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Original Article

The potential of plasma miRNAs for diagnosis and risk estimation of colorectal cancer

Wang-Yang Chen[1], Xiao-Juan Zhao[1], Zhi-Fu Yu[1], Fu-Lan Hu[1], Yu-Peng Liu[1], Bin-Bin Cui[2], Xin-Shu Dong[3], Ya-Shuang Zhao[1]

[1]Department of Epidemiology, Public Health College, Harbin Medical University, Harbin, Heilongjiang 150081, P. R. China; [2]Department of Abdominal Surgery, The Tumor Hospital of Harbin Medical University, Harbin, Heilongjiang Province, P. R. China; [3]Department of Tumor Surgery, The Forth Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang Province, P. R. China

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Abstract: Circulating microRNAs (miRNAs) were recognized to be potential non-invasive biomarkers for colorectal cancer (CRC) detection and prediction. Meanwhile, the association of the expression of plasma miRNAs with the risk of CRC patients has rarely been analyzed. Therefore, we conducted this study to evaluate the value of plasma miRNAs for CRC diagnosis and risk estimation. Fasting blood samples from 100 CRC patients and 79 cancer-free controls were collected. Plasma miR-106a, miR-20a, miR-27b, miR-92a and miR-29a levels were detected by RT-qPCR. Sensitivity and specificity were employed to evaluate the diagnostic value of miRNAs for CRC. Univariate and multivariate logistic regression were employed to analyze the association between miRNAs expression and CRC risk. As results, miR-106a and miR-20a were elevated in the patients with CRC. The sensitivity of miR-106a was 74.00% and the specificity was 44.40%, while the cutoff value was 2.03. As for miR-20a, the sensitivity was 46.00% and specificity was 73.42% when employed 2.44 as cutoff value. High expression of plasma miR-106a increased CRC risk by 1.80 -fold. Plasma miR-106a and miR-20a may as noninvasive biomarkers for detecting the CRC. High expression of miR-106a associated with CRC risk.

Keywords: microRNA, colorectal cancer, plasma, diagnosis, risk factor

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and the second in females worldwide. Due to the improvements in early detection and treatment, CRC related mortality rates have continued to decrease over the years. Nevertheless, over 1.2 million new cancer cases and 608,700 deaths estimated to have occurred per year [1]. In the United States, the 5-year survival rate is 93.2% for stage I as opposed to only 8.1% for stage IV [2]. Therefore, early diagnosis is of vital importance for the treatment and prognosis of CRC patients. Several clinical examinations, including fecal occult blood tests (FOBT), radiologic tests, and endoscopic examinations, are currently used to screening for CRC [3]. However, these existed methods have limitations due to low efficacy, high cost, or invasive trauma [4-6]. Thus, new markers from non-invasive clinical samples are needed.

MicroRNAs (miRNAs) are small non-coding RNAs that post-transcriptionally regulate expression levels of more than 60% of human gene [7]. Growing evidence indicated that miRNA expression profiles in tissues play an important role in the diagnosis of various malignancies [8-12] including CRC [13, 14]. Recent studies has revealed that circulating miRNAs could be detectable in a remarkably stable manner in the blood, plasma, or serum [9, 15, 16], and several studies have reported the potentially diagnostic utility of circulating miRNAs as blood-based biomarkers [17, 18].

In addition to its potential diagnostic value, miRNA can respond for carcinogen exposure by its expression alternation. Circulating miRNAs as accurate biomarkers can reflect the alteration for carcinogenesis induced by chemical carcinogens [19]. Therefore, miRNAs may be considered as factors to evaluate cancer risk, and the association of miRNA with CRC risk has

Table 1. The miRNA-specific primer sequences

MicroRNA	Primer sequence
hsa-miR-92a	TAT TGC ACT TGT CCC GGC C
hsa-miR-16	TAG CAG CAC GTA AAT ATT GGC G
hsa-miR-29a	GGT AGC ACC ATC TGA AAT CGG TT
hsa-miR-106a	GGA AAA GTG CTT ACA GTG CAG GTA G
hsa-miR-27b	CGT TCA CAG TGG CTA AGT TCT GC
hsa-miR-20a	AAA GTG CTT ATA GTG CAG GTA GGG G

became another new focus for its important roles in carcinogenesis [20]. However, few studies were conducted on the association of circulating miRNA with CRC risk based on present reports.

In this study, we aimed to explore the potential diagnostic value of a panel of five miRNAs (miR-106a, miR-20a, miR-27b, miR-92a, and miR-29a), which were selected according to a comprehensive review of relevant literatures and a previously published microarray data of colorectal tumor tissues [21, 22], and to evaluate the association between aberrantly expressed circulating miRNAs and CRC risk.

Materials and methods

Patients and controls

Blood samples of 100 newly diagnosed primary CRC cases were collected before any therapeutic procedures, including surgery, chemotherapy, and radiotherapy after asking the consent of surgeons and patients themselves. All patients were pathologically diagnosed in the Tumor Hospital of Harbin Medical University, from 15 May, 2007 to 1 January, 2008. Clinical data of the CRC patients were derived from medical records. Tumors were staged according to the TNM stage for CRC. Meanwhile, 79 cancer-free controls were recruited from the Second Affiliated Hospital of Harbin Medical University, and their blood samples were collected after asking consent of physician and the patients. The study was approved by Human Research and Ethics Committee Harbin Medical University.

Blood processing and miRNAs extraction

From each participant, 5 to 10 milliliters (ml) of peripheral whole blood was collected into EDTA tube. Plasma was separated from cell components by centrifugation at 2000 g for 10 min at 4°C and 12000 g for another 10 min at 4°C, within eight hours after collection. All the supernatant plasma were separated as aliquots and stored at -80°C until use.

Total RNA was extracted from 250 μl plasma using TRIzol LS reagent (Invitrogen, Carlsbad, California, USA) according to the manufacturer's protocol. Briefly, 750 μl TRIzol LS reagent was added to 250 μl plasma samples, after phase separation by 200 μl chloroform addition and centrifugation. 600 μl 100% isopropanol was added to the aqueous phase and centrifuged to precipitate RNA. RNA pellet was washed by 75% ethanol and air-dried for 5 min, and the final elution volume was 12 μl. The concentrations of all samples were quantified by NanoDrop 2000 spectrophotometer (Thermo, Japan).

Reverse transcription and real-time quantitative PCR

Briefly, 1 μg total RNA from each sample was quantitatively polyadenylated in 20 μl reaction and 3 μl product of polyadenylate was reversely transcribed to cDNA, using polyadenylation polymerase and First-Strand cDNA Synthesis Kit (Tiangen, China) with commercial product reverse primer (Tiangen, China) according to the manufacturer's protocol. RT- control was included in each batch of reactions. The cDNA product was diluted 1:2 with nuclease-free water and stored at -20°C for further analysis.

Quantification of miRNA expression was detected by real-time quantitative polymerase chain reaction (RT-qPCR) in a finally volume of 10 μl with Roche Lightcycler 480 (Roche, Germany) using SYBR Green PCR Master Mix (Kapa, USA). Each reaction contained 5 μl SYBR Green reagents, 0.2 μl each of 10 μM specific forward primer and universal primer, and 3.6 μl DEPC-treated water. The amplification used the following conditions: 95°C for 5 min, followed by 45 cycles at 95°C for 20 s and 60°C for 34 s. The miRNA-specific forward primers were designed according to the miRNA sequence obtained from the miRBase database (http://microrna.sanger.ac.uk/), which was listed in **Table 1**. The universal reverse primer was purchased in Tiangen (Tiangen, China). No-template controls for both RT and PCR, which to ensure target specific amplification, were included on each plate and all PCR reactions were carried

Table 2. The characteristics of subjects

Parameters	Patients	Controls
Sex		
Male	60	44
Female	40	35
Age		
<60	53	38
≥60	47	41
Location		
Colon	35	
Rectum	63	
Unknown	2	
Tumor size		
≤45 mm	44	
>45 mm	43	
Unknown	13	
TNM stage		
I-II	41	
III-IV	38	
Unknown	21	
Lymphnode status		
Positive	43	
Negative	51	
Unknown	6	
Histology		
Adenocarcinoma	73	
Mucinous adenocarcinoma	18	
Polypoid adenocarcinoma	3	
Other	6	

out in triplicate. In order to validate the specificity of the target PCR product, melting curve analysis was performed at the end of PCR cycles. The comparative cycle threshold (CT) method was applied to quantify the expression levels of miRNAs. The expression levels of miRNAs were normalized to miR-16 which was selected as a reference gene [16]. The relative amount of target miRNAs to miR-16 was calculated using the equation $2^{-\Delta CT}$, where ΔC_T= (ΔC_ttarget-ΔC_t*normalizer*). The median of relative amount was chosen to define the cases with high expression or low expression.

Statistical analysis

The Mann-Whitney U test was used to assess the differences of miRNA expression between CRC patients and controls. Sensitivity, specificity, Youden index and receiver operating characteristic (ROC) curve analysis were used to evaluate the diagnostic power of the miRNAs. In addition, univariate and multivariate logistic regression were used to evaluate associations between each miRNA and the risk of CRC. Pearson's X^2 test was used to analyze the relationship between differential expression of miRNAs and clinicopathologic characteristics. The statistical analyses were performed using SPSS 13.0 software (SPSS, Inc., Chicago, IL). All tests were two-sided. *P*-values less than 0.05 were considered statistically significant.

Results

Characteristics and clinical features of subjects

The 100 patients consisted of 60 males and 40 females aged from 27 to 81, with the median age of 58.91. There were 44 males and 35 females included in the cancer free controls. The median age of 79 controls was 60.20, and the range from 27 to 81. The characteristics and clinical features of patients are summarized in **Table 2**.

Expression of plasma miRNAs in CRC and control

The expression levels of plasma miR-106a and miR-20a were statistically significantly higher in CRC patients compared with controls (miR-106a: 17.13 *vs*. 5.59, *P*=0.016; miR-20a: 2.05 *vs*. 1.25, *P*=0.038). (**Figure 1D** and **1E**) No significant differences were observed in the plasma levels of miR-92a, miR-29a and miR-27b between cancer patients and controls (miR-92a: 14.59 *vs*. 15.56, *P*=0.802; miR-29a: 0.16 *vs*. 0.15, *P*=0.548; miR-27b: 0.57 *vs*. 0.45, *P*=0.294) (**Figure 1A-C**; **Table 3**).

Diagnostic value of plasma miR-106a and miR-20a for CRC

When the miR-106a cutoff value was at the first quartile (0.49), the sensitivity and specificity were 80.00% and 31.65%, respectively. While the cutoff value was set at the third quartile (38.59), the corresponding sensitivity and specificity were 29.00% and 81.01%, respectively. The largest Youden Index for miR-106a was 0.184; the corresponding expression level of 2.03 in plasma was as optimal cutoff value. At the cutoff value of 0.36 for miR-20a, the sensitivity was 79.00% and the specificity was

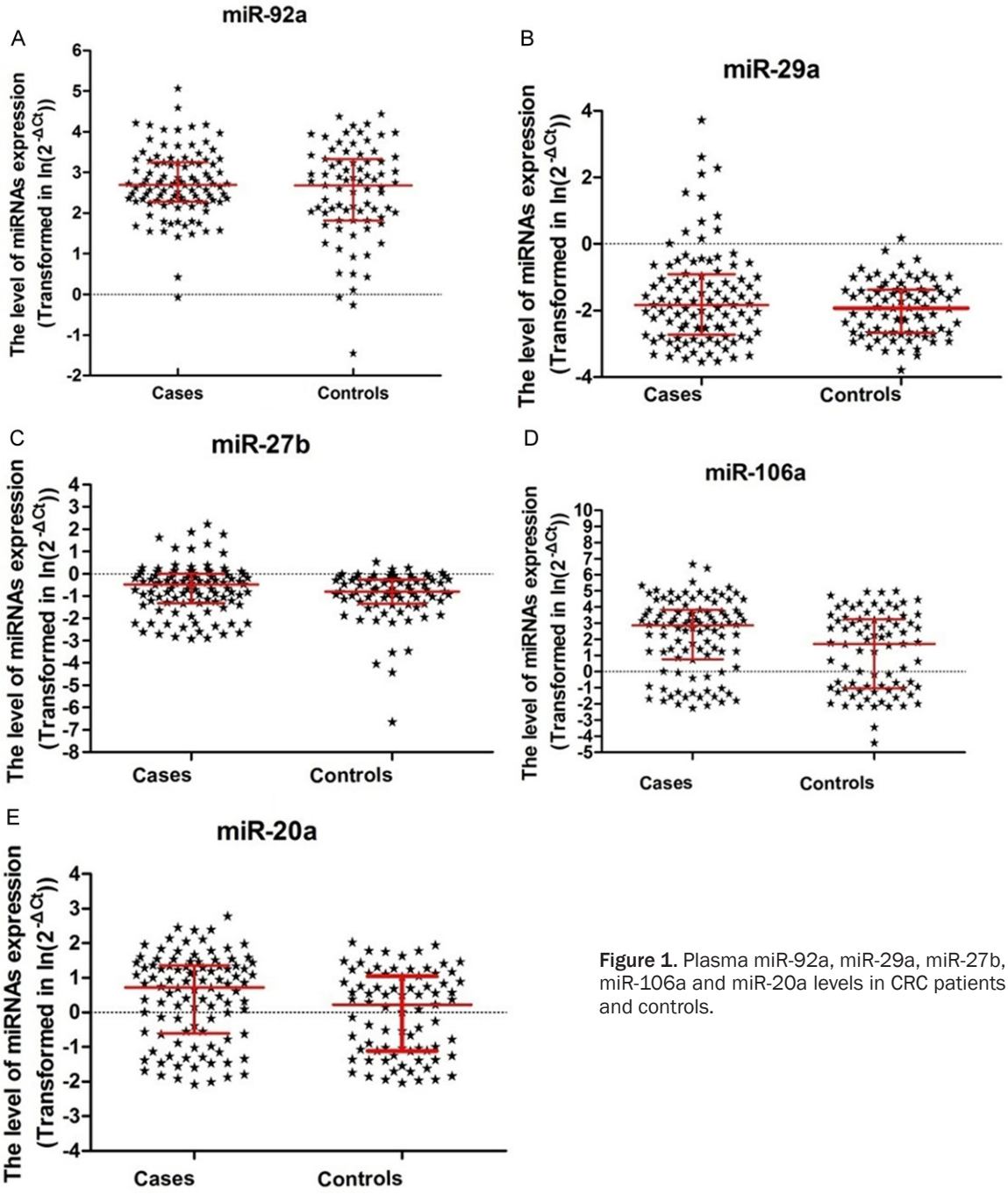

Figure 1. Plasma miR-92a, miR-29a, miR-27b, miR-106a and miR-20a levels in CRC patients and controls.

29.11%, while cutoff value elevated to 3.52, the sensitivity and specificity were 32.00% and 83.54%, respectively. When 2.44 was an optimal cutoff value for miR-20a based on the largest Youden Index of 0.194, the corresponding sensitivity and specificity were 46.0% and 73.42%, respectively (**Table 4**). The area under the ROC (AUC) for miR-106a and miR-20a were 0.605 (95% CI: 0.522-0.688) and 0.590 (95% CI: 0.507-0.674), respectively (**Figure 2A** and **2B**).

Relationship between plasma miRNAs level and clinical features

Higher level of miR-92a expression was statistically associated with tumor size no more than 4.5 mm (P=0.041). Inversely, lower expression of miR-106a was associated with larger tumor (P=0.013). miR-20a expression was significantly associated with differentiation (P=0.037, **Table 5**). The expression of miR-29a in adenocarcinoma group was higher than that in other

Table 3. The expression level of plasma miRNAs between CRC and controls

miRNAs	Case		Control		*P* value*
	Median	Range	Median	Range	
miR-92a	14.59	0.82-157.95	15.56	0.23-200.05	0.802
miR-29a	0.16	0.03-41.36	0.15	0.02-9.25	0.548
miR-27b	0.57	0.03-9.23	0.45	0.00-12.22	0.294
miR-106a	17.13	0.07-776.05	5.59	0.01-148.40	0.016
miR-20a	2.05	0.09-16.08	1.25	0.13-15.45	0.038

*The significance between CRC and controls was test by Mann-Whitney U test.

histological subtype group (56.16% *vs*. 27.78%, 22.22%, *P*=0.026). No statistically significant associations were observed between the five miRNAs and age, gender, TNM stage, lymph node status, or tumor location (**Table 5**).

Association between expression of miRNAs and CRC risk

Univariate logistic regression showed the higher expression of miR-106a was significantly associated with an increased risk of CRC (OR=1.23, 95% CI: 1.02-1.49). The higher level of miR-27b and miR-20a also increased the risk of CRC by 1.20 times (for miR-27b, OR=1.20, 95% CI: 0.99-1.45; for miR-20a, OR=1.20, 95% CI: 0.99-1.45, respectively) (**Table 6**).

Further multivariate logistic regression analysis showed that higher expression of miR-106a increased CRC risk by 1.80 times (OR_{adj}=1.80, 95% CI: 1.12-2.92) after adjusting age, sex. High expression of miR-27b and miR-20a also increased the risk of CRC (for miR-27b, OR_{adj}= 1.55, 95% CI: 0.95-2.52, for miR-20a, OR_{adj}= 1.54, 95% CI: 0.95-2.50) (**Table 6**).

Discussion

MiRNA can exist intact in serum or plasma protected from endogenous RNase activity [9], and its expression profile in human cancer appears to be tissue-specific [15]. Circulating miRNA as a novel biomarker had attracted more attentions on its value of diagnosis, prognosis and treatment. In this study, we analyzed the levels of five miRNAs (miR-92a, miR-29a, miR-27b, miR-106a and miR-20a) in plasma from 100 CRC patients and 79 cancer-free controls. Our results showed that miR-106a and miR-20a were significantly upregulated in the CRC patients comparing with cancer-free controls. Although it had been reported that miR-106a overexpressed in tissue [23, 24], and fecal occult blood [25] of colorectal cancer, this is first study that focus on the miR-106a level in plasma formed that it overexpressed in CRC patients compared with cancer-free controls. Dysregulated miR-106a plays a vital role in the process of oncogenesis and development [7]. For example, overexpressed miR-106a promoted gastric cancer cell proliferation and inhibited apoptosis [8]. Feng et al. [26] revealed that miR-106a could inhibit the expression of transforming growth factor-β receptor 2 (TGFBR2), leading to increased CRC cell migration and invasion. MiR-20a was also confirmed to be significantly higher in CRC tissues than in normal tissues [27]. MiR-20a was upregulated by CCAT2 through TCF7L2-mediated transcriptional regulation in colon cancer [28]. MiR-20a can induce cell invasion and migration by targeting the ABL2 in prostate cancer [29] and contribute chemotherapeutic resistance in colorectal adenocarcinoma cell lines [30]. However, its function in CRC formation was not clear.

In our study, miR-92a and miR-29a were not significantly upregulated in CRC patients compared to controls. However, Huang *et al*. reported miR-92 and miR-29a were upregulated and were identified as plasma diagnostic markers for CRC [16]. At the same time, Cheng H, *et al*. even found that plasma miR-92 was significantly decreased in the TexGen cohort of colon cancer patients [31]. These conflicting conclusions may because of the genetic variations among different ethnic groups as well as the differences of environmental factors and diets. Zhao ZH, *et* al. had revealed the profiles of circulating miRNAs in plasma samples from Caucasian American women or that from African American with early breast cancer were inconsistent, and that differentially expressed miRNAs characterized with potential racial difference [32]. Therefore, more studies with well design are required to identify miRNAs in peripheral blood as noninvasive markers for tumor disease.

Our study showed that expression levels of miR-106a and miR-20a in plasma were increased in CRC patients compared with cancer-free con-

Table 4. The diagnostic value of miR-106a and miR-20a in CRC

Percentile	miR-106a				miR-20a			
	Cutoff	Sensitivity	Specificity	Youden Index	Cutoff	Sensitivity	Specificity	Youden Index
Optimal	2.03	74.0%	44.40%	0.184	2.44	46.00%	73.42%	0.194
25%	0.49	80.0%	31.65%	0.117	0.36	79.0%	29.11%	0.081
50%	11.08	57.0%	58.23%	0.152	1.65	54.0%	54.43%	0.084
75%	38.59	29.0%	81.01%	0.100	3.52	32.0%	83.54%	0.155

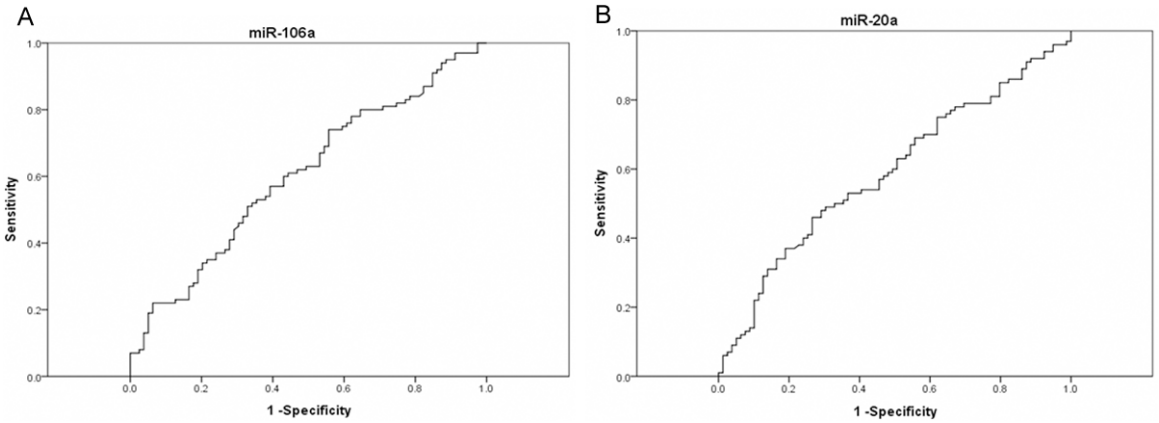

Figure 2. Receiver operating characteristics (ROC) curve analysis using plasma miR-106a (A) and miR-20a (B) for discriminating CRC.

Table 5. The association of plasma miR-106a and miR-20a with clinical characteristics of CRC patients (No. (%)

Parameters	miR-106a			miR-20a		
	Low	High	*P*-value	Low	High	*P*-value
Gender						
Male	26 (44.83)	32 (55.17)	0.224	29 (50.00)	29 (50.00)	1
Female	24 (57.14)	18 (42.86)		21 (50.00)	21 (50.00)	
Age						
<60	24 (48.0)	26 (52.0)	0.689	26 (52.0)	24 (48.0)	0.689
≥60	26 (52.0)	24 (48.0)		24 (48.0)	26 (52.0)	
Location						
Colon	20 (57.14)	15 (42.86)	0.366	20 (57.14)	15 (42.86)	0.366
Rectum	30 (47.62)	33 (52.38)		30 (47.62)	33 (52.38)	
Tumor size						
≤45 mm	17 (38.64)	27 (61.36)	0.013	20 (45.45)	24 (54.55)	0.334
>45 mm	28 (65.12)	15 (34.88)		24 (55.81)	19 (44.19)	
TNM stage						
I-II	26 (63.41)	15 (36.59)	0.229	23 (56.10)	18 (43.90)	0.757
III-IV	19 (50.0)	19 (50.0)		20 (52.63)	18 (47.37)	
Lymphnode status						
Positive	30 (58.82)	21 (41.18)	0.157	28 (54.9)	23 (45.1)	0.418
Negative	19 (44.19)	24 (55.81)		20 (46.51)	23 (53.49)	
Histology						
Adenocarcinoma	40 (54.79)	33 (45.21)	0.278	38 (52.05)	35 (47.95)	0.796
Mucinous adenocarcinoma	7 (38.89)	11 (61.11)		8 (44.44)	10 (55.56)	
Other	3 (33.33)	6 (66.67)		4 (44.44)	5 (55.56)	
Differentiation						
Poor	6 (40.0)	9 (60.0)	0.296	4 (26.67)	11 (73.33)	0.037
Moderate/well	40 (54.79)	33 (45.21)		41 (56.16)	32 (43.84)	

Table 6. The association between miRNAs expression and risk of CRC

miRNA	Univariable analysis			Multivariable analysis		
	OR	95% C.I.	*P*-value	$OR_{adj.*}$	95% CI	*P*-value
miR-92a	1.14	0.95-1.38	0.16	1.14	0.71-1.83	0.59
miR-29a	1.17	0.97-1.41	0.11	1.30	0.79-2.14	0.30
miR-27b	1.20	0.99-1.45	0.06	1.55	0.95-2.52	0.08
miR-106a	1.23	1.02-1.49	0.03	1.80	1.12-2.92	0.02
miR-20a	1.20	0.99-1.45	0.06	1.54	0.95-2.50	0.08

OR_{adj}: adjusted for age and sex.

trols. The AUC of miR-106a and miR-20a were 0.605 and 0.590, respectively. Compared with CEA, which showed 51.9% sensitivity at 90% specificity with AUC of 0.773, the evaluation of miR-106a and miR-20a showed less sensitivity of 22.0% at 90% specificity [33]. Both miR-106a and miR-20a had relatively lower specificity of 44.49% and 73.42%. In some extent, these two miRNAs only presented potential value for auxiliary diagnosis for CRC patients, and further studies with larger samples are needed.

Accumulating evidence indicated that miRNAs associated with clinical parameters, such as higher serum miRNA expression associated with poorly differentiated gastric cancer [34]. In present study, the expression of plasma miR-92 is lower in the patients with a tumor less than 45 mm in diameter and the miR-106a is high expression in the patients with a tumor less than 45 mm in diameter. Some reports showed that circulating miRNAs were associated with tumor size [35, 36], another study did not found the correlation between miRNAs and tumor size [37]. Several reports stated that circulating miRNAs levels were significantly reduced after tumor resection [38, 39]. High expression of miR-20a was also associated with poor differentiation (77.3% *vs.* 26.7%). MiR-20a regulates the Jak-STAT signaling pathways which are closely related to cell differentiation in myeloid leukemia cell [40]. MiR-20a may be a potential factor in the process of CRC progression. Additionally, miR-27b did not show a significant association between their expression levels and clinicopathological characteristics of tumors.

We found that high expression of plasma miR-106a increased the risk of CRC by 1.23 times compared to the low level. After adjusting the age and sex, high expression of miR-106a increased 1.80-fold of CRC risk. High expression miR-27b and miR-20a are the risk of CRC, although they are marginal significance in statistics. More evidence showed that miRNAs play pivotal roles in activating natural killer cells [41], mediating inflammatory response [42], altering the cells microenvironment [43]. Some aberrant expression miRNAs co-existing will increase the predictive value of CRC prognosis [44]. Therefore, we evaluated the combined effects of three miRNAs on CRC risk, and found that the risk effects of CRC noticeably increased with increasing number of putative risk miRNAs. Based on the recent present study, more cohort study will be designed on the association of plasma miRNAs exposure with the cancer risk are needed to confirm our preliminary results.

The limitations of this study should be considered. First, the small sample size caused incredible results by sampling error in case-control study. For example, miR-20a and miR-27b had a marginally statistical significance in CRC risk. Further validations of these markers in large sample in independent studies are necessary. Second, Poly-A method was employed before reverse transcription which would be not completely differ matured miRNA from pre-miRNA. In addition, normalization is a key step for the accurate quantification of miRNA levels with RT-qPCR. A common problem in the circulating miRNA researches is that no consensus endogenous control has been established. We selected miR-16 as endogenous control because of its relatively stable and abundant in plasma/serum [16, 45]. However, several reports showed that aberrant expression of miR-16 in plasma/serum was associated with the risk of lymphoma and prostate cancer [46, 47].

In conclusion, we observed that expression levels of circulating miR-106a and miR-20a were significantly up-regulated in CRC patients plasma compared with cancer-free controls. The aberrant expression of miR-106a and miR-20a will be a potential biomarker for auxiliary diagnosis. High expression of miR-106a, miR-20a and miR-27b may be increased the CRC risk. High level of plasma miR-20a was associated

with poor differentiation. Further good-designed studies with large sample size are required to validate the potential prevention and clinic value of these miRNAs.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Ya-Shuang Zhao, Department of Epidemiology, Public Health College, Harbin Medical University, 157 Baojian Street, Nangang District, Harbin, Heilongjiang 150081, P. R. China. Tel: 86-451-87502823; Fax: 86-451-87502885; E-mail: zhao_yashuang@263.net

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