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Serum miR-21 and miR-210 as promising non-invasive biomarkers for the diagnosis and prognosis of colorectal cancer

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ABSTRACT

Objective: this study aimed to investigate the expression and clinical significance of miR-21 and miR-210 in serum of patients with colorectal cancer (CRC).

Methods: the expression levels of serum miR-21 and miR-210 in 40 CRC patients (CRC group) and 20 healthy patients (control group) were measured by qRT-PCR. Correlation analysis was performed of the relationship between serum miR-21 and miR-210 levels with clinical characteristics, including gender, age, tumor location, tumor size, tumor stage, local invasion and TNM staging. The expression levels of miR-21 and miR-210 in the CRC group were separately measured before and after surgery. ROC analysis was performed to evaluate the diagnostic value of miR-21 and miR-210.

Results: serum miR-21 and miR-210 in the CRC group were much higher than those in the control group. Meanwhile, the levels of serum miR-21 and miR-210 were closely related to tumor size ($p = 0.028$, $p = 0.047$), lymphatic metastasis ($p = 0.038$, $p = 0.028$), TNM staging ($p = 0.014$, $p = 0.047$) and tumor stage ($p = 0.014$, $p = 0.017$),

but independent of gender, age and tumor location. In addition, serum miR-21 and miR-210 in the CRC group (n = 18) after surgery were lower than those before surgery ($p < 0.001$). ROC curves showed that miR-21 (AUC = 0.863) and miR-210 (AUC = 0.818) both had diagnostic efficacy in CRC patients.

Conclusion: miR-21 and miR-210 can be used as novel non-invasive biomarkers for CRC diagnosis and prognosis.

Keywords: miR-21. miR-210. Serum. Colorectal cancer. Biomarkers.

INTRODUCTION

Colorectal cancer (CRC) is one of the most prevalent cancers and the fourth leading cause of cancer-related death worldwide (1,2). Approximately 1.8 million people are newly diagnosed with CRC every year and 880,792 patients died from CRC in 2018 (3). CRC commonly starts with noncancerous adenomas followed by a gradual carcinogenic processes activation and malignant transformation, which depends on diet, environmental, genetic abnormalities and other harmful factors (4). After diagnosis, an estimated 50-70 % of CRC patients already have distant metastasis and the overall survival is largely dependent on an early diagnosis and intervention (5). Accordingly, identifying specific biomarkers is imperative to better understand CRC initiation and prognosis. CEA is the most commonly used blood-based CRC biomarker, it has a low sensitivity and specificity and is therefore a poor screening marker for the detection of recurrent CRC (6). Compared with traditional CRC biomarkers such as CEA and CA19-9, serum miRNAs might be superior for the detection of CRC. Circulating miRNA can withstand unfavorable physiological conditions, such as extreme variations in pH, temperature and multiple freeze/thaw cycles (7). Furthermore, the profiles of circulating miRNAs show consistent expression levels across physiologically healthy individuals (8).

MicroRNAs (miRNAs) are a class of endogenous small non-coding RNAs with a length of 20-24 nucleotides and are generated through multiple enzymatic excisions of miRNA precursors, pri-miRNAs (9). A large number of studies have found that miRNAs are involved in modulating cell functions (10), human protein coding genes

(11), tumorigenesis and tumor development (12-14). miRNAs are generally produced in the nucleus and are able to regulate gene expression in the cytoplasm (15). However, it is reported that miRNAs can also be found in the extracellular environment including serum, suggesting that miRNAs exert their roles both in the intracellular and extracellular environment (16-18). The extracellular miRNAs may originate from the passive leakage from apoptotic or damaged cells and/or from the secretory activity, mainly within extracellular vesicles (including exosomes) (19). Circulating miRNAs stably exist in plasma and serve as potential biomarkers for early diagnosis or monitoring cancer progression for several cancers (20-24). Compared to a single miRNA biomarker, two miRNA biomarkers could effectively increase the predictive value (25,26). Therefore, it is hypothesized that the signature based on circulating miRNAs provides a high sensitivity, success and reproducibility in the diagnosis of different types of cancer with a non-invasive approach (16,27,28). Recent studies have reported that miR-21 or miR-210 can be used for diagnosis and prognosis prediction of several cancers (29-32). miR-21 is known to be up-regulated in CRC, can be detected in serum in CRC patients and is easily detected with a high sensitivity and specificity. However, it is unable to distinguish early CRC patients from advanced CRC patients (33). On the other hand, Qu et al. also demonstrated that the up-regulation of miRNA-210 was correlated with metastasis and tumor progression in CRC (34). However, there is a lack of research on the expression and predictive values of miR-210 and miR-21 in CRC. Therefore, this study aimed to provide a new theoretical basis for the molecular diagnosis and treatment of CRC. An experimental study of the expression patterns and clinical significance of miR-21 and miR-210 in CRC was performed.

MATERIALS AND METHODS

Clinical samples

A total of 126 patients with CRC admitted to the Shaoxing People's Hospital from January 1st, 2017 to January 1st, 2018 and 89 healthy volunteers without a history of cancer and in good health were recruited. A randomized numerical table method was used and finally 40 CRC patients and 20 healthy volunteers were included. All

participants met the following criteria: a) no previous surgery, chemotherapy or radiotherapy; b) no other archenteric complications, inflammation or hematologic disease; c) they had a sterile intestinal condition when undergoing a colonoscopy; and 4) no metabolic diseases with high blood lipid or blood purine. Furthermore, 18 patients were selected for serum sample collection before and after surgery. This study was also authorized by the institutional ethics committee of the Shaoxing People's Hospital and complied with the Declaration of Helsinki on ethical principles for medical research involving human subjects. Written informed consent was obtained from all participants.

Patient database

A total of 40 CRC patients including 18 males and 22 females were enrolled according to the above criteria. Corresponding clinical characteristics, including gender, age, tumor location, tumor size, tumor stage, lymphatic metastasis and Cancer Tumor-Node-Metastasis (TNM) staging are shown in table 1.

Serum sample collection and preparation

Blood samples were collected using EDTA-K2 tubes from 20 healthy individuals and 40 CRC patients. Among the CRC patients, 18 patients underwent surgical treatment and blood samples were collected two weeks after surgery. Blood samples were centrifuged for 10 min at 1,600 g and the supernatants were removed to a new tube and centrifuged again for 10 min at 10,000 g to remove residual blood cells. All plasma samples were divided into two tubes and stored at -80 °C. One sample was used for biochemical analysis and another was used for RNA extraction.

RNA extraction and quantitative real-time-PCR (qRT-PCR)

Total RNA in plasma was extracted using the TRIzol™ reagent (Haling Biotechnology Co., Ltd., Shanghai, China) according to the manufacturer's instructions. RNA concentration, purity and integrity were examined by NanoDrop™ ND-1000 spectrophotometric evaluation and agarose gel electrophoresis. Reverse transcription PCR (RT-PCR) was performed using a PrimeScript™ RT-reagent kit

(Transgen Biotech, Beijing, China). A total of 20 µl of RNA sample was reverse transcribed into cDNA following the instructions of the EasyScript® First-Strand cDNA Synthesis SuperMix kit (AE301-02, Transgen Biotech, Beijing, China). qRT-qPCR was performed using the SYBR® Premix Ex TaqTM I kit (TaKaRa, Dalian, China) under the following conditions: 10 min at 95 °C, 45 cycles of 10 s at 95 °C, 20 s at 60 °C and 1 s at 72 °C. Specific primers were used for RT-qPCR analysis as follows:

miR-21 Forward: 5'-TAGCTTATCAGACTGATGTTGA-3'; miR-21 Reverse: 5'-ATCCAGTGCAGGGTCCGAGG-3''

miR-210 Forward; 5'-AGCCCCTGCCCACCGCACACTG-3'; miR-210 Reverse: 5'-GGACACGGGGCCAGGAGGGTCGCGC-3';

U6 Forward: 5'-CTCGCTTCGGCAGCACA-3'; U6 Reverse: 5'-AACGCTTCACGAATTTGCGT-3'.

Statistical analysis

All data were analyzed using the SPSS 21.0 software (SPSS, Chicago, IL, USA). The count data were expressed as a ratio or percentage. Measurement data were presented as the mean ± standard deviation. The comparisons of measurement data were performed using the Student's *t* test. Receiver operating characteristic (ROC) analysis was used to assess the predictive value for CRC diagnosis. All experiments were performed in duplicate at least three times. Results were expressed as the mean ± SEM. $p < 0.05$ was considered as statistically significant.

RESULTS

Expression levels of serum miR-21 and miR-210 in CRC patients

Levels of serum miR-21 and miR-210 in 40 CRC patients and 20 healthy controls were measured by qRT-PCR. Our result showed that levels of miR-21 and miR-210 in CRC patients were significantly increased before surgery relative to the healthy control group ($p < 0.01$) (Fig. 1A and B). This finding confirmed that there was a close relationship between CRC and the abnormal expression of miR-21 and miR-210.

Subsequently, ROC curves were constructed to evaluate the diagnostic value of the two miRNAs. For miR-21, the area under ROC curve (AUC) was 0.863, and the

sensitivity and the specificity were 88.9 % and 83.3 %, respectively. Furthermore, the optimal cut-off value for miR-21 was 30.83 (Fig. 1C). For miR-210, the AUC value was 0.818, and the sensitivity and the specificity were 88.9 % and 72.2 %, respectively. The optimal cut-off value for miR-21 was 22.15 (Fig. 1D).

Correlation between the levels of serum miR-21, miR-210 and clinicopathological characteristics of CRC patients

The correlation between the levels of serum miR-21 and miR-210 and the clinical pathological characteristics of CRC patients was analyzed. The results shown in table 1 suggested that the serum levels of miR-21 and miR-210 in CRC patients were not associated with gender, age and tumor location ($p > 0.05$). However, they were related to tumor size ($p = 0.028$, $p = 0.047$), TNM staging ($p = 0.014$, $p = 0.047$), tumor stage ($p = 0.014$, $p = 0.017$) and lymph node metastasis ($p = 0.038$, $p = 0.028$).

The relationship between serum miR-21 and miR-210 expression with the prognosis of CRC patients

To determine whether serum miR-21 and miR-210 expression levels were associated with therapeutic efficacy, serum samples were collected from 18 CRC patients two weeks after surgery. As shown in figure 2A and B, serum miR-21 and miR-210 levels in post-operative samples were significantly lower than those in pre-operative serum samples ($p < 0.001$) (Fig. 2A and B).

DISCUSSION

Tumorigenesis and metastasis of CRC are driven by multi-genetic alteration features. A previous study has shown that several miRNAs were altered in CRC and that miR-21 played a crucial role in CRC initiation and progression (35).

In our study, two miRNAs (miR-21 and miR-210) were identified for CRC diagnosis and prognosis. In addition, the expression levels of miR-21 and miR-210 in serum were drastically increased in the CRC group relative to the healthy control group. In fact, high serum miR-21 and miR-210 were closely associated with tumor size, local invasion, TNM staging, lymph node metastasis and tumor stage. However, there was

no significant statistical relationship with other characteristics including age, gender and tumor location. Furthermore, serum miR-21 and miR-210 expression levels were clearly decreased two weeks after surgery.

Exploration of specific serum miRNAs for the diagnosis and prognosis of CRC remains a major challenge in clinical studies. Studies have shown that plasma miR-21 is a potential biomarker for CRC diagnosis (30). Li et al. showed that miR-210 could serve as a promising biomarker for the detection of breast cancer (BC) (36). In addition, a meta-analysis showed that miR-21 was overexpressed in the serum of patients with CRC and the AUC of ROC curve was 0.87. It has been reported that miR-21 could be used as a potential biomarker for CRC diagnosis with a moderate sensitivity and a good specificity (37), which is consistent with our results. Moreover, some studies also reported that elevated levels of miR-21 and miR-210 were associated with several cancers, such as breast cancer, renal cancer, pancreatic cancer and glioma. In this study, we showed the correlation between serum miR-21 and miR-210 levels and clinicopathological characteristics of CRC patients and verified the value of both as diagnostic markers in CRC ROC curve analysis. Our results clearly indicated that increased serum levels of miR-21 and miR-210 in CRC patients were closely related with CRC pathogenesis and progression. The AUC values of these two miRNAs were 0.863 (miR-21) and 0.818 (miR-210), respectively. These results indicate that these two miRNAs have a good predictive value for the diagnosis of CRC

Recently, many potential biomarkers have been effectively applied in clinical trials such as fecal hemoglobin, carcinoembryonic antigen (CEA) and CA19.9, which are not highly promising diagnostic targets for personalized medicine (38). Thus, there is a critical need for specific genetic markers for an individualized and optimized treatment. In our study, miR-21 and miR-210 showed a better diagnostic efficiency, and both AUC values were greater than 0.8 and the sensitivity and specificity were also over 70 %. In addition, serum levels of miR-21 and miR-210 showed a positive correlation with tumor stage and prognosis. miR-21 and miR-210 might function as promising clinical markers for CRC diagnosis and treatment in the future. However, the use of miR-21 or miR-210 expression for the detection and evaluation of different cancers still has its limitations. With regard to the detection of CRC,

evaluating miR-21 and miR-210 expression combined with other clinical accessory examinations and comprehensive analysis is still a promising strategy for CRC diagnosis and prognosis.

In conclusion, miR-21 and miR-210 could be used as potential non-invasive biomarkers for CRC diagnosis. Expression levels of miR-21 and miR-210 in serum could be detected for the assessment of clinical treatment response in CRC patients. However, there are several limitations in our study, such as the small sample size and statistics error, which need to be further improved in subsequent studies for a better CRC diagnosis.

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Table 1. Relationship between serum levels of miR-21 and miR-210 and clinicopathological characteristics of CRC patients

Characteristics	n	MiR-21 expression	p	MiR-210 expression	p
Gender			0.345		0.322
Male	18	54.90 ± 15.53		54.29 ± 20.16	
Female	22	55.76 ± 14.72		55.25 ± 16.23	
Age (years)			0.288		0.432
< 60	15	70.01 ± 15.24		70.02 ± 17.84	
≥ 60	25	69.48 ± 15.42		69.48 ± 18.69	
Tumor location			0.443		0.165
Colon	18	55.21 ± 18.25		56.31 ± 23.29	
Rectal	22	55.05 ± 17.12		55.04 ± 22.54	
Tumor size (cm)			0.028		0.047
< 4.9	17	35.47 ± 18.94		36.52 ± 23.12	
≥ 4.9	23	59.73 ± 17.60		59.31 ± 22.49	
TNM staging			0.014		0.047
T1-T2	13	33.99 ± 14.13		36.02 ± 16.84	
T3	17	49.96 ± 15.21		49.02 ± 17.59	
T4	10	70.37 ± 15.82		71.09 ± 19.21	
Lymphatic metastasis			0.038		0.028
Positive	14	36.13 ± 14.69		35.61 ± 13.90	
Negative	26	60.69 ± 27.86		61.23 ± 26.61	
Tumor stage			0.014		0.017
I	10	17.34 ± 8.94		21.12 ± 9.91	
II	5	54.02 ± 10.54		55.35 ± 12.20	
III	18	59.85 ± 14.54		59.84 ± 15.57	
IV	7	113.27 ± 16.73		114.68 ± 17.16	

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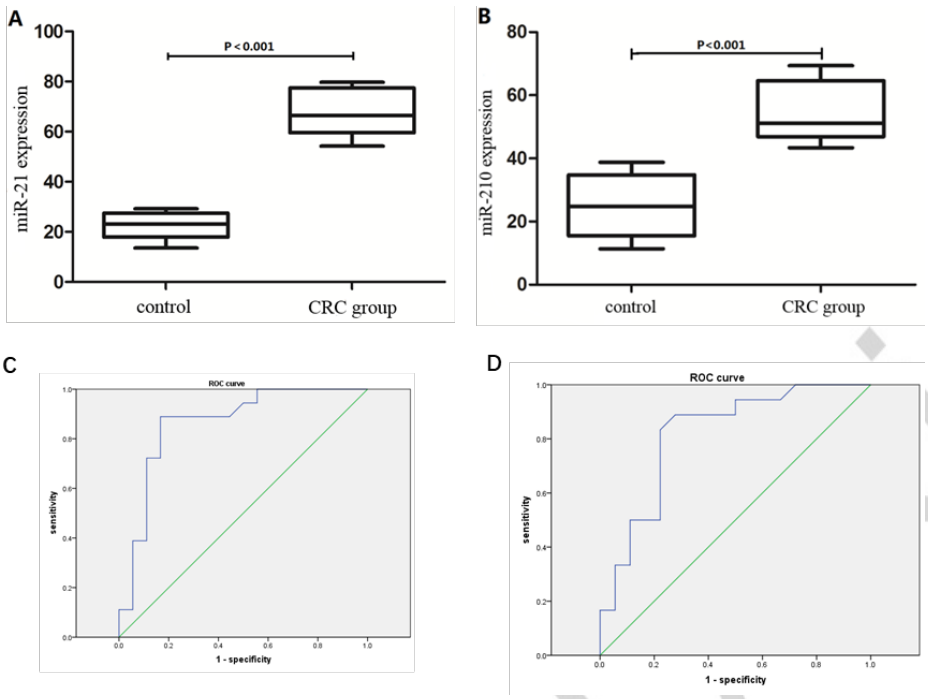

Fig. 1. Expression levels and ROC curves of serum miR-21 (A and C) and miR-210 (B and D) between the control group and CRC group.

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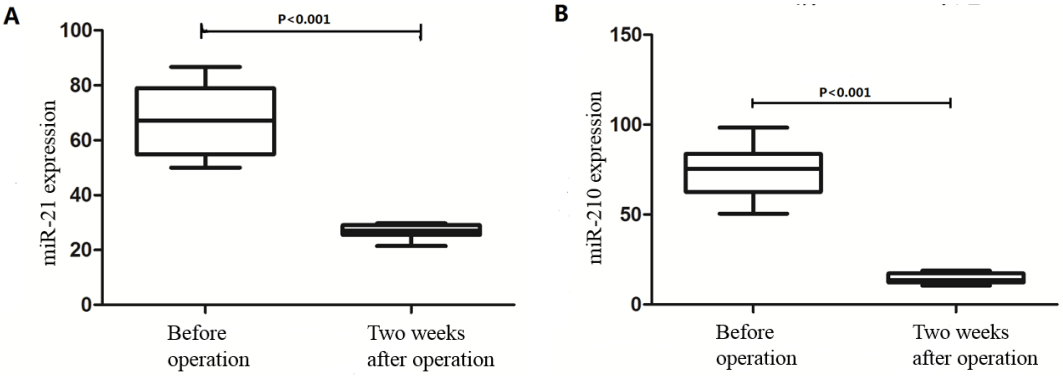

Fig. 2. The expression of miR-21 (A) and miR-210 (B) in serum in the CRC group before surgery and two weeks after surgery.