The expression of lncRNA XIST in hepatocellular carcinoma cells and its effect on biological function

Zhidan Dong¹, Juan Yang², Fuchang Zheng¹, Yao Zhang²

¹Department of General Surgery, Weifang People’s Hospital, Weifang 261041, P.R. China. ²Department of Sterilization and Supply, Weifang People’s Hospital, Weifang 261041, P.R. China.

Summary

Purpose: Hepatocellular carcinoma (HCC) is one of the common cancers, but its relationship with long non-coding (lnc) RNA XIST and microRNA (miR)-488 is still under investigation. Therefore, this study aimed to explore the correlation between miR-488 and XIST in HCC.

Methods: qRT-PCR was employed to quantify the lncRNA XIST and miR-488 in HCC tissues and cells, and miR-488 mimics and lncRNA siRNA vectors were constructed for analysis of the roles of miR-488 and lncRNA XIST in HCC cells. Flow cytometry was applied to determine the cell cycle and apoptosis, Western blot assay to detect apoptosis-related proteins, and the MTT assay to detect cell viability.

Results: lncRNA XIST was highly expressed in HCC, while miR-488 was lowly expressed. Silencing lncRNA XIST gave rise to an increase in G0/G1 phase cells and a decrease in S-phase cells, promoted apoptosis, weakened cell viability, and induced up-regulation of Caspase-3, Caspase-9, and Bax, and up-regulating miR-488 led to similar results. The dual luciferase reporter gene assay confirmed that lncRNA XIST could bind to miR-488, and its inhibition could give rise to up-regulation of miR-488. It was also confirmed that lncRNA XIST was negatively correlated with miR-488.

Conclusion: LncRNA XIST accelerates HCC cell growth by inhibiting miR-488, so inhibiting lncRNA XIST or up-regulating miR-488 has objective potential therapeutic value and may be helpful for the development of new HCC treatment strategies.

Key words: hepatocarcinogenesis, lncRNA XIST, miR-488, cell biological function

Introduction

Hepatocellular carcinoma (HCC) is a common cancer worldwide, with extremely high mortality [1], connected with chronic inflammatory disease [2]. In HCC cells, the levels of pro-inflammatory factor IL-6, and inflammatory pathway protein NF-κB, are significantly up-regulated [3], while diabetes mellitus, hepatitis B virus (HBV), hepatitis C virus (HCV), and excessive drinking are also risk factors for HCC [4]. HCC has a strong drug resistance [5], so patients have poor prognosis. At present, the molecular network of HCC remains unclear, and understanding this kind of molecular network is conducive to gaining accurate prognostic markers and more effective treatment regimens [6].

LncRNA XIST is a RNA with a length of 19296 bp. It is dysregulated in HCC and can accelerate HCC cell growth through pathways such as JPX/XIST/miR-155, XIST/miR-194/MAPK1, XIST/miR-497/PDCD4, and XIST/miR-139/PDK1/AKT [7-10]. MiR-488 is a non-coding RNA located on chromosome 1, with a length of 83 bp, and it is closely related to various diseases. It suppresses the growth and expansion of osteosarcoma cells by binding to the 3’untranslated region of aquaporin-3 (AQP3) [11],

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and its high expression inhibits the bone marrow mesenchymal stem cells from expressing Runx2 protein, Osterix protein, and alkaline phosphatase in osteoporosis, and thus inhibits osteogenic differentiation [12]. Moreover, miR-488 can accelerate fracture healing by inhibiting the expression of DKK1 in osteoblasts [13]. In congenital megacolon, miR-488 is activated by cir-CCDC66 sponge to up-regulate the DCX level and finally promote cell proliferation and migration [14]. MiR-488 is highly expressed in HCC and can be used as a tumor suppressor to affect hepatocarcinogenesis [15].

This study compared the expression levels of IncRNA XIST and miR-488 in tumor/adjacent tissues, finding that IncRNA XIST was highly expressed in HCC, while miR-488 was poorly expressed. There were matching sites between IncRNA XIST and miR-488 according to prediction based on the Starbase database, so this study deduced that the abnormal expression of IncRNA XIST and miR-488 may be related to HCC. At present, the mechanisms of IncRNA XIST and miR-488 are still unclear, so this study has explored the roles of the two in HCC to study the mechanisms.

Methods

Collection of HCC samples

HCC tissues were sampled from 69 patients diagnosed with HCC in our hospital. The inclusion criteria of the patients were as follows: Patients diagnosed with HCC according to clinical or biochemical indicators. The exclusion criteria were as follows: Patients with mental disease, comorbid patients, patients with other tumors, patients who had hepatectomy, had received chemotherapy, radiotherapy or antibiotic therapy, and patients unwilling to cooperate with surgical team. The patients were fully informed of the study, and the study was carried out under the permission of the Ethics Committee of our Hospital. Tissue samples were cut into sections, and stored in -80°C liquid nitrogen until assayed.

Cell culture and transfection

Human normal hepatic cell (THLE-2) and HCC cell lines (HepG2, Hep3B and PLC/PRF/5) purchased from the American Type Culture Collection (ATCC) were transferred into a T25 cell culture flask (Thermo Fisher Company, Shanghai, China), and cultured in an incubator (Binder Company, Germany) under 5% CO2 at 37°C. The culture medium system contained Dulbecco's Modified Eagle Medium (DMEM) (Hyclone Company, Shanghai, China), 10% fetal bovine serum (FBS) solution (Gibco Company, Shanghai, China), and 1% penicillin-streptomycin solution (solution concentration after diluting 100 times) (Solarbio Company, Beijing, China). The experiment was continued after the cells were cultured to cover 80-90% of the culture flask. Before transfection, the culture medium was replaced with a culture medium without fetal bovine serum, and on the day of transfection, the cells were seeded into a 6-well plates at 1×105 cells/well. The miR-488 mimics, NC mimics, XIST siRNA, and NC siRNA were all purchased from Shanghai Sangon Biotech Co., Ltd. The cell lines were transfected with Lipofectamine 2000 transfection kit (Invitrogen, Carlsbad, Calif, USA) in strict accordance with the kit instructions. After 8 h of transfection, the medium was replaced with fresh medium to avoid cell poisoning.

Quantification of IncRNA XIST and miR-488 by qRT-PCR

The total RNA was extracted from tissues and cells using the Trizol method, and the optical density (OD) of the total RNA at 260-280 nm was detected using an ultraviolet spectrophotometer, while the RNA with OD 260/OD280>1.8 was used for subsequent qRT-PCR assays. Reverse transcription and PCR amplification and quantification were conducted using a FastKing one-step reverse transcription-fluorescence quantitative kit (Tiangen Biotech, Beijing Co., Ltd., FP514) and ABI PRISM 7000 (Applied Biosystems, USA). The primers of miR-488 and IncRNA XIST were all designed and synthesized by Shanghai Sangon Biotech Co., Ltd. For miR-488: F: 5′-GG GGC A GC UCA GUA CACAG-3′, R: 5′-CAGTGC GTGTCGTGGAGGT-3′. For IncRNA XIST: F: 5′-CAGAC GTGT GTCGTGGAGGT-3′, R: 5′-CGATCTGTAAGTCCACCA-3′. qRT-PCR was performed under a reaction system consisting of 50 μL of total volume containing 1.25 μL of upstream primer, 1.25 μL of downstream primer, 1.0 μL of probe, 10 pg/μg of RNA template, 5 μL of 50×ROX Reference Dye ROX, and RNase-Free ddH2O added to adjust the volume. The reaction process included reverse transcription at 50°C for 50 min (one cycle) and pre-denaturation at 95°C for 5 min (one cycle), followed by 40 cycles of denaturation at 95°C for 15 s, and annealing at 60°C for 50 s. The results were analyzed using an ABI PRISM 7000 instrument, and the data were normalized using the 2−ΔΔCt method, with U6 and GAPDH as internal references.

Apoptosis-related protein determination by Western Blot assay

The cells were lysed with RIPA protein extraction kit (20 mM of Tris-HCl solution with pH7.5; Solarbio Company) and protein inhibitor (Solarbio Company, Beijing, China), and the solution was repeatedly pipetted until the cells were completely lysed. Then, the solution was centrifuged in a pre-cooling centrifuge at 1.6×104×g for 20 min at 4°C to take the supernatant, and the protein concentration was determined using the bicinchoninic acid (BCA) assay method. The protein was separated out through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a nitrocellulose (NC) membrane, and let to stand at room temperature for 1 h (blocked with 5% skim milk - phosphate buffer saline (PBS) solution). Subsequently, the protein to be detected was let to stand with β-actin antibody at 4°C overnight. The NC membrane was washed with PBS solution three times, added with goat anti-rabbit secondary antibody (HRP conjugant), and then let to standard for 1 h at room temperature. Finally, the NC membrane was washed with PBS solution, and visualized by electrochemiluminescence (ECL) reagent. The internal reference protein was β-actin, and the relative expression

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level of the protein to be detected was recorded as the gray value of the band to be detected/the gray value of β-actin protein band. Caspase-3, Caspase-9, Bax, Bcl2, β-actin primary antibodies and goat anti-rabbit secondary antibody (HRP conjugant) were all purchased from Shanghai Abcam Company.

Determination of cell viability by MTT assay

The transfected cells were seeded into four 96-well plates at 5×10^3 cells/100 μL in each well, with 3 wells in each group. One plate was taken out every 24 h, added with 5 mg/ml MTT solution dissolved in dimethylsulfoxide (DMSO) (Solarbio Company) at 10 μL/well, and then cultured continuously for 1 h. Subsequently, the medium was taken out, and the OD at 570 nm was measured using an enzyme mark instrument. The experiment was repeated 3 times, and cell viability-time curves were drawn.

Detection of apoptosis and cell cycle by flow cytometry

The cells were prepared into suspension through enzymolysis, and the number of cells was controlled to 1×10^6. The cells were immobilized in 70% ice-cold ethanol solution at ambient temperature of 4°C for 30 min, and then the ethanol solution was removed, and the cells were incubated in mixed Annexin V-FITC/7-AAD solution. Subsequently, FACScan flow cytometer (Becton Dickinson Company, USA) was employed to analyze apoptosis, and Phoenix Flow Systems (Innovative Cell Technologies) were applied to analyze the data. The analysis steps of the cell cycle were similar to those of apoptosis, but in cell cycle analysis, after cell immobilization, the mixed Annexin V-FITC/7-AAD solution should be replaced with mixed propidium iodide (PI) (50 ng/ml)/RNase (0.2 mg/ml)/0.1% Triton X-100 solution, and treated at room temperature for 30 min.

Dual luciferase reporter gene assay

PrimGLO-IncRNA XIST-wt and primGLO-IncRNA XIST-mut vectors were constructed and co-transfected into cells with miR-488 mimcs and NC mimcs, respectively. The transfected cells were cultured in 96-well plates for 48 h, and then their luciferase activity was detected using the dual luciferase reporter gene assay system (Promega, Beijing, China).

Statistics

In this study, the data were statistically analyzed using SPSS 20.0 (Asia Analytics Formerly SPSS, China), and presented in figures using GraphPad Prism 6.0.

**Figure 1.** Up-regulation of IncRNA XIST in HCC and down-regulation of miR-488. **A:** LncRNA XIST was up-regulated in HCC tissues; **B:** LncRNA XIST was up-regulated in HCC cells; **C:** MiR-488 was down-regulated in HCC tissues; **D:** MiR-488 was down-regulated in HCC cells (**p<0.01 and ***p<0.001).
The experiments were repeated three times. Measurement data were expressed as mean±standard deviation (Mean±SD). Comparison of HCC tissues and the corresponding non-tumor normal tissues was carried out using the independent-samples T-test. Comparison of IncRNA XIST and miR-488 between different cells was carried out using one-way ANOVA, and post hoc pairwise comparison was carried out using the LSD-t test. Pearson’s correlation analysis was conducted to understand the correlation between miR-488 and IncRNA XIST. All data were analyzed using the two-tailed test, and 95% was used as the confidence interval. P<0.05 indicated a significant difference.

Results

Up-regulation of IncRNA XIST and down-regulation of miR-488 in HCC

In this study, we collected 69 HCC tissue specimens and 69 corresponding non-tumor normal tissue specimens, and quantified IncRNA XIST and miR-488. It was turned out that compared with non-tumor normal tissues, HCC tissues showed increased IncRNA XIST level, but decreased miR-488 level (Figures 1A and 1C). We also determined the expression of IncRNA XIST and miR-488 in normal hepatic cell (THLE-2) and HCC cells (HepG2, Hep3B, and PLC/PRF/5), finding that, compared with THLE-2, those HCC cells showed increased IncRNA XIST level, but decreased miR-488 level (Figure 1B and 1D). The above results demonstrated that IncRNA XIST was actively expressed in HCC, while miR-488 was silently expressed. Since IncRNA XIST and miR-488 had the highest and lowest expression in HepG2 cells respectively, we selected HepG2 for the following experiments.

The role of IncRNA XIST in promoting cell proliferation and inhibiting cell apoptosis

In this study, we constructed IncRNA XIST siRNA vectors to understand the function of IncRNA XIST in HCC cells, and applied flow cytometry to determine the cell cycle and apoptosis. MTT assay was used to determine cell viability, Western blot assay to determine the Caspase-3, Caspase-9, Bax and Bcl2 protein levels. The results of flow cytometry (Figures 2A and 2B) revealed that silencing...
lncRNA XIST resulted in a decrease in S-phase cells and an increase in G0/G1-phase cells accompanied by an increase in apoptosis rate. The results of MTT assay (Figure 2C) revealed that silencing lncRNA XIST resulted in a decrease in cell activity, and the results of Western blot assay (Figure 2D) revealed that silencing of lncRNA XIST induced up-regulation of Caspase-3, Caspase-9 and Bax, and down-regulation of Bcl2. The above results suggested that lncRNA XIST could promote the proliferation of HCC cells and inhibit their apoptosis.

The role of miR-488 in inhibiting cell proliferation and promoting cell apoptosis

We also constructed miR-488 mimics vectors to find out the function of miR-488 in HCC cells, and applied flow cytometry to determine cell proliferation and apoptosis. MTT assay was used to determine cell viability, Western blot assay to determine the Caspase-3, Caspase-9, Bax and Bcl2 protein levels. The results of flow cytometry (Figures 3A and 3B) revealed that up-regulation of miR-488 resulted in a decrease in S-phase cells and an increase in G0/G1-phase cells accompanied by an increase in apoptosis rate. The results of MTT assay (Figure 3C) revealed that up-regulation of miR-488 resulted in a decrease in cell activity, and those of Western blot (Figure 3D) revealed that up-regulation of miR-488 induced up-regulation of Caspase-3, Caspase-9 and Bax, and down-regulation of Bcl2. The above results suggested that miR-488 could inhibit the proliferation of HCC cells and promote their apoptosis.

Targeted inhibition of miR-488 by lncRNA XIST

Matching binding sites with miR-488 were found on the lncRNA XIST sequence fragment through Starbase database prediction (Figure 4A), so we constructed primGLO-XIST-wt and miR-488 mimics vectors to verify whether the two can bind to each other in a targeted manner. The results of dual luciferase reporter gene assay (Figure 4B) revealed that when XIST-wt and miR-488mimics were co-transfected into HepG2 cells, the fluorescence activity of the cells was down-regulated, while other co-transfection combinations did not cause

Figure 3. The role of miR-488 in inhibiting cell proliferation and promoting cell apoptosis. A: Increased miR-488 induced decrease in S-phase cells and increase in G0/G1-phase cells; B: Increased miR-488 resulted in increased apoptosis; C: Increased miR-488 reduced cell activity; D: Increased miR-488 upregulated Caspase-3, Caspase-9 and Bax, but downregulated Bcl2. (*indicates that in comparison with the NC group, p<0.05, and ** indicates that in comparison with the NC group, p<0.01).
significant changes in fluorescence activity, and Pearson’s correlation analysis revealed that lncRNA XIST was negatively correlated with miR-488 mimics (Figure 4C), and miR-488 was up-regulated when lncRNA XIST was interfered by siRNA (Figure 4D). The above results indicated that lncRNA XIST inhibited miR-488 in a targeted way.

Discussion

Hepatocarcinogenesis involves the regulation of various signaling pathways. The following pathways are taken as examples. The p53 pathway interferes with DNA replication and cell division via activation of stress signals, thus giving rise to cell senescence and apoptosis [16], and it can mediate the inhibitory effect of other proteins on the growth of HCC cells [17]. MAPK pathway involves serine and threonine phosphorylation, and regulates cell apoptosis and cell cycle [18], and the expression of MAPK pathway protein in HCC is up-regulated dramatically for the regulation by other factors [19]. PI3K/Akt/mTOR pathway is a crucial signaling pathway that integrates extracellular stimuli and transports them into cells, and it can promote tumor angiogenesis, metastasis and proliferation [20]. In HCC, PI3K pathway is activated to promote tumor formation [21].

In this study, we analyzed the non-coding RNA with abnormal expression in HCC tissues, finding that lncRNA XIST was highly expressed in the tissues, while miR-488 was lowly expressed. Therefore, we speculated that the abnormal expression of lncRNA XIST and miR-488 may be related to hepatocarcinogenesis. We interfered with lncRNA XIST, and found that the interference brought about significantly increased cell apoptosis rate, decreased S-phase cells, increased G0/G1-phase cells, and weakened cell viability. It indicated that lncRNA XIST inhibited apoptosis of HCC cells and promoted them to proceed to S phase. In addition, we also found that the deletion of lncRNA XIST induced up-regulation of Caspase-3, Caspase-9, and Bax, and down-regulation of Bcl2. Those findings demonstrated that lncRNA XIST was a tumor promoting factor for HCC cells, which could promote apoptosis by up-regulating Caspase-3, Caspase-9, and Bax, and down-regulating Bcl2. When the expression of lncRNA XIST was inhibited, miR-488 showed an up-regulation trend instead, which seemed to suggest that miR-488 mediated the effect of lncRNA XIST on HCC cells. Therefore, we overexpressed miR-488 in HCC for further analysis. The results showed that overexpressed miR-488 brought about increased apoptosis rate, constant cell cycle in G0/G1 phase, decreased cell viability, up-regulated Caspase-3, Caspase-9, and Bax, and down-regulated Bcl2, which indicated that miR-488 promoted cell apoptosis and inhibited cell proliferation. The dual luciferase reporter gene assay revealed that lncRNA XIST inhibited the expression of miR-488 in a targeted manner.
Those findings suggested that the lncRNA XIST/miR-488 axis could accelerate HCC cell proliferation and weaken their apoptosis, and this effect may involve multiple signaling pathways. Previous studies have shown that miR-488 regulates the development of various cancers by inhibiting PI3K/Akt/mTOR and MAPK pathway, and activating p53 and TGF-β1 pathway [22-26]. Therefore, the inhibition of lncRNA XIST on miR-488 may change the activity of multiple signaling pathways downstream of miR-488, such as promoting PI3K/Akt/mTOR and MAPK pathway or inhibiting p53 pathway and TGF-β1 pathway, thus inducing a series of molecular and cellular biological changes.

This study explored the relation between lncRNA XIST and hepatocarcinogenesis, focusing on the regulation mechanism of lncRNA XIST mediated by miR-488. In essence, the biological effects of miR on cells need to be transmitted through downstream target proteins. Therefore, in future research, we can further investigate specific downstream target proteins affected by lncRNA XIST/miR-488 and the effects on biological function of cells, and we can also study the changes in the signaling pathway downstream of miR-488.

To sum up, this study has investigated the relationship between lncRNA XIST/miR-488 axis and HCC by studying the biological changes caused by lncRNA XIST and miR-488 in the cancer, and it holds that lncRNA XIST directly regulates miR-488-mediated proliferation and apoptosis through pairing and binding with miR-488. It is through this regulation that lncRNA XIST promotes hepatocarcinogenesis, so targeted inhibition of lncRNA XIST may be helpful for the treatment of HCC patients.

**Conflict of interests**

The authors declare no conflict of interests.

**References**

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