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Correlation analysis of miR-200b, miR-200c, and miR-141 with liver metastases in colorectal cancer patients

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Abstract. – OBJECTIVE: To investigate correlation relationship between serum miR-200b, miR-200c, miR-141 levels with liver metastases in colorectal cancer patients.

PATIENTS AND METHODS: A total of 85 colorectal cancer patients with liver metastases and 54 colorectal cancer patients without liver metastases were divided into experimental group and control. Serum sample was collected before surgery and tested by Real-time PCR to evaluate miR-200b, miR-200c, miR-141 expressions.

RESULTS: The primary site of cancer of two groups was mostly derived from the colon cancer among the control and experimental group (there was no significant difference between the control group (61.1%) and experimental group (63.5%). Most of the invasive depth in the control group was T3 phase (50%), and most of the invasive depth in the experimental group was T4 phase (69.4%); therefore, the difference of the invasive depth between the control and experimental group is significant ($p < 0.05$). The majority of cases in experimental group were in stage IV, while they were in stage II in control. MiR-200b, miR-200c and miR-141 relate with tumor metastasis through epithelial mesenchymal transition (ETM) pathway, and target ZEB1 and ZEB2 genes. MiR-200b, miR-200c, and miR-141 have been confirmed to be related to tumor metastasis. miR-141 levels in serum from experimental group was higher significant compared to control group ($p = 0.024$). miR-200b levels in serum from experimental group was significantly increased compared to control group ($p = 0.031$). The miR-200c levels in serum from experimental group were significantly higher compared to control group ($p = 0.015$). Meanwhile, serum miR-141, miR-200b, miR-200c abnormal expressions in serum were related to tumor occurrence and development. Their levels were positively correlated with liver metastasis.

CONCLUSIONS: The probability of liver metastases in colorectal cancer patients was positively correlated with serum miR-141, miR-200b, and miR-200c expressions, which could be treated as new biomarker for early diagnosis of liver metastases in colorectal cancer.

Key Words:
microRNA, Serum expression, Colorectal cancer, Liver metastasis, Correlation research.

Introduction

Colorectal cancer is a type of malignant tumor with increasing incidence in recent years worldwide[1]. Most liver metastasis of colorectal cancer appears in progress, while about 15% colorectal cancer patients in early period have combined liver metastasis when diagnosed[2]. Therefore, liver metastasis of colorectal cancer has become an international research focus in recent years. It was showed that the occurrence and development of colorectal cancer were closely associated with a variety of oncogenes activation and tumor suppressor genes suppression. The key point of inhibiting liver metastases in colorectal cancer is to screen the molecular targets for the early diagnosis of colorectal cancer liver metastasis, as the clinical process of liver metastases in patients with colorectal cancer is influenced by multiple factors and multiple steps. At present, it was found about 30% of the human gene expressions were regulated by endogenous non-coding small RNA (miRNA). MiRNA widely existed in the body. It was showed that miRNA expression

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was closely related to various tumors occurrence, development, and metastasis[3]. MiR-200b, miR-200c, and miR-141 belonging to miR-200 family were tumor suppressor genes that widely participated in various types of cancer occurrence and development. Previous research revealed that miR-200b and miR-200c significantly declined in breast cancer tissue and cells, whereas miR-200b markedly reduced in colorectal cancer tissue compared with normal control. MiRNA exhibits good stability in serum, thus could be detected by real-time PCR. Therefore, miRNA could be treated as a type of ideal tumor marker[4,5]. According to a previous report, this study intended to use real-time PCR to test miR-200b, miR-200c, and miR-141 expressions in serum from colorectal cancer patients, aiming to analyze their correlations with liver metastasis and provide new basis for early diagnosis of liver metastasis in colorectal cancer.

Patients and Methods

Patients

A total of 139 colorectal cancer patients in the First People's Hospital of Shangqiu City between Jan 2015 and May 2016 were involved in this study. A total of 85 colorectal cancer patients with liver metastases was assigned to the experimental group and 54 patients without liver metastases were assigned to the control group.

Inclusion and Exclusion Criteria

Inclusion criteria: The patients were diagnosed as colorectal cancer by the internal medicine according the pathological reports. No patients inflicted the heart, liver, and kidney dysfunction or incompetence. All of the protocols used in the patients were approved by the Research Ethics Committee of the First People's Hospital of Shangqiu City. All patients gave their informed consent and approved this study before study commencement.

Exclusion criteria: patients with other hematologic disorders, malignant tumors, or severe infection.

Serum Sample Preparation

A total of 5 ml venous blood was collected in the tube at one day before surgery. The blood sample was centrifuged at 3000 r/min for 15 min and the serum was moved to a new Eppendorf (EP) tube to store at -80°C.

Total RNA Extraction

A total of 100 μl serum was added to an RNase-free EP tube and used to extract miRNA by using the miRNeasy Mini Kit Extraction kit (Qiagen, Hilden, Germany). The obtained miRNA was dissolved in RNase-free ddH_2O and reverse transcripted to cDNA. The reaction system was performed at 37°C for 1 h and 85°C for 5 min. Finally, the cDNA was stored at -30°C.

RT-PCR

Real-time PCR (RT-PCR) was used to detect miR-141, miR-200b, and miR-200c levels in the serum. All the primers used in this study were designed and verified by Genecopoeia Inc. (Rockville, MD, USA). MiRNAs were tested on 96-well plate with two replications (Corning, NY, USA). Cel-miR-39 was selected as an internal reference in this study. Meanwhile, the $2^{-\Delta\Delta Ct}$ formula was applied for the calculation.

Statistical Analysis

All data analysis was performed on SPSS 19.0 software (SPSS Inc. Chicago, IL, USA). Measurement data was depicted as $\bar{x} \pm s$ and compared by Student's *t*-test. Enumeration data was tested by χ^2 test. $p < 0.05$ was considered as statistical significance.

Results

Basic Clinical Information Comparison

The primary site of cancer of two groups was mostly derived from the colon cancer among the control and experimental group (there was no significant difference between control group (61.1%) and experimental group (63.5%). Most of the invasive depth in the control group was T3 phase (50%), and most of the invasive depth in the experimental group was T4 phase (69.4%), therefore, the difference of the invasive depth between the control and experimental group is significant ($p < 0.05$). The majority of cases in the experimental group were in stage IV, while they were in stage II in control. No statistical significance was observed in age, gender, clinical staging, and invasive depth between two groups (Table I). Table II illustrated that the miR-200b relates with tumor metastasis through epithelial mesenchymal transition (ETM) pathway, and targets the ZEB1 and ZEB2 genes (Table II). Meanwhile, miR-200c and miR-141 also relate with tumor metastasis through ETM pathway, and both target

Table I. Basic clinical information comparison.

Group	Control	Experimental group
Cases	54	85
Male	31 (57.4)	50 (58.8)
Mean age (year)	(55.8 ± 7.9)	(57.1 ± 6.3)
Primary lesion		
Colon	33 (61.1)	54 (63.5)
Rectum	21 (38.9)	31 (36.5)
Invasive depth		
T1	4 (7.4)	0 (0)
T2	12 (22.2)	0 (0)
T3	27 (50)	26 (30.6)
T4	11 (20.4)	59 (69.4)
Liver metastasis staging	0 (0)	85 (100)
I	10 (18.5)	0 (0)
II	32 (59.3)	0 (0)
III	12 (22.2)	0 (0)
IV	0 (0)	85 (100)

the ZEB1 and ZEB2 genes (Table II). Therefore, the miR-200b, miR-200c, and miR-141 had been confirmed to be associated with tumor metastasis (Table II).

Serum miR-200b, miR-200c, and miR-141 Comparison

Real-time PCR revealed that miR-141 levels in serum from experimental group was higher significant compared to the control group (Table III, Figure 1, Figure 2, $p = 0.024$). The miR-200b level in serum from experimental group was significantly increased compared to the control group (Table III, Figure 1, Figure 3, $p = 0.031$). Moreover, the miR-200c levels in serum from experimental group were also significantly higher compared to that in control (Table III, Figure 1, Figure 4, $p = 0.015$).

Discussion

Endogenous non-coding single short-stranded RNA is called miRNA. MiRNA can restrain oncogenes and tumor suppressor genes transcription and translation by targeted binding with mRNA. Downregulation of miRNA may promote tumor occurrence and development[10]. A large number of studies indicated that a variety of physiological and pathological processes of cancer were closely related to miRNA. It was thought that miRNA can not only regulate gene expression at the post-transcriptional level, it also affect the process of tumor occurrence, growth, and metastasis by regulating other tumor related genes, including oncogenes and tumor suppressor genes[11]. Many studies showed that miRNAs were associated with cancer clinical staging, progress, treatment, and prognosis. Moreover, the relationship between some of miRNAs and cancer progress has been confirmed. It was demonstrated that miRNAs were found overexpressed in lung cancer and colorectal cancer tissues, whereas the expression of different genes was discriminated in different tissues. MiR-128b and miR-150 were found significantly elevated in lung cancer tissue[12]. It was revealed that miR-31 was obviously enhanced in colorectal cancer tissue and associated with tumor staging and degree of infiltration. Furthermore, miR-143 was reported to be reduced only in colon cancer, while it showed no statistical difference with normal mucosa in rectal cancer[13]. It was indicated that miR-21 overexpression was related to tumor vascular invasion and lymph node metastasis in esophageal cancer[14]. It was thought that inflammation immune reaction was the main mechanism of miRNAs. They can bind with the related loci of major histocompatibility complex I chain-related gene A and B antigen, so as to escape from immunological recognition and attack, suggesting that tumor cells

Table II. MiRNA related to tumor metastasis and target genes.

miRNA	Function	Target genes
miR-200b	Epithelial mesenchymal transition (EMT)[7,8]	ZEB[1], ZEB[2]
miR-200c	EMT[9]	ZEB[1], ZEB[2]
miR-141	EMT[7-9]	ZEB[1], ZEB[2]

Table III. Serum miR-200b, miR-200c, and miR-141 comparison ($2^{-\Delta\Delta Ct}$).

Group	Cases	miR-141	miR-200b	miR-200c
Control	54	-0.4897	0.6351	0.5074
Experimental group	85	0.0726	1.3267	1.2621
p-value	–	0.024	0.031	0.015

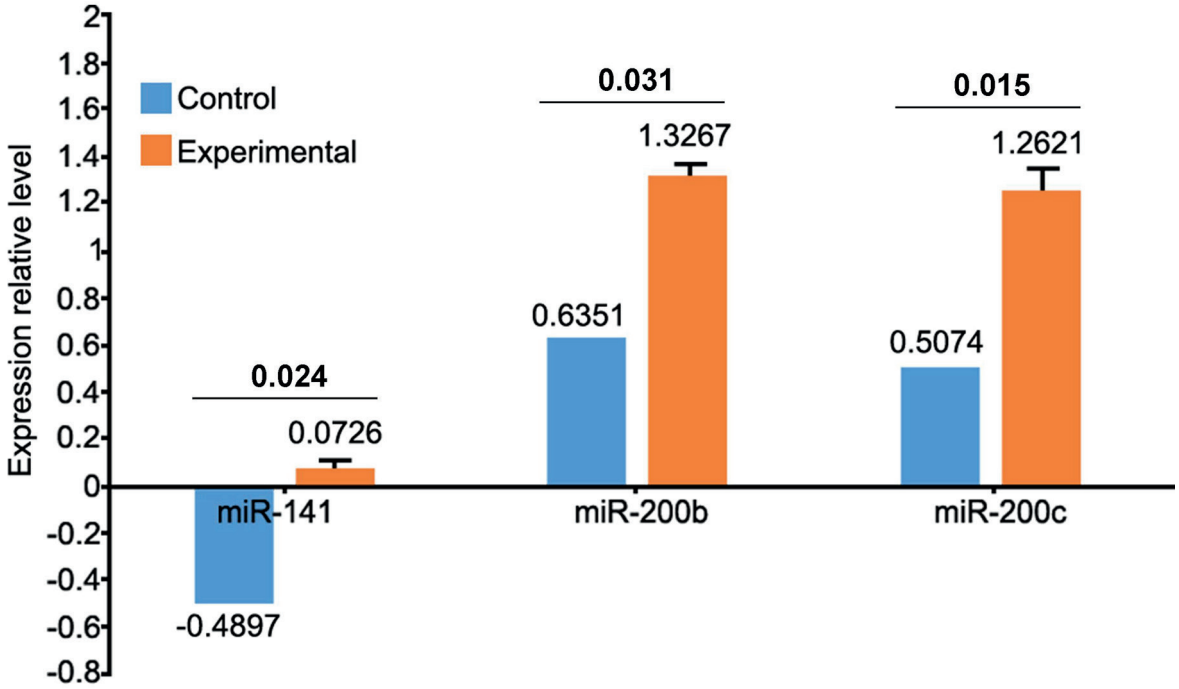

Figure 1. Serum miR-200b, miR-200c, and miR-141 comparison.

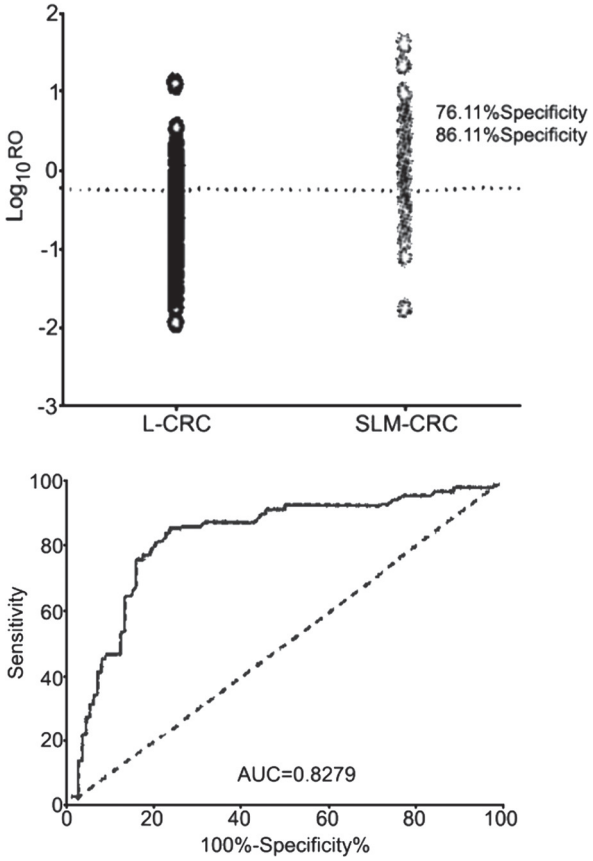

Figure 2. Scatter diagram and ROC curve of miR-141.

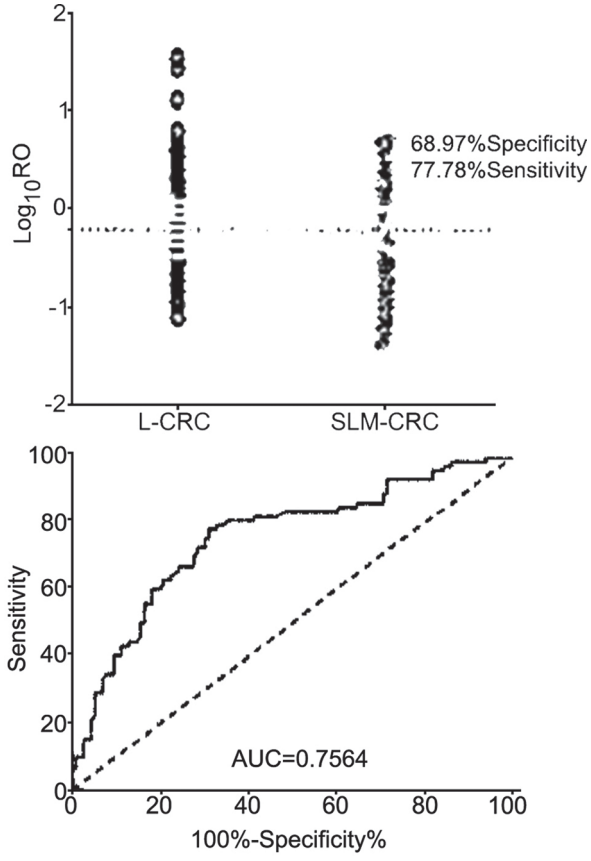

Figure 3. Scatter diagram and ROC curve of miR-200b.

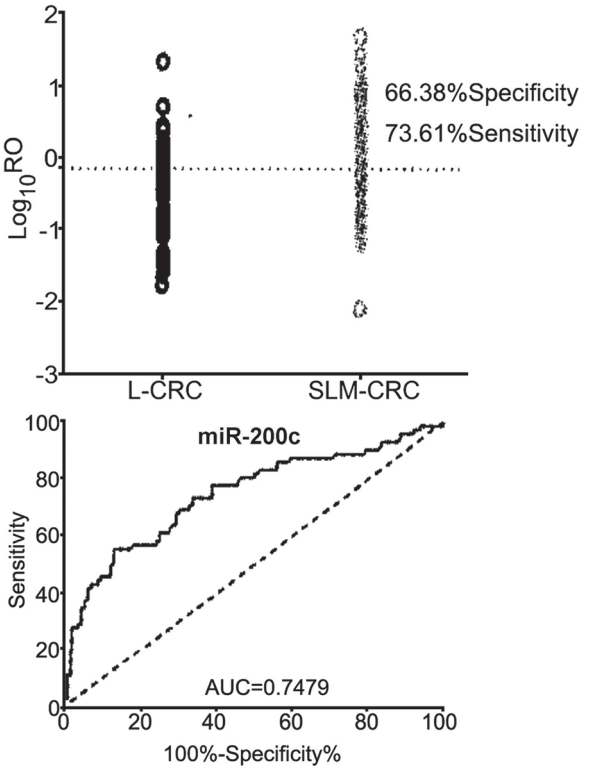

Figure 4. Scatter diagram and ROC curve of miR-200c.

may also escape immune detection upon such mechanism[15-17]. Liu et al[18] reported that tumor related miRNAs in the serum may not change normal miRNAs. It was also found that abnormal expression of miR-200 family existed in the progress process of multiple cancers, and their expression profiles were divergent in different tumor tissues[19]. It was indicated that miR-200b and miR-200c markedly declined in gastric cancer and breast cancer, while miR-200c upregulation can suppress colorectal cancer cells proliferation[20]. It was pointed out that miR-200 family can regulate zinc finger E-box-binding protein (ZEB1) expression. Downregulation of miR-200c increased ZEB1 gene expression and reduced E-cadherin. The reduction of E-cadherin directly induced EMT, thus indirectly promoted cancer cell differentiation and proliferation. This family can target bind with multiple members in RAB family to inhibit breast cancer cell invasion[21]. MiR-141, also belonging to miR-200 family, can regulate EMT in human colorectal cancer metastasis[22,23]. Therefore, monitoring serum miR-141, miR-200b, and miR-200c in tumor patients is of great significance. Following the further understanding of miRNA function, miRNA expression in serum also receives much concern. Tsang et al[24] proposed that serum miRNA expression may be related to tissue hypofunction and malfunction. It was confirmed that a lot of miRNAs existed in serum instead of transforming from other parts[24]. It was found that miRNA level in urine and amniotic fluid could be tested by real-time PCR, providing new approach for the investigation of miRNA expression in serum[25]. Although serum contained a large number of miRNAs, there were multiple digestive enzymes (mainly ribonuclease) in the serum. Therefore, the stability of miRNAs in the serum was unclear. Mitchell et al[26] found miRNAs showed good stability in the serum from human compared with *Caenorhabditis elegans*. Chen et al[27] analyzed miRNA expression in lung cancer cells and found that miRNAs in the serum can escape the digestion of ribonuclease. Meanwhile, the length of time, temperature, and PH value showed no significant impact on the stability of miRNAs. Thus, we considered that miRNAs were stable under the presence of enzyme, and showed high tolerance to acid and alkali, ambient temperature, and indwelling time. They were a kind of ideal tumor markers. Also, numerous studies demonstrated that serum miRNAs exhibited high sensitivity and specificity for the diagnosis of patients. The relative noninvasive process of sampling provided the basis of testing miRNAs expression by real-time PCR in this study[7]. At present, there is still a lack of study about the relationship of serum levels of miR-200b and miR-200c with colorectal cancer. We tested miR-141, miR-200b, and miR-200c levels in serum to analyze their correlation with liver metastasis in colorectal cancer. In this study, real-time PCR was applied to test miRNA content in the serum. Real-time PCR is widely used in clinical research because of its simple operation, high sensitivity, fast and accurate, and relatively low cost[7,28]. It was found that miR-200b and miR-200c expressions in tissue adjacent to tumor were obviously higher than that in tumor lesions, and were closely associated with tumor differentiation, staging, and lymph node metastasis[23]. Hur et al[7] revealed that miR-141 and miR-200c highly expressed in plasma from colorectal cancer patients, while their levels were markedly higher in metastatic patients compared with non-metastatic patients[7]. It was showed that serum miR-141 expression in stage IV patients apparently upregulated compared with patients in other stages[29]. Our results

demonstrated that miR-141, miR-200b, and miR-200c levels elevated in the experimental group compared with control. It was speculated that serum miR-141, miR-200b, and miR-200c levels were positively correlated with liver metastasis in colorectal cancer patients.

Conclusions

To sum up, liver metastasis of colorectal cancer may be related to serum miR-141, miR-200b, and miR-200c levels. They were all highly expressed in serum and could be treated as biomarkers to predict liver metastasis of colorectal cancer.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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