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The ratio of miR-21/miR-24 as a promising diagnostic and poor prognosis biomarker in colorectal cancer

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Abstract. – OBJECTIVE: Optimal management of cancer treatment will be guided by sensitive and specific biomarkers. Searching for potential biomarkers is always a hot spot in cancer research, including colorectal cancer (CRC). MicroRNAs (miRNAs) have been recently proposed as biomarkers for cancers.

PATIENTS AND METHODS: Based on previous miRNA analysis in our hospital and data mining, we hypothesized that the ratio of miR-21/miR-24 (miR-21/24) may serve as plasma biomarkers in CRC patients. The plasma levels of miR-21 and miR-24 were analyzed from the 186 CRC patients before surgery and 97 healthy controls by qRT-PCR. Receiver operating characteristic (ROC) analysis was further used to evaluate the difference in diagnostic accuracy associated with the expression of miR-21, miR-24 and their ratio. Chi-square^2-test or Fisher's exact test was performed to determine the relationship between the ratio of miR-21/24 and clinicopathological parameters. Kaplan-Meier and log-rank testing were performed to evaluate the effect of miR-21/24 ratio on the survival of colon cancer. Hazard ratios (HRs) and corresponding 95% confidence intervals (CIs) were calculated by Cox regression models.

RESULTS: ROC curves revealed that the diagnostic accuracy AUC (area under the curve) in CRC tissue of miR-24, miR-21, and the ratio of miR-21/24 were 0.8971, 0.9128 and 0.9875, respectively. Notably, the ratio of miR-21/24, with the best accuracy among these miRNAs, was significantly correlated with several important prognosis factors in CRC, such as tumor size, TNM stage, lymph metastasis and histologic differentiation (all $p<0.05$). By Kaplan-Meier survival analysis and Cox regression analysis, the ratio of miR-21/24 was shown to be a significant survival risk factor for CRC patients.

CONCLUSIONS: We showed that the plasma ratio of miR-21/24 is a potentially powerful tool for detecting CRC and predicting prognosis.

Key Words:
Colorectal cancer, miR-21, miR-24, Diagnosis, Plasma, Biomarker.

Abbreviations
CRC: colorectal cancer, Mir-RNA: Micro RNA, HR: Hazard ratio, 95% CI: 95% confidence intervals, AUC: area under the curve.

Introduction

Colorectal cancer begins as a tumor or tissue growth on the inner lining of the rectum or colon[1,2]. In the United States, colorectal cancer is the third deadliest of all cancers. An estimated 134,490 new colorectal cancer cases (70,820 in males and 63,670 in females) along with 49,190 colorectal cancer deaths (26,020 and 23,170 in males and females, respectively) were reported in 2016. Colorectal cancer ranks third, only behind prostate cancer and lung cancer, for new cases in males, and behind breast cancer and lung cancer for new cases in females. Colorectal cancer remains a heavy burden on the United States population[3]. A similar trend was also reported in China, and since Western diet style is more popular in China, incidence and mortality of colorectal cancer increased quickly[4]. Currently, CRC is confounded by the difficulty of early diagnosis, due to the lack of reliable cancer-specific diagnostic biomarkers. Profound changes at the cellular and subcellular level, involving DNA, RNA, protein structure and function, are the initial factors for cancer development and progression[5]. MicroRNAs (miRNAs), a class of non-coding RNAs of approximately 22 nucleotides in length, are well

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known to play crucial roles in cancer pathogenesis from initiation to metastasis, primarily through interaction with 3′-untranslated region (3′-UTR) of target mRNA, leading to posttranscriptional inhibition and mRNA degradation[6]. Specific expression profiles of miRNAs have been shown in a variety of human cancers, including CRC[7-10]. Intriguingly, growing evidence has showed that certain miRNAs released from tumor cells are chemically stable and can be detected in a broad range of body fluids, including plasma, which makes quick diagnosis convenient[11,12]. Hence, miRNAs have the promising potential of being novel biomarkers for CRC diagnosis. So far, hundreds of miRNAs have been reported to be associated with CRCs progression and metastasis. MiR-24, one of the novel reported downregulated miRNAs, has been considered as a potential negative biomarker in the diagnosis of the progression of CRC patients, and its plasma level was decreased with the progression of CRC and reached the lowest level in CRC stage IV[13]. MiR-21 is one of most known up-regulated miRNA in colorectal cancer; previous studies have demonstrated that the expression level of circulating miR-21 was able to be detected in CRC cases significantly (p =0.022) with a sensitivity of 82% and a specificity of 56% ((AUC) = 0.633) but was unable to distinguish between early and late cases (AJCC classification) (p =0.194)[14]. A mechanism study suggests that miR-21 can modulate the malignant phenotypes such as proliferation, anti-apoptosis, cell cycle progression and invasion of CRC cells by down-regulating PTEN protein expression[15]. In the current study, we proposed that the ratio of plasma miR-21/24 could be an optional clinical biomarker for CRC diagnosis, and further use clinical resource from our hospital to investigate whether the ratio of miR-21/24 could be a good predictor for prognosis of CRC patients.

Patients and Methods

Patients

Blood samples were collected from 186 CRC patients who had been diagnosed and categorized based on the International Union Against Cancer (UICC) and American Joint Committee on Cancer (AJCC) TNM staging system for CRC established in 2003. Age- and gender-matched 97 healthy individuals with no history of cancer and in good health conditions based on self-report. Subjects were collected from Second Hospital of Tianjin Medical University between December 2010 and November 2016. Patients with other gastrointestinal tract complications, hemolysis, or high blood lipid were excluded. The blood samples were collected from patients before operational treatments, chemotherapy or radiotherapy. All plasma samples were extracted from EDTA-K2 tubes and centrifuged as described previously [16]. After the first centrifugation for 10 min at 1,600 g, the supernatants were carefully removed and transferred to a new tube followed by centrifugation again at 16,000 g for 10 min to remove residual blood cells. The plasma was then divided into small aliquots and snap-frozen at −80°C. Clinical characteristics of the CRC patients are summarized in Table I. The current studies were approved by the Clinical Research Ethics Committee of the Second Hospital of Tianjin Medical University, which abide by the Helsinki Declaration on Ethical principle for medical research involving human subjects. The informed consent from all participants was obtained before blood collection.

RNA Extraction

Total RNAs in plasma were isolated using TRI Reagent BD (Sigma Aldrich, St. Louis, MO, USA) following the instructions from the

Table I. Clinicopathological characteristics of CRC patients and healthy control.

Characteristic	CRC N (%)	Healthy N (%)
Age		
Mean ± SEM (years)	55.4±13.2	54.2±11.5
Gender		
Male/Female	102/84	51/46
Tumor size		
Mean ± SEM (cm)	6.1±2.4	
N.A.		
T Stage		
pT1a	27 (14.5)	N.A.
pT1b	65 (34.9)	N.A.
pT2a	54 (29.0)	N.A.
pT2b	31 (16.7)	N.A.
pT3	9 (4.8)	N.A.
pT4	0 (0)	N.A.
N Stage		
N0	115(61.8)	N.A.
N1	71 (38.2)	N.A.
M Stage		
M0	170 (91.4)	N.A.
M1	16 (8.6)	N.A.
Tumor Grade		
Stage I + II	146 (78.5)	N.A.
Stage III + IV	40 (21.5)	N.A.

manufacturer with modification. In brief, 200 µl of plasma thawed on the ice were added to 750 µl of TRI Reagent (BD Biosciences, Franklin Lakes, NJ, USA) supplemented with 20 µl of acetic acid (5 mol/L). 25 fmol of a synthetic *C. elegans* microRNA cel-miR-39 (Qiagen, Hilden, Germany) were added in as control before chloroform extraction, and then RNA was isopropanol precipitated at -20°C overnight. Finally, the pellet of plasma RNA was resuspended in 15 µl RNase-free water. The quantification and quality of RNA were determined using the Nanodrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA, USA). All samples were stored at -80°C until further analysis.

Reverse Transcription and Quantitative Real-Time-PCR

Reverse transcription of 500 ng tissues and 4 µl plasma RNA were performed using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania) and a reverse transcript primer from RiboBio (Guangzhou, China). Reverse transcription and quantitative Real-Time-Polymerase Chain Reaction (qRT-PCR) analysis were performed with Platinum SYBR Green qPCR Supermix UDG (Invitrogen, Carlsbad, CA, USA) using synthesized primers from RiboBio (Guangzhou, China). Mature miRNAs were detected in accordance with the manufacturer's instructions (LightCycler® 480II, Roche, Basel, Switzerland). The amplification conditions for miRNAs and RNU6B (U6) were as follows: 10 min at 95°C, 45 cycles of 10 s at 95°C, 20 s at 60°C, 1 s at 72°C. Samples were normalized to U6 or cel-miR-39. Relative miRNA expression was calculated using the power formula: $2^{-\cdot CT}$ ($\cdot CT = CT_{miRNA}-CT_{U6}$). The primers were cited by several published documents, they are: miR-24, 5'GCAATGTGGCTCAGTTCAG-3′ (forward), 5' CAGTGCGTGTCGTGGAGT 3′(reverse) [17]; miR-21 (forward, GGACTAGCTTATCAGACTG; reverse, CATCAGATGCGTTGCGTA) and U6-snRNA (forward, ATTGGAACGATACAGAGAAGAT; reverse, GGAACGCTTC ACGAATTT) [18].

Statistical Analysis

Results are expressed as mean ± SEM (standard error of the mean) from at least three independent experiments. Using the GraphPad Prism statistical program, data were analyzed using the Student's 2-tailed *t*-test unless otherwise specified. The Mann-Whitney U test was used to analyze the abundance of miR-21 and miR-24 in the CRC and health groups; receiver operating characteristic (ROC) curves were applied to analyze the diagnostic values of the two microRNAs and its ratio. x^2-test or Fisher's exact test was performed to determine the relationship between the ratio of miR-21/24 and clinicopathological parameters. The Kaplan-Meier and log-rank testing were performed to evaluate the effect of the ratio of miR-21/24 on the survival of colon cancer. Hazard ratios (HRs) and corresponding 95% confidence intervals (CIs) were calculated by Cox regression models. In all cases, a two-tailed *p*-value of < 0.05 was considered to be statistically significant.

Results

miRNA Expression Levels in Plasma of CRC Patients and Healthy Control

We analyzed miR-21 and miR-24 expression levels in plasma from all patients and healthy controls. In accordance with previous studies, miR-21 was significantly upregulated in plasma from CRC patients, while miR-24 was significantly down-regulated in CRC patients compared with healthy control, respectively (Figure 1A and B, $p<0.0001$, Mann-Whitney). To evaluate the discrimination value of miR-21 and miR-24 expression levels in CRC, we performed ROC analysis. ROC curves revealed that both miR-21 and miR-24 served as useful biomarkers for discriminating CRC from healthy subjects with AUC of 0.8971 (95% confidence intervals (CI), 0.8544-0.9397; $p<0.0001$) and 0.9128 (95% CI, 0.8780-0.9476; $p<0.0001$), respectively (Figure 1C and 1D).

The Ratio of Plasma miR-21/miR-24 as a Robust Diagnostic Biomarker in CRC

Considering the downregulation of miR-24 and upregulation of miR-21 in CRC tissues, we analyzed the ratio of miR-21/miR-24 (miR-21/24) in CRC plasma and investigated whether it could provide more accurate prediction of their diagnostic value. As shown in Figure 2A, miR-21/24 was remarkably upregulated in CRC plasma ($p<0.0001$, Mann-Whitney). ROC curve analyses demonstrated that the diagnostic accuracy of miR-21/24 with AUC of 0.9875 was somewhat higher than that of single miR-21 and miR-24 (Figure 2B). Collectively, the ratio of miR-21/24 in plasma might be used as a robust diagnostic biomarker for CRC.

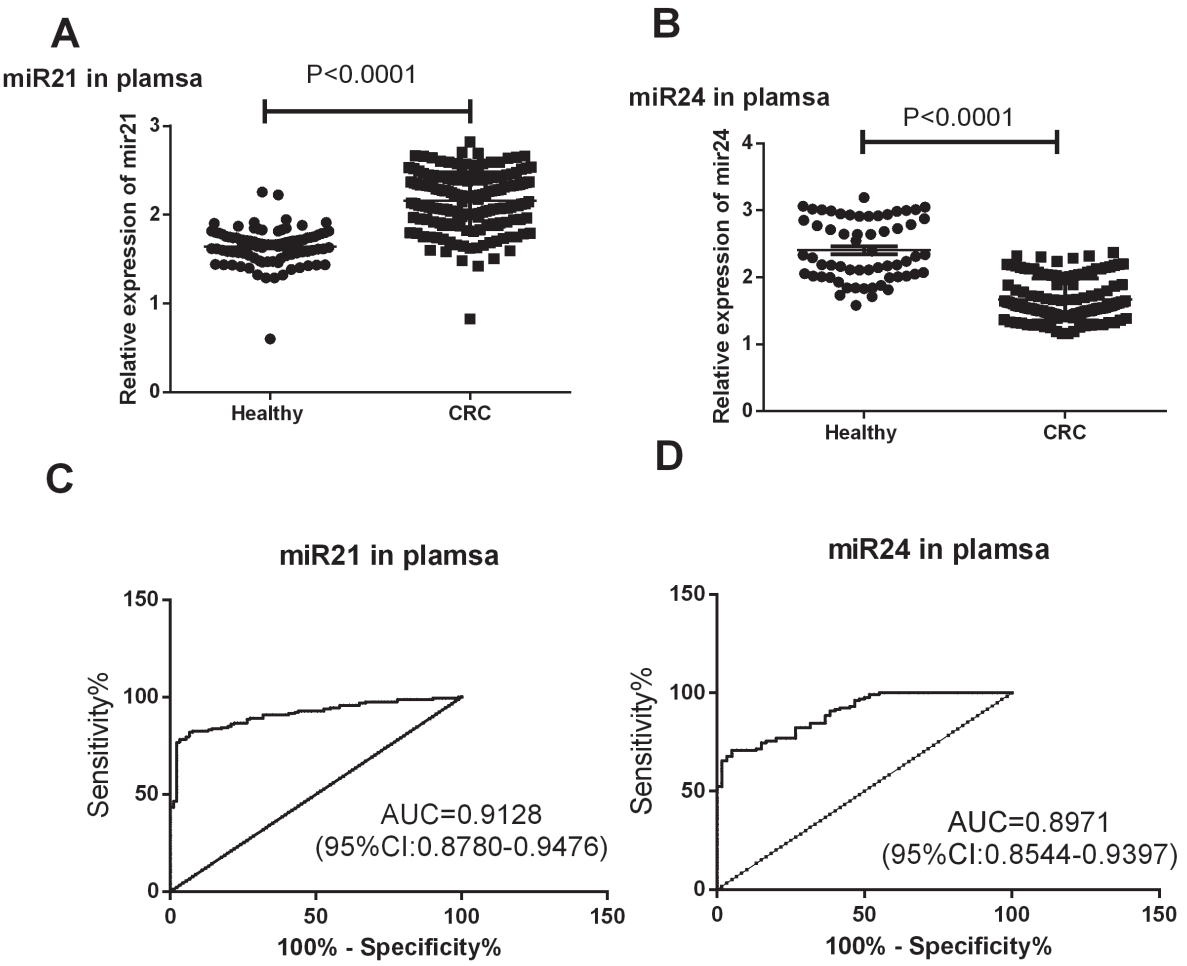

Figure 1. Expression of miR-21 and miR-24 in plasma of CRC patients. Relative miR-21 ***(A)*** and miR-24 ***(B)*** expression levels were determined by qRT-PCR in plasma from paired healthy control and CRC patients. Receiver operating characteristic (ROC) analysis was performed to assess the diagnostic value of plasma miR-21 ***(C)*** and miR-24 ***(D)*** to differentiate between CRCs and healthy subjects.

Association of miR-21/24 Expression with Patient Characteristics

We further evaluated the relationships of miR-21/24 expression with clinicopathological characteristics. Sex, age, tumor size, histologic grade, TNM stage, lymph metastasis and distant metastasis were all included in our analysis, since these clinical features are considered as the key elements of the prognosis of colon cancer patients. x^2-test with Fisher's exact test showed that there was a significant statistical difference between miR-21/24 and tumor size (p<0.0001), TNM staging (p=0.002), histologic differentiation (p=0.003), lymph and distant metastasis (p=0.0001 and 0.003 respectively, Table II). No significant association between miR-21/24 expression and age, as well as gender, was observed.

Correlation of miR-21/24 Expression with Prognosis of Colon Cancer Patients

Overall survival was calculated as the time from the date of surgical resection to the date of last contact or death. Among 186 colon cancer patients, 89 (47.8%) patients died as a result of disease progression during the follow-up. Colon cancer patients with high expression of miR-21/24 had a significantly shorter survival time compared with those with low expression of miR-21/24 (log-rank p < 0.001, Figure 3). In univariate analysis, advanced TNM stage, tumor size, lymph metastasis, histologic differentiation and low expression of miR-21/24 were associated with poor survival. In multivariate analysis, only TNM staging, lymphocyte metastasis and miR-21/24 expression were an independent prognostic factor in colon cancer (Table III).

Table II. Association of serum ratio of miR-21/24 expression with clinicopathological parameters in colorectal cancer.

Characteristics	Ratio of miR-21/24		*p*-value
	Low	High	
Age			0.304
>60	52	44	
≤60	41	49	
Gender			0.302
Male	47	55	
Female	46	38	
Tumor size			<0.0001
>6	42	68	
≤ 6	51	25	
Histologic grade			0.003
Well	41	24	
Moderate	38	37	
Poor	14	32	
TNM staging			0.002
I + II	82	64	
III + IV	11	29	
Lymph metastasis			<0.0001
Yes	21	50	
No	72	43	
Distant metastasis			0.003
Yes	2	14	
No	91	79	

Discussion

Identification of reliable biomarkers for diagnosis and prognosis remains a major challenge in cancer research, especially for CRC. Based on data mining from previous studies of microRNAs in CRC, we focused on the values of relative expression ratio of miR-21 and miR-24 for serving as a useful biomarker. Present study demonstrated high accuracy of the ratio of plasma miR-21/24 in the diagnosis of CRC (AUC=0.9875), it is higher than each of single miRNA. MicroRNA is helpful to distinguish normal from malignant tissues. Similarly to previous reports[19-21], we showed that plasma miR-21 and miR-24 measurement yielded 91% and 89% accuracy in discriminating CRC patients from healthy controls, respectively. Compared with miRNA panel from tumor tissues, the plasma miRNA is more convenient to be obtained[22]. Several potential miRNAs have been reported, such as miR-21, numerous studies have demonstrated that miR-21 could predict CRC incidence with near 90% sensitivity and specificity[23-26], which is similar with our present study. Plasma level of miR-24 was also considered as a novel biomarker for detection of CRC, especially for early stage, which is superior to the currently used clinical biomarkers for CRC detection, such as CEA and CA19-9[13]. Compared with previous researches, most importantly, our results established the miR-21/24 ratio as an extraordinarily accurate diagnostic biomarker for CRC. Normalization is a critical step for the accurate quantification of miRNA levels with qRT-PCR. However, no consensus internal controls currently exist for circulating miRNA. Literature-based tissue housekeeping genes or miRNAs in the blood have been chosen as reference for normalization of miRNAs expression levels, such as miR-16, RNU6B or RNA U6. The facts of miR-16 acting as potential oncomiRs in some types of cancer and degradation of RNU6B in the bloodstream make them not suitable for normalization of serum/plasma sample data[27-29]. Therefore, ratio of two miRNAs could diminish the interference from unqualified reference gene. The concept of the miRNA ratio is relatively novel[30,31], although the combination of multiple miRNAs with greater

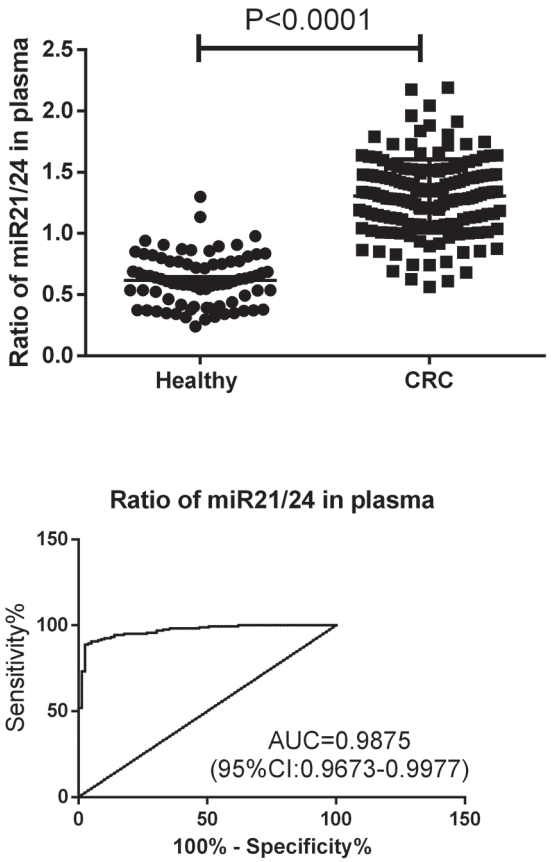

Figure 2. The accuracy of plasma miRNAs in discriminating CRCs from healthy controls. ***A,*** miR-21/24 levels in CRC plasma and healthy controls were analyzed with Mann-Whitney test. ***B,*** miR-21/24 diagnostic accuracy was assessed by ROC analysis.

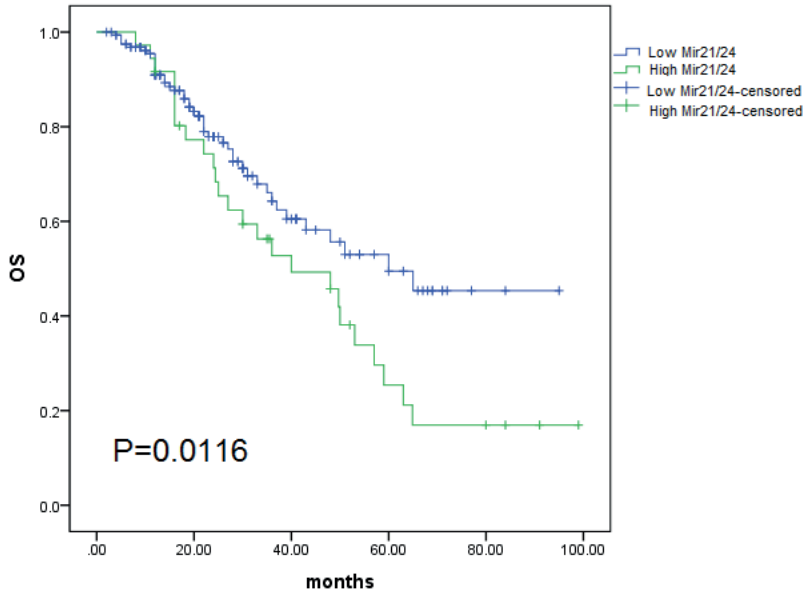

Figure 3. Kaplan-Meier analysis of overall survival for patients with colon cancer according to ratio of miR-21/24 expression.

Table III. Association of serum ratio of miR-21/24 expression with clinicopathological parameters in colorectal cancer.

	Univariate analysis		Multivariate analysis	
Characteristics	**HR (95% CI)**	***p*-value**	**HR (95% CI)**	***p*-value**
Age (year), > 60 *vs.* ≤ 60	0.765 (0.436-2.165)	0.125	0.735 (0.320-2.015)	0.236
Sex, male *vs.* female	1.413 (0.498-2.511)	0.187		
Histologic grade, poor vs. well, moderate	3.424 (1.758-6.322)	0.011		
Tumor size (cm), > 6 *vs.* ≤ 6	2.355 (0.975-4.159)	0.007		
TNM, III + IV vs. I + II	4.115 (2.127-6.290)	0.003	3.576 (0.976-9.524)	0.027
LNM, positive *vs.* negative	3.417 (1.476-5.764)	0.017	2.351 (0.421-6.521)	0.013
miR-21/24, high *vs.* low	5.287 (2.331-9.876)	0.001	3.793 (1.245-7.562)	0.0033

potential prognostic or diagnostic value has been identified by mounting researchers[32]. Using this type of ratio as biomarker has many advantages, such as eliminating the need of internal reference, improving the discrimination and specificity in terms of diagnostic potential, reducing the risk of missing a malignant event, and considering the genetic intratumor heterogeneity of CRC where various molecules may be altered by different mechanisms at different foci[31]. These characteristics make the miRNA ratio as biomarker more attractive in a clinical setting. Here, we demonstrated that the miR-21/24 ratio has diagnostic property in CRC patients. This is the first time that miR-21 and miR-24 are combined into a ratio. Despite that plasma/serum miRNAs have been identified as promising biomarkers for noninvasive diagnosis in various tumor entities, only few data are available on circulating individual miRNAs and a panel of several miRNAs as diagnostic biomarkers of CRC. Wulfken et al[33] reported that miR-1233 was upregulated in CRC tissue and serum, and serum miR-1233 could detect CRC with 77.4% sensitivity but only 37.6% specificity. Previous study demonstrated serum miR-378 (over-expression in CRC patients), miR-451 (down-expression in CRC patients) and combination of the two miRNAs as potential biomarkers for discriminating CRC patients from healthy controls with an AUC of 0.71, 0.77, and 0.86, respectively[34]. In this study, we found that plasma ratio of miR-21/24 had best accuracy for distinguishing CRC patients from healthy individuals than single miRNAs, meanwhile, the accuracy is higher than most known published data. Besides

the importance of early diagnosis biomarkers, the prognosis biomarkers share the same important role. A good prognosis biomarker could provide valuable information for clinicians to determine TNM stage, to perform chemotherapy, radiotherapy and other adjuvant therapy, to predict the possibility of metastasis and survival period for each patient. Some studies suggest single plasma miR-21 concentration could not tell the difference between stage III-IV and early I-II stages[35]. However, in the current study, ratio of miR-21/24 could not only discriminate CRC from healthy controls, but also provide significant correlation with TNM staging, lymph metastasis, tumor size and histological grades, all of these are key elements correlated with CRC prognosis and survival. Recent studies revealed that circulating miRNAs are encapsulated in microparticles secreted actively from cancer cells and function in cell-cell communications. Thus, miRNAs upregulated in tumor tissue may be reflected by overexpression of these miRNAs in the bloodstream. In the present study, we observed that plasma miR-21 was overexpressed in CRC, which might be reflected with tumor growing or metastasis; on the contrary, the miR-24 as a tumor suppressor miRNA might be suppressed gradually by tumor progression. Therefore, combined with these two miRNAs, the ratio could provide accurate prediction value for CRC prognosis.

Conclusions

We showed that the ratio of plasma miR-21/24 is a potentially powerful tool for detection of CRC, more importantly, the ratio of miR-21/24 can provide potential prediction for CRC prognosis, including survival, lymphocyte and TNM staging, which will be helpful in precision treatment for CRC in the future.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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