

Original Article

miR-21 regulates proliferation and invasion of hilar cholangiocarcinoma cells

Chunsheng Nie, Houbin Sun, Hongbin Li, Jia Liu

Department of Interventional Radiology, Harbin Medical University Cancer Hospital, Heilongjiang, China

Received April 19, 2016; Accepted July 22, 2016; Epub September 1, 2016; Published September 15, 2016

Abstract: Previous studies demonstrated difference of micro-RNA expression between hilar cholangiocarcinoma and normal tissues. Further, miR-21 was reported to be overexpressed in hilar cholangiocarcinoma cells. Thus, our study aimed to elucidate association between miR-21 and cell behaviors of hilar cholangiocarcinoma and explore potential mechanisms. miR-21 activator and miR-21 inhibitor were designed and synthesized according to miR-21 sequence. RBE cell line was purchased for cell model. Three groups were prepared for different intervention, including activator group, inhibitor group, and control group. Transient expression model was established with transfection of miR-21 activator or miR-21 inhibitor, respectively. Cell proliferation was analyzed with CCK-8 examination. Cellular migration was examined with cell wound scratch assay. Transwell assay was performed to examine effect of miR-21 on tumor invasion. PTEN expression was determined with Western blot. Compared with control group, miR-21 activator group showed significantly increased proliferation, migration, and invasion ($P < 0.05$), while miR-21 inhibitor group demonstrated reduced proliferation, migration, and invasion ($P < 0.05$). Moreover, PTEN expression group was remarkably inhibited after miR-21 activator transfection, but enhanced after miR-21 inhibitor transfection ($P < 0.05$). miR-21 could influence proliferation, migration, and invasion of hilar cholangiocarcinoma cells via regulating PTEN expression.

Keywords: miR-21, RBE, proliferation, migration, invasion

Introduction

Hilar cholangiocarcinoma (HCCA), also called as Klatskin tumor, was a common malignant tumor in human biliary system. Surgical treatment for HCCA was always challenging due to non-specific lesion sites and infiltrative growth of tumor. Thus, HCCA cannot be eradicated by operation [1]. On the other side, following the development of medical technology and equipment, great progress has been made in diagnosis and treatment of HCCA. For example, the rate of tumor resection gradually increased, and the survival rate was improved [2, 3]. However, clinic application still had limitation since the pathogenesis of HCCA still remained unclear. In addition, the risk of the disease and complexity of mechanisms were elevated by HCCA complications, including primary sclerosing cholangitis, choledochocyst, calculus of bile duct, biliary benign tumor, biliary parasitic disease, and hepatitis, etc. [4].

As a member of PTP gene family, PTEN is an acknowledged tumor suppressor gene. Previous studies showed that PTEN-PI3K-AKT signaling pathway regulated tumor angiogenesis and played a pivotal role in tumor cell behaviors, such as tumor cell cycle, growth inhibition, apoptosis, adhesion, migration, and invasion [5]. Moreover, PTEN could be used as prognostic factor for most tumors [6].

MicroRNA is a kind of small non-coding RNA fragments with a structure of 22 nucleotides. Complete complementary targeting between microRNA and mRNA always results in degradation of target gene [7]. With proven technique developed, more microRNAs became the predictor for tumor in early stage, and some even were transformed into the potential therapeutic targets [8-10]. Therefore, investigating association between microRNA and HCCA is of great clinical significance. What's more, both clinical trials and animal models demonstrated differ-

miR-21 in hilar cholangiocarcinoma

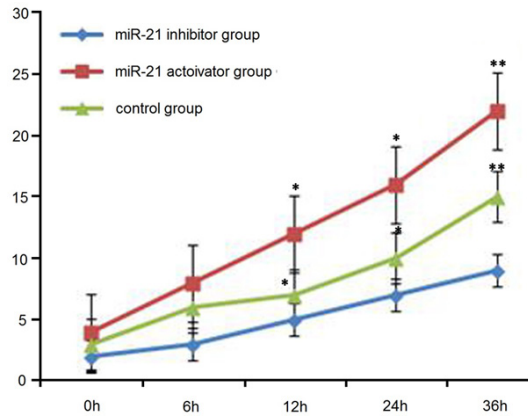


Figure 1. Proliferation of RBE cells in different groups. **: significance compared with control group ($P < 0.01$). *: significance compared with control group ($P < 0.05$).

ence of miR-21 expression between tumor and normal tissues, suggesting miR-21 was a potential target for HCCA [11-13].

Our study was focused on explore the detailed mechanism to better understand the role of miR-21 in HCCA. More specifically, RBE cell transfection model was established for detection of cell behaviors and PTEN expression. We expected to provide more data to promote application of miR-21 in HCCA treatment.

Methods and materials

Materials

RBE cell line was purchased from Sunbio (China) and stored in liquid nitrogen. Reagents for cell culture were purchased from Gibco (USA), including RPMI1640 medium, trypsin, fetal bovine serum, and phosphate buffer. Culture dish was purchased from Corning (USA). Transwell chamber and Matrigel were purchased from BD company (USA). CCK-8 detection kit was purchased from Beyotime Biotechnology (China).

miR-21 activator and miR-21 inhibitor were synthesized in Shanghai Sangon Company (China) as follows: UAGCUUAUCAGACUGAUGUUGA (activator); UGUCGGGUAGCUGACCACAACG (inhibitor).

PTEN antibody was purchased from Santa Cruz (USA). Western blot reagents were purchased from Beyotime Biotechnology (China).

Transfection and Western blot

Frozen RBE cells were resuscitated and transformed into incubator at 37°C for 5 minutes. Then the cells were centrifuged at low speed (1000 rpm/min) for 3 minutes and seeded in RPMI 1640 medium containing 10% fetal bovine serum. The cells were cultured till the number reached 1×10^5 /dish (60 mm) and digested by 0.25% trypsin for transfection. Next, the cells were electrotransfected according to routine protocol (voltage 4 V, 0.004 second). A total of 2 μ g miR-21 activator or miR-21 inhibitor were transfected, respectively. Western Blot was performed to detect PTEN expression at 36 hours after transfection, and β -actin was used as internal marker.

CCK-8 examination for proliferation

Both post-transfection RBE cells and control RBE cells were cultured in incubator with saturation humidity at 37°C and 5% CO₂ for 6 h, 12 h, 24 h, and 36 h, respectively. Then the cells were counted and seeded in 96-well microtiter plate at 1×10^4 /well in 200 μ l for 12 hours. Next, 10 μ l CCK8 reaction solution was added for another 3 hours. Absorbance value (450 nm) was examined using ultraviolet spectrophotometer to calculate rate of cell proliferation and inhibition.

Tumor invasion examined with transwell model

Matrigel (70 μ l/well) was put onto the polycarbonate membrane (bore diameter 8 μ m) in 24-well transwell model at 37°C for 60 minutes. A total of 200 μ l cell suspension at 1×10^5 /ml in BSA-treated medium (Fetal bovine serum free) was added into the upper chamber, while 1000 μ l complete medium was added into the lower chamber. After cultured at 37°C and 5% CO₂ for 6 h, 12 h, and 24 h, respectively, the membrane was stained by crystal violet (0.1%) to identify the penetrated cells under 100 \times fluorescence inversion microscope.

Cell wound scratch assay to examine migration

Cell wound scratch experiment was performed in routine protocol [13]. RBE cells were scratched by a 200 μ l pipette tip and cultured in incubator with saturation humidity at 37°C and 5% CO₂. After indicated time, the cells were photographed and analyzed with Image J software to examine scratch width. Six places were randomly enrolled from scratches for analysis of migration distance.

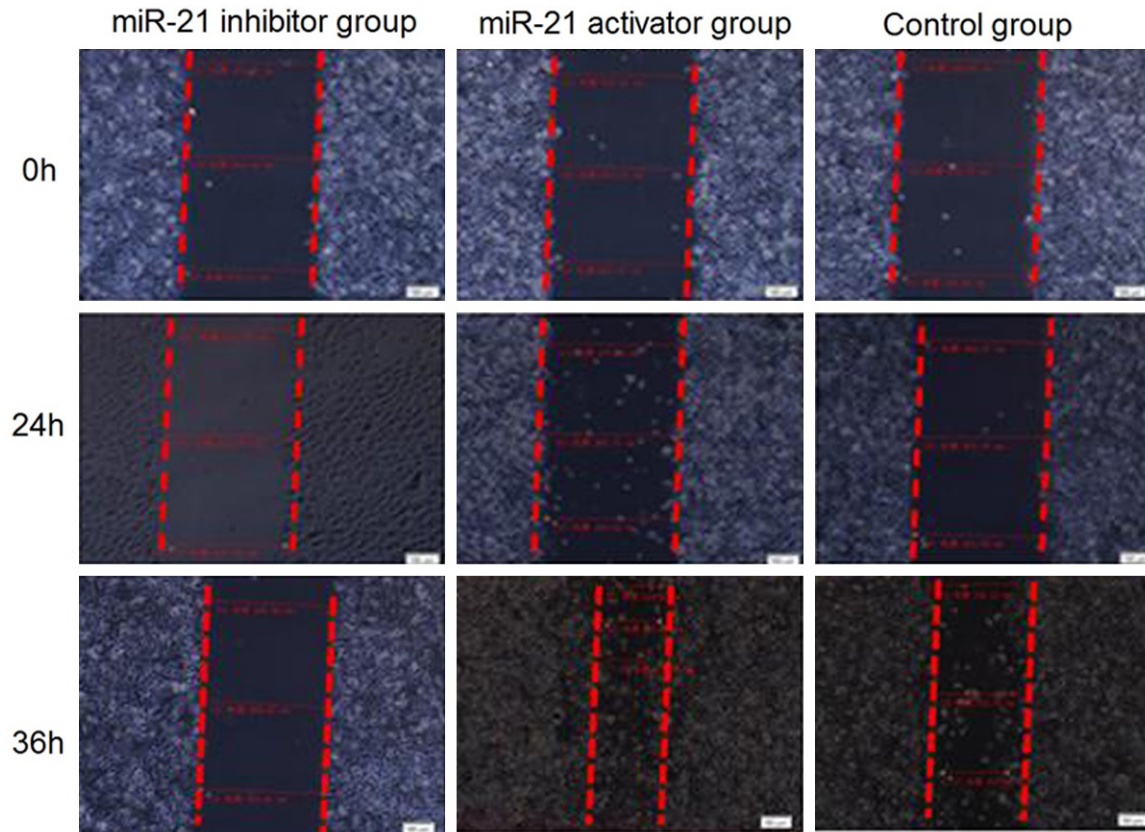


Figure 2. Cell wound scratch assay of three groups at different time points.

Statistical analysis

SPSS13.0 was used for data analysis. Measurement data was compared by t test. All experiments above were repeated at least three times. P value <0.05 was considered to be statistically significant.

Results

miR-21 enhanced proliferation of RBE cells

Proliferation of RBE cells was examined with CCK-8 kit for three different groups (**Figure 1**). Compared with control group, miR-21 activator group exhibited a significant higher level of proliferation ($P<0.05$). More specifically, miR-21 activator significantly enhanced proliferation of RBE cells at 12-hour after transfection with time-dependent ($P<0.01$). In addition, miR-21 inhibition significantly attenuated proliferation of RBE cells at 12-hour after transfection with time-dependent ($P<0.01$). It suggested that miR-21 intervention obviously regulated cell proliferation from 12 hours after transfection.

miR-21 influenced migration of RBE cells

Cell wound scratch assay demonstrated that miR-21 was associated with migration of RBE cells (**Figure 2**). At 24-hour after transfection, migration distances in three groups were $4.23 \pm 1.13 \mu\text{m}$ (miR-21 inhibitor), $24 \pm 6.85 \mu\text{m}$ (miR-21 activator), and $10.32 \pm 3.13 \mu\text{m}$ (control), respectively. At 36-hour after transfection, migration distances in three groups were $7.23 \pm 2.46 \mu\text{m}$ (miR-21 inhibitor), $38 \pm 7.14 \mu\text{m}$ (miR-21 activator), and $18.32 \pm 3.41 \mu\text{m}$ (control), respectively. They elucidated that enhancing miR-21 expression markedly promoted migration of RBE cells ($P<0.05$), while abrogating miR-21 expression significantly attenuated migration of RBE cells ($P<0.05$), suggesting miR-21 could regulate migration of RBE cells. Moreover, the effect of miR-21 on migration of RBE cells showed time dependence.

miR-21 regulated invasion of RBE cells

Invasion of RBE was detected at 12-hour, 24-hour, and 36-hour after transfection,

miR-21 in hilar cholangiocarcinoma

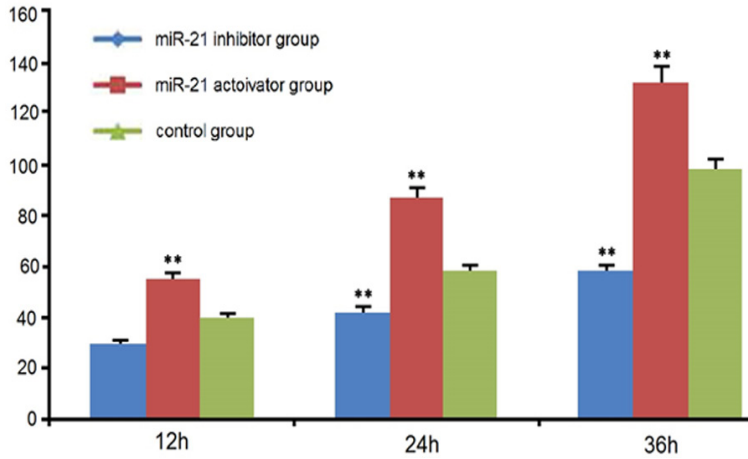


Figure 3. Analysis of crystal violet staining (0.1%) in three groups. **: significance compared with control group ($P < 0.01$).

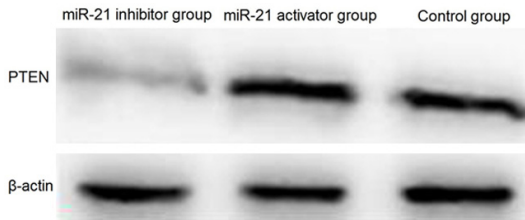


Figure 4. PTEN expression among three groups at 36-hour after transfection.

respectively, by transwell assay. Compared with control group, miR-21 activator group presented a significant higher level of cell invasion (**Figure 3**, $P < 0.05$); Compared with control group, miR-21 inhibitor group exhibited a significant lower level of invasion (**Figure 3**, $P < 0.05$).

miR-21 influenced cell behaviors of RBE cells via regulating PTEN

Western Blot demonstrated significant differences of PTEN expression among three groups at 36-hour after transfection (**Figure 4**). Compared with control group, PTEN in miR-21 activator group significantly decreased, while PTEN in miR-21 inhibitor group significantly upregulated.

Discussion

As a kind of chronic wasting fatal disease, tumor gradually jeopardizes body function so as to cause dysplasia or prosoplasia. Once can-

ceration occurred, elimination of carcinogenic factor cannot stop tumor progressing. Tumor is characterized with infinite growth and invading to adjacent organs [14, 15]. Previous studies have proved that tumor malignancy was depended on proliferation, migration, and invasion of cancer cells, which were also the main action targets of tumor suppressor genes. Moreover, when gene disorder occurred in regulation of gene expression, especially in tumor suppressor genes, tumor metastasis was often triggered to cause spread of cancer cells [16, 17]. Thus, latest

researches are focused on mechanisms underlying regulation of tumor suppressor genes, including microRNA or lncRNA.

Studies have showed difference of miR-21 expression between tumor tissues and normal tissues, and miR-21 might influence migration or invasion of cancer cells. However, it is unclear how miR-21 influence cell behaviors of tumor [18, 19]. Our study proved that miR-21 promoted migration and invasion of cancer cells, verified by Cell wound scratch assay and transwell model, respectively. Furthermore, it was found that miR-21 enhanced proliferation of RBE cells, suggesting miR-21 had more potential effects on tumor. Transfection experiment elucidated that miR-21 expression was positively correlated with RBE cell behaviors, suggesting regulation of miR-21 could be a potential therapeutic target for HCCA.

PTEN was involved in tumor progress via PI3K/PTEN signaling pathway. In addition, studies demonstrated PTEN not only regulated various cell behaviors of tumor, such as cell cycle, proliferation, apoptosis, migration, and invasion, but also influenced multiple tumors, including invasive bladder cancer, papillary thyroid carcinoma, and endometrial cancer [20]. Accordingly, PTEN was proposed as a potential target of miR-21 in our study. Furthermore, Western Blot experiment demonstrated miR-21 expression was negatively correlated with PTEN expression. It indicated miR-21 could inhibit PTEN expression so as to influence cell behaviors of tumor.

In conclusion, our study proved that miR-21 promoted multiple cell behaviors of HCCA via inhibiting PTEN expression. Thus, inhibiting miR-21 expression is of clinical significance for HCCA treatment, and future studies are needed to elucidate the specific mechanisms.

Acknowledgements

This project supported by the National Natural Science Foundation of China Youth Foud (NO. 81400392).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Chunsheng Nie, Department of Interventional Radiology, Harbin Medical University Cancer Hospital, 150 Nangang District Haping Road, Heilongjiang, China. Tel: +86-451-86298000; Fax: +86-451-86298000; E-mail: chunshengnies@sina.com

References

- [1] Allo G, Bernardini MQ, Wu RC, Shih le M, Kalloger S, Pollett A, Gilks CB and Clarke BA. ARID1A loss correlates with mismatch repair deficiency and intact p53 expression in high-grade endometrial carcinomas. *Mod Pathol* 2014; 27: 255-261.
- [2] Bellini MF, Cadamuro AC, Succi M, Proenca MA and Silva AE. Alterations of the TP53 gene in gastric and esophageal carcinogenesis. *J Biomed Biotechnol* 2012; 2012: 891961.
- [3] Agarwal A, Durairajanayagam D, Halabi J, Peng J and Vazquez-Levin M. Proteomics, oxidative stress and male infertility. *Reprod Biomed Online* 2014; 29: 32-58.
- [4] Dufau ML and Tsai-Morris CH. Gonadotropin-regulated testicular helicase (GRTH/DDX25): an essential regulator of spermatogenesis. *Trends Endocrinol Metab* 2007; 18: 314-320.
- [5] Lasko P. The DEAD-box helicase Vasa: evidence for a multiplicity of functions in RNA processes and developmental biology. *Biochim Biophys Acta* 2013; 1829: 810-816.
- [6] Borniger JC, Cisse YM, Cantemir-Stone CZ, Bolon B, Nelson RJ and Marsh CB. Behavioral abnormalities in mice lacking mesenchyme-specific Pten. *Behav Brain Res* 2016; 304: 80-85.
- [7] Pek JW and Kai T. A role for vasa in regulating mitotic chromosome condensation in *Drosophila*. *Curr Biol* 2011; 21: 39-44.
- [8] Li B, Lu Y, Wang H, Han X, Mao J, Li J, Yu L, Wang B, Fan S, Yu X and Song B. miR-221/222 enhance the tumorigenicity of human breast cancer stem cells via modulation of PTEN/Akt pathway. *Biomed Pharmacother* 2016; 79: 93-101.
- [9] Katoh M. Cardio-miRNAs and onco-miRNAs: circulating miRNA-based diagnostics for non-cancerous and cancerous diseases. *Front Cell Dev Biol* 2014; 2: 61.
- [10] Kim JG, Lee SJ, Chae YS, Kang BW, Lee YJ, Oh SY, Kim MC, Kim KH and Kim SJ. Association between phosphorylated AMP-activated protein kinase and MAPK3/1 expression and prognosis for patients with gastric cancer. *Oncology* 2013; 85: 78-85.
- [11] Krstic M, Stojnev S, Jovanovic L and Marjanovic G. KLF4 expression and apoptosis-related markers in gastric cancer. *J BUON* 2013; 18: 695-702.
- [12] Leal MF, Calcagno DQ, Khayat AS, Silva TC, Muniz JA, Assumpcao PP, de Arruda Cardoso Smith M and Burbano RR. hTERT and TP53 deregulation in intestinal-type gastric carcinogenesis in non-human primates. *Clin Exp Med* 2013; 13: 221-224.
- [13] Lee HE, Han N, Kim MA, Lee HS, Yang HK, Lee BL and Kim WH. DNA damage response-related proteins in gastric cancer: ATM, Chk2 and p53 expression and their prognostic value. *Pathobiology* 2014; 81: 25-35.
- [14] Mafficini A, Amato E, Fassan M, Simbolo M, Antonello D, Vicentini C, Scardoni M, Bersani S, Gottardi M, Rusev B, Malpeli G, Corbo V, Barbi S, Sikora KO, Lawlor RT, Tortora G and Scarpa A. Reporting tumor molecular heterogeneity in histopathological diagnosis. *PLoS One* 2014; 9: e104979.
- [15] Park H, Cho SY, Kim H, Na D, Han JY, Chae J, Park C, Park OK, Min S, Kang J, Choi B, Min J, Kwon JY, Suh YS, Kong SH, Lee HJ, Liu ET, Kim JI, Kim S, Yang HK and Lee C. Genomic alterations in BCL2L1 and DLC1 contribute to drug sensitivity in gastric cancer. *Proc Natl Acad Sci U S A* 2015; 112: 12492-12497.
- [16] Sehdev V, Katsha A, Arras J, Peng D, Soutto M, Ecsedy J, Zaika A, Belkhiri A and El-Rifai W. HDM2 regulation by AURKA promotes cell survival in gastric cancer. *Clin Cancer Res* 2014; 20: 76-86.
- [17] Tanaka T, Fujimura A, Ichimura K, Yanai H, Sato Y, Takata K, Okada H, Kawano S, Tanabe S and Yoshino T. Clinicopathological characteristics of human epidermal growth factor receptor 2-positive Barrett's adenocarcinoma. *World J Gastroenterol* 2012; 18: 6263-6268.
- [18] Xiang B, Mi YY, Li TF and Liu PF. Updated meta-analysis of the TP53 Arg72Pro polymorphism and gastric cancer risk. *Asian Pac J Cancer Prev* 2012; 13: 1787-1791.
- [19] Zhang HQ, He B, Fang N, Lu S, Liao YQ and Wan YY. Autophagy inhibition sensitizes cisplatin cytotoxicity in human gastric cancer cell line

miR-21 in hilar cholangiocarcinoma

- SGC7901. *Asian Pac J Cancer Prev* 2013; 14: 4685-4688.
- [20] Alexandrova E, Miglino N, Hashim A, Nassa G, Stellato C, Tamm M, Baty F, Brutsche M, Weisz A and Borger P. Small RNA profiling reveals de-regulated phosphatase and tensin homolog (PTEN)/phosphoinositide 3-kinase (PI3K)/Akt pathway in bronchial smooth muscle cells from asthmatic patients. *J Allergy Clin Immunol* 2016; 137: 58-67.