LncRNA MINCR promotes the development of liver cancer by regulating microRNA-107/β-catenin

Hui Li, Rui Yuan, Haibin Wang, Chunmei Li, Jia Wei

Department of Liver and Infectious Diseases, Liver Disease Research Center, the second People’s Hospital of Yunnan Province, Kunming 650021, China.

Summary

Purpose: To investigate the level of long noncoding ribonucleic acid (lncRNA) MINCR in hepatocellular carcinoma (HCC), and to further investigate whether it can promote the development of HCC through modulating microRNA-107/β-catenin.

Methods: MINCR level in 52 pairs of HCC tumor tissues as well as adjacent tissues and HCC cell lines was detected by quantitative real-time polymerase chain reaction (qRT-PCR). MINCR knockdown model was constructed using lentivirus in the carcinoma cell lines. Furthermore, cell counting kit-8 (CCK-8), plate cloning and apoptosis assay were used to analyze the effect of MINCR on the biological function of HCC cells. Finally, cell recovery experiment was performed to explore its potential mechanism and the association between MINCR and microRNA-107/β-catenin.

Results: qPCR results showed that the level of MINCR in HCC was remarkably higher than that in adjacent tissues, and the difference was statistically significant. Compared with patients with low level of MINCR, patients with high level of MINCR had lower overall survival rate. Similarly, the cell proliferation ability of sh-MINCR group was remarkably decreased while the apoptosis ability was oppositely increased when compared with the short hairpin RNA (shRNA) group. In addition, studies have demonstrated that the levels of microRNA-107 and MINCR in HCC tissues were negatively related. In our study, dual-luciferase reporting assay verified that MINCR can be targeted by microRNA-107 through certain binding site. In addition, MINCR was confirmed to be able to further regulate the malignant progression of HCC through microRNA-107/β-catenin.

Conclusions: The level of MINCR was remarkably increased in HCC, which was associated with poor prognosis of HCC patients. Moreover, MINCR was capable of promoting the proliferation and inhibiting apoptosis of liver cancer cells via regulating microRNA-107/β-catenin.

Key words: MINCR, miRNA-107, HCC, proliferation

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies in China, and its incidence ranks fourth among the major malignancies. Due to its high grade of malignancy, rapid progress and poor prognosis, the mortality rate ranks second among all major malignancies [1-3]. Clinically, the onset of most HCC is insidious, and specific early clinical manifestations and typical symptoms are often absent. Once detected, HCC is in the middle and late stage, making the patients lose the opportunity for effective treatment. Moreover, the late recurrence rate is high and metastatic, and the 5-year survival rate is lower than 20%. Therefore, early clinical diagnosis and treatment are particularly important [4,5]. Cirrhosis and liver fibrosis caused by chronic hepatitis virus infection are the most important risk factors for HCC. However, the exact pathogenesis of HCC has not been fully elucidated. It may be the interaction of genetic and environmental factors, which leads to abnormal expression
of key genes and involves multiple signal transduction disorders such as cell growth, differentiation and apoptosis, and finally induces malignant transformation of hepatocytes [6-8]. Therefore, the in-depth exploration of genes related to the pathogenesis of HCC and their use as clinical diagnostic markers and therapeutic intervention targets for HCC have not only important clinical application value, but also provide new ideas for the study of the pathogenesis of HCC [9].

Long non-coding RNAs (lncRNAs) are a class of RNA molecules with a nucleotide length of more than 200 nt, which can be located in the cytoplasm and/or nucleus, but lack the ability to encode proteins [10,11]. At first, since the number, type and function of lncRNAs were not clear, they were called “dark matter” in the genome, non-coding “junk” deoxyribonucleic acid (DNA) or “noise” of gene transcription [12,13]. In recent years, studies have shown that lncRNAs can be widely involved in various cell biological processes such as embryonic development, chromatin remodeling, cell proliferation, differentiation, apoptosis and nuclear transport in the form of RNA, and regulate the level of related genes at multiple levels such as epigenetic, transcriptional and post-transcriptional levels. Meanwhile, the abnormal level of lncRNAs is closely related to the occurrence and development of many diseases, including malignant tumors [13-15]. At present, there are few reports about the role of lncRNA MINCR in the development of tumors [16,17]. Therefore, this study comprehensively analyzed the level and biological effects of lncRNA MINCR in HCC, and preliminarily explored the molecular mechanism of its tumor regulatory effect. Recent studies have shown that lncRNAs can also serve as competing endogenous RNAs (ceRNA or micro ribonucleic acids (miRNAs) sponges), and competitively bind miRNAs through their miRNA response elements (MREs), inhibit the function and activity of miRNAs, and thus regulate the mRNAs level of target genes of miRNAs at the post-transcriptional level [18,19]. In recent years, microRNA-107 has been studied to varying degrees in a variety of tumors, but no studies have been reported in HCC [19,20]. Therefore, this study investigated whether lncRNA MINCR mediated the occurrence and development of HCC through the regulation of mirna-107/-catenin, hoping to provide experimental basis for its clinical application.

**Methods**

**Patients and colon cancer samples**

In this study, 52 pairs of HCC tumor tissues and their corresponding adjacent tissues were collected from surgically treated HCC cases and then stored at -80°C. This study was approved by the ethics committee of the second People’s Hospital of Yunnan Province. Signed written informed consents were obtained from all participants before the study.

**Cell lines and reagents**

Six human HCC cells (Bel-7402, HepG2, MHCC88H, SMMC-7221, Huh7, Hep3B) and one human normal liver cell line (LO2) were purchased from American type culture collection (ATCC, Manassas, VA, USA), and Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were purchased from American Life Technologies (Gaithersburg, MD, USA). The HCC cell line was cultured in a DMEM high glucose medium containing 10% FBS, penicillin (100 U/mL), and streptomycin (100 µg/mL). All cells were cultured in incubator at 37°C and 5% CO₂, and were passaged with 1% trypsin+ethylenediaminetetraacetic acid (EDTA) for digestion when grown to 80%-90% confluence.

**Transfection**

The lentivirus containing the MINCR knockdown sequence (sh-MINCR) and the negative control group (shRNA) and were purchased from Shanghai Jima Company (Shanghai, China). Cells were plated in 6-well plates and grown to a cell density of 40%, then transfected according to the manufacturer’s instructions, and cells were harvested 48 h later for quantitative real-time polymerase chain reaction (qRT-PCR) analysis and cell function experiments.

**Cell proliferation assay**

After 48 h of transfection the cells were collected and plated into 96-well plates at 200 cells per well. The cells were cultured for 24 h, 48 h, 72 h and 96 h respectively, and then added with cell counting kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan) reagent. After incubation for 2 h, the optical density (OD) value of each well was measured in a microplate reader at 490 nm absorption wavelength.

**Colony formation assay**

48 h after transfection, cells were collected and 200 cells were seeded in each well of a 6-well plate and cultured in DMEM medium containing 5% FBS for 2 weeks. The medium was changed after one week and then changed twice a week. The medium should not be replaced as much as possible in the previous week to avoid cell adhesion. After 2 weeks, cells were washed twice with phosphate buffer saline (PBS) and fixed in 2 mL of methanol for 20 min. After the methanol was aspirated, the cells were washed with PBS, stained with 0.1% crystal violet staining solution for 20 min, photographed and counted.

**Flow cytometry**

Liver cells in logarithmic growth phase were plated into 6-well plates. After 24 h of drug treatment, the cells
were collected, washed twice with PBS, resuspended in the binding solution, incubated at room temperature for 15 min in the dark, and 5 μL of Annexin V-FITC (fluorescein isothiocyanate)/propidium iodide (PI) was added. After mixed gently, cell apoptotic rate was measured by flow cytometry.

QRT-PCR

After the cells were treated accordingly, 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA) was used to lyse the cells, and total RNA was extracted. The initially extracted RNA was treated with DNase I to remove genomic DNA and re-purify the RNA. RNA reverse transcription was performed according to the Prime Script Reverse Transcription Kit (TaKaRa, Tokyo, Japan) instructions, and qRT-PCR was performed according to the SYBR® Premix Ex TaqTM (TaKaRa, Tokyo, Japan) kit instructions. The PCR reaction was performed using the StepOne Plus Real-time PCR System (Applied Biosystems, Foster City, CA, USA). The following primers were used for qRT-PCR reaction: MINCR: F: 5'-TGTGGCAAAACTTGAATGGA-3', R: 5'-GGGGGAGGACAAGAGAAAGA-3'; β-catenin: F: 5'-GGGGAAGGATTTTTTATACCA-3', R: 5'-CTGAAATTAGGGCCCAATT-3'; GAPDH: F: 5'-GCGTGGGTTTTTGTATCCA-3', R: 5'-CTGAAATTAGGGCCCAATT-3'; miRNA-107: F: 5'-AGCAGCATTGTACAGGG-3', R: 5'-GTGCAGGGTCCGAGGT-3'. U6: F: 5'-CTCGCTTCGGCACA-3', R: 5'-AACGCTTCACGAATTTGCGT-3'. Each sample was subjected to a three-hole repeated experiment and repeated twice. The Bio-Rad (Hercules, CA, USA) PCR instrument was used to analyze and process the data with the software iQ5 2.0. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 genes were used as internal parameters, and the gene level was calculated by the $2^{-ΔΔCt}$ method.

Western blot

The transfected cells were lysed using cell RIPA lysis buffer, shaken on ice for 50 min, and centrifuged at 14,000 g for 15 min at 4°C. Total protein concentration was calculated by bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL, USA). The extracted proteins were separated using a 10% dodecyl sulfate, sodium salt-polyacrylamide gel electrophoresis (SDS-PAGE) gel and subsequently transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). Western blot analysis was performed according to standard procedures. The primary antibodies were β-catenin and GAPDH, and the secondary antibodies were anti-mouse and anti-rabbit, all purchased from Cell Signaling Technology (Danvers, MA, USA).

Dual-luciferase reporter assay

A reporter plasmid was constructed in which a specific fragment of the target promoter was inserted in front of the luciferase level sequence. The transcription factor plasmid was co-transfected in Bel-7402 and HepG2 cells with the reporter plasmid. The activity of the luciferase was determined 48 h later using the Promega luciferase (Madison, WI, USA) kit in which a specific luciferase substrate was added which could react with the substrate to generate fluorescence, and then the intensity of the fluorescence was detected.

Statistics

Statistical analysis was performed using GraphPad Prism (Version X; La Jolla, CA, USA). Statistical differences between the two groups were analyzed using Student’s t-test. Comparison between multiple groups was done using one-way ANOVA test followed by Post Hoc test (least significant difference). The survival curves were plotted using the Kaplan-Meier method and were compared using log-rank test. Independent experiments were repeated at least three times for each experiment and data were expressed as mean±standard deviation. P<0.05 was considered statistically significant.

Figure 1. MINCR is highly expressed in hepatocellular carcinoma tissues and cell lines. A: qRT-PCR was used to detect the difference in the expression of MINCR in hepatocellular carcinoma tumor tissues and adjacent tissues; B: qRT-PCR was used to detect the expression level of MINCR in osteosarcoma cell lines; C: Kaplan-Meier survival curve of patients with hepatocellular carcinoma based on MINCR expression; the prognosis of patients with high expression was significantly worse than that of the bottom expression group. Data are mean±SD, *p<0.05, **p<0.01, ***p<0.001.
Results

MINCR was highly expressed in hepatocellular carcinoma tissues and cell lines

In order to determine the level of MINCR in HCC, we detected the difference of MINCR level in HCC tumor tissues and adjacent tissues by qRT-PCR. The results showed that MINCR was elevated in HCC tissues compared with adjacent tissues (Figure 1A), suggesting that MINCR may play an oncogene role in HCC. In addition, MINCR was remarkably higher in 6 human HCC cells than in human normal liver cell lines (LO2), and the difference was statistically significant (Figure 1B). Based on the mRNA results of MINCR in 52 pairs of HCC tissues and paracancer tissues, we divided the level of MINCR into high level group and low level group, and analyzed the relationship between MINCR level and poor prognosis of patients with HCC. As shown in Figure 1C, high level of MINCR was closely related to poor prognosis of HCC. These results suggested that MINCR was highly expressed in HCC tissues and cell lines and was related to the prognosis of this disease.

Knockdown of MINCR inhibited cell proliferation, and promoted cell apoptosis

To investigate the function of MINCR in HCC, we constructed a knockdown MINCR lentiviral vector. After transfecting the MINCR lentiviral vector in the Bel-7402 and HepG2 cell lines, the qRT-PCR assay was performed to verify the interference efficiency (Figure 2A). Cell proliferation and apoptosis were detected by CCK-8, plate cloning and flow cytometry in knockdown of MINCR in Bel-7402 and HepG2 cell lines, respectively. The results showed...
that the cell proliferation ability was remarkably reduced in the MINCR-silencing group sh-MINCR compared to shRNA (Figure 2B, 2C). In addition, the ability of apoptosis in sh-MINCR in the MINCR-silence group was remarkably increased (Figure 2D). In sum, we could conclude that MINCR knock-down inhibited cell proliferation and promoted cell apoptosis in HCC.

Direct targeting of MINCR and microRNA-107

As shown in Figure 3A, in order to further verify the targeting of microRNA-107 to MINCR, we cloned the MINCR sequence into the luciferase reporter plasmid pmirGLO, and also constructed the mutation vector pmirGLO-MINCR-mut, and then pmirGLO-MINCR-wt, pmirGLO-MINCR-mut or pmirGLO and microRNA-107 were co-transfected into Bel-7402 and HepG2 cells for luciferase reporter gene experiments. The results demonstrated that MINCR can be targeted by microRNA-107 through this binding site.

MicroRNA-107 was lowly expressed in hepatocellular carcinoma tissues and cell lines

QRT-PCR experiments showed the level of microRNA-107 in 52 pairs of HCC tumor tissues and their corresponding paracancer tissues, as well as HCC cell lines. The results showed that miR-107 was remarkably decreased in HCC tissues compared with paracancer tissues (Figure 3B). Sub-

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**Figure 3.** Direct targeting of miR-107 by MINCR. A: Dual luciferase reporter assays demonstrate direct targeting of MINCR and miR-107. B: qRT-PCR was used to detect the difference in expression of miR-107 in hepatocellular carcinoma tumor tissues and adjacent tissues. The qRT-PCR verified the mRNA expression level of miR-107 after transfection of MINCR in hepatocellular carcinoma cell lines. D: There was a significant negative correlation between the expression levels of MINCR and miR-107 in hepatocellular carcinoma tissues. E: Silencing MINCR expression significantly increased miR-107 expression levels. Data are mean±SD, *p<0.05, **p<0.01, ***p<0.001.
sequently, we used qRT-PCR to find that microRNA-107 was remarkably expressed in HCC cells in hepatic cell lines (Figure 3C). In addition, the level of MINCR and microRNA-107 were detected by qRT-PCR, and the results revealed that MINCR and microRNA-107 showed a negative correlation between gene levels in HCC tissues (Figure 3D). Subsequently, after constructing the MINCR knockdown lentiviral vector in the Bel-7402 and HepG2 cell lines, the level of microRNA-107 in the MINCR-silencing group sh-MINCR was remarkably increased (Figure 3E).

Overexpression of microRNA-107 inhibited cell proliferation, and promoted cell apoptosis

To further investigate the function of microRNA-107 in HCC, the transfection efficiency of the microRNA-107 vector in the Bel-7402 and HepG2 cell lines was verified by qRT-PCR. The difference was statistically significant (Figure 4A). After over-expressing microRNA-107 in Bel-7402 and HepG2 cell lines, CCK-8, plate cloning and flow cytometry assay were used to detect cell proliferation and apoptosis. The results showed that, compared with miR-NC, the cell proliferation ability of microRNA-107 mimics was remarkably decreased in the microRNA-107 overexpression group (Figure 4B, 4C). In addition, the apoptotic ability of microRNA-107 mimics was remarkably increased in the microRNA-107 overexpression group (Figure 4D).

MicroRNA-107 modulated β-catenin level in CRC tissues and cell lines

To further explore the ways in which MINCR promoted the malignant progression of HCC, we found a possible relationship between MINCR and
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microRNA-107/β-catenin through relevant bioinformatics analysis. After overexpression of microRNA-107 in Bel-7402 and HepG2 cell lines, the levels of β-catenin were detected by qRT-PCR and Western Blot, and it was found that microRNA-107 mimics can inhibit the level of β-catenin (Figure 5A, 5B). The level of microRNA-107 and β-catenin was detected by qRT-PCR, and it was confirmed that microRNA-107 and β-catenin were negatively correlated in HCC tissues (Figure 5C). To further validate the targeting of β-catenin to microRNA-107, a luciferase reporter assay was performed. The results demonstrated that microRNA-107 can be targeted by β-catenin through this binding site (Figure 5D).

Discussion

Although a large number of studies have shown that IncRNAs play an important role in the occurrence and development of tumors, the precise molecular mechanism is still not very clear [11-13]. Compared with miRNAs, the regulatory mechanism of IncRNAs in cancer formation is very diverse and complicated [15]. LncRNAs can regulate the downstream target genes and regulate the specificity and accuracy of the signal [14,15].

Recent studies have found that IncRNAs can also act as ceRNA or miRNA sponges to competitively bind miRNAs and inhibit the function and activity of miRNAs. They are also involved in the biological processes of tumor cell proliferation, invasion, metastasis and angiogenesis [14-16]. Studies have shown that MINCR is remarkably overexpressed in oral squamous cell carcinoma, and its overexpression is correlated with poor prognosis of patients. In addition, studies have found that abnormal level of MINCR is associated with poor prognosis of lung cancer patients [16,17]. In this study, IncRNAs related to the malignant progression of HCC were studied by knockdown of cell lines in MINCR established by lentivirus, and MINCR was selected as the candidate IncRNA, and the relationship between MINCR and the occurrence and development of HCC was finally determined. The up-regulated level of MINCR can promote the generation of malignant progression of HCC. Through tissue verification, we found that the level of MIN-
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CR in HCC was remarkably higher than that in adjacent tissues, and was positively correlated with poor prognosis of HCC. Therefore, we believe that MINCR might play a role in promoting cancer in HCC. In order to further study the molecular mechanism of MINCR in the development of HCC and to prove that MINCR is a disease-related gene, in vitro cell line experiments were conducted. CCK-8 assay and plate cloning assay were performed, and it was found that, compared with shRNA group, the silenced bel-7402 and HepG2 cell lines of MINCR could promote the proliferation of HCC and inhibit apoptosis.

At present, studies on the mechanism of lncRNAs’ biological effects of regulation and modification mainly claim that lncRNA can competitively bind miRNA, thus affecting the regulation of its target mRNA. Previous studies predicted the possible interaction between miRNA-107 and MINCR through bioinformatics analysis [12-14]. MiRNA-107 is a key molecule in the MiRNA family. In this study, it was found that miRNA-107 was lower expressed in HCC tissues than in adjacent tissues, and miRNA-107 inhibited proliferation in HCC cells. In this study, we used bioinformatics, dual luciferase reporter gene and other molecular biology experiments to verify the direct binding of MINCR to downstream miRNA-107. In order to further explore the role of MINCR and microRNA-107 in the level regulation of HCC cell lines, the levels of MINCR and microRNA-107 were just negatively correlated.

Based on the above results, MINCR can be used as an oncogene to promote the proliferation, invasion and metastasis of HCC cells, and the mechanism found was that MINCR promoted the malignant progression of HCC through the microRNA-107/β-catenin signal pathway in HCC. However, one IncRNA molecule regulates the function of multiple miRNAs molecules, and one miRNA molecule can regulate the level of multiple target genes, which inevitably involves the cooperation and cross-talk of multiple signaling pathways. In addition, the level of IncRNAs themselves may also be regulated by the downstream transcription factors of the signaling pathway. Given that there are only a few IncRNAs with important biological function in HCC, the direction of future efforts is looking for liver disease specificity to IncRNAs molecules, and exploration of its potential function and signal mechanism not only helps understand the molecular mechanism of HCC occurrence, development, but also provide targeted IncRNA HCC diagnosis and treatment with the new development direction.

Conclusions

The level of LncRNA MINCR was remarkably increased in HCC, which was remarkably correlated with poor prognosis of HCC. In addition, MINCR may promote malignant progression of HCC by regulating microRNA-107 /β -catenin.

Conflict of interests

The authors declare no conflict of interests.

References

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