients had short survival <50 months (range 10-33 months) from diagnosis until the patient's disease specific death, and the remaining five patients had long survival ≥50 months (range 50-152 months) after diagnosis. We performed unsupervised hierarchical clustering of the 610 filtered miRNAs among the 10 CRC samples. The analysis revealed that almost all CRC tumors were grouped according to their survival outcome (Fig. 1). The CRC patients with short survival were grouped separately from the patients with long survival.

Deregulated expression of miR-185 and miR-133b are associated with overall survival and metastasis in CRC patients. To identify the most significant miRNAs that could distinguish the patient survival, we performed significance analysis of microarray (SAM) analyses based on the data obtained from the screening approach. We found 7 over-expressed and 356 under-expressed miRNAs in the short survival group compared to the long survival group (data not shown).

Table I. Clinical and histopathological information of the 50 colorectal cancer patients studied.

Case no.	Sex (M/F)	Age at diagnosis (years)	Stage	Location	Metastasis detected	Reccurence detected	Follow-up	
							Time (months)	Outcome
1	F	78	2	Rectum	No	N/A	153	Dead
2	F	77	4	Right colon	Liver	N/A	6	Dead-DOD
3	M	73	3	Rectum	Liver	N/A	33	Dead-DOD
4	M	80	3	Left colon	No	No	50	Dead-N/A
5	M	54	3	Left colon	Liver and lung	Yes	8	Dead-DOD
6	M	78	2	Rectum	Lung	Yes	62	Dead-DOD
7	F	74	2	Right colon	No	Yes	56	Dead-DOD
8	F	64	3	Rectum	No	No	83	Dead
9	F	38	2	Transverse colon	N/A	Yes	24	Dead-DOD
10	M	81	3	Rectum	No	Yes	10	Dead-DOD
11	F	81	1	Right colon	No	N/A	133	Dead
12	F	60	3	Left colon	No	No	152	Alive
13	M	75	4	Right colon	Lung	No	10	Dead-DOD
14	M	87	3	Left colon	Liver	No	10	Dead-DOD
15	M	75	3	Right colon	Lung	N/A	2	Dead-DOD
16	M	62	2	Left colon	No	No	152	Alive
17	M	71	2	Left colon	No	No	152	Alive
18	M	54	3	Left colon	Liver and lung	Yes	10	Dead-DOD
19	F	80	2	Left colon	No	N/A	19	Dead-N/A
20	F	78	2	Left colon	No	No	151	Alive
21	F	68	4	Rectum	Liver and lung	No	5	Dead-DOD
22	F	52	3	Right colon	Liver and lung	No	43	Dead-DOD
23	M	75	3	Left colon	Liver	No	69	Dead-DOD
24	F	48	2	Right colon	No	No	176	Alive
25	M	72	4	Left colon	Liver	No	12	Dead-DOD
26	F	81	3	Rectum	No	N/A	41	Dead-N/A
27	M	53	4	Rectum	Liver	No	11	Dead-DOD
28	F	61	3	Rectum	Lung	No	64	Dead-DOD
29	M	66	3	Right colon	Liver	Yes	23	Dead-DOD
30	F	79	1	Right colon	No	No	74	Dead
31	M	76	1	Right colon	No	No	167	Alive
32	F	70	1	Rectum	No	N/A	83	Dead-N/A
33	M	78	3	Rectum	No	No	170	Alive
34	M	75	1	Left colon	No	N/A	196	Alive
35	M	80	2	Left colon	Liver	N/A	104	Dead-DOD
36	M	76	2	Rectum	No	No	168	Alive
37	M	80	4	Left colon	Liver	No	3	Dead-DOD
38	F	55	1	Rectum	No	No	168	Alive
39	M	87	1	Left colon	No	No	85	Dead
40	F	73	1	Right colon	No	No	141	Dead
41	F	69	2	Right colon	Liver	Yes	29	Dead-DOD
42	F	74	2	Rectum	No	No	91	Dead
43	F	79	2	Left colon	Liver	Yes	25	Dead-DOD
44	F	83	2	Right colon	No	No	154	Dead
45	M	85	2	Transverse colon	No	No	29	Dead
46	F	61	3	Rectum	No	No	204	Alive
47	M	79	3	Rectum	No	No	61	Dead
48	M	76	3	Left colon	Liver and lung	No	36	Dead-DOD
49	F	37	1	Rectum	No	No	212	Alive
50	M	N/A	3	Left colon	N/A	N/A	N/A	Alive

M, male; F, female; DOD, dead of disease; N/A, not available.

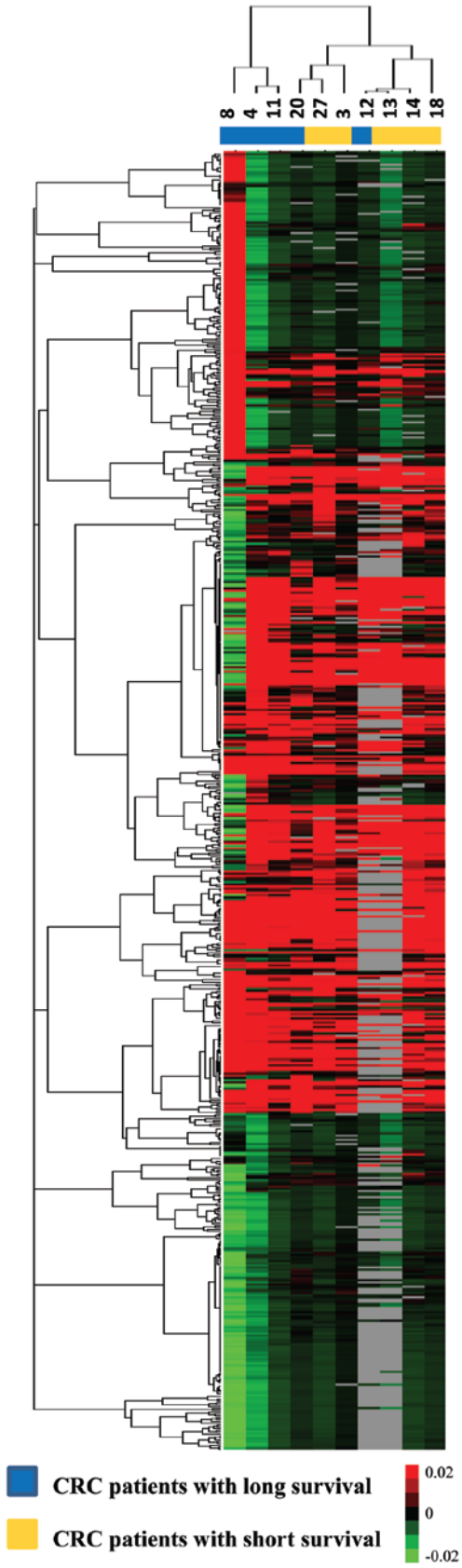


Figure 1. Unsupervised clustering analysis of miRNA expression. Heat maps showing unsupervised clustering of miRNA profiling data of 10 colorectal cancer patients using a SYBR Green qRT-PCR array method. Samples were clustered based on un-centered correlation and complete linkage. The red and green colors indicate relatively high and low expression, respectively, while grey indicates missing values. The two major subgroups mainly separate CRC patients with short and long survival.

To verify the array results and to evaluate the significance of the finding, we performed TaqMan qRT-PCR analyses on six selected miRNAs from among the differentially expressed miRNAs in a cohort of 50 clinical samples. The selected miRNAs consisted of three over-expressed (*miR-185, miR-320b* and *miR-663b*) and three under-expressed miRNAs (*miR-133b, miR-615-5p* and *miR-892b*) in CRC patients with short survival compared to patients with long survival (Fig. 2). In addition, we also assessed the expression level of *miR-21* in the validation cohort because high expression of *miR-21* has been associated with poor survival in colon adenocarcinoma (24); however, it was not significantly different between the two groups based on our screening data.

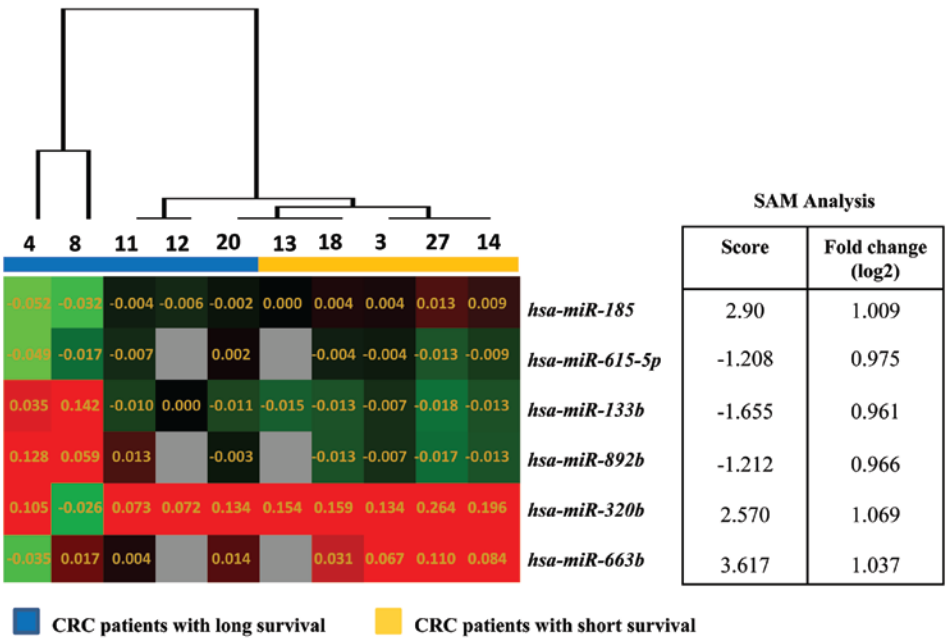
Using Kaplan-Meier survival plots and log-rank analyses, we evaluated the association of each individual miRNA expression with overall survival. We found significant associations with survival for *miR-185* and *miR-133b* (Fig. 3A), whereas m*iR-320b, miR-21, miR-892b, miR-663b* and *miR-615-5p* were not found statistically significant (data not shown). Patients with high expression of *miR-185* (p=0.001; log-rank test) and low expression of *miR-133b* (p=0.028; log-rank test) were found to have significantly shorter survival (Fig. 3A).

We also evaluated the selected miRNAs for their association with clinical parameters. Interestingly, we found that increased expression of *miR-185* (p=0.007; Fisher's exact test) and decreased expression of *miR-133b* (p=0.036; Fisher's exact test) were also associated with the development of metastatic disease (Table II and Fig. 3B). No correlation was detected between stage, recurrence, age or gender and expression of these miRNAs (Table II). In addition, no significant association was found for the remaining five miRNAs for these parameters (data not shown).

Discussion

In this study, we report the deregulation of two miRNAs (*miR-185* and *miR-133b*) correlates with patient survival and metastasis in colorectal cancer, suggesting that these miRNAs (or their targets) may have prognostic implications in CRC.

In a cohort of 50 CRC samples, we observed that high expression of *miR-185* is significantly associated with poor survival and metastasis of CRC patients. Increased expression of *miR-185* has also been observed in clear cell renal cell carcinoma compared to normal kidney tissues, and its expression is inversely correlated with its putative target *PTPN13* (31). PTPN13 (protein tyrosine phosphatase, non-receptor type 13) is a Fas-associated protein tyrosine phosphatase and putative tumor suppressor gene that can inhibit PI3K/AKT signaling, suppress cell growth and induce apoptosis (32). Importantly, somatic *PTPN13* mutations have been found in ~9% of CRCs (33), suggesting a critical role in the pathogenesis of CRC. The role of *PTPN13* in colorectal cancer progression and its regulation by *miR-185* need further investigations. In contrast to its oncogenic role, *miR-185* has also been demonstrated to suppress tumor growth and progression in non-small lung cancer (34), ovarian, pediatric renal and breast cancer cell lines (35). These findings suggest that *miR-185* may target different genes in different cell types, which contributes to different biological processes.



SAM Analysis

	Score	Fold change (log2)
hsa-miR-185	2.90	1.009
hsa-miR-615-5p	-1.208	0.975
hsa-miR-133b	-1.655	0.961
hsa-miR-892b	-1.212	0.966
hsa-miR-320b	2.570	1.069
hsa-miR-663b	3.617	1.037

Figure 2. Clustering of the six selected miRNA expressions based on SAM (significance analysis of microarray) analysis of the SYBR-green qRT-PCR array data. The selected miRNAs consist of three over-expressed (*miR-185*, *miR-320b* and *miR-663b*) and three under-expressed miRNAs (*miR-133b*, *miR-615-5p* and *miR-892b*) in CRC patients with short survival compared to patients with long survival. Relative expression value of miRNAs for each sample is shown in the data points. The red and green colors indicate relatively high and low expression, respectively, and grey represents missing data points.

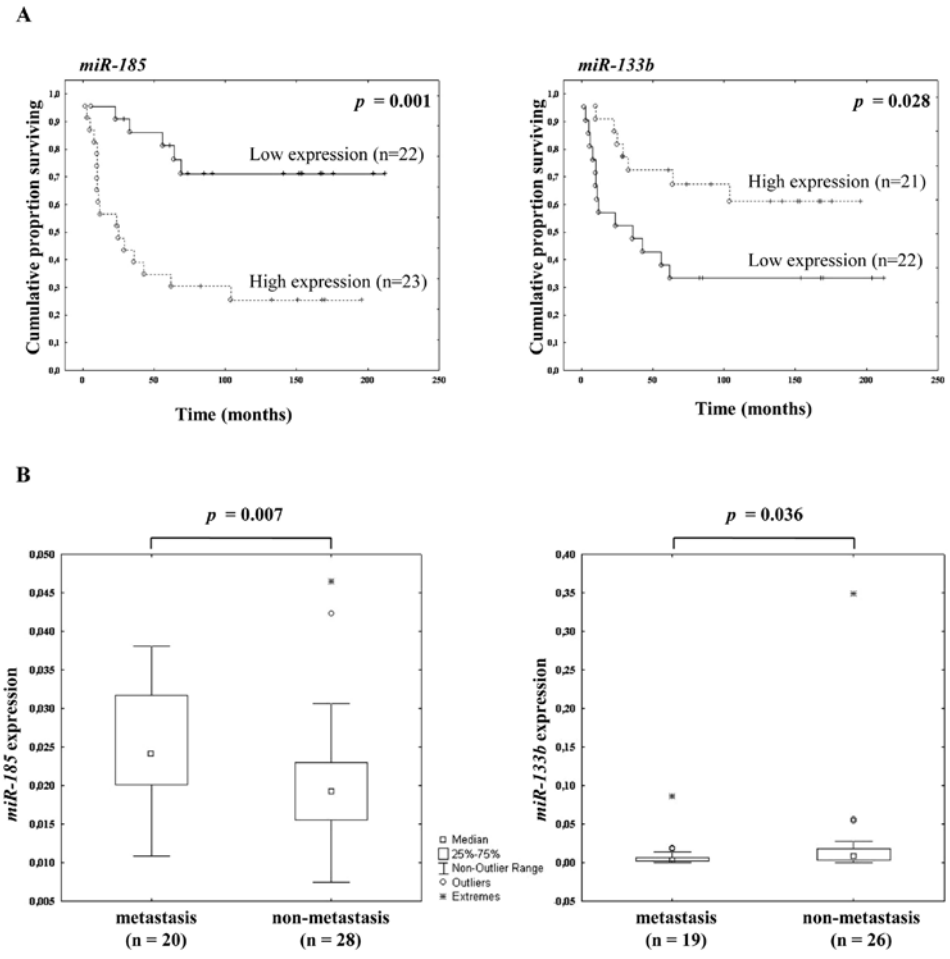


Figure 3. Association between miRNA expression levels and clinical outcomes. (A) Kaplan-Meier curves showing significant associations of *miR-185* and *miR-133b* expressions with overall survival in CRC patients. Expression levels of the two miRNAs were measured in 50 CRC patients by TaqMan quantitative RT-PCR. Differences in the two survival groups were calculated using log-rank test. (B) Box plots showing expression levels of *miR-185* and *miR-133b* in colorectal cancer patients with metastasis and without metastasis. Significance of differences was calculated using Fisher's exact test.

Table II. Correlation between clinical characteristics and expression levels of *miR-185*, *miR-133b*, *miR-663b*, *miR-21*, *miR-320b*, *miR-615-5p* and *miR-892b*.

		miR-185			*miR-133b*			*miR-663b*			*miR-21*			*miR-320b*			*miR-615-5p*			*miR-892b*		
Parameter	No. cases	High	Low	p-value	High	Low	p-value	High	Low	p-value	High	Low	p-value	High	Low	p-value	High	Low	p-value	High	Low	p-value
Age																						
≥75	26	15	11	0.396	13	10	0.555	16	10	0.156	15	11	0.562	14	12	0.778	13	13	1.0	13	10	0.556
<75	23	10	13		10	13		9	14		10	12		11	12		12	11		10	12	
Gender																						
Female	23	10	13	0.571	13	9	0.385	9	14	0.156	10	13	0.396	14	9	0.256	14	9	0.256	12	10	0.768
Male	27	15	12		11	14		16	10		15	11		11	16		11	16		11	12	
Metastasis																						
Positive	20	15	5	**0.007**	6	13	**0.036**	10	10	1.0	9	11	0.561	12	8	0.38	11	9	0.770	7	11	0.357
Negative	28	9	19		17	9		14	14		15	12		12	16		13	15		15	11	
Disease stage																						
I-II	25	11	14	0.572	14	9	0.178	11	14	0.572	15	9	0.156	13	12	1.0	13	12	1.0	10	13	0.376
III-IV	25	14	11		9	15		13	11		10	15		12	13		12	13		13	9	
Recurrence																						
Positive	9	7	2	0.127	4	5	0.713	4	5	1.0	5	4	1.0	6	3	0.45	4	5	0.716	5	4	1.0
Negative	30	13	17		15	13		15	15		14	15		14	16		16	14		13	14	

p-values were determined by two-sided Fisher's exact test.

Besides *miR-185*, low expression of *miR-133b* was also found to be associated with poor survival and the development of metastases in CRC patients. *miR-133b* is also down-regulated in several cancer types, including colorectal cancer (17), lung cancer (36), bladder cancer (37), gastric cancer (38), esophageal squamous cell carcinoma (39) and squamous cell carcinoma of tongue (40). In addition, *miR-133b* has been reported to have prognostic potential for predicting bladder cancer progression (41). Functionally, *miR-133b* targets oncogenic *FSCN1* in esophageal squamous cell carcinoma (39), pro-survival molecules *MCL-1* and *BCL2L2* in lung cancer (36), and *MET* proto-oncogene in colorectal cancer cells (42) affecting cell proliferation and invasion. Taken together, these findings suggest that *miR-133b* may suppress cancer progression in several cancer types. However, the significance of the role of *miR-133b* and its target(s), such as MET, in CRC progression remains to be further investigated in a larger series of clinical samples.

Recently, Schetter and coworkers showed that high expression of *miR-21* was associated with poor survival and therapeutic outcome in colon adenocarcinoma patients (24). However, we did not observe significant difference in expression levels between the long and short survival groups of CRC patients using both SYBR-green and TaqMan qRT-PCR assays. The discrepancy may be due to the differences in location of tumor site or tumor histological subtype. In line with this statement, the association of poor survival and *miR-21* over-expression was only documented in colon cancer (24,43), but not in rectal cancer (43). In addition, high expression of *miR-21* was also detected in adenocarcinoma sub-type (24). Due to the limited number of cases, we could not ascertain its expression in relation to tumor site and histological subtype. Interestingly, Nielsen *et al* observed that the expression of *miR-21* was predominantly found in fibroblast-like cells located in the stromal compartment of the colon tumors (43). The cellular context of the tumors analyzed may also contribute to the discrepancy.

The significant correlation of *miR-185* and *miR-133b* to overall survival and metastasis may prove useful for future development as prognostic markers, to identify CRC patients at high risk of advanced disease. Given that circulating miRNAs exist and are stable in serum and plasma of human samples (44), *miR-185* and *miR-133b* have a potential as novel non-invasive biomarkers for prognostication and treatment stratification of CRC patients after surgery. However, it remains to be determined if these miRNAs exist in serum samples in reproducible quantities and whether their expressions are related to the clinical outcome of CRC patients.

In conclusion, we report two miRNAs that are associated with overall survival and metastasis of CRC patients, suggesting their potential prognostic values in this disease type. The findings may provide important insights into understanding the progression of colorectal cancer.

Acknowledgements

We thank the members of the Medical Genetics group for their helps and suggestions. This study was supported by the Swedish Research Council (523-2009-3517), Åke Olsson's Foundation for Haematological Research, Åke Wiberg's Foundation, Axel and Signe Lagerman's Donation Foundation, King Gustaf V Jubilee Fund, Karolinska Institutet and Stockholm County Council.

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