Int J Clin Exp Med 2016;9(8):15547-15553
www.ijcem.com /ISSN:1940-5901/IJCEM0020938

Original Article

MiRNA-30a inhibits cell proliferation and migration by targeting IRS2 in human cholangiocarcinoma

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Received September 12, 2015; Accepted April 18, 2016; Epub August 15, 2016; Published August 30, 2016

Abstract: MicroRNAs are small non-coding RNAs that play a crucial role in malignant progression including proliferation, metastasis and invasion by suppressing target gene expression. Altered expression of miR-30a has been reported in some tumors including cholangiocarcinoma (CCA). In this study, we analyzed the expression of miR-30a in CCA tissues. We found that miR-30a was downregulated in cholangiocarcinoma tissues. Overexpression of miR-30a suppressed cell proliferation and migration, and induced cell cycle arrested in RBE and HCCC-9810 cells. Furthermore, bioinformatic analysis combined with real-time PCR and western blot demonstrated that IRS2 was a direct target gene of miR-30a. Furthermore, our results also shown that miR-30a suppressed the activation of AKT and ERK1/2. Taken together, these data suggest that miR-30a may act as a tumor suppressor and might be a potential target in cancer therapy of cholangiocarcinoma.

Keywords: MiRNA-30a, cholangiocarcinoma, proliferation, IRS2

Introduction

Cholangiocarcinoma (CCA) is a biliary tract malignancy, which are the second most common of primary liver cancers [1]. Over the past years, the incidence and mortality rate of CCA are increasing worldwide, with a very low 5-year survival rate. Although advanced in surgical techniques, chemotherapies and radiotherapies, the prognosis is still very poor [2, 3]. Therefore, it is urgent to uncover the molecular mechanisms underlying its growth and early metastasis.

MicroRNAs, a group of short non-coding RNAs, which regulate gene expression by the inhibition of the translation and/or decreasing of the stability of target mRNAs [4]. MiRNAs are deregulated in several diseases including cancers, where play important regulatory roles in cell growth, proliferation, differentiation and cell death [5]. To date, dysregulated miRNAs and their roles in cholangiocarcinoma development have attracted much attention. Some microRNAs such as miR-124 [6], miR-26a [7], and miR-34a [8] have been reported to participate in the initiation and progression of CCA. Recent studies have shown that dysregulation of miR-30a could inhibit cell proliferation, migration and invasion in a variety of cancer cells [9, 10]. However, the regulation of miR-30a in CCA and the mechanism remain largely unknown.

In this study, we investigated the role of miR-30a in cholangiocarcinoma (CCA). Our results showed that miR-30a was significantly downregulated in CCA tissues compared with adjacent normal tissues. Moreover, overexpression of miR-30a and knockdown of IRS2 both inhibited the proliferation and migration of cholangiocarcinoma cell lines. Our findings suggested that miR-30a has a tumor suppressive effect in intrahepatic cholangiocarcinoma by inhibiting cell proliferation and migration.

Materials and methods

Cell lines and cell culture

Two human cholangiocarcinoma cell lines (RBE and HCCC-9810) were purchased from Cell

Bank of Type Culture Collection Committee of the Chinese Academy of Sciences (Shanghai). Cells were cultured in RPMI-1640 medium (HyClone, Logan, UT, USA) at 37°C, 5% CO_2. Medium was supplemented with 10% fetal bovine serum (Gibco, NY, USA), 100 U/ml penicillin and 100 U/ml streptomycin.

Cell transfection

Cells were grown in the appointed medium 12-16 h before transfection. miR-30a mimics or non-specific controls (miR-NC) were designed and synthesized by Ribio Corporation (Guangzhou, China). RBE and HCCC-9810 cells were transfected with either 50 nM miR-30a mimics or miR-NC using Lipofectamine 2000 transfection reagent (Invitrogen) according to the instructions provided by the manufacturer. The IRS2 specific siRNA and scrambled siRNA (siR-NC) were also designed and synthesized by Ribio Corporation (Guangzhou, China). And cell transfection of siRNA were similar to above. Then cells were incubated at 37°C for 48 hours prior to further analysis.

RNA Isolation and qRT-PCR

Total RNA isolation from cell lines by TRIzol reagent (Life Technologies, CA, USA) according to the manufacturer's protocol. One Step PrimeScript miRNA cDNA Synthesis Kit (Takara, Dalian, China) was used to perform reverse transcription. And strictly following the manufacturer's instruction. Expression of miR-30a was assessed by quantitative real-time RT-PCR, and the small nuclear RNA U6 was used as an internal control. miR-30a and U6 detection primer were provided by Guangzhou RuiBo Biotechnology Company. To quantify mRNA levels, SYBR Premix Ex Taq (Takara, Dalian, China) was applied. To normalize mRNA expression levels, GAPDH was used as an endogenous internal control. Data analysis was performed using the $2^{-\Delta\Delta Ct}$ method.

Western blot

After transfected with miR-30a mimics and miR-NC in RBE and HCCC-9810 cells for 48 h, the cells were lysed to extract the proteins from the lysate. The proteins were separated in 12% SDS-PAGE and then transferred to a PVDF membrane. After blocking in 5% nonfat milk, the membranes were incubated with the following primary antibodies: IRS21:1000 (Cell Signaling Technology) and b-actin antibody 1:2500 (Sigma) at 4°C overnight. After washing off the primary antibodies, the membrane was incubated with HRP-conjugated secondary antibody for 1 h; ECL kit was used to develop the immunoreactive bands.

Cell proliferation assay

MTS assay was adopted to assess the impact of miR-30a on the RBE and HCCC-9810 cells proliferation. Cells in logarithmic grow phase, 6×10^3/ml, were seeded in 96-well microplates, 100 μl/well, and cultured overnight to allow attachment. After transfected with miR-30a mimics and miR-NC in RBE and HCCC-9810 cells under standard condition for 12 h, 24 h, 48 h and 72 h incubation. WST-8 was metabolized producing a chromogen and the absorbance at 450 nm was measured after 2 h reaction using a DU640 spectrophotometer (Beckman Coulter, CA, USA).

Cell cycle assay

RBE and HCCC-9810 Cells were cultured in a 6-well plate at 1×10^6 cells/well, after transfection for 48 h. For cell cycle analysis, cells were washed with PBS and fixed in 75% ethanol at -20°C for 24 h, then PBS washes for three times and stained with 50 μg/m propidium iodide (PI) for 30 min away from light. Then, the cell cycle was analyzed with a FACS Epics XL (Beckman Coulter, CA, USA).

Wound healing assay

Wound healing assays were performed to evaluate cell migration activity. Briefly, transfected cells were seeded onto the 6-well plate growing to 80-90% confluency and starved for 24 h, and then the cell monolayers were carefully wounded with a 200-μl pipette. After washing with PBS for three times, and cultured in serum free 1640 medium for 24 h at 37°C and 5% CO_2. Cell migration was monitored under a microscope TE2000 (Nikon, JAPAN). The experiment was performed in triplicate.

3'-UTR reporter assay

The IRS2 3'-UTR (2000 bp) was cloned into a pmirGLO Dual Luciferase miRNA Target expres-

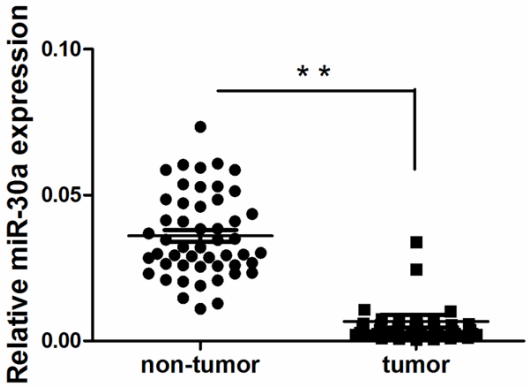

Figure 1. miR-30a is down-regulated in human cholangiocarcinoma tissues. Expression of miR-15a in 50 pairs cholangiocarcinoma tissues and their adjacent non-tumor tissues by real-time PCR. **P<0.001.

sion vector (Promega Corporation, Madison, WI, USA). For luciferase reporter assays, HEK-293T cells at 50% confluence in 96-well plates were co-transfected with miR-30a or miR-NC using Lipofectamine™ 2000. Twenty-four hours later, cells were co-transfected with 50 ng of firefly luciferase reporter containing wild-type or mutant 3'-UTR of target gene. After transfected for 48 h, the firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega). Luciferase activity was normalized for transfection efficiency using the corresponding Renilla luciferase activity. All experiments were performed at least three times.

Statistical analysis

Data are presented as mean ± standard deviation (SD). The Student's t-test was used for statistical analysis, and *P* values of less than 0.05 were regarded as statistically significant.

Results

Expression of miR-30a was down-regulated in human cholangiocarcinoma tissues

To investigate the role of miR-30a in human cholangiocarcinoma pathogenesis, we different, we examined the expression of miR-30a in 50 pairs tumor and nontumor tissues of human cholangiocarcinoma samples by real-time PCR. The result showed that the miR-30a expression was down-regulated in cholangiocarcinoma tissues compared with the corresponding adjacent normal tissues (**Figure 1**). These data suggested that aberrant expression of miR-30a inhibited proliferation capability of human cholangiocarcinoma cells.

miR-30a inhibit cell proliferation and regulated cell cycle in vitro

To further confirm the role of miR-30a during cholangiocarcinoma progression, miR-30a mimic and miR-NC was transfected into RBE and HCCC-9810 cells, respectively. CCK-8 assay showed that miR-30a introduction can suppress the cell growth in RBE and HCCC-9810 cells (**Figure 2A**). To determine whether cell cycle was a contributing factor to cell growth inhibition, we analyzed the effect of miR-30a expression on cell cycle of human cholangiocarcinoma cells. As shown in **Figure 2**, miR-30a transfected contributed to delay the cell cycle progression and induced S phase arrested in RBE and HCCC-9810 cells, which compared with miR-NC transfected cells (P<0.01). These observations suggest that miR-30a had an important role in reducing the cell growth of cholangiocarcinoma cells by inhibiting cell cycle.

miR-30a suppresses the migration of CCA cells

To further investigate the effect of miR-30a on RBE and HCCC-9810 cells migration, we transfected the cells with miR-30a mimics or miR-NC and then used wound healing assay to detect the function of miR-30a on cell migration. As shown in **Figure 3**. The miR-30a mimics suppressed the potential of RBE and HCCC-9810 cells migration. These results suggested that miR-30a could inhibit cholangiocarcinoma cells migration.

miR-30a directly targets IRS2 in CCA cells

Based on the Sanger miRNA database and TargetScan software, we predicted the targeted gene of miR-30a (**Figure 4A**), and found that the 3'-UTR of the IRS2 gene contains one 7-mer miR-30a-binding seed sequences that are conserved. We therefore used 3'UTR luciferase reporter assay to verify whether miR-30a directly targets IRS2. Luciferase reporter assays demonstrated that miR-30a significantly repressed activity of reporter vectors harboring wild-type 3'-UTRs of IRS2, whereas mutations of putative miR-30A-bingding sites in these 3'-UTR regions partia lly mitigated repression of

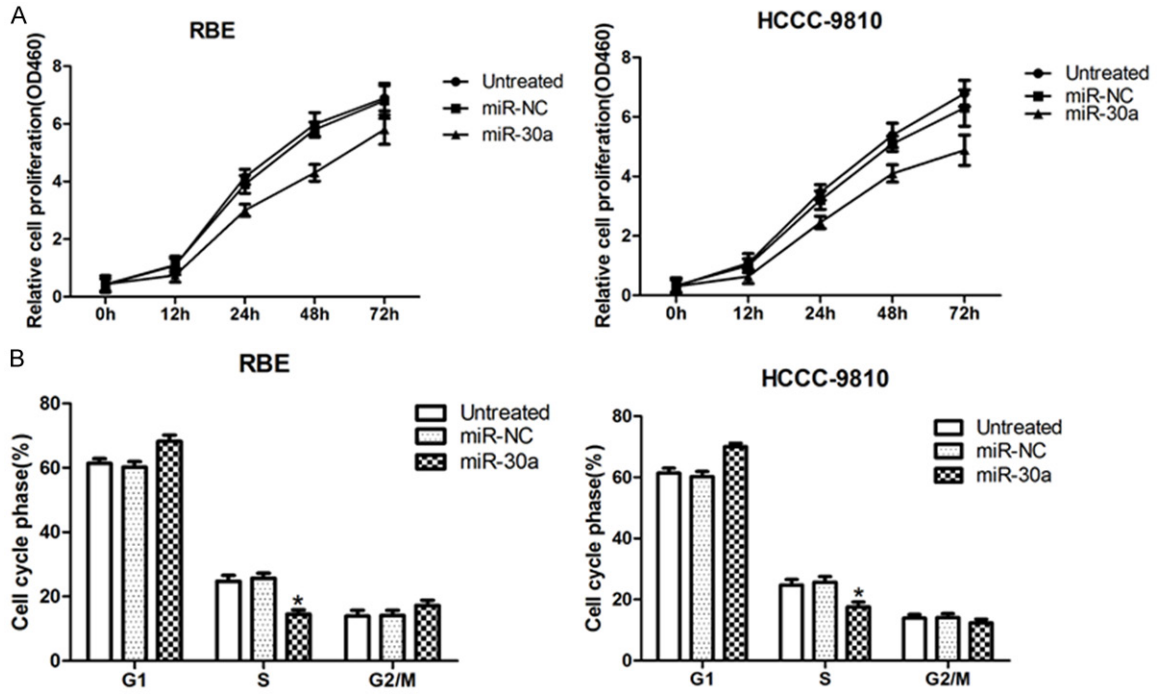

Figure 2. miR-30a inhibits cholangiocarcinoma cell proliferation and induced cell cycle arrested. A. CCK-8 assay was employed to evaluate the effect of miR-30a on cell proliferation in RBE and HCCC-9810 cells. B. The effect of miR-30a on cell cycle progression of in RBE and HCCC-9810 cells were done by flow cytometry. *P<0.05 vs. miR-NC group.

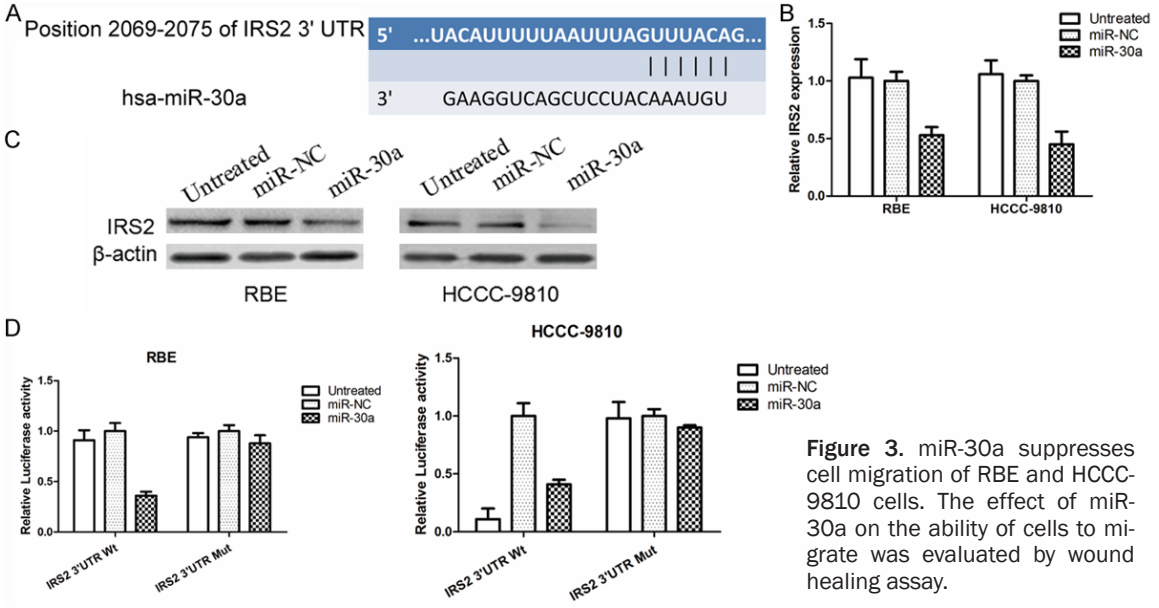

Figure 3. miR-30a suppresses cell migration of RBE and HCCC-9810 cells. The effect of miR-30a on the ability of cells to migrate was evaluated by wound healing assay.

miR-30a, when compared to the parental vector (**Figure 4B**). Furthermore, Real-time PCR and western blot analyses showed that mRNA and protein levels of IRS2 were dramatically down-regulated in RBE and HCCC-9810 cells when transfected with miR-30a mimics compared to miR-NC group. Therefore, our data suggested that miR-30a repressed IRS2 mRNA and protein levels via direct targeting of the 3'-UTR of IRS2.

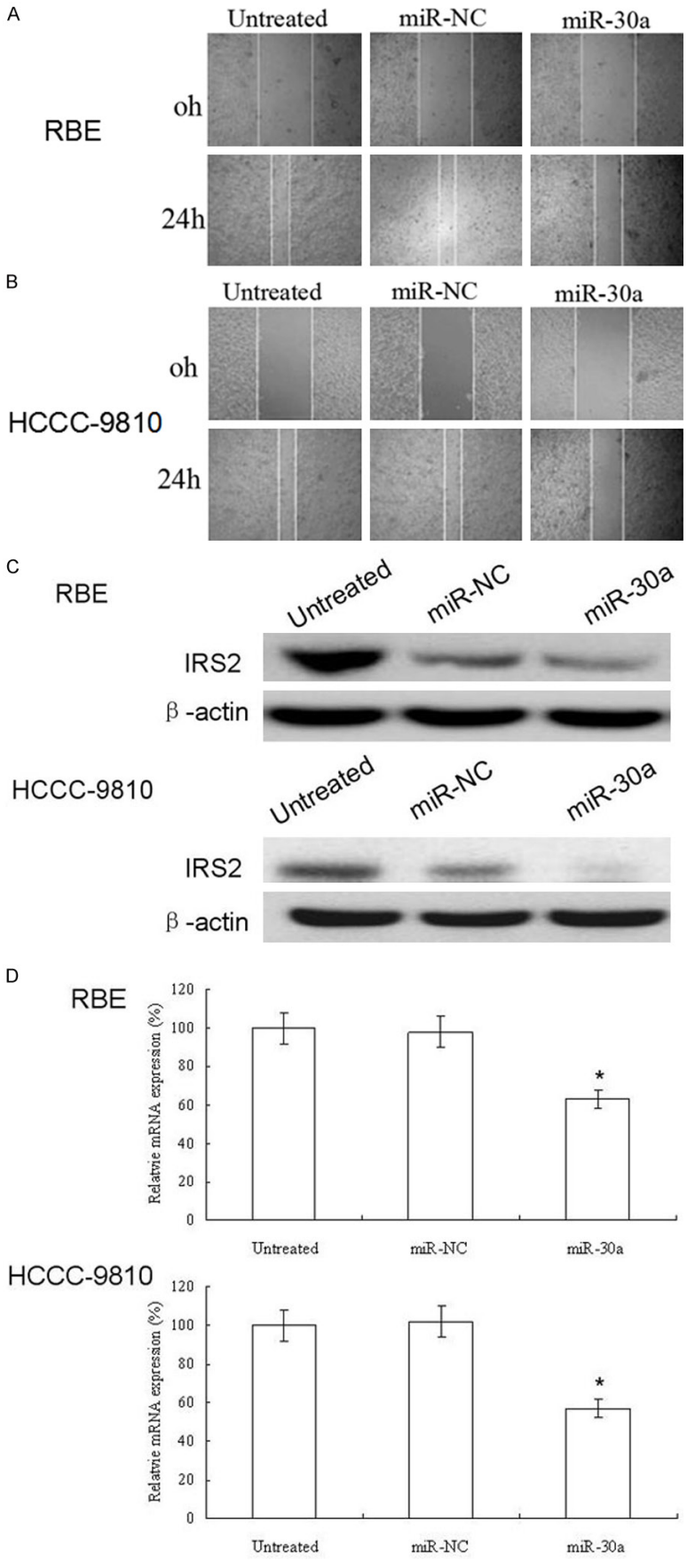

Figure 4. IRS2 is one of direct target of miR-30a. A. The predicted miR-30a binding site within the IRS2 3'-UTR; B. mRNA levels were detected by real-time PCR in RBE and HCCC-9810 cells after transfected with miR-30a mimic or miR-NC; C. Protein levels were detected by western blot analyses in RBE and HCCC-9810 cells after transfected with miR-30a mimic or miR-NC; D. Relative firefly luciferase activity was evaluated by 3'-UTR report assay after cotransfected with IRS2 WT 3'-UTR or Mut 3'-UTR, either miR-30a mimics or miR-NC. *P<0.05 vs. miR-NC group.

MiR-30a regulate PI3K/AKT and MAPK pathway

To further explore the underlying mechanisms of miR-30a regulating IRS2, we investigated whether miR-30a could regulate downstream signaling of IRS2. It is well-documented that the IRS2 is a critical adaptor of the insulin signaling cascade, which activated PI3K/Akt and MAPK pathways [11]. So we investigated the effects of miR-30a on Akt and Erk1/2 phosphorylation. We transfected RBE and HCCC-9810 cells with miR-30a mimics and miR-NC, followed by immunoblot analysis of Akt and Erk1/2 phosphorylation. As showed in **Figure 5**, the phosphorylation of Akt and Erk1/2 down-regulated by miR-30a compared to miR-NC group. These data demonstrated that PI3K/AKT and MAPK relevant pathway involved in miR-30a inhibited proliferation and migration in CCA cells.

Discussion

In this study, we founded that a significant downregulation of miR-30a expression in human cholangiocarcinoma tis-

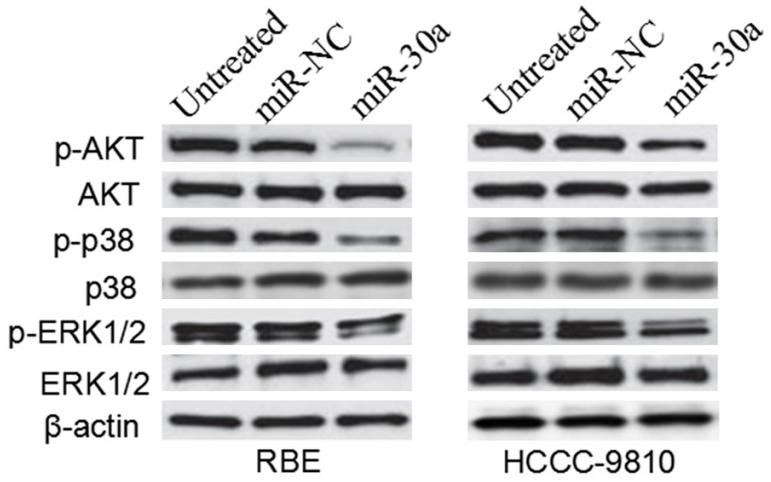

Figure 5. MiR-30a regulate PI3K/AKT and MAPK pathway in CCA cells. phosphorylation of AKT, p38 MAPK and ERK1/2 were detected by western blot analyses after transfected with miR-30a or miR-NC in RBE and HCCC-9810 cells.

sues as compared with adjacent normal tissues. These results also confirmed by another study that miR-30a expression was significantly downregulated in liver cancer tissues. In addition, we found that over-expression of miR-30a induced cell cycle arrested, inhibited the cell proliferation and migration of CCA cell lines in vitro. These indicated that miR-30a inhibited cell growth and migration of cholangiocarcinoma cell through induction cell cycle arrested.

IRS2 was a cytoplasmic signaling molecule that mediates effects of insulin, insulin-like growth factor 1, and other cytokines by acting as a molecular adaptor between diverse receptor tyrosine kinases and downstream effectors [12, 13]. It has been report that overexpression of IRS2 can promote cellular proliferation, cell motility and invasion in neuroblastoma and mesothelioma cells [14, 15]. IRS2 expression is up-regulated in hepatocellular carcinoma, down-regulation of IRS-2 expression increased cell and liver tumor progression [16]. In the current study, to investigate the underlying mechanisms of miR-30a in CCA cells, we predicted the target gene of miR-30a in CCA cells, and found that IRS2 might be the potential target of miR-30a. The results of 3'UTR report assay, real-time PCR and western blot suggested that IRS2 was a direct target of miR-30a. The result indicated that miR-30a might function as a tumor suppressor partly mediated by repressing IRS2 expression.

Akt is an important protein kinase in the signaling pathway and a downstream target protein of P13K. The p-Akt is the active form of phosphorylated Akt and closely related to tumor development and progression [17]. The MAPK signaling is mediated by p38 MAPK, ERK1/2 and JNK, which are important in the control of cell proliferation, apoptosis, migration and invasion [18]. It was well known that PI3K/AKT and p38MAPK/JNK pathway were downstream pathway of IRS2. We decided to investigate the effect of miR-30a overexpression on PI3K/AKT and p38MAPK/JNK pathway. In current study, we found that miR-30a suppressed the phosphorylation of Akt and Erk1/2. Demonstrated that AKT and MAPK relevant pathway involved in miR-30a inhibited proliferation and migration in CCA cells.

In conclusion, this study shows that miR-30a function as a tumor suppressor miRNA in human cholangiocarcinoma via inhibiting IRS2 expression. Over- expression of miR-30a has the effect of suppressing cholangiocarcinoma cell growth and migration, and induced cell cycle arrested in vitro by regulating IRS2, PI3K/AKT and regulated p38MAPK/JNK pathway. Our findings prompt important roles of miR-30a in cholangiocarcinoma etiology and have potential as a new therapeutic target for treating cholangiocarcinoma patients.

Disclosure of conflict of interest

None.

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