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Expression and Clinical Pathological Significance of miR-200a in Concurrent Cholangiocarcinoma Associated with Hepatolithiasis

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Approximately 2–10% of the patients with hepatolithiasis may develop cholangiocarcinoma (CCA). Despite recent advances in the treatment of cancers, the 5-year survival rate for CCA patients currently remains poor, primarily due to early local invasion and distant metastasis of the cancer. This study aimed to investigate miR-200a expression in combined hepatolithiasis and CCA as well as its correlation with the clinical features of CCA.

Material/Methods: miR-200a expression in combined hepatolithiasis and CCA was detected by real-time reverse transcription PCR (qRT-PCR). Its correlation with the clinicopathology of CCA was analyzed by t-tests. The effect of miR-200a on the proliferation CCA cells was determined by MTT assay. The effect of miR-200a on the invasive ability of CCA cells was assessed by Boyden chamber test.

Results: The expression level of MiR-200a in patients with combined hepatolithiasis and CCA was significantly decreased compared with patients with only hepatolithiasis ($P<0.01$). Furthermore, miR-200a expression in hepatic duct cancer RBE cells was substantially reduced compared with hepatolithiasis group ($P<0.01$). Correlation analysis showed that abnormal expression of miR-200a was only associated with the differentiation degree and metastasis of CCA. MiR-200a transfection significantly inhibited the proliferation and invasion of REB cells ($P<0.01$).

Conclusions: MiR-200a may suppress the proliferative and invasive ability of REB cells. The reduced miR-200a expression might be correlated with the development and progression of CCA.

MeSH Keywords: Cholangiocarcinoma • MicroRNAs • Tumor Necrosis Factor Ligand Superfamily Member 13

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Background

Cholangiocarcinoma (CCA) refers to a highly malignant tumor with poor prognosis that is derived from the extrahepatic bile duct, including the hepatic hilar region to the lower part of the common bile duct [1]. Hepatolithiasis, also known as intrahepatic bile duct stone, is a type of bile duct stone that may cause severe complications and is the leading cause of death due to benign biliary tract disease. High incidence rate of hepatolithiasis has been reported in East and Southeast Asia, including China, Japan, Indonesia, Philippines, Thailand, Malaysia, Korea, and other countries [2]. In addition, it has also been reported that 2–10% of patients with hepatolithiasis may develop intrahepatic CCA [3,4]. Although the mechanism of CCA remains unclear, the intrahepatic cholestasis that often occurs concurrently with hepatolithiasis and the physical stimuli to the bile duct wall caused by stone itself or bacterial infection may cause atypical hyperplasia of bile duct epithelial cells and ultimately lead to adenocarcinoma [5,6].

Intrahepatic CCA associated with hepatolithiasis is usually difficult to detect in early stages. The 5-year survival rate of CCA is still poor despite great advances in the treatment of cancer [7,8], primarily due to early local invasion and distant metastasis of the tumor [9]. Therefore, it is urgent to understand the molecular mechanism of the occurrence, progression, and metastasis of CCA, and to provide novel therapeutic targets in order to develop effective treatment strategies.

MicroRNAs (miRNAs) are small, evolutionary conserved, single-stranded, non-coding RNA. Growing evidence suggests that abnormal expression of miRNA is closely associated with the occurrence and development of tumors. It has been found that the expression of MiR-200a is significantly decreased in a wide variety of tumor tissues, and it can inhibit the proliferation and invasion of many types of tumor cells, suggesting that reduced miR-200a expression is closely related to the occurrence and development of cancer [10–13]. There are currently few reports on miR-200a expression in combined hepatolithiasis and intrahepatic CCA, and scant research attention has been focused on its effect on the proliferation and invasion of hepatic duct cancer cells. In this study, we detected miR-200a expression in combined hepatolithiasis and intrahepatic CCA tissue by real-time reverse transcription PCR (qRT-PCR), and analyzed its correlation with the clinical features of CCA. We also investigated the effect of miR-200a on the proliferation and invasion of hepatic duct cancer cells transfected with miR-200a mimics in order to provide an experimental basis for discovering novel therapeutic targets for the treatment of CCA.

Material and Methods

Subjects and sample collection

This study included 50 patients with intrahepatic CCA who received liver resection in Nanfang Hospital, Southern Medical University between January 2011 and December 2011. All postoperative specimens were examined using H&E staining by 2 experienced pathologists to confirm the diagnosis of intrahepatic CCA.. No patients had received preoperative radiotherapy or chemotherapy. The corresponding para-carcinoma tissues were also collected as controls. The study protocol was approved by the Research Ethics Committee of our hospital, and all patients signed the informed consent before the study.

Cell lines

REB cell line was purchased from Shanghai Haocheng Biotech Co., Ltd. (Shanghai, China). Human intrahepatic biliary epithelial cells were purchased from Shanghai Bioleaf Biotech Co., Ltd. (Shanghai, China). REB cells and human intrahepatic biliary epithelial cells were maintained in RPMI-1640 medium supplement with 10% fetal bovine serum (FBS).

Instruments and reagents

Milli-Q Advantage system was purchased from Millipore (Martillac, France). CO_2 incubator was obtained from Thermo (Waltham, MA). FBS was bought from Gibco (Grand Island, NY). RPMI-1640 medium was purchased from Hyclone (Logan, UT). Lipofectamine2000 transfection reagents and TRIzol were purchased from Invitrogen (Grand Island, NY). RNA extraction kit and SYBR Green PCR kit were purchased from Tiangen Biotech co., Ltd. (Beijing, China), and MiR-200a and negative control (NC) sequences were synthesized by Ambion (Grand Island, NY). The Boyden chamber was purchase from Kylin-Bell Lab Instruments Co., Ltd. (Haimen, China).

Real-time reverse transcription PCR (qRT-PCR)

Total RNA was extracted from the tissues and reverse transcribed into cDNA. The cycling conditions consisted of an initial, single cycle of 15 min at 95°C, followed by 40 cycles of 10 s at 95°C and 20 s at 60°C. Primer sequences were as follows: miR-200a forward 5'-TATGCCAATGCTAGACCTCCCACT-3', reverse 5'-GTCCTATGCAGTCCACGCTCGGAG-3'; U6 forward 5'-GCTTCGGCAGCACATATACTAAAAT-3', and reverse 5'-CGCTTCACGAATTTGCGTGTCAT-3'. The relative amount of miRNAs was normalized against the U6 snRNA, and the fold change was calculated by the $2^{-\Delta\Delta}$Ct method.

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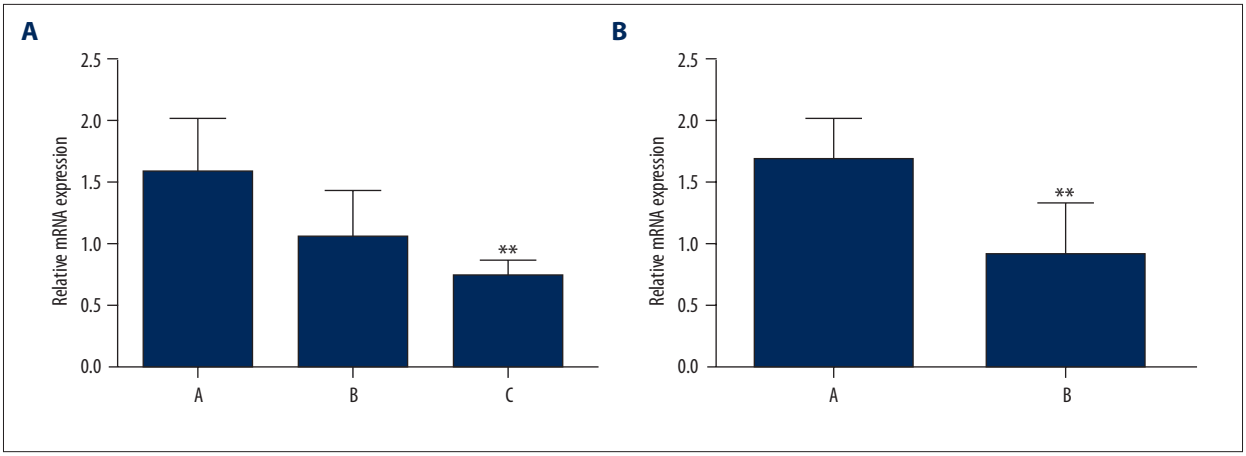

Figure 1. miR-200a expression detected by qRT-PCR. (**A**) A, para-carcinoma tissue; B hepatolithiasis tissue; C combined hepatolithiasis and CCA. ** P<0.01 compared with group A and B. (**B**) A, intrahepatic bile duct epithelial cells; B, RBE cells. ** P<0.01 compared with group A.

Table 1. Correlation between miR-200a expression and the clinicopathological parameters of CCA.

Parameter	n	miR-200a	*P* value
Age (years)			
<60	33	0.67±0.11	0.773
≥60	27	0.75±0.07	
Gender			
Male	30	0.77±0.09	0.718
Female	30	0.70±0.13	
T stage			
T1	20	0.71±0.11	0.583
T2	20	0.65±0.05	
T3	20	0.75±0.10	
Differentiation			
High/moderate	30	1.05±0.15	0.000
Low	30	0.45±0.09	
Lymph node metastasis			
Yes	21	0.59±0.07	0.009
No	39	0.94±0.11	

Cell transfection

Cells in the log phase were transfected with miR-200a mimics or control using Lipofectamine2000 reagent according to the manufacture's instruction.

MTT assay

Cells were inoculated in 96-well culture plates. A total of 20 μL of MTT was added to each well and plates were incubated at 37°C for 4 h. Another 150 μL of DMSO was added and OD value of each well was determined at 490 nm wavelength.

Invasive ability assay

Invasive ability assay was performed using the Boyden chamber as described previously [14,15]. Briefly, 1×10^5 cells in 300 μL of medium were seeded in the upper chamber, and 500 μL of medium was added to the lower chamber. After 12 h of incubation, the cells in the lower chamber were stained

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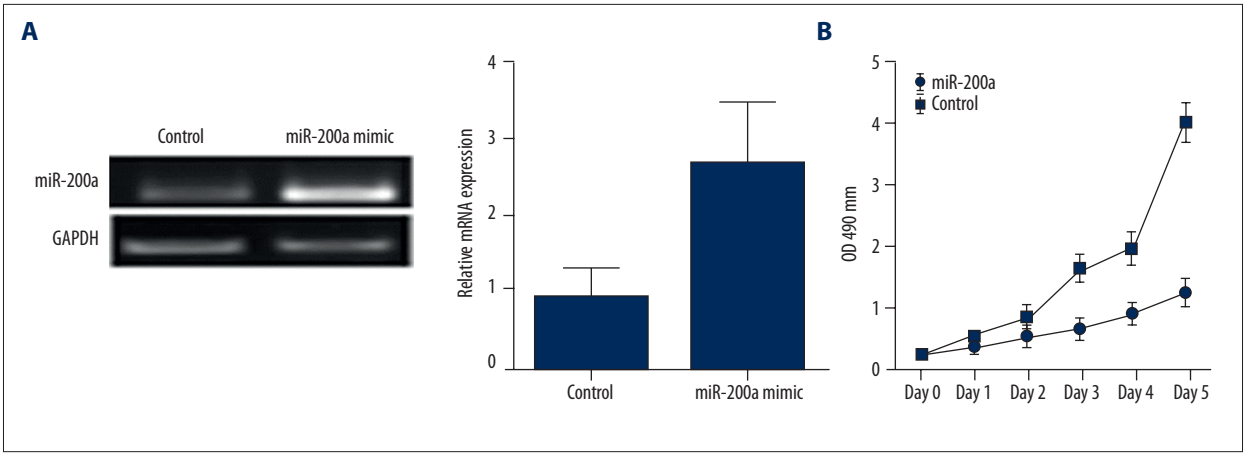

Figure 2. Effect of miR-200a on the proliferation of REB cells. A, miR-200a expression detected by qRT-PCR; B, the proliferation of REB cells detected by MTT assay.

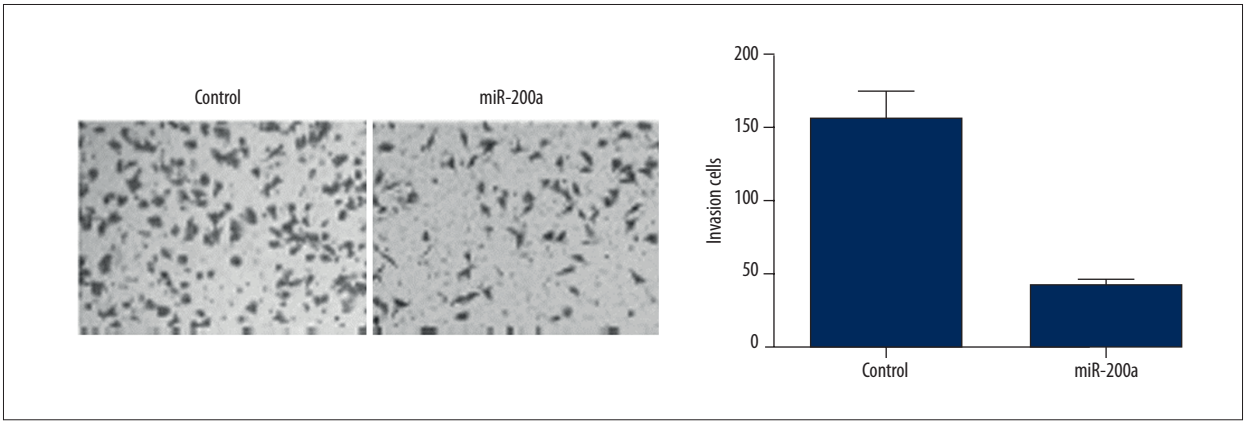

Figure 3. Effect of miR-200a on the invasion of REB cells.

with 0.1% crystal violet for 30 min and the cell number was determined.

Statistical analyses

All statistical analyses were performed using GraphPad software (La Jolla, CA). Differences among multiple groups were analyzed by one-way ANOVA or t-tests. P<0.05 was considered as statistically significant.

Results

MiR-200a expression in hepatolithiasis tissue, combined hepatolithiasis and intrahepatic CCA, and CCA cells

The relative expression level of miR-200a in the normal paracarcinoma tissue, hepatolithiasis tissue, and combined hepatolithiasis and intrahepatic CCA was 1.56±0.45, 1.04±0.38, and 0.74±0.13, respectively. As shown in Figure 1A, MiR-200a expression in combined hepatolithiasis and intrahepatic CCA was significantly reduced compared with the other 2 groups ($P<0.01$). In addition, MiR-200a expression in RBE cells (0.91±0.41) was significantly lower than that in intrahepatic bile duct epithelial cells (1.68±0.52) ($P<0.01$) (Figure 1B).

Correlation between miR-200a expression and clinicopathological parameters of CCA

As shown in Table 1, the relative expression level of miR-200a in the lymph node metastasis group was markedly lower compared with the non-metastasis group ($P<0.01$). miR-200a expression in the high/moderate differentiation group was also higher than that in the low differentiation group ($P<0.01$).

Effect of miR-200a on the proliferation of REB cells

As shown in Figure 2A, miR-200a expression in REB cells was significantly elevated after transfection with miR-200a mimics ($P<0.01$). Moreover, the proliferative ability of REB cells was significantly decreased after the transfection of miR-200a mimics ($P<0.01$) (Figure 2B).

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Effect of MiR-200a on the invasion of REB cells

As shown in Figure 3, REB cells transfected with miR-200a mimics were significantly lower compared with the control group ($P<0.01$), suggesting the inhibitory effect of miR-200a on the invasive ability of REB cells.

Discussion

CCA is a highly malignant tumor with poor prognosis. Although surgery in combination with adjuvant chemotherapy has achieved good therapeutic effect in patients at early stages, the long-term survival rate of CCA patients remains poor, primarily because concurrent cholangiocarcinoma associated with hepatolithiasis is seldom detected at early stages. Increasing evidence shows the abnormal expression of a variety of miRNAs in cancer tissues, suggesting that these miRNAs may be closely associated with the occurrence and development of tumors [16–21]. Bai et al. [16] confirmed that miR-409-3p expression is significantly reduced in colorectal cancer tissues, and miR-409-3p can inhibit the invasion and metastasis of colorectal cancer cells. Huang et al. [20] found that miR-10b was overexpressed in non-small cell lung cancer, and silencing miR-10b can significantly inhibit the proliferation of non-small cell lung cancer cells and induce the apoptosis of these cells. Zhang et al. [21] revealed that miR-183 expression in non-small cell lung cancer was significantly elevated and that miR-183 can promote the occurrence and development of the cancer. Cao et al. proved that miR-324-5p can suppress liver cancer cell invasion by downregulating ETS1 and SP1 [22].

Several studies have reported abnormal expression of miRNAs in CAA [23,24]. Our study mainly focused on miR-200a expression and effect on cholangiocarcinoma caused by hepatolithiasis. It has been reported that miR-200a expression is significantly reduced in several tumor tissues, and it can markedly inhibit the proliferation and invasion of a variety tumor cells, suggesting that its low expression is closely associated with the occurrence and development of cancer [10–13]. Previous studies have confirmed that miR-200a may regulate the development of renal cancer cells through targeting the transforming growth factor beta 2 (TGFβ2) [25]. In endometrial carcinoma, miR-200a can mediate the proliferation of cancer cells by targeting phosphatase and tensin homolog (PTEN) [26]. MiR-200a can inhibit the proliferation and migration of liver cancer cells through metastasis-associated colon cancer 1 (MACC1) and can be used as a prognostic factor for liver cancer [27]. Furthermore, miR-200a can suppress the proliferation of breast cancer cells by targeting mitochondrial transcription factor A (TFAM) [28]. Currently, there are few reports on miR-200a expression in combined hepatolithiasis and intrahepatic CCA, or its effect on the proliferation and invasion of hepatic duct cancer cells. In this study, the expression of miR-200a in combined hepatolithiasis and intrahepatic CCA was detected by qRT-PCR. It was found that miR-200a expression in combined hepatolithiasis and intrahepatic CCA was significantly decreased compared with that in hepatolithiasis tissues and normal para-carcinoma tissue ($P<0.01$). Moreover, the expression of miR-200a in REB cells was significantly reduced compared to the control group ($P<0.01$). Further, the correlation of miR-200a with the clinical features of CCA was analyzed and it was shown that miR-200a expression was only associated with the differentiation and metastasis degree of CCA, but not with sex, age, or T stage of patients. Functional experiments showed that the proliferative and invasive ability of REB cells transfected with miR-200a mimics was significantly lower than that in the control group ($P<0.01$), suggesting its inhibitory effect on the proliferation and invasion of hepatic duct cancer cells.

Conclusions

Our results revealed that miR-200a expression in concurrent CCA associated with hepatolithiasis was significantly decreased. The inhibitory effect of miR-200a on the proliferation and invasion of CCA cells was also confirmed, suggesting the reduced expression level of miR-200a might be associated with the occurrence and development of CCA. Although the molecular mechanism behind the inhibitory effect of miR-200a on the proliferation and invasion of CCA cells needs to be further investigated, the current study has provided an experimental basis for the search for novel therapeutic targets for the treatment of CCA.

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