MicroRNA214 expression inhibits HCC cell proliferation through PTK2b/ Pyk2

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ARTICLE INFO

ABSTRACT

MicroRNAs (miRNAs/miRs) are crucial regulatory molecules that act as the most significantly downregulated microRNAs in hepatocellular carcinoma (HCC). PTK2b/Pyk2 is a non-receptor protein tyrosine kinase, which plays an important role in the development and metastasis of cancer. In this study, we explored the expression level and functional relationship between MicroRNA-214 (miR-214) and PTK2b/Pyk2 in liver cancer cells. For this purpose, we analyzed the expression of miR-214 and PTK2b/Pyk2 in 38 cases of HCC and paired non-neoplastic tissue specimens using real-time PCR. MTT, cell cycle and construct recombinant plasmids analysis were used to explore the effects of miR-214 and PTK2b/Pyk2 on liver cancer cell proliferation. Results showed that the expression level of mir-214 in liver cancer tissues and liver cancer cell lines was significantly lower than that in normal tissues and cells, while the expression of PTK2b/Pyk2 was significantly increased. The overexpression of mir-214 or inhibition PTK2b/Pyk2 inhibited the proliferation of HCC cells. This research showed that mir-214 has an inhibitory effect on liver cancer through the expression of PTK2b/Pyk2.

DOI: http://dx.doi.org/10.14715/cmb/2022.68.1.4

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Introduction

Hepatocellular carcinoma (HCC), as one of the most common malignancies in the world, ranks 3rd in the mortality rate of all cancers (1). HCC progression involves quite a lot of risk factors, and there remains tremendous difficulty in the treatment of HCC (2). Thus, figuring out the potential molecular mechanism and the novel therapeutic targets is of great significance for the treatment of HCC.

Dysregulation or dysfunction of miRNAs may be involved in the progression of cancers, which may closely correlate with the development, progression and clinical prognosis of the HCC. At present, various studies have focused on the roles of miRNAs in HCC, including miR-224, miR-106b and miR-21, which may participate in the progression of HCC (1, 3). However, the specific molecular mechanism of miRNA down-regulation in the development and progression of HCC remains unknown. Previously, miR-214 is one of the miRNAs with the most obvious down-regulation in HCC patients (4). β-catenin and hepatoma-derived growth factor (HDGF), as the target genes of miR-214, can curb the growth and invasion of hepatic carcinoma (5). Nevertheless, there is no adequate evidence showing the other potential target genes and the molecular functions of miR-214 in HCC progression.

PTK2B/Pyk2, as the member of focal adhesive kinase family, can transmit the cell signals by phosphorylation of the downstream substrate (6, 7). Generally, PTK2B/Pyk2 has three major functions relating to cell survival, proliferation and migration (8, 9). Furthermore, PTK2B/Pyk2 exerts a key role in the development and progression of multiple cancers, and at present, the effects of PTK2B/Pyk2 on the cancer are mainly summarized as follows: (i) PTK2B/Pyk2 is excessively expressed in many cancers; (ii) The abnormal expression of
PTK2B/Pyk2 in some cancer is closely associated with the poor prognosis; (iii) In many animal models, the interference of PTK2B/Pyk2 expression can block the development and metastasis of tumors (10).

In this study, PTK2B/Pyk2 is considered as one of the functional downstream targets of miR-214, and to be involved in the HCC progression.

Materials and methods

Subjects and HCC samples

In Yichang Central People’s Hospital, 38 primary HCC patients who were admitted for treatment between March 2019 and June 2021 were enrolled into this study and diagnosed according to the diagnostic criteria of HCC. These patients had no history of chemotherapy prior to the surgery. Among these patients, there were 26 males (68.42%) and 12 females (31.58%). As for the laboratory examination, 32 patients were positive to HBV (84.2%) and 27 with AFP>100 ng/mL (71.05%). Among all patients, 28 patients were diagnosed with one tumor lesion (28/38, 73.68%), while 10 patients with multiple tumors (10/38, 26.32%). Histological examination showed that 18 patients were classified into stage 1 (18/38, 47.37%), and the remaining patients into stage 2 or 3 (20/38, 52.63%). Follow-up of patients was performed at the same clinic of the hospital, with an average of follow-up of 21.6 months. HCC tissues and non-tumor tissues were preserved at -80°C. The written informed consent for the collection of liver samples was obtained from the patients, and the study protocol was reviewed and approved by the Ethics Committee of Yichang Central People’s Hospital.

Cell culture

HCC cell lines (HepG2 and Hep3B) and the normal liver cell line (HL-7702) were provided by the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Science. All cells were cultured in the DMEM medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C and 5% CO2.

Construction of the expression vectors of pcDNA3.1-miR-214 and PGCsi-Pyk2 shRNA

Plasmids of pcDNA3.1-miR-214 and PGCsi-Pyk2 shRNA were constructed by Sangon Biotech (Shanghai) Co., Ltd. These plasmids carried out XhoI/BamHI sequence: 5′-GGATCCCTCAACATCAGTCTGATAAGCTACGATCTCAAATCAGTCTGATAAGCTA-3′, 5′-ACCGGTTCAACAATCAGTCTGATAAGCTATCGATCCATCAGTCTGATAAGCTACCCGGTG-3′. Plasmids were digested and recombined by using the BamHI enzyme, and the target segment of miR-214 was inserted into the pcDNA 3.1 plasmid and PGCsi-Pyk2 shRNA carrying the interference sequence of PTK2B/Pyk2. Then, the plasmids were integrated into the competent cells for amplification, extraction and virus packaging.

RNA extraction and qRT-PCR

Total RNA was extracted from the collected tissue samples by using the RNA extraction kit (Bio-Rad), and prepared for the cDNA synthesis by using the Prime-Script RT kit (Takara Biotechnology Co., Ltd., Dalian, China). With the cDNA, qPCR was performed. miR-214 expression was determined by using the ABI Prism 7000, and the relative expression by using the MX 3000P PCR apparatus (Stratagene, San Diego, CA) in the following settings: Initial denaturation at 95°C for 30 s, 40 cycles of annealing and extension at 95°C for 5 s, and extension at 60°C for 30 s: miR-214, forward ATCCAGTGCCTGTCGG, reverse TGCTACAGCAGGCACAGAC; Pyk2, forward GCAACAAAACTAAGCACAAGTCCA, reverse AATCAAAGTCCCA GAAGCATAACC.

Cell proliferation determined by MTT assay

Cells were inoculated into the 96-well plate at a density of 5000/well, and transfected by the pcDNA3.1-miR-214 and PGCsi-Pyk2 shRNA, or the Lipo2000 according to the standard protocol. Cell proliferation was determined by using the CCK-8 kit (7Sea Biotech, Shanghai, China) at 24 h, 48 h and 72 h after transfection by detecting the OD value using the FLUOstar OPTIMA (BMG Labtech GmbH, Ortenberg, Germany) at a wavelength of 450 nm.
Cell cycles
Lipo 2000 was used for the transfection of HepG2 and Hep3B cell lines using the pcDNA3.1- miR-214 and PGCsi-Pyk2 shRNA. At 48 h after transfection, 1 × 10⁶ cells were rinsed in PBS and fixed in 70% cold ethanol overnight at 4°C. Following several washes in PBS, cells were incubated with 0.1 mg/mL RNase A and 0.05 mg/mL PI (Sigma-Aldrich, Darmstadt, Germany) at 4°C for 30 min, and sorted according to the fluorescent signals. The distribution of the cell cycles was analyzed by using the ModFit LT version 4.0 (Verity Software House, Inc., Topsham, ME, USA).

Invasion experiment of carcinoma cells
HepG2 and Hep3B cells were treated with the mitomycin (20 mg/mL, 30 min, Sigma Aldrich, USA) to block the proliferation, and then transferred into the upper chamber of the Transwell plate (10⁵ cell/well, Corning, USA) in the serum-free medium. The lower chamber was filled with the medium supplemented with 10% FBS. Following 5 hours of incubation, the medium was removed, and cells that invaded into the lower chamber were fixed in 4% PFA, stained in crystal violet and imaged. According to the number of cells in the lower chamber, the invasive capability of cells was determined.

Statistical analysis
SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. Student t-test or One-way ANOVA followed by Tukey’s post hoc test was adopted for the data analysis of three repeats. The difference with \( P < 0.05 \) had statistical significance.

Results and discussion
Expression of miR-214 and PTK2B/Pyk2 gene in the HCC cell lines and hepatocellular carcinoma
To investigate the roles of miR-214 and PTK2B/Pyk2 in HCC, we initially measured the expressions of miR-214 and PTK2B/Pyk2 genes in the HepG2 and Hep3B cells. Results showed that miR-214 was down-regulated in the HepG2 and Hep3B cell lines, significantly lower than those in the HL-7702 cell line (Figure 1A, \( P < 0.05 \)), but PTK2B/Pyk2 was up-regulated (Figure 1B, \( P < 0.05 \)). To further validate the results above and explore the correlation between miR-214 and PTK2B/Pyk2, we detected the expressions of miR-214 and PTK2B/Pyk2 in the HCC and non-tumor tissues and found that miR-214 was down-regulated in the HCC tissues, while PTK2B/Pyk2 was up-regulated (Figure 1 C and D, \( P < 0.05 \)).

Effect of pcDNA3.1- miR-214 and PGCsi-Pyk2 shRNA on the proliferation of HCC cells
To explore the biological functions of miR-214 and PTK2B/Pyk2 and the interactions, we detected the proliferation of cells by using the MTT assay. As a result, in HepG2 and Hep3B cell lines transfected by pcDNA3.1- miR-214, miR-214 expression was up-regulated, while PTK2B/Pyk2 was down-regulated (Figure 2A and B, \( P < 0.05 \)). However, transfection of PGCsi-Pyk2 shRNA resulted in only the down-regulation of PTK2B/Pyk2 expression, without any effect on the miR-214 (Figure 2 A and B, \( P < 0.05 \)). Assay of the cell proliferation revealed that HepG2 and Hep3B cell proliferation was curbed by the up-regulation of miR-214 and down-regulation of PTK2B/Pyk2 (Figure 2 C and D, \( P < 0.05 \)).
Transfection of pcDNA3.1- miR-214 and PGCsi-Pyk2 shRNA alters the invasion of HCC cells

Transwell assay was carried out to determine the invasion of HepG2 and Hep3B cells after transfection of pcDNA3.1- miR-214 and PGCsi-Pyk2 shRNA. After 5 h, cells were stained by using crystal violet. In comparison with the HepG2 and Hep3B cells without any transfection, we found that the transfection significantly decreased the invasion of HCC cells (Figure 4 A and B, P < 0.05).

Transfection of pcDNA3.1- miR-214 and PGCsi-Pyk2 shRNA inhibited the proliferation of HCC cells by regulation of cell cycle

HepG2 cells were transfected by the pcDNA3.1-miR-214 and PGCsi-Pyk2 shRNA, and the distribution of cell cycles by flow cytometer, and we found that most of the HepG2 cells transfected by pcDNA3.1-miR-214 or PGCsi-Pyk2 shRNA stayed at G0/G1 (Figure 3 A and B). Thus, miR-214 overexpression or silencing PTK2B/Pyk2 might induce the arrest of the cell cycle at G1.

PTK2B/Pyk2 is a highly conservative pathway critical to the regulation of embryonic development and maintenance of the cellular or tissue homeostasis, and the abnormal expression may be a major cause of cancer (13). Furthermore, it is also involved in the HCC progression. During the proliferation, PTK2B/Pyk2 can regulate the adhesion signals, initiate the downstream signal pathway, activate the transcription factor and regulate the expression of proliferative cytokines to accelerate the proliferation (13, 14). Hence, the progression of HCC is in close association with the PTK2B/Pyk2. As reported, PTK2B/Pyk2 may correlate with the HCC, which

Figure 2. Expressions of miR-214 and PTK2B/Pyk2 in the HepG2 and Hep3B cells transfected by pcDNA3.1-miR-214 or PGCsi-Pyk2 shRNA. A, Expressions of miR-214 in the HepG2 and Hep3B cells transfected by pcDNA3.1-miR-214 or PGCsi-Pyk2 shRNA; B, Expressions of PTK2B/Pyk2 in the HepG2 and Hep3B cells transfected by pcDNA3.1-miR-214 or PGCsi-Pyk2 shRNA; C, Proliferation of HepG2 cells transfected by pcDNA3.1-miR-214 or PGCsi-Pyk2 shRNA; D, Proliferation of Hep3B cells transfected by pcDNA3.1-miR-214 or PGCsi-Pyk2 shRNA

Figure 3. The arrest of the cell cycle in HepG2 and Hep3B transfected by pcDNA3.1-miR-214 or PGCsi-Pyk2 shRNA

Figure 4. Decreases in the invasion of HepG2 and Hep3B cells transfected by pcDNA3.1-miR-214 or PGCsi-Pyk2 shRNA

Previous studies have reported the down-regulation of miR-214 in the majority of HCC tissues, and similarly, the results of this study also confirmed that miR-214 was down-regulated in vivo or in vitro. miR-214 down-regulation is in a close correlation with the invasion of portal veins and early recurrence, and also one of the pivotal clinical factors deciding the prognosis of HCC patients (11, 12). Thus, miR-214 down-regulation may correlate with the prognosis and increased mortality rate of HCC patients. To predict the prognosis of HCC patients, miR-214 expression may be an efficient index.
makes it necessary to establish the correlation between PTK2B/Pyk2 and HCC for elucidating the molecular mechanism of HCC (15).

Previous studies have shown that miR-214 down-regulation can promote the metastasis of intrahepatic cholangiocarcinoma by targeting the Twist gene (16), and also suppress the growth and invasion of cervical cancer cells by GALNT7 (17). Thus, one miRNA may target different genes to regulate the post-transcription gene expression. In light of this, we carried out more work to establish the target and function of miR-214 in cancer progression. Hence, research on the interaction between miR-214 and PTK2B/Pyk2 is significant for the prophylaxis and treatment of HCC.

In this study, miR-214 down-regulation may be pivotal to the regulation of PTK2B/Pyk2. Besides, miR-214 down-regulation and the consequent overexpression of PTK2B/Pyk2 enhance the invasion of HCC. As a result, miR-214 and its downstream effectors may be novel prognostic indicators or the therapeutic targets of HCC.

Acknowledgments
None

Conflict interest
The authors declare no conflict of interest.

References