

## RESEARCH ARTICLE

# MicroRNA-21 Regulates the Invasion and Metastasis in Cholangiocarcinoma and May Be a Potential Biomarker for Cancer Prognosis

Qiang Huang<sup>1&</sup>, Lei Liu<sup>1&</sup>, Chen-Hai Liu<sup>1</sup>, Hao You<sup>1</sup>, Feng Shao<sup>1</sup>, Fang Xie<sup>1</sup>, Xian-Sheng Lin<sup>1</sup>, San-Yuan Hu<sup>2\*</sup>, Chuan-Hai Zhang<sup>1</sup>

### Abstract

**Background:** MicroRNAs are noncoding RNA molecules that posttranscriptionally regulate gene expression. The aim of this study was to determine the role of microRNA-21 in cholangiocarcinomas and its relationship to cholangiocarcinoma RBE cell capacity for invasion and metastasis. **Methods:** MicroRNA-21 expression was investigated in 41 cases of cholangiocarcinoma samples by *in situ* hybridization and real-time PCR. Influence on cholangiocarcinoma cell line invasion and metastasis was analyzed with microRNA-21 transfected cells. In addition, regulation of reversion-inducing-cysteine-rich protein with kazal motifs (RECK) by microRNA-21 was elucidated to identify mechanisms. **Results:** *In situ* hybridization and real-time quantitative PCR results for patients with lymph node metastasis or perineural invasion showed significantly high expression of microRNA-21 ( $P < 0.05$ ). There was a dramatic decrease in cholangiocarcinoma cell line invasion and metastasis ability after microRNA-21 knockdown ( $P < 0.05$ ). However, overexpression significantly increased invasion and metastasis ( $P < 0.05$ ). Real-time PCR and Western-blot analysis showed that microRNA-21 could potentially inhibit RECK expression in RBE cells. Survival analysis showed that patients with higher expression levels of microRNA-21 more often had a poor prognosis ( $P < 0.05$ ). **Conclusions:** MicroRNA-21 may play an important role in cholangiocarcinoma invasion and metastasis, suggesting that MicroRNA-21 should be further evaluated as a biomarker for predicting cholangiocarcinoma prognosis.

**Keywords:** MicroRNA-21 - cholangiocarcinoma - invasion - metastasis

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### Introduction

Cholangiocarcinoma (CCA) is a highly malignant tumor derived from bile duct epithelial cells (Sawanyawisuth, 2009). It represents 10% to 15% of the total hepatobiliary tumors and its incidence and mortality are increasing worldwide. Most patients with bile duct cancer are diagnosed when the disease is advanced, the overall prognosis is poor, usually only survive for several years (Blechacz et al., 2008; Blechacz et al., 2011). However, in recent years, multiple studies have been conducted to investigate the genes and their products that are involved in cholangiocarcinoma metastasis (Andersen et al., 2012; Shen et al., 2010).

MicroRNAs are a class of naturally occurring small non-coding RNAs that control gene expression by targeting mRNAs for translational repression or cleavage (Pillai et al., 2005; Iorio et al., 2012). Recently, the role for MicroRNAs in the establishment and progression of cholangiocarcinoma has become evident, and some

miRNAs have been identified as oncogenes or tumor suppressor genes in cholangiocarcinoma (Li et al., 2012; Zhang et al., 2012). Specifically, microRNA-21 stands out as the microRNA most often overexpressed in very diverse types of malignancy. Moreover, microRNA-21 may play an important role in many cancer-related processes such as apoptosis, invasion, and metastasis (Meng et al., 2007; Markou et al., 2008; Slaby et al., 2007). Florin et al. also found microRNA-21 was always overexpressed in cholangiocarcinoma cell (Florin et al., 2009). However, the precise role played by microRNA-21 in cholangiocarcinoma invasion and metastasis is unknown. In a bioinformatic analysis, RECK has been known to contain the microRNA-21 binding site, which acts as a tumor suppressor gene regulating various aspects associated with cancer cell invasion, metastasis (Liu et al., 2010; Han et al., 2011). In this study, based on these various aspects of the microRNA-21 function, we hypothesized that microRNA-21 played an important role in the progression of cholangiocarcinoma and

<sup>1</sup>Department of General Surgery, Affiliated Provincial Hospital of Anhui Medical University, Hefei, Anhui, <sup>2</sup>Department of General Surgery, Qilu Hospital of Shandong University, Jinan, Shandong, China \*Equal contributors \*For correspondence: 1452504862@qq.com

potential regulated RECK expression. Furthermore, we evaluated whether or not microRNA-21 as a biomarker for earlier diagnosis and predicting the survival of cholangiocarcinoma patients.

## Materials and Methods

### Human tissues

Surgical specimens from patients who had been surgically treated for cholangiocarcinoma at Anhui Provincial Hospital, Hefei, from 1 January 2005 to 1 January 2010 were employed. All without radiotherapy or chemotherapy before operation. All tumors were clinically and histologically diagnosed as cholangiocarcinoma. Inclusion criteria for all cases included: (i) unambiguous histology and absence of mixed tumour types; (ii) sufficient viable tissue available for RNA extraction; (iii) absence of any treatment prior to surgery; and (iv) age of tissue block less than 7 years (Jaime et al., 2011). Eventually leaving 41 cases specimens meet the requirements, there were 25 cases of men, 16 women, from 32 to 80 years old (The median age 60±2.6 years). The clinicopathological characteristics of the patients are given in Table 1. From each block, 15 slices of 5 µm each were collected in one 1.5 mL tube for RNA extraction and microRNA analysis.

### Cell lines and cultures

The cholangiocarcinoma RBE cell were obtained from the Cell Bank of the Chinese Academy of Sciences. All cells were grown in RPMI 1640 medium and with 10% fetal bovine serum at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>.

### In situ hybridization of microRNA

In situ hybridization was done according to the manufacturer's protocol for formalin-fixed, paraffin embedded tissue. The probes were 3', 5'-labeled with digoxigenin with the DIG tailing kit (Exiqon). The staining was carried out as previously described. After deparaffinization, the specimens were subjected to proteinase K (40 µg/ml) digestion for 20 mins. The postfixed tissues were subsequently incubated overnight with the locked nucleic acid-modified probes. For the immunodetection, tissues were incubated overnight at 4°C in anti-DIG-AP FAB fragment (1:1000). The final visualization was carried out with BCIP/NBTP. The stained sections were reviewed and scored under a light microscope (Olympus America) independently by two investigators. The staining intensity and percentage of tissue staining were recorded (staining intensity: 1= no staining or weak staining, 2 = intermediate staining, 3 = strong staining; percentage: 1 = <10%, 2 = 10-30%, 3 = >30%). Both scores multiplied. If more than 3 points that case was considered to be high expressed, otherwise it is low expressed (Lorenzo et al., 2007).

### RNA Extraction and Real-time RT-PCR

Total RNA was extracted as described previously (Rosenfeld et al., 2007). Briefly, the samples was incubated several times in xylene at 60°C to remove excess paraffin and then washed several times with ethanol.

Protein degradation was performed by incubation of the sample in a proteinase K solution at 55°C for a few hours. The RNA was extracted using acid phenol/chloroform and then precipitated with ethanol, DNase was introduced to digest DNA. The expression of mature microRNAs was determined by real-time PCR using the TaqMan microRNA assay. U6 small nuclear RNA was used as an internal control. The following primers were used: microRNA-21, 5'-ACACTCCAGCTGGGTAGCTTATCAGACTGATG-3' (forward); 5'-CTCAACTGGTGTCTGTGGA-3' (reverse); and U6, 5'-CTCGCTTCGGCAGCACA-3' (forward); 5'-AACGCTTCACGAATTTGCGT-3' (reverse). To quantify expression of RECK mRNA was quantified by real-time PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal control. PCR primers used were as follows, RECK, 5'-TTCAGAACCCCACTACTG-3' (forward) and 5'-ATGCAAGATGCTTTGATGC-3' (reverse); and for GAPDH, 5'-TGCACCACCAACTGCTTAGC-3' (forward) and 5'-GGCATGGACTGTGGTCATGAG-3' (reverse). All PCRs were done in triplicates.

### Transfections and In vitro Invasion and metastasis Assays

Transfection with microRNA-21, lipo (Liposome control group), microRNA-21-NC (Negative control group) and microRNA-21-IN (microRNA-21 inhibition group) was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturers' instructions. Cells were collected for experiments after were transfected in 48 h. Cell We used a Transwell insert to determine the effect of microRNA-21 on cholangiocarcinoma cell invasion and metastasis in vitro. Cells transfected with either microRNA-21 or microRNA-21-IN plated on transwell chambers precoated with Matrigel. Medium containing 10% fetal bovine serum in the lower chamber served as the chemoattractant. After the cells were incubated for 24 h at 37°C in a humidified incubator, the noninvading cells were removed with cotton swabs. The invasive cells attached to the lower surface of the membrane insert were fixed in 100% methanol at room temperature for 2 mins and stained with toluidine blue. The number of invasive cells on the lower surface of the membrane was then counted under a microscope.

### Western blotting

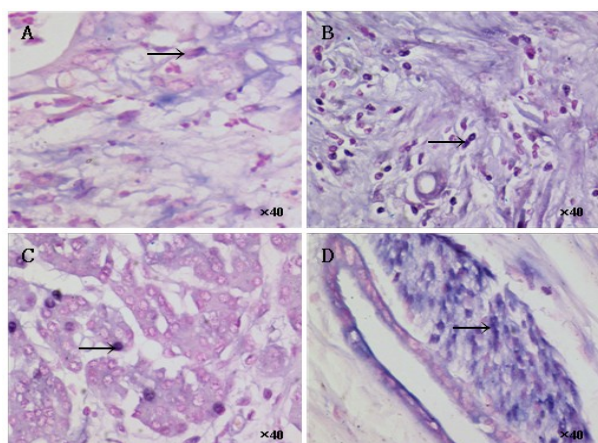
For isolating the proteins, cells harvested in 6-well plates were washed once in PBS and lysed in the lysis buffer. SDS-PAGE and Western blotting were done according to standard procedures. Western blotting of GAPDH on the same membrane was used as a loading control. The signals were detected by secondary antibodies labeled with ECL Detection System, and signal intensity was determined by Labworks 4.6.

### Statistical analysis

These analyses were performed with SPSS Version 17.0 statistical software package. Differences between two groups were compared using Pearson's chi-square test for qualitative variables and the Mann-Whitney U test. The overall Survival and relapse-free Survival curves were calculated using the Kaplan-Meier method and analyzed

**Table 1. Correlation of Clinicopathologic Parameters and MicroRNA-21 Expression**

Characteristic	n	microRNA-21		$\chi^2$	P
		High	Low		
Gender				0.322	$P>0.05$
Male	25	15	10		
Female	16	11	5		
Age				0.588	$P>0.05$
<60 years	14	10	4		
≥60 years	27	16	11		
Tumor location				0.51	$P>0.05$
Hepatic portal	11	6	5		
Middle or inferior	30	20	10		
Differentiation				6.365	$P<0.05$
Well	10	3	7		
Moderate or Poor	31	23	8		
Histological type				0.256	$P>0.05$
Adenocarcinoma	37	23	14		
Others	4	3	1		
Lymph metastasis				0.552	$P<0.05$
Positive	15	13	2		
Negative	26	13	13		
Perineural invasion				11.896	$P<0.05$
Positive	20	18	2		
Negative	21	8	13		



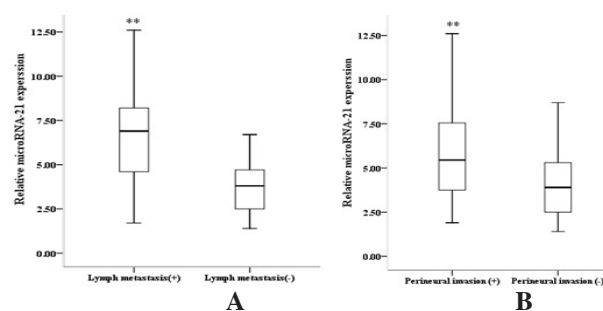
**Figure 1. Detection of microRNA-21 in Cholangiocarcinoma Tissues by in Situ Hybridization.** The positive rate for microRNA-21 was significantly higher in lymph node metastasis group than in that without ( $P<0.05$ ; Figure 1A,B). The positive rate for microRNA-21 was higher in the Perineural invasion group than that without ( $P<0.05$ ; Figure 1C,D) (Magnification:  $\times 400$ )

using the Log-rank test. The level of statistical significance was set at  $P\leq 0.05$ .

## Results

### *In situ hybridization showed microRNA-21 expression in cholangiocarcinoma*

To visualize microRNA-21 expression in different tumor, we performed in situ hybridization in formalin-fixed, paraffin-embedded tissue (Figure 1). The positive rate for microRNA-21 expression was significantly higher in the cholangiocarcinoma with lymph node group (Figure 1B) than that without (Figure 1A). The positive rate for microRNA-21 was higher in the Perineural invasion group



**Figure 2. Expression of microRNA-21 in Cholangiocarcinoma Tissues.** Mature microRNA-21 expression was analyzed by TaqMan-quantitative real-time PCR and normalized to U6 expression. The ratios of tumor to normal tissue for microRNA-21 expression were presented as relative T/N ratio of microRNA-21 expression.  $**P < 0.05$ . ( $P=0.038, 0.010$ ; Figure 2A,B)

(Figure 1D) than that without (Figure 1C); microRNA-21 expressions was significantly higher in tumors showing in tumor differentiation degree, lymph node metastasis, perineural invasion ( $P<0.05$ ). It has nothing to do with others factor, such as gender, age, tumor location, tumor type ( $P>0.05$ ) (Table 1).

### *qRT-PCR data for microRNA-21 validate In situ hybridization findings*

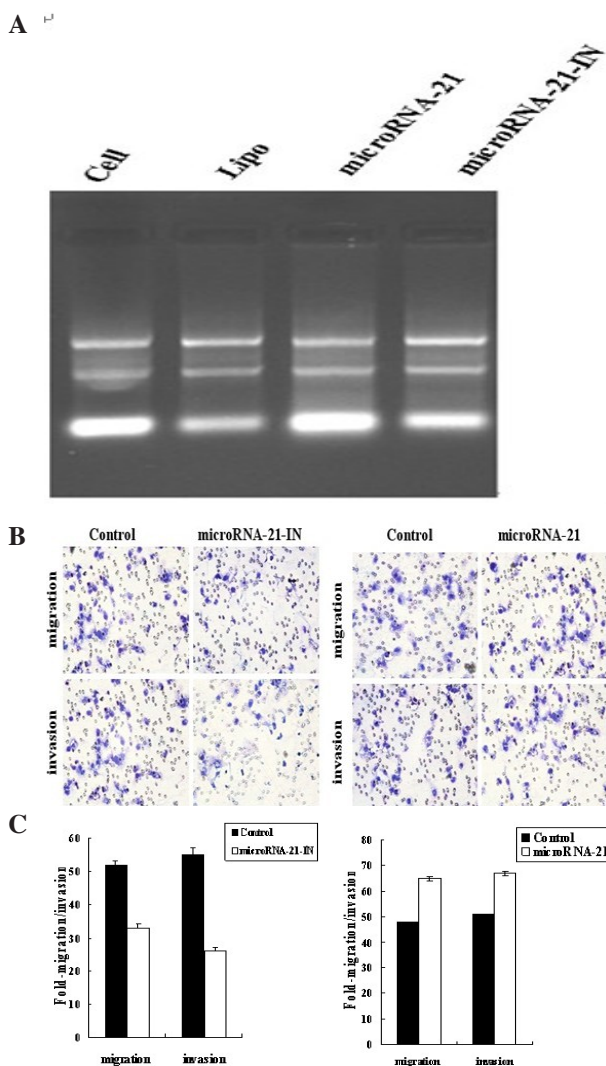
Total RNA was isolated from cholangiocarcinoma tissues, and the microRNA-21 levels were determined by real-time PCR (Figure 2). The correlation of T/N ratios for microRNA-21 expression with the clinicopathologic factors listed in Table 1 was examined. The patients with Perineural invasion or lymph node metastasis were significantly higher than those without them ( $P=0.038, 0.010$ ; Figure 2A,B).

### *microRNA-21 can effect cell invasion and metastasis in cholangiocarcinoma*

The four cell groups microRNA-21 levels were determined by real-time PCR (Figure 3A). We next assayed whether microRNA-21 can change the capacity of cholangiocarcinoma cells for invasion and metastasis. RBE cells were suitable for the invasion and metastasis assay, because they showed good invasion to the Matrigel membranes. The cell invasion and metastasis assay showed that knockdown of microRNA-21 resulted in decreased RBE cell invasion and metastasis rate compared with the control cells ( $P<0.05$ , Figure 3 B, C). In contrast, ectopic expression of microRNA-21 in RBE cells resulted in significant increased cell invasion and metastasis in transwell assays ( $P<0.05$ , Figure 3 D, E). In addition, we found overexpression or knockdown of miRNA-21 have an obviously effect on cell proliferation or cell death in the first 48h.

### *RECK, a putative target of microRNA-21*

Bioinformatic analyses found that the RECK gene, among a number of others, may be a potential microRNA-21 target. We chose RECK for further analysis because it was previously implicated in promoting cholangiocarcinoma progression, specifically, its can effect cell invasion and metastasis ability (Ziyan et al.,

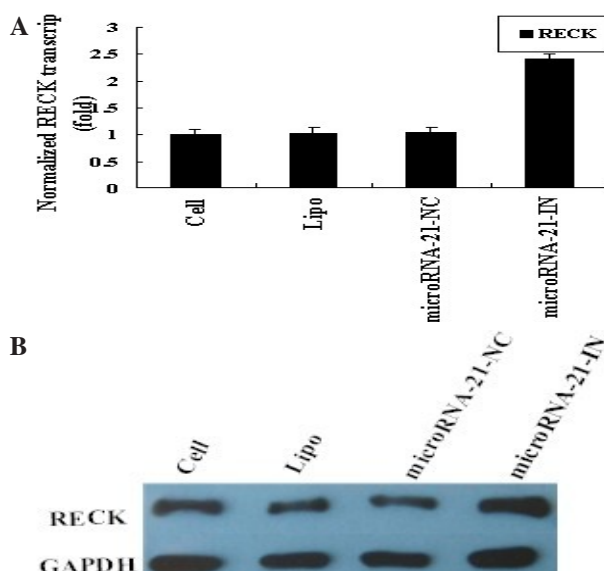


**Figure 3. microRNA-21 Inhibitor Transfection Leads to Reduced Cell Invasion in Cholangiocarcinoma.** The four cell groups microRNA-21 levels were determined by real-time PCR (Figure 3.A). RBE cell transfected with microRNA-21-IN plated on transwell chambers precoated with Matrigel. Representative photographs (Figure 3B) and quantification (Figure 3C) are shown. RBE cell transfected with microRNA-21 can increased cell invasion and migration in transwell assays (Figure 3 D, E). Magnification:  $\times 400$

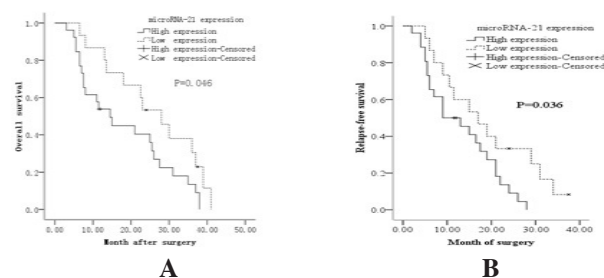
2011). Indeed, we found that RBE cells transfected with microRNA-21-IN had dramatically increased RECK levels by 10 fold, compared with the control cells ( $P < 0.001$ , Figure 4).

*Association of microRNA-21 expression with overall survival and relapse-free survival*

We then performed Kaplan-Meier analyses to determine whether microRNA-21 expression was associated with overall survival and relapse-free survival in the cholangiocarcinoma patients. These Patients who had high microRNA-21 expression presented mean 3-year overall survival of 15%, whereas patients who had low microRNA-21 express presented mean 3-year overall survival of 33%.The difference was statistically significant ( $P = 0.046$ , Figure 5A). Similarly,a statistically significant association of microRNA-21 with relapse-free survival was also found ( $P = 0.036$ , Figure 5B).



**Figure 4. Regulation of RECK mRNA Expression by MicroRNA-21 in Cholangiocarcinoma.** RBE cells were grown and transiently transfected with microRNA-21,lipo(Liposome control group), miR-21-NC(Negative control group)and microRNA-21-IN(miRNA-21 inhibition group), respectively and then subjected to RNA extraction and real-time PCR analysis (Figure 4A). Cells lysates were prepared and analyzed by Western blotting.The membranes were blotted by anti- RECK IgG and anti-GAPDH IgG (Figure 4B)



**Figure 5. Association of MicroRNA-21 Expression with Overall Survival and Relapse-free Survival of the Patients with Cholangiocarcinoma.** A, Overall survival (OS). B, Relapse-free survival (RFS)

**Discussion**

The current studies showed that a number of miRNAs were differentially expressed in cancer and normal tissues. In this regard, microRNA-21 has been suggested to function as an oncogene because it is overexpressed in many types of malignancy such as lung cancer (Liu et al., 2012), ovarian cancer (Iorio et al., 2007), colon cancer (Slaby et al., 2007), gastric cancer (Chan et al., 2008) and esophageal squamous cell carcinoma (Yukiharu Hiyoshi et al., 2009). Particularly, it had a high expression in the more aggressive cholangiocarcinoma cell and tissue samples (Florin et al., 2009). In vitro, to visualize microRNA-21 expression in different tumors, we performed in situ hybridization and real-time quantitative PCR in formalin-fixed, paraffin-embedded tissue (Figure 1, 2). The results showed the microRNA-21 expression in patients with lymph node metastasis or Perineural invasion was significantly higher than that in those without them,as suggested that abnormal expression of

microRNA-21 may be involved in the cholangiocarcinoma malignant process, and it may be involved in tumor invasion and metastasis relapse mechanisms. Due to the critical functions of its target proteins in various signaling pathways, microRNA-21 play a important role in the tumor proliferation and apoptosis by regulate some target proteins (Cioffi et al., 2010; Dong et al., 2012). So our next step will be to study which signaling pathways should influence cholangiocarcinoma invasion and metastasis ability.

We therefore further explored the role of microRNA-21 in cholangiocarcinoma cell lines in vivo. The cell invasion and metastasis assay showed that knockdown of microRNA-21 resulted in reduction RBE cell invasion and metastasis rate when compared with the control cells ( $P < 0.05$ , Figure 3A, B). In contrast, ectopic expression of microRNA-21 in RBE cells resulted in significant increased cell invasion and metastasis in transwell assays ( $P < 0.05$ , Figure 3C, D). Our current findings showed that microRNA-21 could significantly increase tumor cell invasion and metastasis capacity. This further suggests that microRNA-21 may play an role in regulation of cholangiocarcinoma cell invasion and metastasis.

Because RECK contains the binding sites of microRNA-21 in its 3'-UTR. So we focused our attention on RECK. Based on these various aspects of the microRNA-21 function, we hypothesized that microRNA-21 played an important role in the progression of cholangiocarcinoma and regulated RECK expression. Indeed, our current study revealed that RBE cells transfected with miR-21-IN had dramatically increased RECK expression, which is associated with suppression of the metastasis and invasion of cholangiocarcinoma. However, it is not clear whether inhibition of RECK expression by microRNA-21 is direct or indirect. Therefore, further studies are needed for clarification.

Furthermore, the association between microRNA-21 expression and prognosis has been shown in some types of cancer. It has been shown that high expression of microRNA-21 was associated with poor survival in patients with colon cancer (Schetter et al., 2008), pancreatic cancer (Dillhoff et al., 2008). In our study, microRNA-21 expressions was significantly higher in tumors with high tumor differentiation degree, lymph node metastasis, perineural invasion (Table 1). Survival analysis showed that the patients with higher expression levels of microRNA-21 often had poor prognosis (Figure 5A, B). Although the observation period was too short to evaluate the prognosis of 41 patients enrolled in the present study, these findings still show that a high expression of microRNA-21 be associated with a poor survival in cholangiocarcinoma patients and microRNA-21 could be further evaluated as a biomarker. Thus, microRNA-21 expression was an independent prognostic factor for cholangiocarcinoma patient.

In conclusion, we demonstrate for the first time that microRNA-21 is overexpressed in cholangiocarcinoma tissues, and knockdown of microRNA-21 could inhibit cellular invasion and metastasis and dramatically increase RECK levels in vitro. Furthermore, the microRNA-21 may play an important role in cholangiocarcinoma Occurrence

and development. Meanwhile, our study also provides some essential information for prediction of patient prognosis and identification of new treatment targets for future cholangiocarcinoma management. These findings raise the possibility that anti-microRNA-21 may have some potential therapeutic values in cholangiocarcinoma. Therefore, microRNAs, in particular microRNA-21, may serve as a potentially useful target for cholangiocarcinoma therapy.

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