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Effect of LncRNA TINCR on Hepatocellular Carcinoma Hep-3B Cell Growth and Chemosensitivity

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ABSTRACT

Present study was aimed to investigate the effect of LncRNA TINCR on the proliferation, invasion, migration and chemotherapy sensitivity of hepatocellular carcinoma Hep-3B cells and its mechanism. In present study Hep-3B cells cultured in vitro were divided into control group (normal culture), NC-siRNA group (transfected with NC-siRNA), and TINCR-siRNA group (transfected with TINCR-siRNA), In addition, cells treated with cisplatin after transfection of TINCR-siRNA were designated as the TINCR-siRNA + cisplatin group, and only cells treated with cisplatin were designated as cisplatin group. The expression levels of TINCR and miR-646 in the cells of the control group, NC-siRNA group and TINCR-siRNA group were tested by RT-PCR, and the cell proliferation, invasion and migration ability of the three groups were detected by the CCK-8 method and Transwell chamber experiment. Cell proliferation and apoptosis of NC-siRNA group, TINCR-siRNA group, cisplatin group and TINCR-siRNA+cisplatin group were checked by CCK-8 method and flow cytometry; and targeted binding relationship between TINCR and miR-646 was measured by dual luciferase reporter gene experiment. According to results of our study, compared with the control group there was no significant difference in the parameters of Hep-3B cells after transfection of NC-siRNA ($P > 0.05$), but the expression level of TINCR and cell proliferation, invasion and migration ability were significantly reduced, and the expression level of miR-646 in the cells was significantly increased, which were statistically significant compared with the control group or the NC-siRNA group ($P<0.05$). In addition, transfection of TINCR-siRNA can also enhance the antiproliferative and pro-apoptotic effect of cisplatin on Hep-3B cells. Double luciferase reporter gene experiments confirmed that TINCR can target miR-646. We concluded Down-regulating the expression of TINCR can inhibit the proliferation, invasion and migration of hepatocellular carcinoma Hep-3B cells and enhance its sensitivity to chemotherapy with cisplatin, and the mechanism may be related to the targeted regulation of miR-646 expression.

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Authors' Contribution
GY, WK and SZ performed the experiment. YS, JZ and RY recorded and concluded the experimental data. GY an SZ wrote the manuscript.

Key words
Hepatocellular carcinoma, LncRNA TINCR, Cell proliferation, Invasion, Migration, Cisplatin, miR-646.

INTRODUCTION

Hepatocellular carcinoma is the world's sixth most common malignant tumor, also the second leading cause of cancer death (Tang *et al*., 2018). With a still increasing global burden, it may soon have a morbidity exceeding 1 million cases per year, seriously threatening people's health (Llovet *et al*., 2018). Chemotherapy plays an important role in the treatment of patients with intermediate and advanced hepatocellular carcinoma, but chemotherapy resistance is an important factor affecting its treatment. Therefore, in-depth exploration of the molecular mechanism of occurrence and development of hepatocellular carcinoma to search new effective therapeutic targets for suppressing the malignant progress of hepatocellular carcinoma and improving the sensitivity of tumor cells to chemotherapeutics has attracted much attention. Many cell transcripts do not code for proteins, and many are long non-coding RNAs (lncRNAs). LncRNAs are commonly identified as non-coding components of RNA that are longer than 200 nucleotides. Extremely, studies indicate that through various pathways, lncRNAs control gene transcription (Marchese *et al*., 2017). Studies (Gramantieri *et al*., 2018; Wu *et al*., 2018; Huang *et al*., 2018) have shown existence of abnormally expressed lncRNAs in hepatocellular carcinoma, and these LncRNAs may not only participate in the regulation of tumor cell function, but are also closely related to tumor cell resistance. LncRNA TINCR, an important member of LncRNAs, has been proven to be capable of promoting epithelial-mesenchymal transition in breast cancer and trastuzumab resistance (Dong *et al*., 2019). Studies by Tian *et al*. (2017) and Zhang *et al*. (2019) have pointed out that TINCR is abnormally highly expressed in hepatocellular

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carcinoma tissues, and the change in its expression level is closely related to poor prognosis in patients. However, the effect of TINCR on the growth and chemosensitivity of hepatocellular carcinoma cells remains unclear. This study aims to explore the effects of down-regulation of TINCR on the proliferation, invasion, migration and cisplatin chemosensitivity of hepatocellular carcinoma Hep-3B cells.

MATERIALS AND METHODS

Materials

Micro-ultraviolet spectrophotometer and constant temperature CO_2 incubator (Thermo, USA), inverted microscope (Olympus, Japan), flow cytometer (Becton Dickinson, USA), PCR instrument, gel imaging analysis system and microplate reader (Bio-Rad, USA). Reverse transcription kit (Life Technologies, USA), RT-PCR kit (DaKaRa, Dalian), dual luciferase reporting system detection kit (Promega, USA), Annexin V-FITC/PI apoptosis detection kit (Dalian Meilun Biotechnology), CCK-8 kit (Dojindo, Janpan), Matrigel and Transwell chamber (Corning, USA), DMEM medium and penicillin-streptomycin double antibody (Hyclone, USA), BCA protein concentration determination kit, ECL reagent and RIPA lysate (Shanghai Beyotime Biotechnology), fetal bovine serum and trypsin (Gibco, USA), Trizol reagent (Shanghai Yubo Biotechnology), Lipofectamine® 3000 transfection reagent (Invitrogen, USA), human hepatoma cell line Hep-3B (China Center for Type Culture Collection), small RNA interference sequence TINCR-siRNA targeting TINCR and its negative control NC-siRNA (Shanghai GenePharma), miR-646 mimic and its negative control miR-NC (Guangzhou RiboBio), cisplatin (Qilu Pharmaceutical), PCR primers (Shanghai Sangon Biotech).

Hep-3B cell culture and treatment

Hep-3B cells were routinely cultured in a DMEM medium containing 100 U/mL penicillin-streptomycin double antibody and 10% fetal bovine serum in a 5% CO_2 cell incubator at 37°C. Hep-3B cells in logarithmic growth phase were planted to a 6-well cell plate at a density of 5×10^5 cells/well, and then routinely cultured in a cell incubator. When the cell fusion reached 70%, transient transfection was performed with reference to Lipofectamine® 3000 transfection reagent. The cells transfected with NC-siRNA were taken as NC-siRNA group, the cells transfected with TINCR-siRNA were taken as TINCR-siRNA group, and the cells normally cultured without transfection were taken as the control group. In addition, the cells treated with 5μg/mL cisplatin for 48 h after transfection with TINCR-siRNA were taken as TINCR-siRNA + cisplatin group, while cells only treated with 5 μg/mL cisplatin for 48 h were taken as the cisplatin group. After 6 h of transfection, fresh medium was replaced. After 48 h further culture, cells from each group were collected for subsequent experiments.

Detection of TINCR and miR-646 expression in Hep-3B cells by RT-PCR method

After the total RNA of Hep-3B cells were extracted using Trizol method, cell concentration and integrity were detected by ultraviolet spectrophotometer. According to the reverse transcription kit instructions, RNA was reverse-transcribed into cDNA. Using cDNA as a template, PCR amplification was performed according to the RT-PCR kit instructions. Using GAPDH or U6 as an internal reference, expression levels of TINCR and miR-646 in Hep-3B cells were detected by $2^{-\Delta\Delta ct}$ method. Where, the primer sequences used in the experiment were as follows: TINCR forward 5'-TGTG-GCCCAAACTCAGGGATACAT-3', reverse: 5'-AGATGA-CAGTGGCTGGAGTTGT-CA-3'; miR-646 forward 5'-ACACTCCAGCTGGGAAGCAGCTG-CCTC-3', reverse: 5'-CTCAACTGTGCTGCATTAGTT-AGCTCAGA-3'; GAPDH forward 5'-ATTCCATGGCACCGTCAAGGCTGA-3', reverse: 5'-TTCTCCATGGTGGTGAAGACGCCA-3'; U6forward 5'-CTCGCTTCGGCAGCACA-3', reverse: 5'--AACGCTTCACGAATTTGCGT-3'.

Detection of Hep-3B cell proliferation by CCK-8 method

After the treatment, cells in the control group, NC-siRNA group and TINCR group were inoculated into 96-well cell plates at 5×10^4 per well, with 3 parallel wells in each group. After routine culture in a cell incubator for a required period, 10 μL of CCK-8 reagent was added to each well; after 4 h incubation, OD value of the cells was detected using a microplate reader; where, the detection wavelength was 450 nm. In the later period, cell proliferation activity of NC-siRNA group, TINCR-siRNA + cisplatin group, cisplatin group and TINCR-siRNA group was detected by the same method. The experiment was repeated three times.

Detection of Hep-3B cell invasion and migration by Transwell chamber experiment.

Invasion experiment: Before the experiment, Matrigel diluted 1:8 was evenly spread to the upper Transwell chamber and allowed to fully solidify at room temperature. Cells in the control group, NC-siRNA group, and TINCR-siRNA group were collected in a serum-free medium and made into cell suspension at a concentration of 10^5 cells/mL. Then, 100 μL of cell suspension and 500 μL of serum-containing medium were added into the

upper and lower chambers. After routine incubation in a cell incubator for 24 h, remove the chamber and immerse it in 4% paraformaldehyde stationary liquid for 30 min fixation. After staining with 0.1% crystal violet for 10 min, the number of transmembrane cells was counted with a microscope. The results were expressed as average number of cells in 3 randomly selected fields. Migration experiment: It is not necessary to coat Transwell chamber using Matrigel in the experiment. The remaining steps were the same as those in invasion experiment.

Detection of Hep-3B cell apoptosis by flow cytometry

For the cisplatin group, TINCR-siRNA group, NC-siRNA group and TINCR-siRNA + cisplatin group cells after trypsin digestion and collection were washed twice with pre-chilled phosphate buffer, then 200 μL of binding buffer was added to suspend the cells. 5 μL of Annexin V-/FITC and PI staining solution were added to a sample tube containing 10^5 cells; after staining at room temperature for 15 min in the dark, apoptosis rate of each group was detected using Becton Dickinson Calibur. The experiment was repeated three times.

Detection of the targeted binding relationship between TINCR and miR-646Double using dule-luciferase reporter gene experiment

TINCR 3'UTR fragment was cloned, amplified and recombined into a luciferase vector as a TINCR wild-type (TINCR-WT) vector. In addition, after site-specific mutagenesis, TINCR and miR-646 targeted binding sites were cloned and recombined into a luciferase vector as a TINCR-MUT vector. According to the instructions of the transfection reagent, the constructed TINCR-MUT and TINCR-WT vectors were co-transfected with miR-646 mimic and its negative control miR-NC into Hep-3B cells. After 48 h of transfection, luciferase activity of each group of cells was detected by referring to the instructions of the luciferase activity detection kit. The experiment was repeated three times.

Data analysis

The data obtained in the experiment are expressed in the form of $\pm s$. SPSS 22.0 software was used for statistical analysis. One-way analysis of variance was used for comparison between multiple groups. SNK-q was used for multiple comparisons between groups. Independent sample t-test was used for comparison between two groups. $P<0.05$ indicates statistically significant difference.

RESULTS

Table I shows the expression level of TINCR, effect of downregulation of TNCR expression on proliferaion, invasion and migration apoptosis and expression of miR646 in hepatocellular carcinoma Hep-3B cells. The expression level of TINCR in Hep-3B cells after transfection of NC-siRNA is not significantly different from that of the control group ($P> 0.05$), but the expression level of TINCR in Hep-3B cells after transfection with TINCR-siRNA is significantly lower than that in the control group or NC-siRNA group.

The down-regulation of TINCR expression inhibited the proliferation and atenuated the invasion and migration of hepatocellular carcinoma Hep-3B cells. The cell proliferation activity of NC-siRNA group is not statistically different from that of control group ($P>0.05$); TINCR-siRNA group has significantly reduced cell proliferation activity compared to the control group or NC-siRNA group ($P <0.05$). The cell invasion and migration ability of NC-siRNA group is not statistically different from the control group ($P> 0.05$); compared with the control group or NC-siRNA group, TINCR-siRNA group has significantly reduced cell invasion and migration ability ($P <0.05$).

Table I.- Expression level of TINCR in Hep-3B cells of each group.

Groups	mRNA
Control	1.00±0.08
NC-siRNA	0.95±0.07
TINCR-siRNA	0.24±0.03*
F	399.910
P	<0.001

Note: compared with control or NC-siRNA group, *$P<0.05$.

Table II.- Cell proliferation activity (OD) of each group.

Groups	24 h	48 h	72 h
Control	0.48±0.03	0.79±0.05	1.19±0.10
NC-siRNA	0.51±0.04	0.83±0.06	1.22±0.13
TINCR-siRNA	0.36±0.03*	0.64±0.03*	0.75±0.06*
F	50.029	38.700	61.289
P	<0.001	<0.001	<0.001

Note, compared with control or NC-siRNA group, *$P<0.05$.

Table III.- Invasion and migration ability of cells in each group (number of transmembrane cells).

Groups	No. of invasive cells	No. of migrating cells
Control	75.64±4.48	96.85±6.54
NC-siRNA	72.38±5.05	98.62±7.23
TINCR-siRNA	48.25±2.66*	61.36±3.72*
F	114.798	109.562
P	<0.001	<0.001

Note, compared with control or NC-siRNA group, *$P<0.05$.

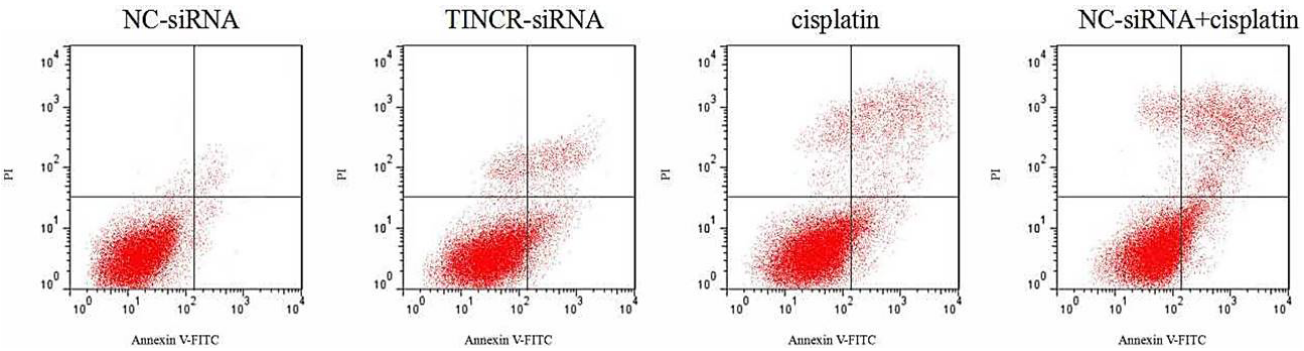

Fig. 1. Apoptosis of cells in each group detected by flow cytometry.

Down-regulation of TINCR expression can enhance chemosensitivity of hepatocellular carcinoma Hep-3B cells to cisplatin

Figure 1 and Table IV show that compared with the NC-siRNA group, TINCR-siRNA group, cisplatin group, TINCR-siRNA + cisplatin group have significantly reduced cell proliferation activity and significantly increased apoptotic rate (P <0.05); compared with the cisplatin group or TINCR-siRNA group, TINCR-siRNA + cisplatin group has significantly reduced cell proliferation activity and significantly increased apoptosis rate (P <0.05).

Table IV.- Proliferation activity and apoptosis rate of cells in each group.

Groups	Proliferative activity (OD value)	Apoptosis rate / %
NC-siRNA	0.81±0.06	7.36±1.15
TINCR-siRNA	0.62±0.04*	16.12±2.03*
cisplatin	0.57±0.03*	20.54.±2.38*
TINCR-siRNA+cisplatin	0.46±0.02*#	33.25±2.76*#
F	118.339	223.535
P	<0.001	<0.001

Note, compared with NC-siRNA group, *P<0.05; compared with TINCR-siRNA or cisplatin group, #P<0.05.

Table V.- Expression level of miR-646 in cells of each group.

Groups	miR-646
Control	1.00±0.08
NC-siRNA	1.12±0.09
TINCR-siRNA	5.52±1.25*
F	113.584
P	<0.001

Note, compared with control or NC-siRNA group, *P<0.05.

Down-regulation of TINCR expression increased miR-646 expression in hepatocellular carcinoma Hep-3B cells

Table V shows that expression level of miR-646 in the NC-siRNA group is not statistically different from that in the control group (P> 0.05); compared with the control group or NC-siRNA group, TINCR group has significantly increased expression level of miR-646 (P <0.05).

TINCR-WT 3'UTR 5'- ...GUUGUCAGAGCUGCUU...-3'

miR-646 3' -....AGUCUCCGUCGACGAA...-5'

TINCR-MUT 3'UTR 5'- ...GUUGUCAGACGACCAA...-3'

Fig. 2. Complementary binding sites between TINCR and miR-646.

Table VI.- Luciferase activity of cells in each group.

Groups	TINCR-WT	TINCR-MUT
miR-NC	1.00±0.06	1.00±0.05
miR-646 mimics	0.36±0.03*	0.97±0.06
F	28.622	0.152
P	<0.001	0.266

Note, compared with miR-NC group, *P<0.05.

TINCR can bind to miR-646 in a targeted manner

LncBase Predicted v.2 software prediction result in Figure 2 shows that TINCR and miR-646 have complementary binding sites. At the same time, the dual-luciferase reporter gene experiment result in Table VI shows that compared with miR-NC group, miR-646 mimics reduces luciferase activity in plasmid cells transfected with TINCR-WT (P<0.05), but it has no significant effect on luciferase activity in plasmid cells transfected with TINCR-MUT (P> 0.05).

DISCUSSION

LncRNAs are a class of RNA having a length of more than 200 amino acids, which have no protein coding function, but play an important role in regulating gene transcription. Participating in cell proliferation, invasion and migration, they are closely related to tumor occurrence and development (Peng *et al*., 2017; Yang *et al*., 2018). TINCR is an LncRNA closely related to epidermal cell differentiation. Located on chromosome 19, it has a size of 3.7 kb. Abnormally expressed in many tumors, TINCR is closely related to tumor occurrence and development as well as drug resistance formation (Zhang and Feng, 2018). Some studies have pointed out that TINCR has low expression in colorectal cancer tissues, and its loss of expression can promote tumor cell proliferation and metastasis by activating Wnt/β-catenin pathway (Zhang *et al*., 2016). Up-regulating its expression can inhibit cancer cell proliferation and promote apoptosis by PPAR signaling pathway regulation via miR-107/CD36 axis (Zhang *et al*., 2019). Highly expressed in non-small cell lung cancer, TINCR can promote tumor cell proliferation and migration by activating MAPK pathway (Zhu and He, 2018). In addition, HER-2 + breast cancer patients with highly expressed TINCR respond poorly to trastuzumab treatment and have short survival time. However, knocking down TINCR expression can reverse the tumor cells' drug resistance to trastuzumab by targeted regulation of miR-125b expression (Dong *et al*., 2019).

Some studies have confirmed that TINCR is up-regulated in hepatocellular carcinoma tissues, and its expression level is closely related to patients' tumor size, TNM stage and short survival time (Tian *et al*., 2017; Zhang *et al*., 2019). Nevertheless, the role of TINCR in the occurrence and development of hepatocellular carcinoma as well as formation of chemotherapy resistance remains unclear. After down-regulating TINCR expression in this study, it was found that hepatocellular carcinoma Hep-3B cells had significantly reduced proliferation, invasion and migration ability. The results suggest that down-regulating TINCR expression can inhibit the proliferation, invasion and migration of hepatocellular carcinoma Hep-3B cells. Cisplatin is a commonly used chemotherapeutic drug for hepatocellular carcinoma in the clinic (Yang *et al*., 2019), which was taken as the research object of chemotherapy resistance in this study. It was found that down-regulating TINCR expression could enhance the antiproliferative and pro-apoptotic effects of cisplatin on hepatocellular carcinoma Hep-3B cells. This indicates that down-regulating TINCR expression can enhance chemosensitivity of hepatocellular carcinoma Hep-3B cells to cisplatin. It is suggested that TINCR plays an important role in promoting the growth of hepatocellular carcinoma cells and the formation of resistance to cisplatin chemotherapy. As a competitive endogenous RNA, LncRNA plays a role in tumor regulation by adsorbing miRNA (Pi *et al*., 2019). miRNAs are a class of endogenous small RNAs consisting of 22~24 amino acids, which can affect cell biological behavior by interacting with target mRNA and are involved in the occurrence and development of many tumors including hepatocellular carcinoma (Li *et al*., 2018; Wong *et al*., 2018). MiR-646 is a member of the miRNAs family, which has been reported to be underexpressed in tumors such as nasopharyngeal, gastric cancers and play an important inhibitory role in tumor cell growth (Song *et al*., 2019; Zhang *et al*., 2017). In addition, miR-646 can also be regulated by circ-u0081143 in a targeted manner, and participate in the molecular mechanism of circ-u0081143 by promoting gastric cancer cell proliferation, invasion and resistance to cisplatin chemotherapy (Xue *et al*., 2019). Studies (Pan *et al*., 2019) have pointed out that miR-646 is down-regulated in hepatocellular carcinoma tissues and plays a role in inhibiting cancer cell proliferation, invasion and promoting apoptosis. Further detection in this study revealed that down-regulation of TINCR could cause increased expression of miR-646 in hepatocellular carcinoma Hep-3B cells. At the same time, bioinformatics software predicted the existence of complementary binding sites between TINCR and miR-646; dual luciferase reporter gene experiment confirmed that miR-646 can be targeted to 3'UTR region of TINCR. The results indicate that TINCR can perform targeted regulation of miR-646 expression. It is suggested that down-regulating TINCR expression may inhibit proliferation, invasion, migration of hepatocellular carcinoma Hep-3B cells and enhance chemosensitivity of cancer cells to cisplatin by targeted up-regulation of miR-646 expression.

CONCLUSION

To conclude, down-regulating TINCR expression can inhibit the proliferation, invasion, migration of hepatocellular carcinoma Hep-3B cells and enhance its chemosensitivity to cisplatin. The mechanism may be related to the targeted regulation of miR-646 expression. The results initially revealed the role of TINCR in the occurrence and development of hepatocellular carcinoma and formation of cisplatin resistance, thus providing new clues for the pathogenesis and treatment of hepatocellular carcinoma.

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Statement of conflict of interest

The authors have declared no conflict of interests.

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