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LncRNA TATDN1 induces the progression of hepatocellular carcinoma *via* targeting miRNA-6089

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Abstract. – OBJECTIVE: To clarify the potential effect of long non-coding RNA (lncRNA) TATDN1 on accelerating the proliferative rate and cell cycle progression of hepatocellular carcinoma (HCC) via sponging miRNA-6089, thus participating in the progression of HCC.

PATIENTS AND METHODS: TATDN1 expression in HCC tissues and normal tissues was first determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The correlation between TATDN1 expression to metastasis and overall survival of HCC was analyzed. The cellular level of TATDN1 in HCC cell lines was examined as well. Regulatory effects of TATDN1 on cell cycle progression and viability of HepG2 and SMMC7721 cells were evaluated. Subsequently, a potential target of TATDN1 was screened out and verified by Dual-Luciferase reporter gene assay. The expression pattern and biological function of the target gene miRNA-6089 in HCC were also determined. In a similar way, LIX1L was verified to be the target of miRNA-6089 and tested for its biological function in HCC.

RESULTS: TATDN1 was upregulated in HCC tissues and cell lines. The overexpression of TATDN1 accelerated the proliferative rate and cell cycle progression of HCC. MiRNA-6089, the target gene of TATDN1, was lowly expressed in HCC. The overexpression of miRNA-6089 partially reversed the promotive role of TATDN1 in regulating the proliferation and cell cycle of HCC cells. Subsequently, LIX1L was verified to be the target of miRNA-6089. The overexpression of LIX1L partially reversed the regulatory effect of miRNA-6089 on the proliferative rate and cell cycle progression of HCC.

CONCLUSIONS: TATDN1 accelerates the proliferative rate and cell cycle progression of HCC by degrading miRNA-6089 to upregulate LIX1L.

Key Words:
HCC, TATDN1, MiRNA-6089, LIX1L, Proliferation, Cell cycle.

Introduction

Hepatocellular carcinoma (HCC) is the most common malignant subtype of liver tumors and one of the top ten fatal cancers[1]. About 90% of HCC patients experience recurrence and metastasis after the initial treatment, and ultimately die from it[2]. Therefore, potential therapeutic and prognostic markers for HCC are needed to be searched and explored[3].

Molecular targeting therapy for cancer-based on tumor genomics and precision medicine of big data platform has been well concerned in recent years[4]. Some targeted therapy drugs for HCC (such as the first-line drug Sorafenib, and the second-line drug Regaginib) have already been applied and achieved promising outcomes[5]. In recent years, researches on tumor-related non-coding RNAs have attracted much more attention.

Long noncoding RNA (lncRNA) lacks an open reading frame that does not encode proteins or only encode a short peptide[6]. It has been considered transcriptional noise in human genome sequences for a long time[7]. So far, evidence has identified the regulatory effects of lncRNA on physiological and pathological processes[8,9]. Certain lncRNAs have been identified to participate in the progression, invasion, metastasis and recurrence of HCC[10,11]. For example, HULC is the earliest discovered lncRNA that is abnormally expressed in HCC. Downregulation of

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HULC markedly altered expressions of cyclin D1, c-jun and hepatocyte growth factor, suggesting its crucial function in the development of HCC[12]. LncRNA-UFCl is highly expressed in liver cancer and can promote the tumor cell proliferation[13]. Wang F. et al[14] found that the upregulation of lncRNA PVT1 in liver cancer cells accelerates the proliferative rate and maintains the stemness of liver cancer cells.

LncRNA TATDN1 is highly expressed in NSCLC, and its downregulation weakens tumor cell abilities to proliferate and invade[15]. Wang L. et al[16] pointed out that TATDN1 stimulates lung cancer cells to proliferate and enhances cisplatin-resistance *via* miR-451/TRIM66 axis. Although TATDN1 may exert an important role in lung cancer, its research on HCC has not been reported.

Previous research indicated the upregulation of TATDN1 in HCC tissues. In this paper, we aim to elucidate the potential function of TATDN1 in the pathogenesis of HCC.

Patients and Methods

General Data

HCC tissues and paracancerous tissues (n=24) were collected from HCC patients undergoing surgery. The inclusive criteria were: (1) No history of other malignancies; (2) Patients did not receive preoperative treatment and underwent surgical resection for the first time; (3) HCC was pathologically diagnosed by two independent pathologists and (4) patients had complete clinical and follow-up data. Tissues samples were immediately placed in liquid nitrogen within 15 min *ex vivo*. Patients volunteered to participate in the research and signed written informed consent. This study was approved by the Ethics Committee of Renji Hospital Affiliated to Shanghai Jiaotong University School of Medicine.

Cell Culture

L02 cell line and HCC cell lines (PLC/PRF/5, Bel-7404, HepG2 and SMMC7721) were provided by the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured at 37°C, 5% CO_2 incubator with Roswell Park Memorial Institute-1640 (RPMI-1640; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 U/mL penicillin and 0.1 mg/mL streptomycin.

Transfection

Cells were seeded one day before transfection. At the other day, transfection of relative plasmids or miRNA mimics at a final dose of 50-100 nm was performed using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA).

RNA Extraction

Tissues or cells were lysed in 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA). After maintenance at room temperature for 5 min, 200 μL of chloroform was added, mixed and let stand at room temperature for 5 min. The supernatant was transferred into a new RNase-free centrifuge tube after centrifugation at 4°C, 12000 rpm for 15 min. Isopropanol with the same volume of the supernatant was added for harvesting RNA precipitate by centrifugation. The extracted RNA was air dried and quantified. RNA samples with A260/A280 of 1.8-2.0 were dissolved in 10-20 μL of diethyl pyrocarbonate (DEPC) water.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

RNA was subjected to a reverse transcription system using a PrimeScript RT reagent Kit to obtain a complementary deoxyribose nucleic acid (cDNA). Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was carried out in accordance with the instruction of SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). The total qRT-PCR system was 10 μL and performed as pre-denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Primer sequences were as follows: MiRNA-6089, F: 5′-ACACTCCAGCTGGGGGAGGCCGGGGTGGGGCG-3′, R: 5′-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCCGCCCCG-3′; U6, F: 5′-CTCGCTTCGGCAGCAGCACATATA-3′, R: 5′-AAATATGGAACGCTTCACGA-3′; TATDN1, F: 5′-ACCGGAGGAAAATCCCTAGC-3′, R: 5′-AAATCCCCATAAAAGGGGCGGCTG-3′; GAPDH,F:5′-GAAGAGAGAGACCCTCACGCTG-3′, R: 5′-ACTGTGAGGAGGGGAGATTCAGT-3′; LIX1L, F:5′-GGGAGGGGCACTCTCCGAGC-3′, R: 5′-GCGAAGCTCCTCACCACGGC-3′.

Dual-Luciferase Reporter Gene Assay

The wild-type and mutant-type TATDN1/LIX1L sequences containing the binding sites of miRNA-6089 were inserted into the pGL3 Dual-Luciferase vector for constructing TATDN1 WT/LIX1L WT and TATDN1 MUT/LIX1L MUT. Cells were co-transfected with miRNA-6089 and wild-type/mutant-type vectors. Transfected cells were lysed at 24 h for Luciferase activity deter-

mination using Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Cell Cycle Assay

Transfected cells for 24 hours were seeded in 6-well plates. After incubation overnight, cells were subjected to fixation with ethanol at 4°C overnight and dye with 1 mL of DNA staining solution for 30 min. Finally, cell cycle progression was determined using MACS flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

Viability Determination

Cell density was adjusted to 3-5×10^3/mL and inoculated in 96-well plates. After cell adherence, 100 µL of medium with 10 µL of Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) was applied per well and the optical density $(OD)_{450}$ value was recorded 1 h later.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Intergroup differences that were asymptotically normal were analyzed by *t*-test. The comparison between groups was made using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). Otherwise, a non-parametric test was performed. $p<0.05$ was considered statistically significant.

Results

Great Abundance of TATDN1 in HCC

First, we examined TATDN1 expression in HCC tissues and paracancerous tissues (n=24). QRT-PCR data showed a higher level of TATDN1 in tumor tissues relative to controls (Figure 1A). Meanwhile, TATDN1 expression was determined in metastatic and non-metastatic HCC patients. TATDN1 was highly expressed in metastatic HCC (Figure 1B). Based on the collected follow-up data, the Kaplan-Meier curve indicated the prognostic value of TATDN1 in HCC (AUC=0.8898, cut-off value=2.078, Figure 1C). Moreover, HCC patients with high-level TATDN1 tended to have shorter overall survival relative to those with low level (Figure 1D). It is suggested that TATDN1 may predict a poor worse of HCC.

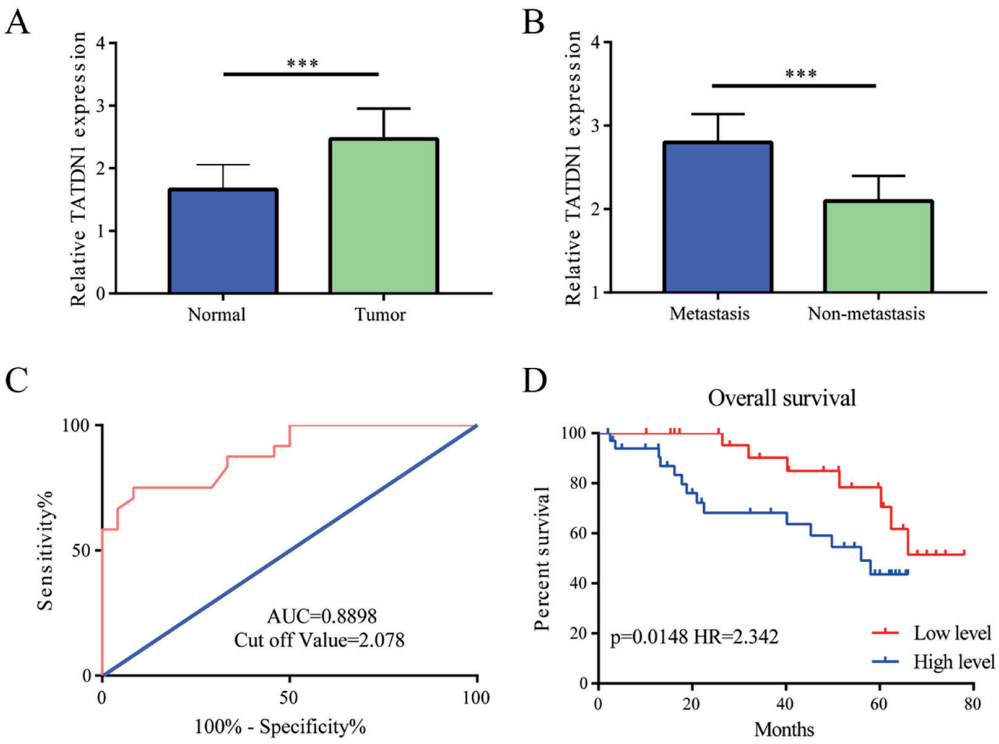

Figure 1. Great abundance of TATDN1 in HCC ***A,*** QRT-PCR data showed a higher level of TATDN1 in tumor tissues relative to controls (n=24). ***B,*** TATDN1 was highly expressed in metastatic HCC relative to non-metastatic HCC. ***C,*** Kaplan-Meier curve indicated the prognostic value of TATDN1 in HCC (AUC=0.8898, cut-off value=2.078). ***D,*** HCC patients with high-level TATDN1 had shorter overall survival relative to those with low level.

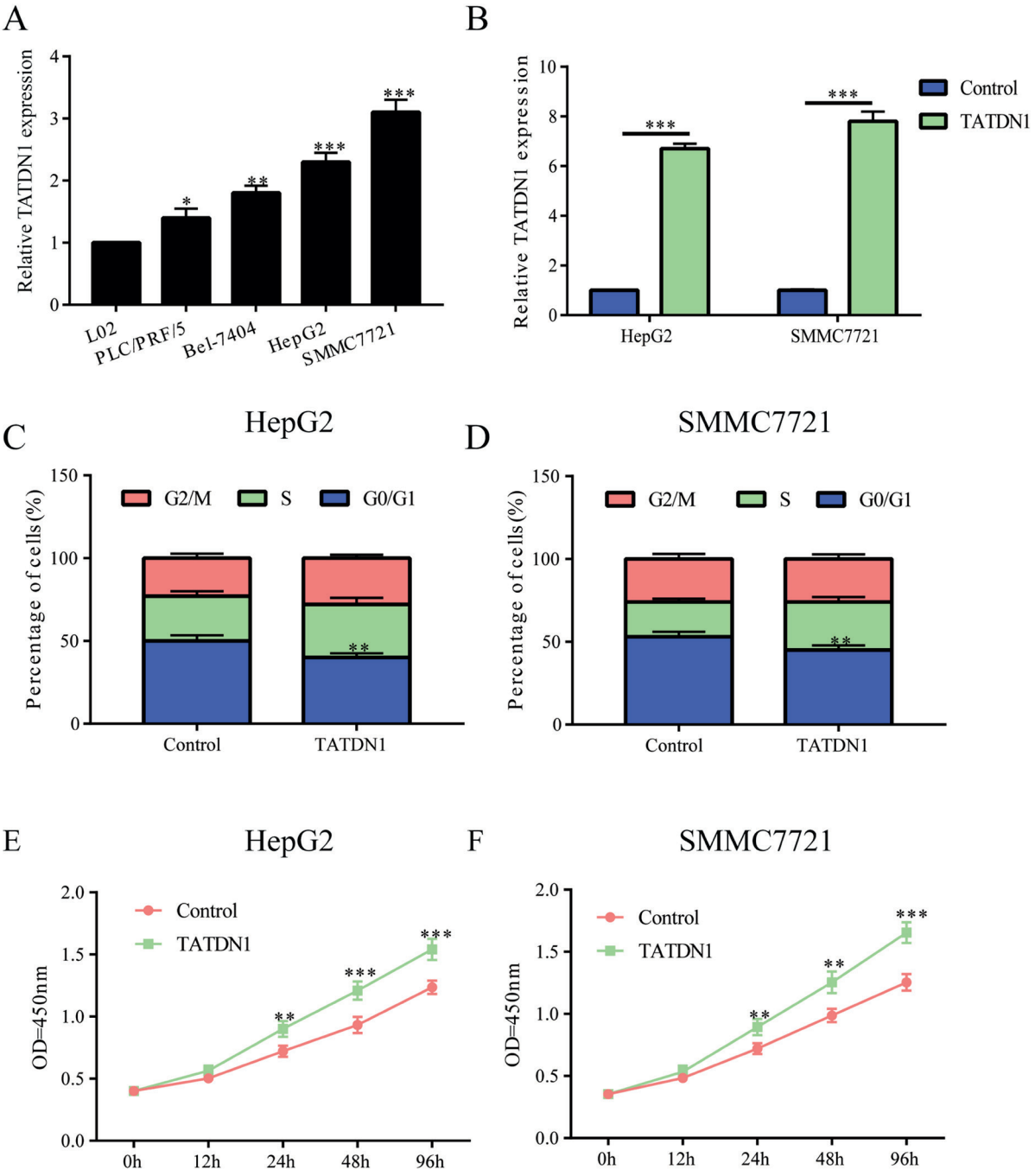

Figure 2. TATDN1 accelerated proliferative rate and cell cycle of HCC. ***A,*** Compared with L02 cells, TATDN1 was highly expressed in HCC cell lines. ***B,*** Transfection of the constructed pcDNA-TATDN1 sufficiently upregulated TATDN1 expression in HepG2 and SMMC7721 cells. ***C, D,*** Flow cytometry results showed that TATDN1 overexpression accelerated cell cycle progression of HepG2 and SMMC7721 cells. ***E, F,*** TATDN1 overexpression elevated viability of HepG2 and SMMC7721 cells.

TATDN1 Accelerated Proliferative Rate and Cell Cycle of HCC

TATDN1 expression in HCC cells was further determined. Compared with L02 cells, TATDN1 was highly expressed in HCC cell lines (Figure 2A). Transfection of the constructed pcDNA-TATDN1 sufficiently upregulated TATDN1 expression in HepG2 and SMMC7721 cells (Figure 2B). Flow cytometry results indicated that TATDN1 overexpression accelerated

cell cycle progression of HCC (Figure 2C, 2D). Besides, the viability of HCC cells was elevated by TATDN1 overexpression (Figure 2E, 2F).

TATDN1 Sponged MiRNA-6089 in HCC

To further explore the possible mechanism of TATDN1 in HCC, we predicted that miRNA-6089 was the target gene of TATDN1 through online prediction (Figure 3A). Subsequently, Dual-Luciferase reporter gene assay confirmed their binding (Figure 3B). By detecting miRNA-6089 level, it was lowly expressed in HCC tissues (Figure 3C). After transfection of miRNA-6089 mimics, the miRNA-6089 level was markedly upregulated in HCC cells (Figure 3D). We speculated that TATDN1 may exert its function in HCC by sponging miRNA-6089. The cells were transfected with the negative control, pcDNA-TATDN1 or pcDNA-TATDN1+miRNA-6089 mimics, respectively. The upregulation of miRNA-6089 partially inhibited the promotive effect of TATDN1 on cell cycle progression of HCC (Figure 3E). Identically, the promoted proliferation of HCC due to TATDN1 overexpression was reversed by miRNA-6089 (Figure 3F). Therefore, we suggested that TATDN1 exerted its function in HCC by sponging miRNA-6089.

MiRNA-6089 Degraded LIX1L to Exert Its Function

Through bioinformatics analysis, we found that LIX1L was a potential target gene of miRNA-6089

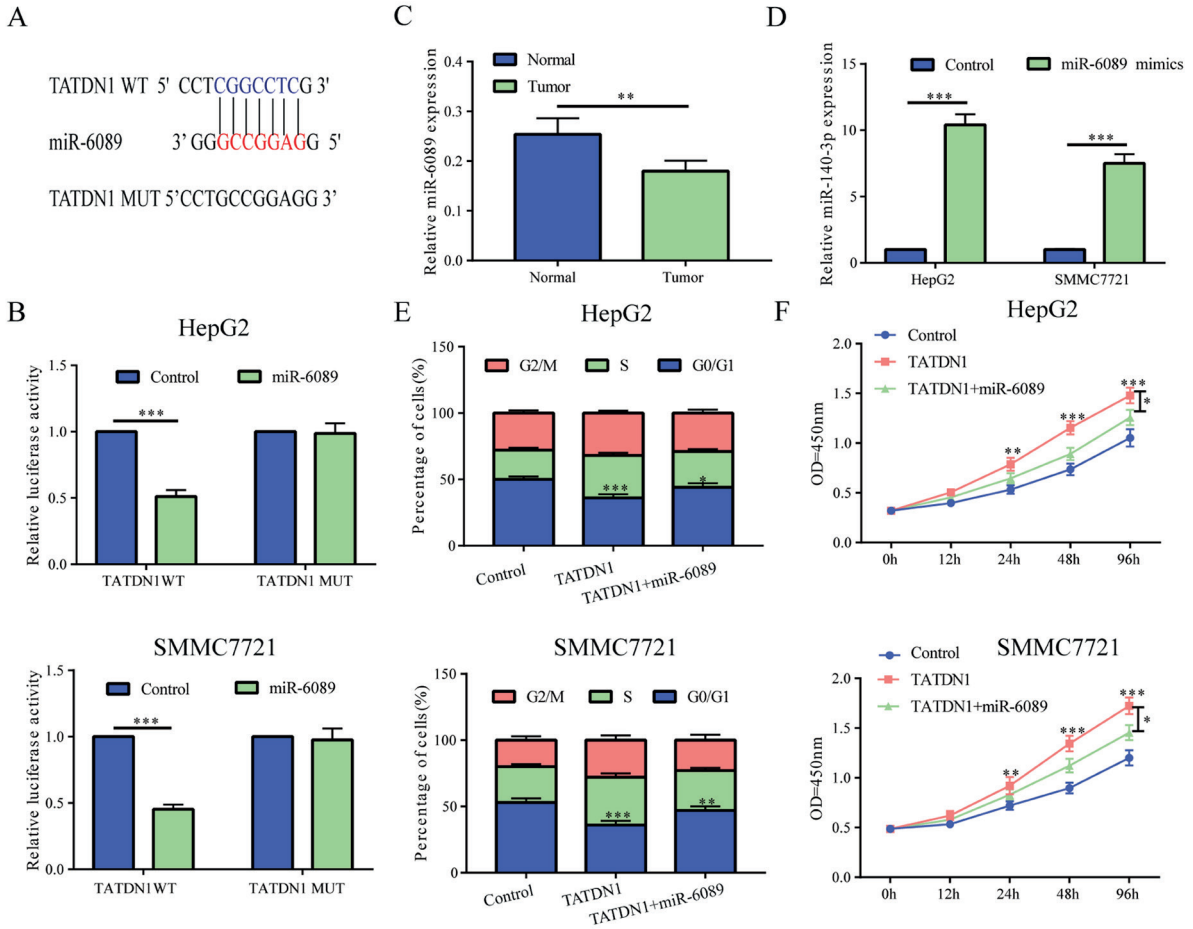

Figure 3. TATDN1 sponged miR-6089 in HCC. ***A,*** Predicted binding sequences between TATDN1 and miR-6089. ***B,*** Dual-Luciferase reporter gene assay showed the binding between TATDN1 and miR-6089. ***C,*** MiR-6089 was lowly expressed in HCC tissues relative to normal ones. ***D,*** Transfection of miR-6089 mimics upregulated miR-6089 level in HepG2 and SMMC7721 cells. ***E,*** Upregulation of miR-6089 partially inhibited the promotive effect of TATDN1 on cell cycle progression of HepG2 and SMMC7721 cells. ***F,*** Upregulation of miR-6089 partially inhibited the promotive effect of TATDN1 on viability of HepG2 and SMMC7721 cells.

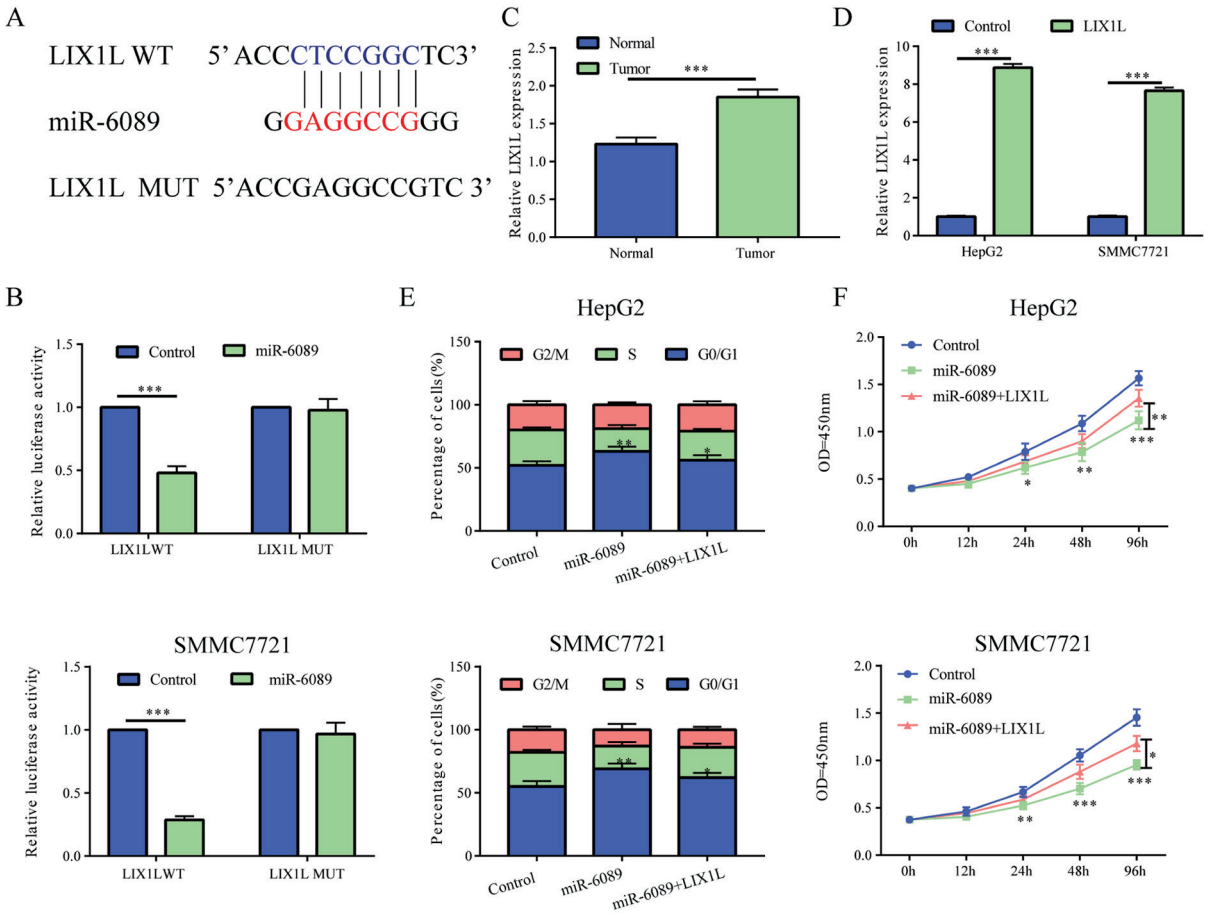

Figure 4. MiR-6089 degraded LIX1L to exert its function. ***A,*** Predicted binding sequences between LIX1L and miR-6089. ***B,*** Dual-Luciferase reporter gene assay revealed the binding between LIX1L and miR-6089. ***C,*** LIX1L was highly expressed in HCC tissues relative to normal ones. ***D,*** Transfection of pcDNA-LIX1L upregulated LIX1L level in HepG2 and SMMC7721 cells. ***E,*** Upregulation of LIX1L partially suppressed the inhibitory effect of miR-6089 on cell cycle progression of HepG2 and SMMC7721 cells. ***F,*** Upregulation of LIX1L partially suppressed the inhibitory effect of miR-6089 on viability of HepG2 and SMMC7721 cells.

(Figure 4A). To verify whether there is a binding relationship between these two genes, we performed the Dual-Luciferase reporter gene assay and confirmed their binding (Figure 4B). LIX1L was lowly expressed in HCC (Figure 4C). Transfection of pcDNA-LIX1L significantly upregulated LIX1L level in HepG2 and SMMC77219 cells (Figure 4D). Co-overexpression of miRNA-6089 and LIX1L in HCC cells partially reversed the inhibitory role of miRNA-6089 in cell cycle progression (Figure 4E). Similarly, the upregulation of LIX1L also reversed the inhibited proliferation of HCC cells overexpressing miRNA-6089 (Figure 4F). We believed that TATDN1/miRNA-6089/LIX1L axis promoted the proliferative rate and cell cycle of HCC.

Discussion

Since the competitive endogenous RNA (ceRNA) hypothesis proposed in 2011, research on ceRNA has been extensively performed. The interaction of mRNA, miRNA and lncRNA exerts a great biological significance, especially in tumorigenesis. The ceRNA hypothesis suggests that lncRNAs directly regulate target gene expressions. More importantly, lncRNAs may contain the core seed sequences that sponge corresponding miRNAs, thus further influencing the mRNA abundances[17]. The ceRNA hypothesis provides a novel direction in tumor diagnosis and treatment[18].

In gastric cancer, HOTAIR serves as a ceRNA to sponge miR-331-3p and further upregulate the

post-transcriptional level of HER2, indicating its oncogenic effect[19]. MALAT1 sponges miR-363-3p and miR-206 to regulate the occurrence and progression of gallbladder carcinoma through two different pathways[20]. In addition, lncRNA HULC is an important regulator in the progression of HCC. Compared to normal liver tissues and hepatocytes, HULC is overexpressed in HCC and absorbs miR-200a-3p to upregulate ZEB1. As a result, upregulated HULC leads to EMT and malignant behaviors of HCC cells[21]. In this paper, lncRNA TATDN1 was highly expressed in HCC, indicating a poor prognosis. To further elucidate the potential function of TATDN1 in cellular behaviors of HCC cells, we examined the influence of overexpressed TATDN1 on the proliferation and cell cycle. As the results indicated, the overexpression of TATDN1 markedly accelerated the proliferative rate and cell cycle progression of HCC cells. Subsequently, miRNA-6089 was predicted to be the target gene of TATDN1. However, the role of miRNA-6089 in HCC is rarely reported.

Our study determined the expression level of miRNA-6089 in HCC. The lowly expressed miRNA-6089 in HCC inhibited HCC cells from proliferating and partially reversed the regulatory effect of TATDN1 on HCC cells.

Next, LIX1L was searched as the potential downstream of miRNA-6089 through online prediction. LIX1L (Limb expression 1-like), a potential RNA-binding protein, is widely expressed in various tissues. It exerts a great abundance in gastric cancer, lung cancer, breast cancer and other tumors, indicating a crucial role in tumor development. We first examined its expression in HCC cells, which remained higher relative to controls. Dual-Luciferase reporter gene assay further confirmed the binding of LIX1L to miRNA-6089. A series of rescue experiments demonstrated that LIX1L promoted the proliferative rate and cell cycle progression of HCC cells. The inhibitory effects of miRNA-6089 on cellular behaviors of HCC cells were speculated by LIX1L. Based on the above results, we speculated that TATDN1/miRNA-6089/LIX1L axis exerted a regulatory function in HCC.

Conclusions

We found that TATDN1 accelerates the proliferative rate and cell cycle progression of HCC by degrading miRNA-6089 to upregulate LIX1L. This study provides a novel direction and therapeutic target for clinical treatment of HCC.

Conflict of interest

The authors declare no conflicts of interest.

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