Plasma miR-200c and miR-18a as potential biomarkers for the detection of colorectal carcinoma

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Abstract. It has been demonstrated that there are abundant stable microRNAs (miRNAs) in plasma/serum, which can be detected and are potentially disease-specific. The aim of this study was to investigate whether plasma miR-200c and miR-18a can be used as biomarkers for the detection of colorectal carcinoma (CRC). This study was divided into three parts: i) confirmation of higher miR-200c and miR-18a levels in primary CRC tissues compared to normal colorectal tissues; ii) evaluation of plasma miR-200c and miR-18a expression by comparing 78 patients with 86 healthy volunteers and iii) comparison of miR-200c and miR-18a levels in paired pre-and post-operative plasma in cancer patients who underwent curative CRC resection. Results showed that the expression of miR-200c and miR-18a was significantly higher in CRC compared to normal tissues. The plasma levels of miR-200c and miR-18a were significantly higher in CRC patients compared to controls. miR-200c yielded an area under the receiver-operating characteristics (ROC) curve (AUC) of 0.749 and miR-18a yielded an AUC of 0.804 when distinguishing CRC patients from the controls. Combined ROC analyses using the two miRNAs revealed an elevated AUC of 0.839 with 84.6% sensitivity and 75.6% specificity in discriminating CRC. Plasma levels of miR-200c and miR-18a were significantly lower in post-operative compared to pre-operative samples. The results of this study suggest that plasma miR-200c and miR-18a are significantly elevated in the plasma of CRC patients and that they may serve as non-invasive molecular markers for CRC screening.

Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide with an estimated one million new cases and 500,000 deaths annually (1). Early detection of CRC is vital to reduce the mortality of this disease. Several CRC screening tests, such as carcinoembryonic and carbohydrate antigens 19-9, fecal occult-blood testing (FOBT) and colonoscopy, have been used to detect CRC (2,3). However, none of these methods has been established as a well-accepted screening tool, due to the low sensitivity, high cost or invasive nature. Thus, novel non-invasive biomarkers are required to improve the detection of CRC.

Previous studies showed that microRNAs (miRNAs), which are involved in tumorigenesis and in the development of various types of cancer, are detectable in the plasma/serum (4,5). Mitchell *et al* (4) clearly demonstrated that circulating miRNAs originate from cancer tissues (4). Additional studies have demonstrated the presence of circulating miRNAs and their potential use as novel biomarkers of various types of cancer, such as pancreatic (6), colorectal (7), breast (8) and gastric cancers (9). These studies have provided new opportunities for a non-invasive examination for the early diagnosis of various types of cancer.

miR-200c and miR-18a are significantly upregulated in CRC tumor tissues compared to their adjacent normal tissues (10,11). miR-18a is significantly higher in the plasma of pancreatic cancer patients and is a potential screening biomarker for pancreatic cancer (12). Additional studies have demonstrated that miR-200c was detectable at a higher level in the plasma of CRC patients (7). In CRC tissues, patients with overexpressed miR-200c or miR-18a exhibited a poorer clinical prognosis (10,11). However, to the best of our knowledge, this is the first study to report whether the circulating miR-200c and miR-18a in plasma samples are potential non-invasive markers for CRC.

In this study, we investigated whether the circulating miR-200c and miR-18a in plasma samples might be used to screen for CRC by comparing findings in CRC patients and volunteer controls. Results clearly demonstrated their potential usefulness. The findings of this study provided evidence that the plasma levels of miR-200c and miR-18a may be used to distinguish CRC patients from healthy individuals with a clinically satisfactory degree of sensitivity and specificity.

Materials and methods

Patients and tissue samples. Plasma samples were collected from 164 patients, including 78 CRC patients and 86 normal

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controls between January, 2010 and April, 2011 (Table I). Exclusion criteria included inflammatory bowel disease, a family history of familial adenomatous polyposis, hereditary non-polyposis colon cancer or previous colonic surgery. Normal controls were asymptomatic individuals recruited from a colonoscopy screening programme at the First Department of General Surgery of the Affiliated Hospital of the North Sichuan Medical College (Nanchong, China). Plasma samples of CRC patients were collected prior to surgical resection. The normal control samples were collected prior to colonoscopy and at least 2 weeks following the initial colonoscopy. Tumors were staged according to the 6th edition of the International Union Against Cancer (UICC) Tumor-Nodes-Metastasis (TNM) staging system. Patients who had undergone neoadjuvant therapy prior to surgery were excluded. To investigate the changes in plasma-based miRNA levels following removal of the lesion, plasma samples were also collected at least 1 month after surgery. Forty two pairs of tissue samples were collected from CRC patients. The tumor and adjacent normal samples (≥4 cm apart from the tumor) were biopsied during surgical resection. Samples were stored at -80˚C for subsequent analysis. The study was approved by the Medical Ethics Committee of the North Sichuan Medical College, and written informed consent was obtained from the patients.

miRNA quantitation by real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Total RNA was extracted from tissues using the miRNeasy mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Total RNA containing small RNA was extracted from 500 μl of plasma using TRIzol LS reagent (Invitrogen, Carlsbad, CA, USA) and the miRNeasy Mini kit (Qiagen). according to the manufacturer's instructions. The final elution volume was 30 μl. The RNA concentration was measured with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). For miRNA qPCR, reverse transcription was performed using the QuantiMir™ RT kit (System Biosciences, Mountain View, CA, USA). The complementary DNA (cDNA) served as the template for SYBR-Green real-time PCR using Power SYBR-Green PCR Master mix (Applied Biosystems, Foster City, CA, USA). The reactions were performed in triplicate on the iCycler iQ™ Multi-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using miRNA-specific primers (Applied Biosystems). The amplification profile was denatured at 95˚C for 10 min, followed by 50 cycles of denaturation at 95˚C for 15 sec, annealing at 60˚C for 30 sec, and extension at 72˚C for 1 min. The comparative cycle threshold (C_T) method was applied to quantify the expression levels of miRNAs. The relative amount of miR-200c and miR-18a to small nuclear U6 RNA (RNU6B) was calculated using the equation $2^{-\Delta C_T}$, where $\Delta C_T = (C_{T\ miR\text{-}200c/miR\text{-}18a} - C_{T\ RNU6B})$. The fold change of the gene expression was calculated using the equation $2^{-\Delta\Delta C_T}$.

Statistical analysis. The difference in miRNA levels between paired tissue samples was determined using the Wilcoxon matched-pairs test. Expression levels of plasma miRNAs were compared using the Mann-Whitney U or the Kruskall-Wallis test. Receiver-operating characteristics (ROC) curves were established to evaluate the diagnostic value of plasma miRNAs to distinguish between tumors and controls. Statistical analyses were carried out using the SPSS 16.0 software (SPSS, Chicago, IL, USA). Two-sided P-values were calculated, and P<0.05 was considered to indicate a statistically significant difference.

Table I. Patient characteristics for plasma miRNA analysis.

Characteristics	Patients	
	Normal controls (n=86)	CRC (n=78)
Age (years)		
Mean ± SD	60.3±11.8	61.4±13.6
Gender		
Male	53	43
Female	33	35
Histological type		
Well, moderate		44
Poor, mucinous		34
Location		
Colon		31
Rectum		47
Lymph node status		
Positive		37
Negative		41
TNM stage		
I, II		36
III, IV		42

CRC, colorectal cancer; SD, standard deviation; TNM, Tumor-Nodes-Metastasis.

Results

Patient characteristics. Patient characteristics are summarised in Table I. A total of 164 participants including 78 CRC patients and 86 healthy individuals were included in this study. No significant differences of age or gender were found in CRC patients and normal controls (Student's t-test, P=0.564; χ^2 test, P=0.399).

Enhanced expression of miR-200c and miR-18a in CRC tissue samples. Of the 42 pairs of CRC tissue samples, 36 (85.7%) demonstrated higher miR-200c expression in tumor tissue compared to adjacent normal tissue (P=0.001; Fig. 1A), with an average increase of 2.02-fold. Thirty-seven (88.1%) pairs of tissue samples demonstrated higher miR-18a expression in the tumor tissue compared to adjacent normal tissue (P<0.001; Fig. 1B), with an average increase of 2.66-fold.

Increased levels of plasma miR-200c and miR-18a in CRC patients. Concentration of miR-200c and miR-18a was investigated in an independent group of 164 plasma samples that included 78 CRC patients and 86 healthy controls (Table I). miR-200c and miR-18a concentration significantly increased

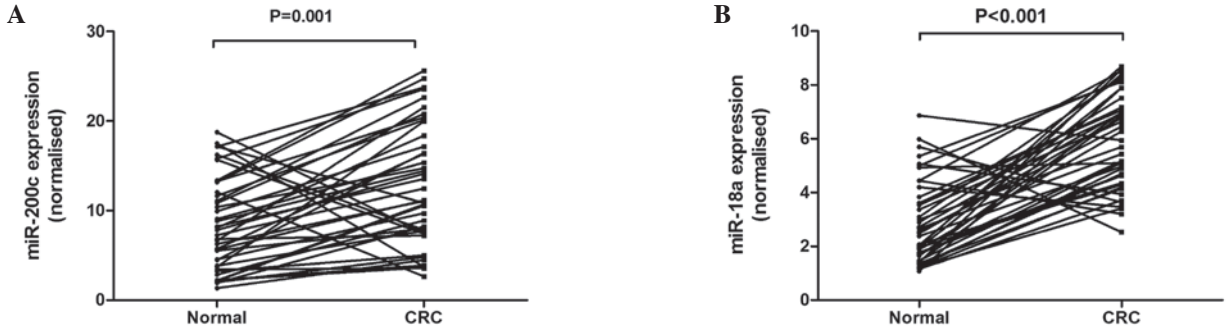

Figure 1. Levels of (A) miR-200c and (B) miR-18a in 42 pairs of colorectal tumors and adjacent normal tissues. P-values indicate significant differences in miRNA level between paired samples determined using the Wilcoxon matched-pairs test.

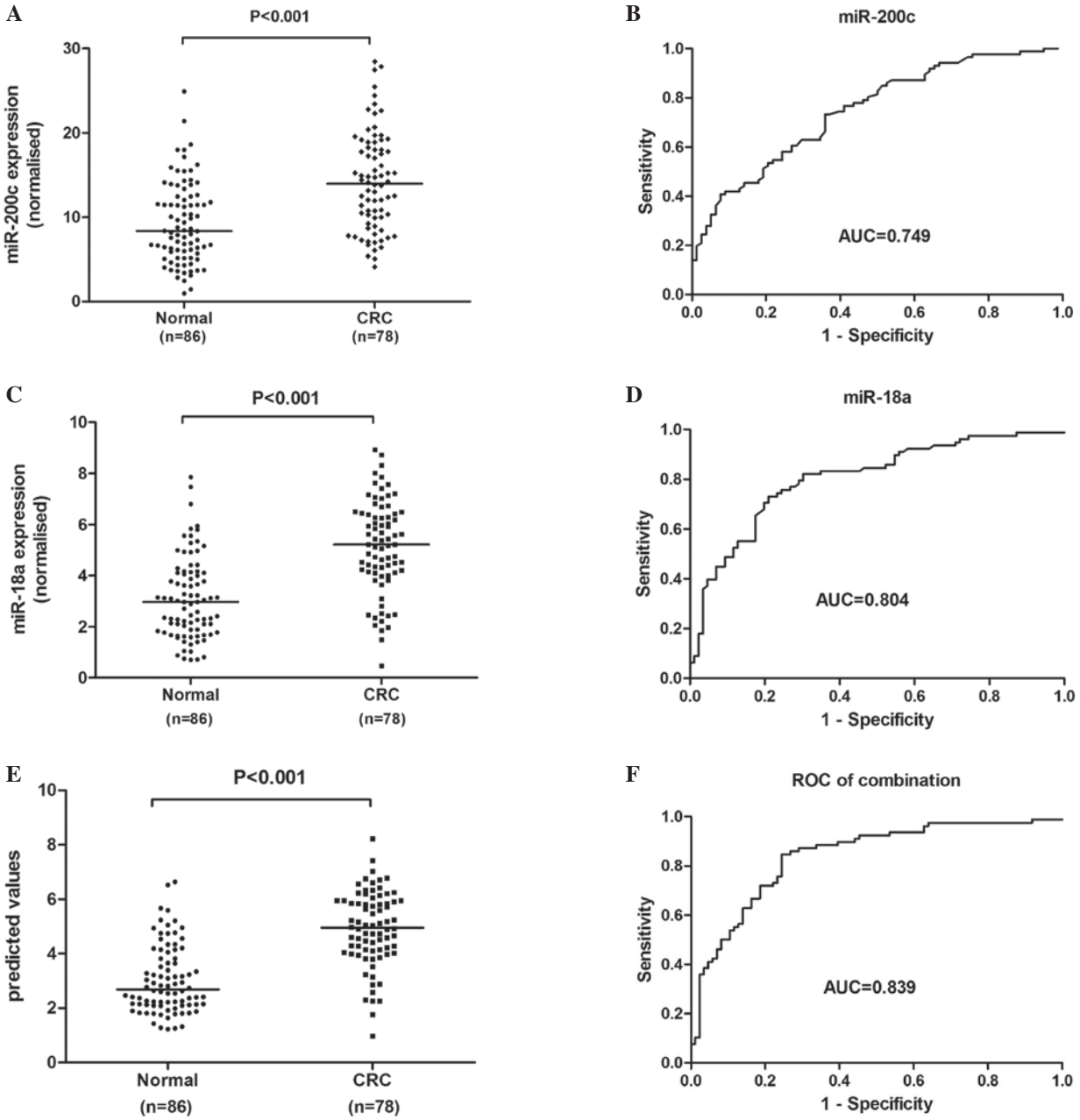

Figure 2. Validation of miR-200c and miR-18a in plasma samples (n=164). Scatter plots of plasma levels of (A) miR-200c and (C) miR-18a in healthy subjects (n=86) and colorectal cancer (CRC) patients (n=78). The expression levels of miRNAs were normalized to RNU6B. The line shows the median value. Mann-Whitney U test was used to determine statistical significance. Receiver-operating characteristics (ROC) curve analysis using (B) plasma miR-200c and (D) plasma miR-18a for discriminating CRC is shown. Scatter plots of predicted value after (E) logistic regression and (F) combination ROC curve for discriminating CRC from controls are shown.

Table II. Correlation between plasma miR-200c expression and clinicopathological characteristics of CRC patients.

Characteristics	miR-200c expression Low (n=39)	miR-200c expression High (n=39)	P-value
Age (years)			
Mean ± SD	63.1±14.5	59.7±12.6	0.281
Gender			0.255
Male	19	24	
Female	20	15	
Histological type			0.361
Well, moderate	20	24	
Poor, mucinous	19	15	
Tumor location			0.247
Colon	18	13	
Rectum	21	26	
Lymph node status			0.496
Positive	17	20	
Negative	22	19	
TNM stage			0.364
I, II	20	16	
III, IV	19	23	

CRC, colorectal cancer; SD, standard deviation; TNM, Tumor-Nodes-Metastasis.

Table III. Correlation between plasma miR-18a expression and clinicopathological characteristics of CRC patients.

Characteristics	miR-18a expression Low (n=39)	miR-18a expression High (n=39)	P-value
Age (years)			
Mean ± SD	62.1±13.7	60.7±13.6	0.655
Gender			0.495
Male	23	2O	
Female	16	19	
Histological type			0.171
Well, moderate	25	19	
Poor, mucinous	14	20	
Tumor location			0.488
Colon	14	17	
Rectum	25	22	
Lymph node status			0.112
Positive	14	23	
Negative	25	16	
TNM stage			0.069
I, II	22	14	
III, IV	17	25	

CRC, colorectal cancer; SD, standard deviation; TNM, Tumor-Nodes-Metastasis.

in the plasma of CRC patients compared to normal controls (P<0.001, Mann-Whitney U test) (Fig. 2A and B).

Sensitivity of plasma miR-200c and miR-18a towards the detection of CRC. ROC curve analyses demonstrated that the plasma levels of miR-200c and miR-18a were useful biomarkers for distinguishing between CRC patients and controls with ROC curve areas of 0.749 [95% confidence interval (CI), 0.675-0.822] and 0.804 (95% CI, 0.736-0.872), respectively (Fig. 2B and D). At the cut-off value of 7.6 for miR-200c (relative expression in comparison to RNU6B), the sensitivity was 64.1% and specificity 73.3%. At the cut-off value of 2.3 for miR-18a (relative expression in comparison to RNU6B), the sensitivity and specificity were 73.1 and 79.1%, respectively. miR-18a demonstrated a stronger discriminating ability compared to miR-200c, with no statistical significance (P=0.243). The predicted values of logistic regression showed a significant difference between these two groups (P<0.001, Fig. 2E) and combination ROC analysis demonstrated an increased AUC value to 0.839 (Fig. 2F, miR-18a, P=0.013; miR-200c, P=0.022) with a sensitivity and specificity of 84.6 and 75.6%, respectively.

Correlation between plasma miR-200c and miR-18a expression, and clinicopathological characteristics. The correlation between plasma-based miR-200c/miR-18a and clinicopathological characteristics is shown in Tables II and III. The expression levels of plasma miR-200c and miR-18a were classified as low or high on the basis of the median value. No significant correlation was found between the two miRNAs and gender, age, histological type, tumor location, lymph nodal status or Tumor-Nodes-Metastasis (TNM) classification (P>0.05), while the levels of miR-18a demonstrated an elevation trend in patients with more advanced TNM stage (P=0.069).

Follow-up of plasma miR-200c and miR-18a levels following removal of the lesion. Plasma miR-200c and miR18a levels were measured in a subgroup of CRC patients (n=21), following the resection of lesions. A significant decrease in miR-200c (P=0.021, Fig. 3A) and miR-18a (P=0.004, Fig. 3B) levels following the resection was noted.

Discussion

miRNAs have been reported to be involved in tumorigenesis, acting as either oncogenes (13,14) or tumor suppressors (15). Several studies have described altered expression of miRNAs in cancer tissues compared to normal tissues, suggesting miRNAs to be potential novel diagnostic and prognostic markers (16,17).

Studies investigating plasma miRNAs comprise a promising field for clinical application. Tumor-derived miRNA in the plasma was first described by Mitchell *et al* (4). According

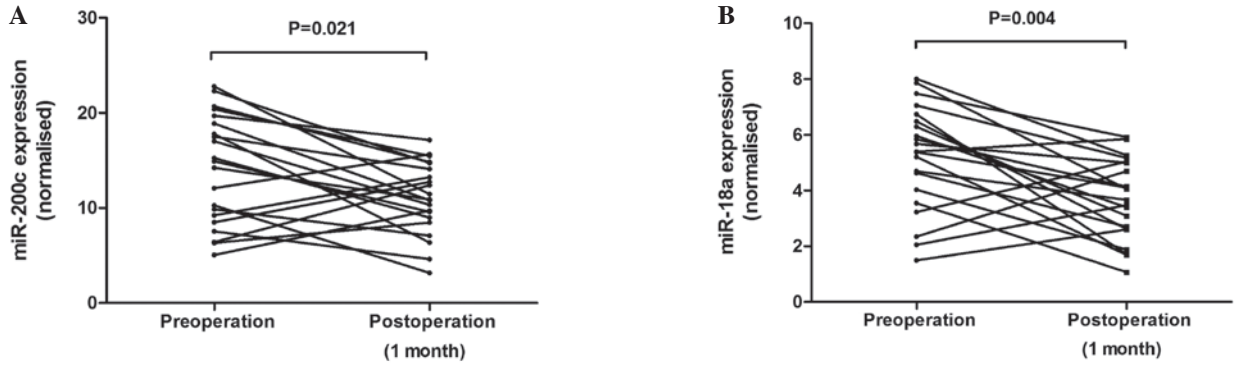

Figure 3. Changes of (A) miR-200c and (B) miR-18a plasma levels in patients with CRC (n=21) prior to (pre-operation) and 1 month following (1 month post-operation) surgical removal of the tumor are shown. The expression levels of the miRNAs were normalised to RNU6B. Statistically significant differences were determined using the Wilcoxon matched-pairs test.

to this study, high stability was exhibited after prolonged incubation at room temperature and/or multiple freezing-thawing processes (4). Additionally, the characteristics of miRNAs, such as tissue-specific miRNA signatures and the availability of numerous copies/cells, indicated potential advantages as biomarkers compared to those of other nucleic acids, such as circulating DNA and mRNA. An increasing number of studies also suggest the potential use of miRNAs in the early detection of patients with several malignancies, such as gastric, pancreatic, colorectal and breast cancer (6-9).

Recent studies have focused ons the role of circulating miRNAs in the plasma of CRC patients (7,18). Huang *et al* (18) reported that the combined analyses of miR-29a and miR-92a in the plasma were able to distinguish CRC patients from healthy individuals. However, the investigation of plasma miR-200c and miR-18a for the detection of CRC has not been previously reported. In this study, we have systematically established the feasibility of miR-200c and miR-18a in the plasma, as non-invasive biomarkers for CRC screening.

This study has shown that miR-200c and miR-18a were overexpressed in colorectal tumor tissues compared to their corresponding adjacent non-tumor tissues. In line with their enhanced expression levels in primary tumor specimens, miR-200c and miR-18a were also detected at high levels in the plasma samples of CRC patients. Based on ROC curves, we selected cut-off values that best differentiated CRC patients from healthy individuals. miR-200c was found to have a sensitivity of 64.1% and a specificity of 73.3%, whereas miR-18a had a sensitivity of 73.1% and a specificity of 79.1% for CRC. miR-18a demonstrated a higher discriminating ability compared to miR-200c, while not statistically significant (P=0.243). Combined ROC analyses using these two targets may yield an increased AUC of 0.839, with 84.6% sensitivity and 75.6% specificity in discriminating CRC patients from normal controls (miR-18a, P=0.013; miR-200c, P=0.022), indicating the additive effect in the diagnostic value of these two miRNAs.

miR-18a is part of the miR-17-92 gene cluster, located on chromosome 13q13. As a known oncomir, miR-17-92 cluster is able to promote cell proliferation, suppress apoptosis, induce tumor angiogenesis and accelerate tumor progression (19,20). miR-18a has been found to be significantly upregulated in gastric cancer (21), hepatocellular, pancreatic and colorectal carcinomas (11), suggesting its importance in tumorigenesis. In CRC tissues, patients with overexpressed miR-18a exhibited a poorer clinical prognosis. Recently, Morimura *et a*l (12) reported that plasma miR-18a was a potential marker for pancreatic cancer that was not associated with clinical factors. Our findings were comparable to those of Morimura *et al* (12).

miR-200c is a member of the miR-200 family, previously shown to inhibit the epithelial-to-mesenchymal transition (EMT) by targeting the transcriptional repressors of cadherin 1 (CDH1) and zinc finger E-box binding homeobox 1 (ZEB1), suggesting that miR-200c is able to prevent tumor progression by negatively regulating ZEB transcriptional repressors and preventing EMT (22,23). A recent study demonstrated high expression of serum miR-200c associated with poor prognosis in patients with lung cancer (24). miR-200c was also detectable at a higher level in the plasma of CRC patients, and patients with overexpressed miR-200c exhibited a poorer clinical prognosis (7,10). These results indicate that miR-200c acts as an oncogene or a tumor suppressor, depending on the circumstances. This study suggested that similar to miR-18a, the expression of miR-200c, did not correlate with gender, age, histological type, tumor location, lymph nodal status or TNM classification (P>0.05), and yielded low predictive value compared to miR-18a. However, miR-200c was able to improve the differentiation power of miR-18a between CRC patients and the controls, resulting in an increased sensitivity and specificity.

In this study, we also measured circulating miRNAs in paired-plasma samples obtained prior to and 1 month after surgical removal of the tumors, to confirm the release of circulating miRNAs. Results showed that both miR-18a and miR-200c concentrations decreased significantly following removal of the lesion. These findings suggest that the high levels of miR-18a and miR-200c in the plasma of CRC patients are derived from neoplastic cells.

Plasma miRNA assays possess several potential clinical uses: screening patients at high risk for CRC and monitoring disease recurrence during the follow-up period after CRC resection. miRNA biomarkers might also be powerful and useful for confirming the completeness of tumor resection and for evaluating the efficacy of adjuvant therapies when the elimination clearance of plasma miRNAs can be elucidated.

Although the results of this study are promising, there are several limitations. First, further validations of the usefulness of these markers in large cohorts are necessary, since the sample size used in this study was small. Second, although miR-200c and miR-18a elevation in plasma are likely to be derived from CRC, it is uncertain whether this elevation is specific for CRC, and whether it can be used to distinguish sporadic from familial types of CRC. Thus, additional studies are required to examine familial and sporadic cases. Third, despite the significantly elevated levels of plasma miR-200c and miR-18a in CRC, it is important to examine whether its plasma level changes in patients with adenoma with various degree of dysplasia. The determination of whether plasma miR-200c and miR-18a can be used for the detection of pre-malignant lesions, such as adenoma, is likely to add more value to the use of this marker for CRC prevention.

In conclusion, this study has demonstrated the feasibility of using plasma miR-18a and miR-200c as non-invasive tools for the detection of CRC. These data serve as a basis for further investigation, preferably in large-scale prospective studies, before these two miRNAs can be incorporated into routine clinical practice as non-invasive screening tools for colorectal neoplasia.

Acknowledgements

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