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Proliferation and migration of hepatocellular carcinoma are accelerated by LINC01287 *via* the miR-559/TCF12 axis

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Abstract. – OBJECTIVE: To uncover the role of LINC01287 in the progression of hepatocellular carcinoma (HCC) and the indicated molecular mechanism.

PATIENTS AND METHODS: Relative levels of LINC01287 and miR-559 in 32 pairs of HCC tissues and normal ones, as well as HCC cell lines were detected by quantitative real-time polymerase chain reaction (qRT-PCR). Receiver operating characteristic (ROC) curves and Kaplan-Meier curves were depicted for assessing the diagnostic and prognostic potentials of LINC01287 in HCC, respectively. Proliferative and migratory capacities in HCC cells influenced by LINC01287 were assessed by cell counting kit-8 (CCK-8) and transwell assay, respectively. The regulatory loop LINC01287/miR-559/TCF12 was ascertained by Dual-Luciferase reporter assay. The involvement of the regulatory loop in the progression of HCC was examined via rescue experiments.

RESULTS: LINC01287 was upregulated in HCC tissues and cell lines, whereas miR-559 was downregulated. LINC01287 displayed certain diagnostic and prognostic potentials in HCC. Knockdown of LINC01287 could inhibit proliferative and migratory capacities in HCC cells. The regulatory loop LINC01287/miR-559/TCF12 was responsible for the aggravation of HCC.

CONCLUSIONS: LINC01287 drives proliferative and migratory capacities in HCC via targeting the miR-559/TCF12 axis.

Key Words:
Hepatocellular carcinoma (HCC), LINC01287, Proliferation, Migration.

Introduction

Liver cancer is classified into primary and secondary cancer. Primary liver cancer originates from liver epithelium or mesenchymal tissues, which is highly malignant with an extremely low 5-year survival[1]. Hepatocellular carcinoma (HCC) histologically covers 90% of liver cancer cases[2]. Currently, therapeutic strategies for HCC mainly include surgical resection, liver transplantation, radiofrequency ablation, interventional therapy, radiotherapy and chemotherapy[3,4]. The obtained clinical experiences have shown that chemotherapy efficacy for HCC is poor because of low sensitivity, drug resistance and side effects[5]. The pathogenesis of HCC remains largely unclear. It is generally considered that both genetic changes and external environmental factors result in the carcinogenesis of HCC. Therefore, great efforts should be made to clarify molecular mechanisms of HCC, thus improving the prognosis in HCC patients.

Nucleotide length of lncRNAs exceeds 200 nt. LncRNAs are expressed both in the cytoplasm or nucleus. They used to be interference signals in gene expressions. Later, increasing evidence has proven the diverse regulatory effects of lncRNAs on life activities[6]. Through complementary base pairing, lncRNAs interact with other nucleic acids and proteins, thereafter participating in cell function regulation. Biologically functional lncRNAs have been well concerned[7] and lncRNAs have been found to be able to influence the progression and prognosis in HCC[8]. It is reported that LINC00346 mediates HCC progression *via* the CDK1/CCNB1 axis[9]. At present, diagnostic strategies and prognosis assessments for HCC lack high sensitivity and specificity. A large number of HCC patients lose the optimal opportunity for surgery due to aggravated disease progression. As a result, screening and assessment for HCC as early as possible are of great significance.

Song et al[10] showed that LINC01287 induces breast cancer cells to proliferate and migrate

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through the Wnt signaling as an oncogene. LINC01287 is able to trigger the malignant growth and migration in HCC[11]. In this paper, we found that LINC01287 was upregulated in HCC tissues than normal ones. *In vitro* knockdown of LINC01287 could weaken proliferative and migratory capacities in HepG2 and SMMC-7721 cells through the miR-559/TCF12 axis. Our findings enrich the research on the pathogenesis of HCC, and provide a novel target for HCC treatment.

Patients and Methods

Clinical Specimen and Cell Culture

Paired HCC tissues and adjacent normal ones were surgically resected from 32 HCC patients in Yantai Mountain hospital from May 2017 to March 2019. All specimens were stored at -80°C. Inclusion criteria: (1) HCC patients were confirmed by pathology and the resection margin was negative, and they were treated by radical resection. (2) Postoperative follow-up data were complete. Exclusion criteria: (1) patients combined with other tumors, acute and chronic hepatitis and other fetal diseases. (2) HCC patients with preoperative chemoradiotherapy. This investigation was approved by the Ethics Committee of Yantai Mountain hospital. Signed written informed consents were obtained from all participants before the study.

Human immortalized normal hepatocyte line (LO2) and HCC cell lines (HepG2, Hep3B, Huh7 and SMMC-7721) were provided by American Type Culture Collection (ATCC; Manassas, VA, USA). They were cultivated in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) at 37°C with 5% CO_2. 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 μg/mL streptomycin and 100 U/mL penicillin (Sigma-Aldrich, St-Louis, MO, USA) were supplemented in the medium.

Transfection

Transfection plasmids were constructed by GenePharma (Shanghai, China). Cells were cultivated in 6-well plates to 60-70%, and they were subsequently cultured in serum-free medium. Plasmid transfection (50-100 nM) was conducted using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). Six hours later, complete medium was replaced for further cultivation.

Cell Counting Kit-8 (CCK-8)

Cells were inoculated in a 96-well plate with 1-2×10^5 cells per well, and six replicates were set. At the indicated time points, 10 μL of CCK-8 solution (Dojindo Molecular Technologies, Kumamoto, Japan) was applied per well for 1 h incubation and optical density at 450 nm was measured.

Transwell Assay

Cells were collected and suspended in serum-free medium at the density of 1-10×10^5 cells/mL. 100-200 μL of suspension and 500 μL of complete medium were respectively applied in the top and bottom chamber. After 24 h cell culture, transwell chambers were taken out. Cells in the bottom were subjected to methanol fixation for 15 min, and crystal violet staining for 20 min. Migratory cells were counted in 5 randomly selected fields per sample.

qRT-PCR

Extraction of total RNAs was conducted using TRIzol (Invitrogen, Carlsbad, CA, USA). RNA concentration and purity were detected using a spectrophotometer (NanoDrop2000 ND-1000, NanoDrop, Thermo Fisher Scientific, Waltham, MA, USA). Qualified RNA samples were stored at -80°C. 1 μg RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA). The obtained cDNA was subjected to qRT-PCR, and relative level was calculated using $2^{-\Delta\Delta ct}$ method. Primer sequences were listed as follows: LINC01287: forward: 5'-GGTTGATGTAAGGACCTCGT-3'; reverse: 5'-GAGACCTTGTTTCATGTGTCG-3'; miR-559: forward: 5'-CCTGGGACCCCATTATCCTT-3'; reverse: 5'-TGCTGTCCACAGTGTGTTTG-3'; TCF12: forward: 5'-GGTGGCTTGCAAAGTCAGTC-3'; reverse: 5'-AGCACTTTCTTTATGCAAGA-3'. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH): forward: 5'-TCAAGATCATCAGCAATGCC-3'; reverse: 5'-CGATACCAAAGTTGTCATGGA-3'. U6: forward: 5'-ATACAGAGAAAGTTAGCACGG-3'; reverse: 5'-GGAATGCTTCAAAGAGTTGTG-3'.

Dual-Luciferase Reporter Assay

Wild-type and mutant-type Luciferase vectors were constructed based on the predicted binding sites in the 3'UTR of LINC01287 or miR-559, which were co-transfected into cells with miR-559 mimics or NC. After 24 h transfection, cells

were lysed, and centrifuged at 10,000 g for 5 min. The upper layer supernatant was used for measuring Luciferase activity (Promega, Madison, WI, USA).

Statistical Analysis

Statistical analyses were conducted using Statistical Product and Service Solutions (SPSS) 17.0 (SPSS, Chicago, IL, USA). Data were expressed as mean±SEM (Standard Error of Mean). The independent sample *t*-test was conducted to compare the differences between groups. Diagnostic and prognostic potentials of LINC01287 in HCC were assessed by depicting receiver operating characteristic (ROC) and Kaplan-Meier curves, respectively. $p<0.05$ was considered as statistically significant.

Results

Expression Level of LINC01287 in HCC Tissues and Cell Lines

Expression levels of LINC01287 were detected in HCC and normal tissues by qRT-PCR. It is shown that LINC01287 was markedly upregulated in HCC tissues (Figure 1A). Further analyses uncovered a higher abundance of LINC01287 in advanced HCC cases and metastatic HCC cases (Figure 1B, 1C). Depicted ROC curves proved the diagnostic potential of LINC01287 in HCC (AUC=0.8647, cut-off value=0.9727, Figure 1D). Meanwhile, survival analysis suggested that highly expressed LINC01287 was unfavorable to the overall survival in HCC (p=0.0173, HR=3.001, Figure 1E). LINC01287 expression in HCC cell lines was detected as well. Compared with normal hepatocytes, LINC01287 was upregulated in HCC cell lines, especially HepG2 and SMMC-7721 cells (Figure 1F). They were utilized for establishing LINC01287 knockdown models by transfection of si-LINC01287 (Figure 2A, 2D).

Knockdown of LINC01287 Weakened Proliferative and Migratory Capacities in HCC

In HepG2 and SMMC-7721 cells with LINC01287 knockdown, CCK-8 assay showed lower viability than those of controls (Figure 2B, 2E). Besides, migratory cell number decreased after knockdown of

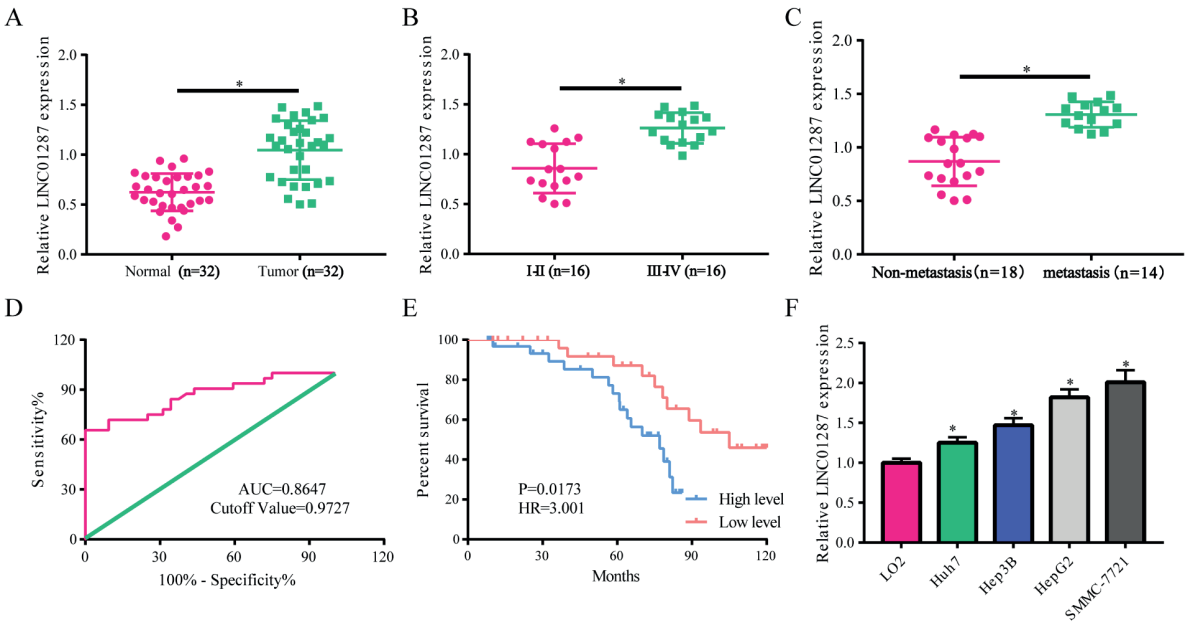

Figure 1. Expression level of LINC01287 in HCC tissues and cell lines. **A,** LINC01287 was highly expressed in HCC tissues than normal ones; **B,** LINC01287 was highly expressed in advanced HCC cases than early stage cases; **C,** LINC01287 was highly expressed in metastatic HCC cases than non-metastatic cases; **D,** ROC curves depicted for the diagnostic potential of LINC01287 in HCC (AUC=0.8647, cut-off value=0.9727); **E,** Kaplan-Meier curves depicted for the prognostic potential of LINC01287 in HCC (HR=3.001, p=0.0173); **F,** LINC01287 was highly expressed in HCC cell lines than normal hepatocytes. *$p<0.05$.

LINC01287 in HCC cells (Figure 2C, 2F). It is indicated that LINC01287 stimulated proliferative and migratory capacities in HCC.

MiR-559 Was the Target Gene of LINC01287

To elucidate the molecular mechanism of LINC01287 on triggering proliferative and migratory capacities in HCC, we predicted potential targets of LINC01287 through bioinformatic analysis. Compared with normal tissues, LINC01287 was downregulated in HCC tissues (Figure 3A). The binding sites in the 3'UTR of LINC01287 and miR-559 were identified (Figure 3B). Luciferase activity was reduced by overexpression of miR-559 in the wild-type LINC01287 vector, indicating that LINC01287 could target miR-559 through the predicted binding sites (Figure 3C). In HepG2 and SMMC-7721 cells with LINC01287 knockdown, miR-559 was remarkably upregulated (Figure 3D). On the contrary, miR-559 was downregulated in HCC cells overexpressing LINC01287 (Figure 3E, 3F).

TCF12 Was the Target Gene of MiR-559

Using the bioinformatic tool, TCF12 was predicted to be the target gene of miR-559 (Figure 4A). In a similar way, Dual-Luciferase reporter assay verified the binding between miR-559 and TCF12 (Figure 4B, 4C). In HepG2 and SMMC-7721 cells overexpressing miR-559, TCF12 level was markedly downregulated, displaying a negative interaction between miR-559 and TCF12 (Figure 4D, 4E). Knockdown of miR-559, conversely, upregulated TCF12 in HCC cells (Figure 4F, 4G).

LINC01287 Regulated HCC Cell Functions by Upregulating TCF12

To verify whether LINC01287 regulates HCC cell functions by upregulating TCF12 through the sponge effect on miR-559, we performed further verification. Transfection efficacy of pcDNA-TCF12 was examined in HepG2 and SMMC-7721 cells (Figure 5A). The reduced viability in HCC cells with LINC01287 knockdown was reversed by overexpression of TCF12 (Figure 5B). As expected, overexpression of TCF12 abolished the attenuated migration in HCC cells with LINC01287 knockdown (Figure 5C, 5D). It is concluded that LINC01287/miR-559/TCF12 axis aggravated the progression of HCC.

Discussion

Tumor staging, liver function and physical tolerance should be taken into consideration of developing therapeutic strategies for HCC. Liver cancer is the rare type of malignant tumor that can be cured by organ transplantation, a complicated surgery requiring a close cooperation among oncologists, surgeons, hepatologists and anesthesiologists[12]. Diagnosis as early as possible and timely treatment of HCC display a huge impact on prolonging the survival[13]. In recent years, searching for HCC-specific molecular biomarkers has been highlighted. With the rapid progression made on molecular biology technology, accumulating lncRNAs relevant to HCC have been identified. The regulatory mechanisms of HCC-specific lncRNAs in cancer progression, however, should be further validated.

Similar to lnRNAs, miRNAs are noncoding RNAs with a shorter transcript (22 nt). About one third of human genomes are regulated by miRNAs, and one miRNA can regulate expression levels of more than 200 target genes[14]. The interaction between lncRNAs and miRNAs is capable of influencing gene expressions. The newly proposed ceRNA theory is a vital mechanism in noncoding RNA regulation. Through the common miRNA response elements, lncRNAs or mRNAs competitively bind the corresponding miRNAs, which is known as ceRNAs[15]. The ceRNA regulatory network is complicated. Its intervention may lead to pathological changes.

Our results showed that LINC01287 was upregulated in HCC tissues, while miR-559, the target gene binding LINC01287, was lowly expressed. A negative correlation between expression levels of LINC01287 and miR-559 has been identified in HCC cells. Wang et al[16] reported that miR-599 is a prognostic biomarker for gastric cancer[16]. By targeting THRB, miR-599 is involved in the progression of papillary thyroid carcinoma[17].

We thereafter predicted potential targets for miR-559 by bioinformatic analysis, and TCF12 was selected. TCF12 is a member of the helix-loop-helix family, presenting the DNA-binding ability[18]. Functionally, TCF12 is responsible for cell growth and differentiation[19]. Through literature review, TCF12 is a cancer-associated gene. In gallbladder specimens, TCF12 is upregulated and its level is closely linked to

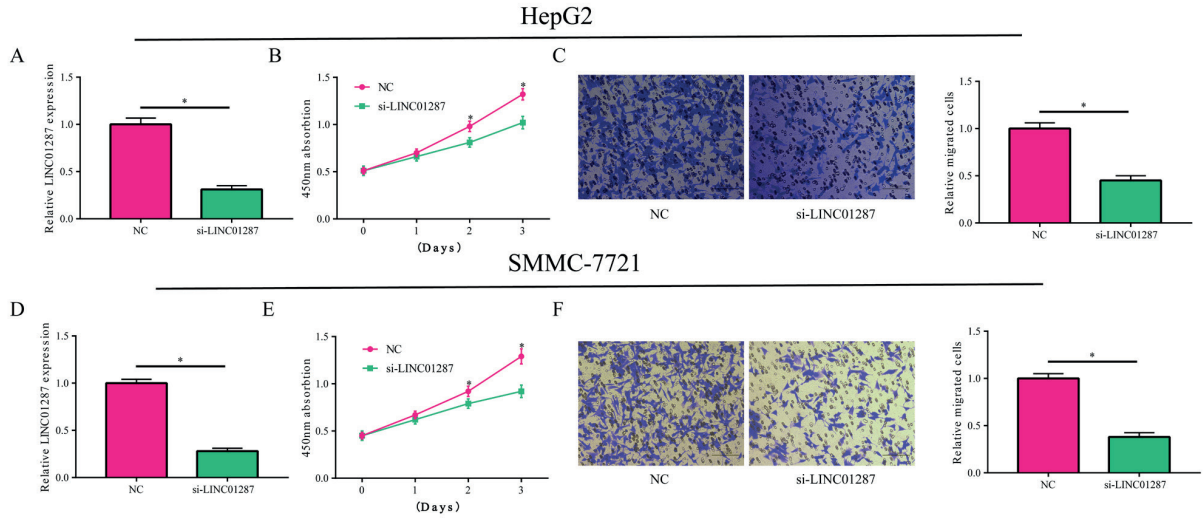

Figure 2. Knockdown of LINC01287 weakened proliferative and migratory capacities in HCC. **A,** Transfection of si-LINC01287 significantly downregulated LINC01287 in HepG2 cells; **B,** Transfection of si-LINC01287 significantly decreased viability in HepG2 cells; **C,** Transfection of si-LINC01287 significantly decreased migration in HepG2 cells, (magnification: 40×) **D,** Transfection of si-LINC01287 significantly downregulated LINC01287 in SMMC-7721 cells; **E,** Transfection of si-LINC01287 significantly decreased viability in SMMC-7721 cells; **F,** Transfection of si-LINC01287 significantly decreased migration in SMMC-7721 cells, (magnification: 40×) *p<0.05.

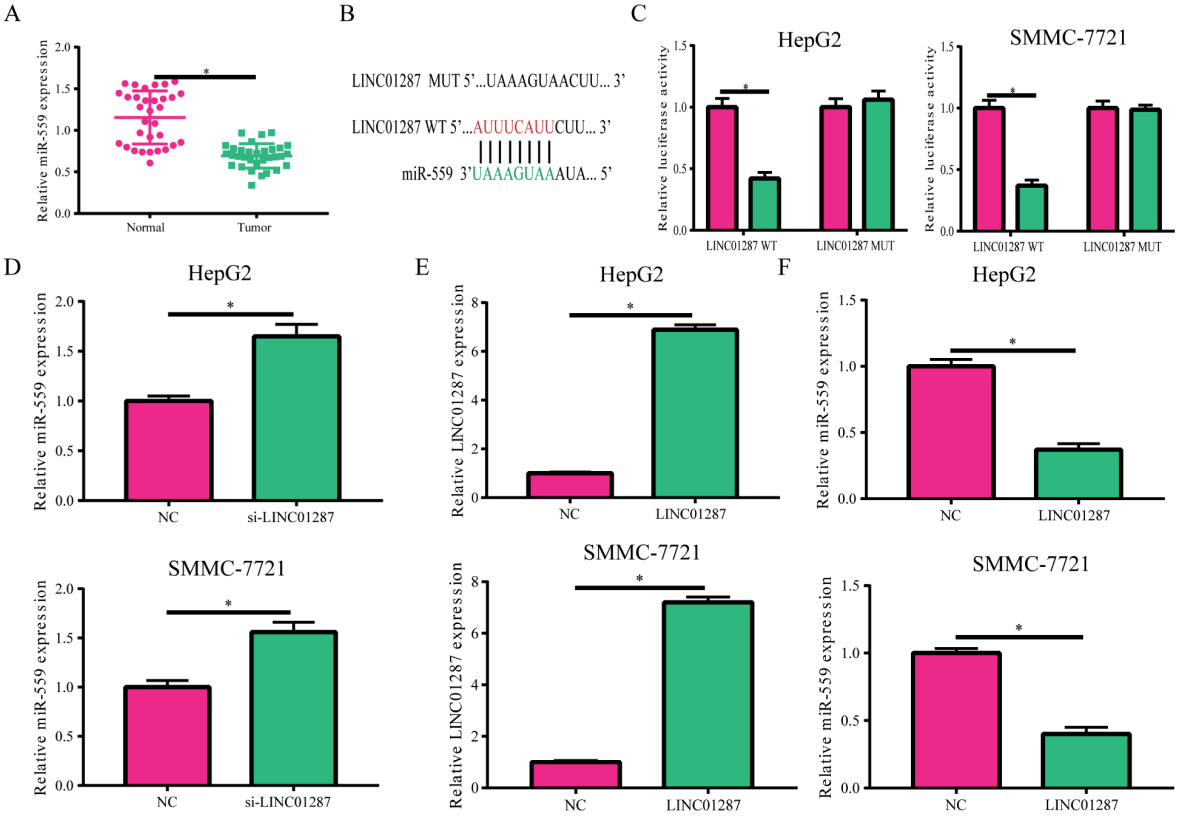

Figure 3. MiR-559 was the target gene of LINC01287. **A,** MiR-559 was lowly expressed in HCC tissues than normal ones; **B,** Binding sites in the 3'UTR of LINC01287 and miR-559; **C,** Luciferase activity in HepG2 and SMMC-7721 cells co-transfected with wild-type LINC01278/mutant-type LINC01278 vector and miR-559 mimics/NC, respectively; **D,** MiR-559 was significantly upregulated in HepG2 and SMMC-7721 cells transfected with si-LINC01287; **E,** Transfection of pcDNA-LINC01287 significantly upregulated LINC01287 in HepG2 and SMMC-7721 cells; **F,** MiR-559 was significantly downregulated in HepG2 and SMMC-7721 cells transfected with pcDNA-LINC01287. *p<0.05.

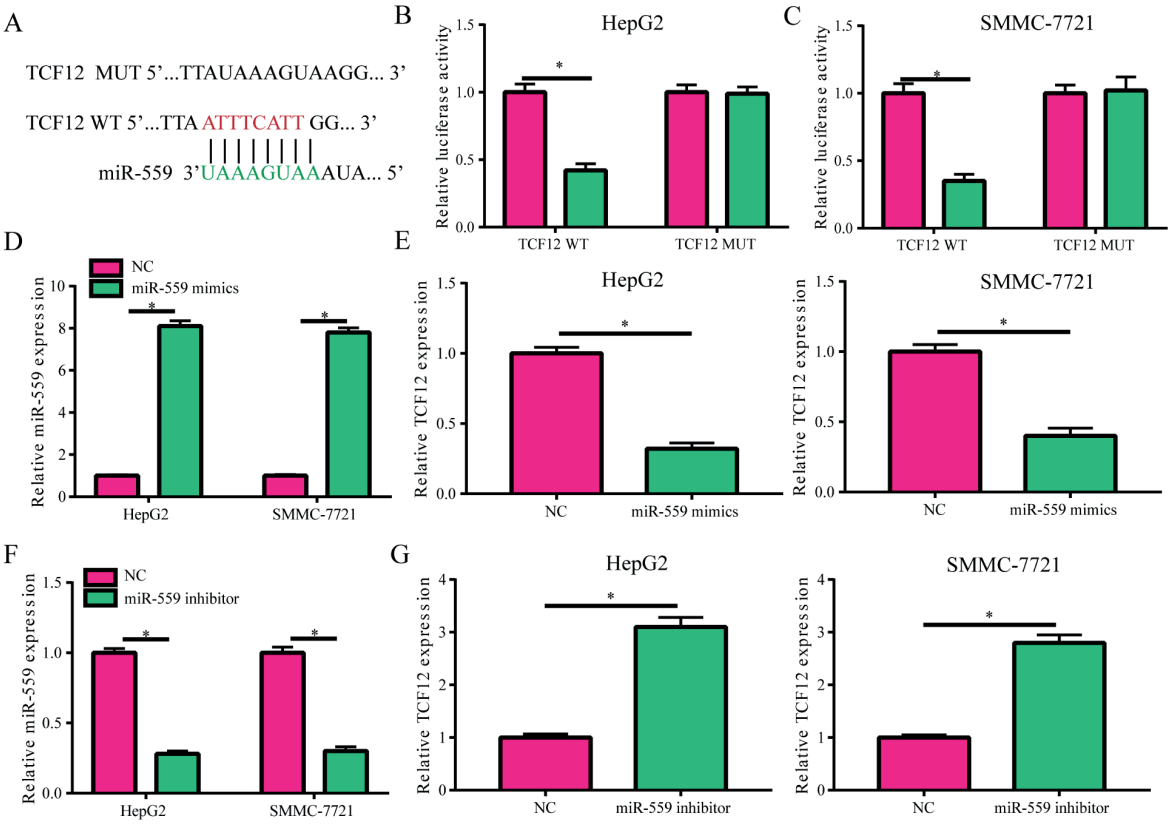

Figure 4. TCF12 was the target gene of miR-559. **A,** Binding sites in the 3'UTR of TCF12 and miR-559; **B,** Luciferase activity in HepG2 cells co-transfected with wild-type TCF12/mutant-type TCF12 vector and miR-559 mimics/NC, respectively; **C,** Luciferase activity in SMMC-7721 cells co-transfected with wild-type TCF12/mutant-type TCF12 vector and miR-559 mimics/NC, respectively; **D,** Transfection of miR-559 mimics significantly upregulated miR-559 in HepG2 and SMMC-7721 cells; **E,** TCF12 was significantly downregulated in HepG2 and SMMC-7721 cells transfected with miR-559 mimics; **F,** Transfection of miR-559 inhibitor significantly downregulated miR-559 in HepG2 and SMMC-7721 cells; **G,** TCF12 was significantly upregulated in HepG2 and SMMC-7721 cells transfected with miR-559 inhibitor. *p<0.05.

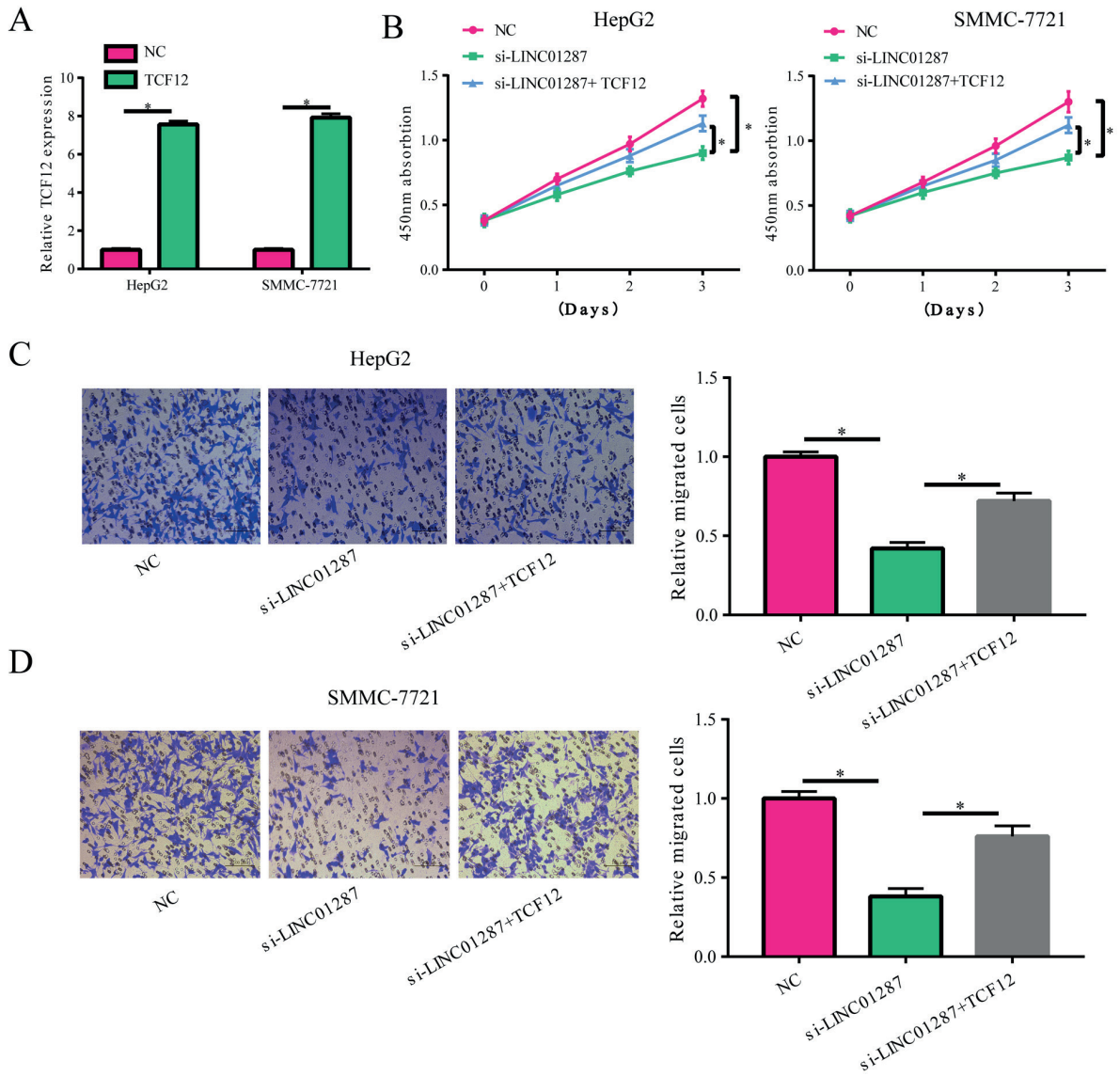

Figure 5. LINC01287 regulated HCC cell functions by upregulating TCF12. **A,** Transfection of pcDNA-TCF12 significantly upregulated TCF12 in HepG2 and SMMC-7721 cells; **B,** Decreased viability in HepG2 and SMMC-7721 cells transfected with si-LINC01287 was reversed by overexpression of TCF12; **C,** Decreased migration in HepG2 cells transfected with si-LINC01287 was reversed by overexpression of TCF12, (magnification: 40×) **D,** Decreased migration in SMMC-7721 cells transfected with si-LINC01287 was reversed by overexpression of TCF12, (magnification: 40×) *$p<0.05$.

overall survival[20]. TCF12 is highly expressed in tumor-associated fibroblasts, which is able to induce extracellular matrix remodeling, and promote both *in vitro* and *in vivo* invasiveness and metastasis in breast cancer[21]. We therefore speculated that LINC01287 served as a sponge for miR-559, and thus upregulated TCF12. Rescue experiments confirmed our speculation that LINC01287/miR-559/TCF12 axis was responsible for driving the progression of HCC.

Conclusions

This study, for the first time, suggested that LINC01287 was significantly elevated in HCC, and promoted the progress of HCC through the miR-559/TCF12 axis. Our findings provide a novel idea for the diagnosis and targeted therapy of HCC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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