

# Down-regulation of microRNA-21 inhibits cell proliferation and invasion of high-invasion liver cancer stem cells

Y.-C. LI<sup>1</sup>, F.-M. XU<sup>1</sup>, G.-O. ZHANG<sup>2</sup>, S.-B. LI<sup>1</sup>, Y.-Y. WEN<sup>3</sup>, F. ZENG<sup>4</sup>

<sup>1</sup>Department of Infectious Diseases, Zhoushan Hospital, Zhoushan, China

<sup>2</sup>Department of Hepatobiliary Surgery, Zhoushan Hospital, Zhoushan, China

<sup>3</sup>Department of Pathology, Zhoushan Hospital, Zhoushan, China

<sup>4</sup>Prenatal Diagnosis Laboratory, Zhoushan City Maternal and Child Health Care, Zhoushan, China

**Abstract. – OBJECTIVE:** Cancer stem cells (CSCs) play critical roles in tumorigenesis, tumor recurrence and metastasis. This study aims to investigate the effects of small interfere microRNA-21 RNA (miR-21 RNAi) on cell proliferation, invasive ability of high-invasion liver cancer stem cells (H-ILCSCs), HCCLM3 and HL-7702 cells.

**MATERIALS AND METHODS:** pLVX-shRNA2 lentiviral vector system was established, packaged and transfected into H-ILCSCs, HCCLM3 and HL-7702 cells. Cell counting kit-8 (CCK-8) assay was performed to observe cell viabilities of cells. Transwell assay was conducted to evaluate the invasion potential of H-ILCSCs, HCCLM3 and HL-7702 cells. Quantitative PCR (qPCR) assay was used to examine the miR-21 levels in different cell lines.

**RESULTS:** pLVX-anti-miR21 lentiviral vector system was successfully established. miR-21 levels were down-regulated in anti-miR-21 gene steady expression cell lines compared to untreated cells ( $p < 0.05$ ). miR-21 levels were significantly lower in H-ILCSC2-LV-anti-miR-21 group compared to HCCLM3-anti-miR-21 and HL7702-anti-miR-21 ( $p < 0.05$ ). miR-21 inhibition significantly decreased cell proliferation and invasion compared to untreated cells ( $p < 0.05$ ). Cell proliferation and invasive ability of H-ILCSC2-LV-anti-miR-21 group were significantly higher compared to HCCLM3-anti-miR-21 and HL7702-anti-miR-21 ( $p < 0.05$ ). There were even not effects of miR-21 RNAi treatment on the cell proliferation and invasion of HL-7702 cells.

**CONCLUSIONS:** The down-regulation of miR-21 significantly inhibited the cell proliferation and invasion abilities of H-ILCSCs and HCCLM3 cells, and illustrated higher effects on H-ILCSCs.

*Key Words:*

High-invasion liver cancer stem cells, micrRNA-21, Proliferation, Invasion.

## Introduction

The cancer stem cells (CSCs) play critical roles in the tumorigenesis, tumor recurrence, tumor metastasis, chemotherapy resistance, which are also closely correlated with the grade of malignancy<sup>1,2</sup>. Therefore, the investigations for CSCs as the targeting cells for tumor therapy become a hot topic in the tumor field. Liver cancer stem cells (LCSCs) could trigger the unlimited proliferation, self-renewal and tumor differentiation of hepatic cancer cells, and are the most common risk factors for tumor recurrence and metastasis<sup>3,4</sup>. Normally, LCSs are divided into high- or low-invasive cancer stem cells (H/L-ILTSCs), which are key points for the progression of tumor<sup>5</sup>. Meanwhile, the H-ILTSCs initiate the invasion and metastasis of tumors. Therefore, the regulation of the H-ILTSCs or the other high-invasive liver cancer cells is critical for the liver cancer. MicroRNAs are a series of endogenous and highly conserved non-coding single-stranded RNAs that closely correlated with the tumor progression<sup>6,7</sup>. The previous study<sup>8</sup> reported that the microRNAs play important roles as the tumor suppressors or oncogenes in the cancer progression. Following the development of the CSCs-associated microRNAs researches, many microRNAs have been involved in the maintaining of stem cell characteristics of CSCs<sup>9</sup>. Meanwhile, the microRNAs may also play critical roles in regulating or modulating the cancer stem cell differentiation or proliferation<sup>10</sup>. Among the microRNAs, the microRNA-21 (miR-21) is considered to be the most important miRNA that over-expresses in many CSCs and various types of carcinomas<sup>11-13</sup>. Han et al<sup>14</sup> reported

that miR-21 could activate the endothelial mesenchymal transition (EMT) and promote the tumor invasion and metastasis, playing critical roles in regulating the associated functions of stem cells. Yu et al<sup>15</sup> also proved that miR-21 induce the differentiation of the colon cancer stem cells by down-regulating transforming growth factor  $\beta$  receptor 2. However, seldom miR-21 was reported in the regulation of the liver cancer stem cells (LCSCs). Previous reseraches<sup>16,17</sup> reported that miR-21 higher-expressed in LCSCs compared to liver cancer cells and promote the tumor metastasis. Jiang et al<sup>18</sup> also reported that miR-21 causes the invasion, migration and hepatic carcinogenesis. Therefore, we investigated the effects of miR-21 on the H-ILCSCs development, and attempted to provide the novel diagnostic and therapeutic method. In this study, the lentiviral expression vector, pLVX-anti-21, was established to interfere the miR-21 expression in H-ILCSCs, liver cancer cell line (HCCLM3) and normal hepatic cell line (HL-7702). Meanwhile, the effects of interfere miR-21 on the cell proliferation, invasive ability of H-ILCSCs, HCCLM3 and HL-7702 cells, were observed in this study.

## Materials and Methods

### Cells and Culture

The high invasion liver cancer cell line, HCCLM3, normal hepatic cell line, HL-7702, the high invasion liver cancer stem cell line, H-ILCSCs, were purchased from Typical Culture Preservation Center of Shanghai Academy of Science (Shanghai, China). The H-ILCSCs were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco BRL. Co. Ltd., Grand Island, NY, USA) supplementing with 2% growth factor B27 (Gibco BRL. Co. Ltd.) and 20 ng/ml epithelial growth factor (EGF)/basic fibroblast growth factor (bFGF, Peprotech Co. Ltd., Rocky Hill, NJ, USA) (final concentration). The HCCLM3 cells were cultured in DMEM (Gibco BRL. Co. Ltd., Grand Island, NY, USA) supplementing with 10% fetal bovine serum (FBS, HyClone, South Logan, UT, USA). The HL-7702 cells were cultured in Roswell Park Memorial Institute 1640 (RPMI-1640) (Gibco BRL. Co. Ltd., Grand Island, NY, USA) supplementing with 10% fetal bovine serum (FBS, HyClone, South Logan, UT, USA). All of the above cells were cultured at 37°C and 5% CO<sub>2</sub>, supplementing with 1% (volume: volume) streptomycin and penicillin, and adjusting the

humidity of 100%. This study was approved by the Ethical Committee of Zhoushan City Maternal and Child Health Care (Zhoushan, China).

### Lentiviral Vectors Construction

pLVX-shRNA2 lentiviral vector (Clontech. Laboratories Inc., Palo Alto, CA, USA) was used to construct the pLVX-anti-miR-21 RNAi. Meanwhile, the oligonucleotides for the miR-21 were constructed and synthesized by the Shaihai Generay Biotech. Co. Ltd. (Shanghai, China). The oligonucleotide sequence for miR-21 mimics were listed as the followings: miR-21-sense (BamH I site), 5'-GGATCCGGGTAGCTTATCAGACTGATGTTCAAGAGACATCAGTCTGATAAGC TACCC-3', miR-21-antisense (EcoR I site), 5'- GAATTCGGGTAGCTTATCAGACTGATGTC TCTTGAACATCAGTCTGATAAGCTACCC-3'. Then, the miR-21 related DNA double-chain was artificially synthesized by using above sequences due to the template sequences according to PCR amplification kit (TaKaRa Bio. Inc., Otsu, Shiga, Japan). Finally, the synthesized double-chain sequences were digested using BamH I and EcoR I endonuclease (TaKaRa Bio. Inc., Dalian, China) and subcloned into pLVX-shRNA2 lentiviral vector to form the H-ILCSCs-LV-anti-miR-21, HCCLM3-LV-anti-miR-21, HL-7702-LV-anti-miR-21, respectively.

### Lentiviral Vector Packaging

A total of 24 h before the transfection of above plasmids, the 293T cells (Typical Culture Preservation Center of Shanghai Academy of Science, Shanghai, China) were seeded into a 10-cm dish (Corning Costar, Corning, CA, USA). The H-ILCSCs-LV-anti-miR-21 plasmid, HCCLM3-LV-anti-miR-21 plasmid, HL-7702-LV-anti-miR-21 plasmid, and packing plasmids, including PG-p1-V-SVG, PG-P2-REV, PG-P3-RRE, were transfected by using RNAi-mate lentiviral vector packaging system (GenePharma Co., Ltd, Shanghai, China), according to manufacturer's instruction. A total of 72 h post the transfection, the supernatant was harvested by centrifuging at 4000 r/min at 4°C for 5 min. The supernatants were passed by using a 0.45  $\mu$ m syringe filter (Sigma-Aldrich, St. Louis, MO, USA), and were cleared by centrifuging again at 20000  $\times$ g at 4°C for 1 h. The titers of the about lentivirus were evaluated by examining the expression of the green fluorescent protein (GFP) due to instruction of manufacturer (Clontech. Laboratories Inc., Palo Alto, CA, USA). The packaged

lentivirus was designated the H-ILCSCs-LV-anti-miR-21 plasmid, HCCLM3-LV-anti-miR-21 plasmid, HL-7702-LV-anti-miR-21 plasmid. Finally, the viral titers were calculated using the following formula: viral titer (TU/ml) = GFP positive cell counts/dilution times.

### **H-ILCSCs, HCCLM3 and HL-7702 Infecting with LV-anti-miR-21 Viral Vectors**

The H-ILCSCs, HCCLM3 and HL-7702 cells were infected with the LV-anti-miR-21 viral vectors, at a multiplicity of infection ratio of 20, supplementing with the 8 µg/ml polybrene (GenePharma Co. Ltd, Shanghai, China). The infection efficiency of the viral vectors in H-ILCSCs, HCCLM3 and HL-7702 cells were evaluated by employing the inverted microscope (Mode: CKX53, Olympus, Tokyo, Japan) for the GFP fluorescence.

### **Cell Counting Kit-8 (CCK-8) Assay**

Cell viabilities of the H-ILCSCs, HCCLM3 and HL-7702 cells were determined by utilizing the CCK-8 assay kits (Beyotime Biotech. Shanghai, China) according to the manufacturer's instruction. The exponentially growing H-ILCSCs, HCCLM3 and HL-7702 cells ( $5 \times 10^4$  cells/ml) were seeded into one well of a 96-well plate (Corning Costar, Corning, NY, USA) and incubated for 72 h. At 24 h, 36 h and 48 h, the CCK-8 solution (10 µl/ml medium) was added to three randomly selected wells and incubated at 37°C for 4 h. The cell viability was represented by optical density (OD) values detected at 450 nm with a microplate reader (Mode: MK3, Thermo Electron Corp., Waltham, MA, USA).

### **Transwell Assay**

In order to observe the invasion potential of H-ILCSCs, HCCLM3 and HL-7702 cells, 100 µl DMEM medium containing  $1 \times 10^5$  cells were seeded into the upper chamber of a transwell system (Corning, Corning, NY, USA). The lower chamber of the transwell system contained 800 µl complete DMEM medium supplemented with 10% fetal bovine serum (FBS). All the cells were incubated in chambers and allowed to migrate through the porous membrane at 37°C for 48 h. Cells in the upper surface of the chamber were completely removed and then chambers were fixed with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) for 20 min. Post washing with phosphate-buffered solution (PBS, Beyotime Biotech. Shanghai, China) for 3 times, the cham-

bers were stained with 1% (w/v) crystal violet for 5 min. Cells numbers in each chamber were evaluated by using a microscope (Model: CX41, Olympus, Tokyo, Japan) and analyzed by utilizing the image-proplus (IPP) software 6.0 (Media Cybernetics Inc., Silver Spring, MD, USA).

### **Quantitative PCR (qPCR) Assay**

The total RNAs were extracted employing the TRIzol method according to the instruction of manufacturer (Western Biotech., Chongqing, China). The cDNA templates were synthesized by using the AMV Reverse Transcriptase kit (Promega, Madison, WI, USA) according to the manufacturers' instruction. The final reaction mix of 20 µl volume contained 10 µl qPCR master Mix (Biomics Biotech., Nantong, China), 0.5 µl of each primer (miR-21: forward, 5'-ACACTCCA-GCTGGGTAGCT TATCAGACTGA-3', reverse, 5'-CTCAACTGGTGTCTGGAGTCGGCA-ATTC AGTTGAGTCAACATC-3'. U6: forward, 5'-CTCGCTTCGGCACA-3', reverse, 5'-AAC-GCTTCACGAATTTGCGT-3'. Primers were synthesized by the Western Biotech., Chongqing, China), 1 µl cDNA template, and 8 µl RNase free H<sub>2</sub>O (TaKaRa, Dalian, China). The thermal cycling parameters for qPCR amplification were set as the follows: denaturation at 94°C for 15 min, followed by 40 cycles of amplification of 94°C for 15 s, 55°C for 30 s and 70°C for 30 s. The relative expression levels of target genes were calculated using the PCR Detection System (Agilent Technologies, Santa Clare, CA, USA) and the formula of  $2^{-\Delta\Delta Ct}$  according to the previous study described<sup>19</sup>.

### **Statistical Analysis**

The data were illustrated as mean ± standard deviation (SD) and analyzed with SPSS software 13.0 (SPSS Inc., Chicago, IL, USA). For every experiment or test, data were obtained from at least six repeats. The Student's *t*-test was used for statistical analysis between two groups. Tukey's post-hoc test was used to validate ANOVA for comparing measurement among groups. A statistical significance was defined when  $p < 0.05$ .

## **Results**

### **Lentiviral Vector pLVX-Anti-21 was Successfully Established**

The recombinant plasmid pLVX-miR21 was established by cloning the BamH I and EcoR I



site into the vector. The image for the established pLVX-anti-miR-21 plasmid was illustrated as Figure 1A. Meanwhile, the pLVX-anti-miR21 plasmid was digested at the EcoR I site, and a liner fragment DNA with the length of 8000 bp was obtained (Figure 1A). This result suggested that the target fragment DNA was inserted into the pLVX-miR-21 plasmid successfully. Meanwhile, the target gene sequence was also sequenced and blasted with the GenBank (data not shown), which also suggests that the lentiviral vector pLVX-miR-21 was successfully established.

#### ***pLVX-Anti-miR21 Lentivirus Illustrated Higher Infection Efficiency***

In order to identify the optimal dosage of the pLVX-miR-21 lentivirus for infecting the cells, the infection efficiency was evaluated. The results indicated that the dosage of 1  $\mu$ l illustrated the optimal efficiency in 293 T cells (Figure 1B). Therefore, 1  $\mu$ l pLVX-anti-miR21 lentivirus was applied to infect the H-ILCSCs, HCCLM3 and HL-7702 cells. The results showed that levels of the GFP fluorescence in pLVX-anti-miR21 lentivirus infected H-ILCSCs (Figure 2A), HCCLM3 cells (Figure 2B), HL-7702 cells (Figure 2C) were significantly increased compared to the blank pLVX-GFP vector, and the GFP fluorescence density even achieved to 90%. Moreover, after passaging for several generations, the H-ILCSCs, HCCLM3 and HL-7702 cell lines steadily expressing and inhibiting miR-21 cell lines were successfully established.

#### ***miR-21 Levels Were Down-Regulated in anti-miR-21 Gene Steady Expression Cell Lines***

The results indicated that miR-21 levels in the LV-anti-miR-21 group were significantly decreased compared to the Untreated and Mock vector group in H-ILCSCs cells (Figure 3A,  $p < 0.01$ ). Meanwhile, the miR-21 levels in LV-anti-miR-21 group were also significantly declined compared to the Mock group in the HCCLM3 cells ( $p < 0.01$ ). However, we also discovered that the miR-21 levels were significantly lower in H-ILCSCs-LV-anti-miR-21 group compared to the HL-7702-LV-anti-miR-21 group (Figure 3A,  $p < 0.05$ ). The miR-21 levels were also significantly lower in HCCLM3-LV-anti-miR-21 group compared to HL-7702-LV-anti-miR-21 group (Figure 3A,  $p < 0.05$ ). Meanwhile, miR-21 levels were significantly lower in H-ILCSC2-LV-anti-miR-21 group compared to HCCLM3-anti-miR-21 group (Figure 3A,  $p < 0.05$ ).

#### ***miR-21 Inhibition Significantly Decreased Cell Proliferation***

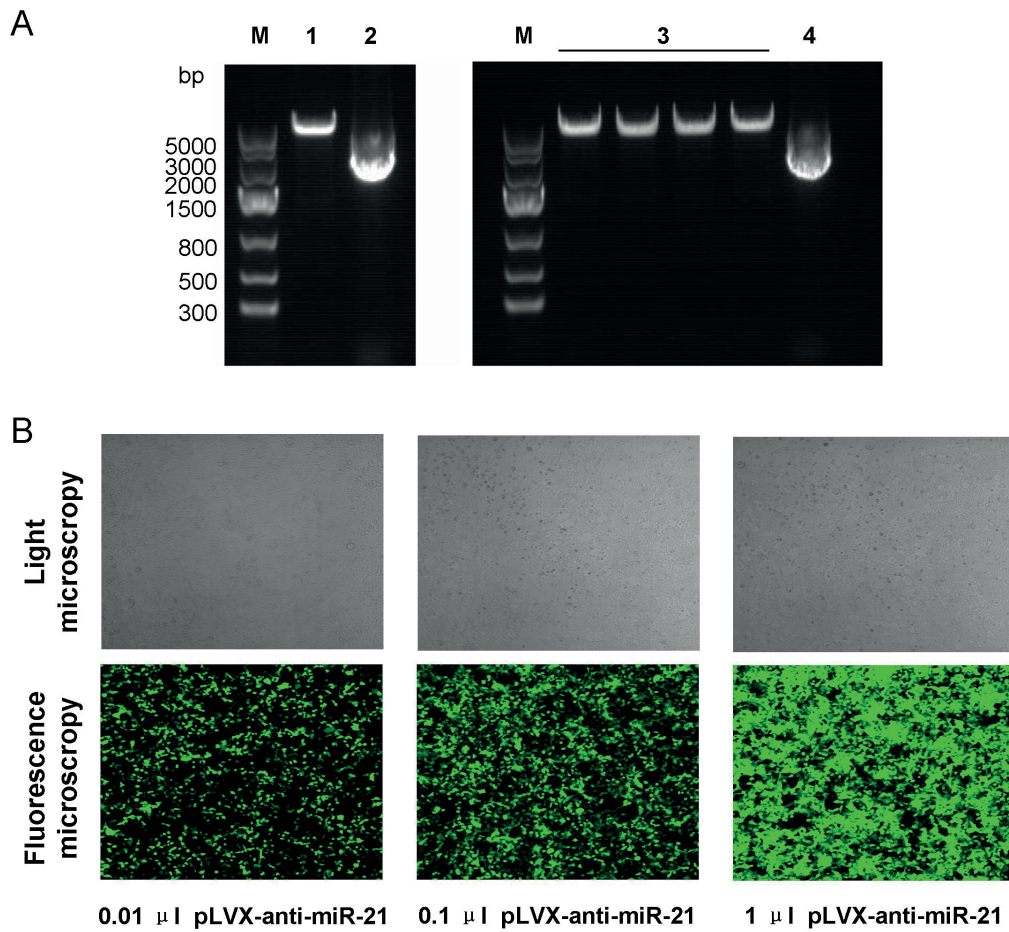
The results showed that H-ILCSCs cells proliferation in the LV-anti-miR-21 group was significantly lower compared to the Untreated and Mock vector group at 24, 36 and 48 h, respectively (Figure 3B,  $p < 0.01$ ). Also, the cell proliferation of LV-anti-miR-21 group was significantly lower compared to Untreated and Mock vector group in HCCLM3 cells at different time points (Figure 3B,  $p < 0.05$ ). However, there were no significant differences for the cell proliferation between LV-anti-miR-21 group and the Untreated group (or Mock vector group) in HL-7702 cells (Figure 3B,  $p > 0.05$ ). Moreover, the cell proliferation in LV-anti-miR-21 group of H-ILCSCs was also higher significantly compared that of HCCLM3 cells and HL-7702 cells (Figure 3B,  $p < 0.05$ ) at 24 h, 36 h and 48 h, respectively. The cell proliferation in HCCLM3 cells was higher significantly compared to that of HL-7702 cells (Figure 3B,  $p < 0.05$ ).

#### ***miR-21 Down-Regulation Inhibited Proliferation of Cells***

The results indicated that the miR-21 RNAi treatment significantly inhibited the cell proliferation of the H-ILCSCs and HCCLM3 cells compared to the blank pLVX-GFP group (Table I,  $p < 0.01$ ). Meanwhile, the miR-21 RNAi treatment significantly inhibited cell proliferation of H-ILCSCs compared to the blank pLVX-GFP group at different time points (Table I,  $p < 0.05$ ). Furthermore, the cell proliferation inhibition rates in H-ILCSCs-pLVX-antiR-21 group were significantly higher compared to the HCCLM3-pLVX-anti-miR21 group and HL-7702-pLVX-anti-miR21 group at 24 h, 36 h and 48 h, respectively (Table I,  $p < 0.05$ ). Meanwhile, cell proliferation inhibition rates in H-ILCSCs-pLVX-anti-miR21 group were significantly higher compared that that of HCCLM3-pLVX-anti-miR21 group (Table I,  $p < 0.05$ ).

#### ***miR-21 Down-Regulation Suppressed the Invasive Ability of Cells***

In order to evaluate the invasive ability of the H-ILCSCs, HCCLM3 and HL-7702 cells, the Transwell assay was conducted. The results showed that the invasions in LV-anti-miR-21 treatment group were significantly suppressed compared to that in Untreated and Mock vector group of the H-ILCSCs cells, HCCLM3 cells and HL-7702 cells, respectively (Figure 4). Moreover, the invasive ability of the H-ILCSCs-LV-anti-miR-21 group was significantly higher compared to that



**Figure 1.** Identification of the pLVX-miR21 plasmid and evaluation for the transfection efficiency. A. Graphs for the pLVX-miR21 plasmid, including circle and liner plasmid (by digesting with endonuclease). B. Evaluation for the transfection efficiency of pLVX-miR-21 vector in 293 T cells at different concentrations. H-ILCSCs: high-invasion liver cancer stem cells.

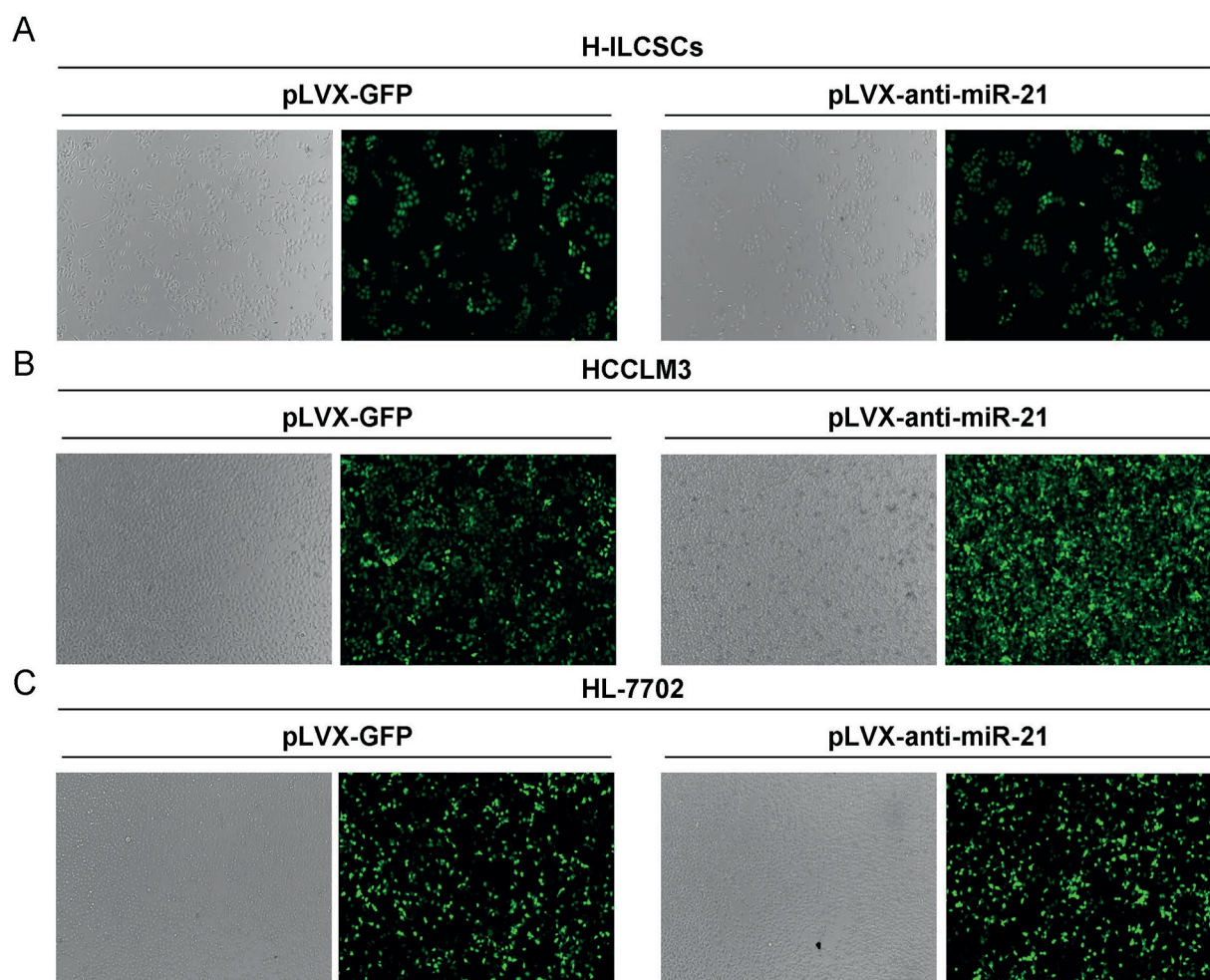
**Table I.** Cell proliferation rate in the H-ILCSCs, HCCLM3, HL-7702 cells undergoing miR-21 transfection (mean  $\pm$  SD).

Groups	Cell proliferation inhibition rate (%)		
	24 h	36 h	48 h
H-ILCSCs-pLVX-GFP	7.64 $\pm$ 4.35	9.32 $\pm$ 4.70	16.46 $\pm$ 10.68
H-ILCSCs-pLVX-MI21-RNAi	39.54 $\pm$ 7.79 <sup>**, &amp;&amp;, @</sup>	42.04 $\pm$ 5.13 <sup>**, &amp;&amp;, @</sup>	44.18 $\pm$ 7.26 <sup>**, &amp;&amp;, @</sup>
HCCLM3-pLVX-GFP	6.28 $\pm$ 6.79	7.28 $\pm$ 5.19	5.31 $\pm$ 4.22
HCCLM3-pLVX-MI21-RNAi	20.06 $\pm$ 7.84 <sup>**, &amp;</sup>	19.70 $\pm$ 2.44 <sup>**, ##, &amp;</sup>	23.09 $\pm$ 6.74 <sup>**, ##, &amp;</sup>
HL-7702-pLVX-GFP	8.49 $\pm$ 5.60	9.94 $\pm$ 7.25	9.19 $\pm$ 9.39
HL-7702-pLVX-MI21-RNAi	8.84 $\pm$ 6.69	11.25 $\pm$ 6.81	10.99 $\pm$ 6.75

<sup>\*\*</sup> $p$ <0.01 vs. pLVX-GFP group, <sup>&&</sup> $p$ <0.01, <sup>&</sup> $p$ <0.05 vs. HL-7702 group, <sup>@</sup> $p$ <0.05, vs. HCCLM3-pLVX-MI21-RNAi group.

of HCCLM3- and HL-7702-LV-anti-miR-21 group (Figure 4). The invasive ability of HCCLM3-LV-anti-miR-21 group was also significantly higher compared to that of HL-7702-LV-

anti-miR-21 group (Figure 4). Moreover, the invasive inhibition rates in LV-anti-miR-21 treatment group were also significantly increased compared to that of pLVX-GFP group (Table



**Figure 2.** Examination for the miR-21 steadily expressing cell lines. **A**, GFP fluorescence observation in H-ILCSCs cells. **B**, GFP fluorescence observation in HCCLM3 cells. **C**, GFP fluorescence observation in HL-7702 cells. H-ILCSCs: high-invasion liver cancer stem cells. H-ILCSCs: high-invasion liver cancer stem cells.

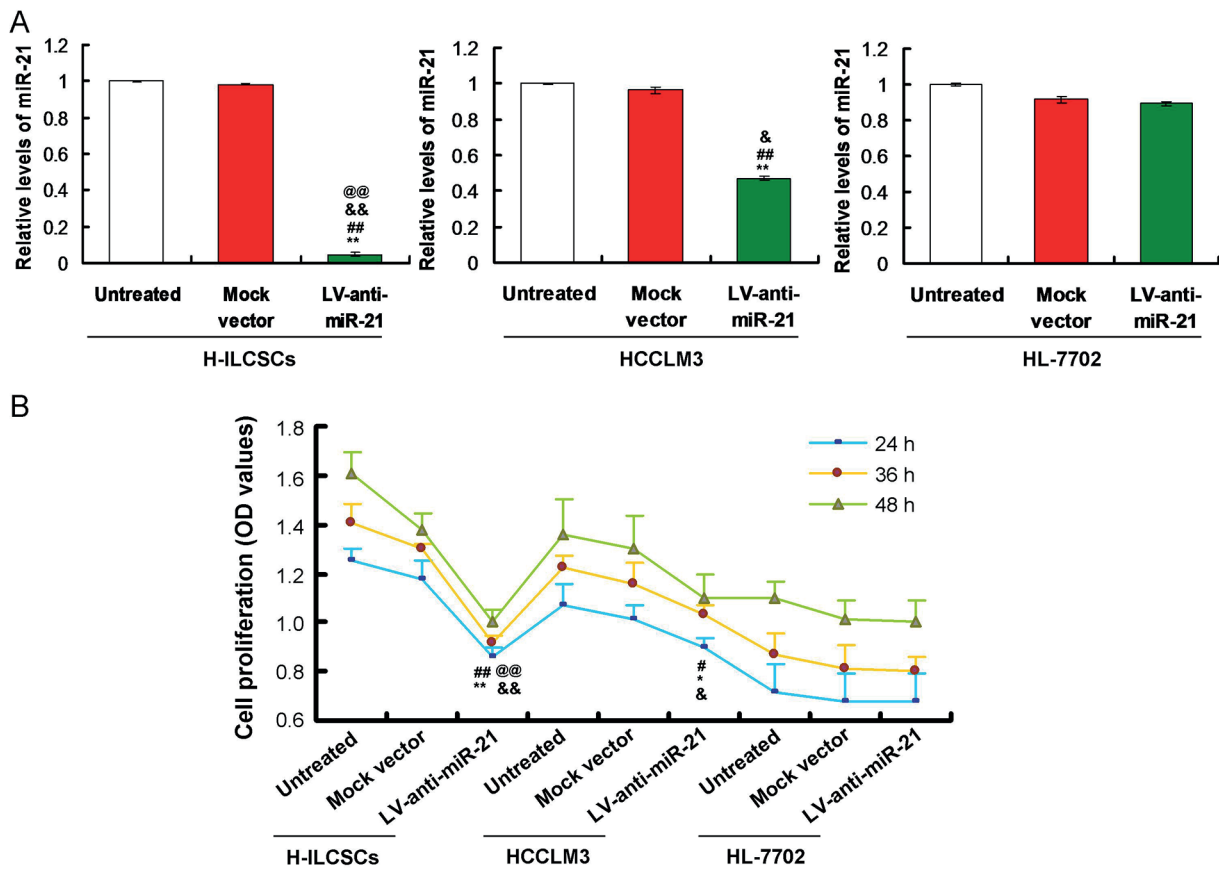
II,  $p < 0.01$ ). Also, the invasive inhibition rates in H-ILCSCs-LV-anti-miR-21 group were significantly higher compared to that of HCCLM3-LV-anti-miR-21 group and HL-7702-LV-anti-miR-21 group (Table II,  $p < 0.05$ ). The invasive inhibition rate in HCCLM3-LV-anti-miR-21 group was higher significantly compared to that of HL-7702-LV-anti-miR-21 group (Table II,  $p < 0.05$ ).

### Discussion

The production of the liver cancer cells always causes the reoccurrence or progression post the liver cancer operation<sup>3,20</sup>, suggesting that inhibiting tumor cell proliferation or eliminating the tumor cells is the key for treating liver cancer in clinical. We focused on the miR-21 as the targeting site

to perform the investigation on H-ILCSCs cells. Nowadays, the miRNA blocking technology or miRNA downregulating is a common applied method in investigating functions of miRNAs<sup>21</sup>. In this study, lentiviral vector was used to express targeting gene<sup>22</sup>, and which could stably express the miR-21 RNAi in each cell line. In this study, we successfully established the pLVX-miR21-RNAi plasmid and pLVX-miR21-RNAi lentiviral vector system. The pLVX-miR21-RNAi virus was infected to the high-invasive live cancer stem cell line (H-ILCSCs), high-invasive liver cell line (HCCLM3) and normal liver cell line (HL-7702), respectively, achieving to the transfection efficacy more than 90%. This result suggests that pLVX-miR21-RNAi virus illustrated better tropism to the above cell lines, which consists of the characteristics of the lentiviral vectors<sup>23</sup>. We





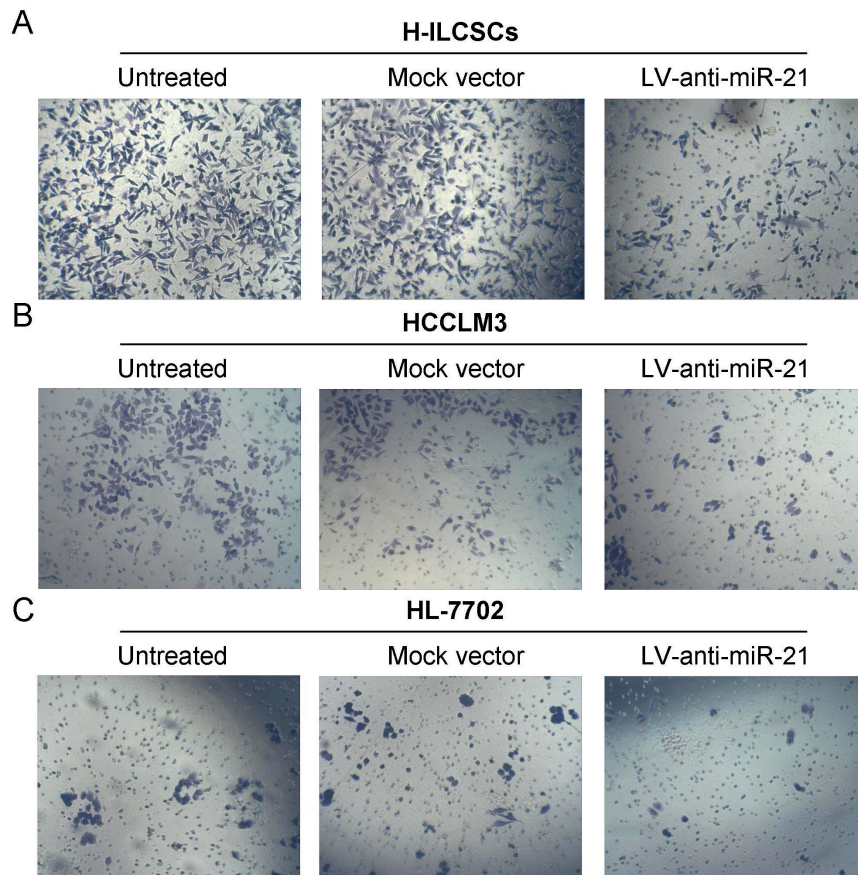
**Figure 3.** Observation for the miR-21 levels and proliferation in different cell lines. **A**, miR-21 levels in H-ILCSCs, HCCLM3 and HL-7702 cells. **B**, Cell proliferation in H-ILCSCs, HCCLM3 and HL-7702 cells. \* $p < 0.05$ , \*\* $p < 0.01$  vs. Untreated group, # $p < 0.05$ , ## $p < 0.01$  vs. Mock group, & $p < 0.05$ , && $p < 0.01$  vs. HL-7702-pLVX-anti-miR21 group, @ $p < 0.05$  vs. HCCLM3-pLVX-anti-miR21 group. H-ILCSCs: high-invasion liver cancer stem cells.

found that the miR-21 levels were downregulated in the anti-miR-21 gene steady expressed H-ILCSCs, HCCLM3 and HL-7702 cell lines. However, the miR-21 levels in H-ILCSCs were significantly lower compared to that of HCCLM3 group and HL-7702 group, and miR-21 levels in HCCLM3 group were significantly lower compared to that of HL-7702 group. Meanwhile, there were even no effects of miR-21 RNAi on the expression of miR-21 in HL-7702 cells. These results suggest that the high-invasive live cancer stem cell line or higher invasive liver cell line are sensitive to the miR-21 RNAi stimuli, and the normal liver cell line is resistant to miR-21 RNAi stimuli. We also indicated that the cell proliferation in LV-anti-miR-21 groups was significantly lower compared to that of Untreated group and Mock vector group in H-ILCSCs cells and HCCLM3 cells, at 24, 36 and 48 h, respectively. However, there are no effects of LV-anti-MIR21 on the proliferation

of HL-7702 cells. Cell proliferation in LV-anti-miR-21 group of H-ILCSCs was also higher significantly compared that of HCCLM3 cells and HL-7702 cells. The above results suggest that anti-miR21 RNAi illustrated even not effects on normal liver cell proliferation; however, significantly inhibited the proliferation of high-invasive liver stem cell or liver cell lines. Moreover, anti-miR21 RNAi treatment significantly inhibited the invasion of the H-ILCSCs cells and HCCLM3 cells, however, showing no effects on the invasion of normal liver cells. These findings are very significant and important for the anti-tumor drug development and application in clinical<sup>24</sup>.

## Conclusions

Liver cancer stem cells (LCSCs) tend to be at the leading edge of the liver cancer field<sup>25</sup>.



**Figure 4.** Images for the invasion of the H-ILCSCs, HCCLM3, HL-7702 cells undergoing anti-miR-21 RNAi treatment. **A**, Invasive images of H-ILCSCs cells. **B**, Invasive images of HCCLM3 cells. **C**, Invasive images of HL-7702 cells. H-ILCSCs: high-invasion liver cancer stem cells.

**Table II.** Invasive inhibition rate in the H-ILCSCs, HCCLM3, HL-7702 cells undergoing miR-21 RNAi transfection (mean  $\pm$  SD).

Group	Invasive inhibition rate (%)
H-ILCSCs-pLVX-GFP	9.08 $\pm$ 4.47
H-ILCSCs-pLVX-MI21-RNAi	78.70 $\pm$ 1.26**&&@
HCCLM3-pLVX-GFP	9.54 $\pm$ 4.77
HCCLM3-pLVX-MI21-RNAi	57.46 $\pm$ 6.65**&
HL-7702-pLVX-GFP	6.20 $\pm$ 3.56
HL-7702-pLVX-MI21-RNAi	11.12 $\pm$ 4.28*

\*\* $p$ <0.01 vs. pLVX-GFP group, && $p$ <0.01, & $p$ <0.05 vs. HL-7702 group, @ $p$ <0.05, vs. HCCLM3-pLVX-MI21-RNAi group.

However, there are also many issues needed to be resolved, including biomarker screening, cell sorting and culture, liver cancer examination and therapy. The present work was based on our previous research findings<sup>26</sup>, and proposed the concept of high-invasive liver cancer stem cells (H-ILCSCs) for the first time. In this study, we successfully established the pLVX-miR21-RNAi lentiviral vector system, which stably expressed

the miR-21 RNAi in the H-ILCSCs, HCCLM3 and HL-7702 cells. The down-regulation of the miR-21 could significantly inhibit the cell proliferation and invasion abilities of the H-ILCSCs and HCCLM3 cells, illustrating no effects on HL-7702 cells. Meanwhile, the H-ILCSCs is the most sensitive cell to the miR-21 RNAi treatment for cell proliferation and invasion among three cells. Our study provided the candidate molecule for



the gene therapeutic application of miR-21 in liver cancer in clinical, and initiated the beginning for investigating the function of miR-21 on the liver cancer stem cells or liver cancer cells.

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### Conflict of Interest

The Authors declare that they have no conflict of interest.

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