JBUON 2021; 26(5): 1964-1969
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
Email: editorial_office@jbuon.com

ORIGINAL ARTICLE

LINC00355 triggers malignant progression of hepatocellular carcinoma *via* the sponge effect on miR-217-5p with the involvement of the Wnt/β-catenin signaling

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Summary

Purpose: *To uncover the biological role of LINC00355 in regulating the proliferative and apoptotic potentials in hepatocellular carcinoma (HCC), and the underlying mechanism.*

Methods: *LINC00355 levels in HCC tissues and cell lines were detected by quantitative real-time polymerase chain reaction (qRT-PCR). After knockdown of LINC00355 or miR-217-5p in Hub7 and Hep3B cells, proliferative and apoptotic potentials were assessed by cell counting kit-8 (CCK-8), colony formation assay and flow cytometry. The interaction between LINC00355 and miR-217-5p was determined by dual-luciferase reporter assay and Pearson correlation test. Western blot analysis was conducted to illustrate the regulatory effects of LINC00355 and miR-217-5p on the Wnt/β-catenin signaling.*

Results: *LINC00355 was upregulated in HCC tissues and cell lines. Knockdown of LINC00355 reduced viability in Hub7 and Hep3B cells, which was much pronounced on days 3 and 4. Clonality was attenuated by transfection of shLINC00355 as well. In addition, apoptosis rate increased by knockdown of LINC00355 in HCC cells. Protein levels of β-catenin, GSK3β, c-myc and cyclin D1 were downregulated in Hub7 and Hep3B cells transfected with shLINC00355. MiR-217-5p was the target gene binding LINC00355. It displayed exactly opposite regulations on HCC cell phenotypes and protein levels of vital genes in the Wnt/β-catenin signaling to those of LINC00355.*

Conclusions: *LINC00355 is upregulated in HCC specimens, LINC00355 triggers proliferative rate and inhibits apoptosis in HCC cells by negatively regulating miR-217-5p and activating the Wnt/β-catenin signaling.*

Key words: *LINC00355, MiR-217-5p, Wnt/β-catenin, HCC*

Introduction

Liver cancer is a common malignant solid tumor. There were 841,000 new cases and 782,000 deaths of liver cancer globally in 2018 [1-4]. In China, the incidence of liver cancer is high. Hepatocellular carcinoma (HCC) is the most common liver cancer subtype, accounting for 80-90% of the cases [1,3,4]. Because HCC is highly invasive and prone to metastasize, its prognosis is not satisfying even though great strides have been made toward improving the diagnostic and therapeutic results [5-7]. Therefore, it is necessary to clarify the molecular mechanism of HCC and to develop biomarkers and therapeutic targets [8-10].

Long noncoding RNAs (lncRNAs) lack the ability to encode proteins, and they are extensively involved in biological functions [11]. LncRNAs and miRNAs/miRs are the two types of noncoding RNAs that have been well studied [11,12]. LncRNAs exceed 200 nucleotides, and they are vital regulators in the body development, cell differentiation and tumor

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Received: 19/05/2021; Accepted: 28/07/2021

progression [13,14]. Typically, lncRNAs exert sponge effect on miRNAs, thus regulating the expressions and functions of target genes binding miRNAs [15]. Previous studies have shown that LINC00355 plays a carcinogenic role in bladder cancer, head and neck neoplasms and lung adenocarcinoma, which is closely linked to tumor prognosis [16-18].

MiRNAs are highly conserved noncoding RNAs containing 20-25 nucleotides, which are derived from hairpin-structured premiRNAs [19,20]. Through completely or incompletely complementary base pairing, miRNAs degrade or inhibit the translation of target mRNAs, respectively [20,21]. MiRNAs are involved in cell growth, apoptosis, differentiation, metabolism and immunity, which are considered as potential targets for tumor diagnosis and treatment [22,23]. This study aimed to elucidate the biological functions of LINC00355 and miR-217-5p in regulating the proliferative and apoptotic potentials in HCC.

Methods

Patients and HCC samples

HCC and paracancerous tissues (3 cm away from the tumor) were collected. Patients did not have preoperative chemotherapy or radiotherapy, and were diagnosed by postoperative pathology. Collected tissues were preserved at -80°C for RNA extraction. TNM staging in HCC was defined according to the criteria of Union Internationale Contre le Cancer (UICC). This study was approved by the research Ethics Committee of Shanghai Xuhui District Central Hospital and complied with the Helsinki Declaration and its latter amendments. Sample collection was conducted after obtaining signed informed consent form from the patients or their families.

Cell lines and reagents

HCC cell lines (Bel-7402, HepG2, Huh7, Hep3B) and normal hepatocytes (LO2) were purchased by American Type Culture Collection (ATCC) (Manassas, VA, USA). They were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Rockville, MD, USA) at 37°C with 5% CO_2. Ten percent fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 100 μg/mL streptomycin and 100 U/mL penicillin (Life Technologies, Gaithersburg, MD, USA) were added in the medium. Cell passage was conducted when the cells were grown to 80-90% confluence using 1×trypsin+ethylenediaminetetraacetic acid (EDTA).

Transfection

Cells in 6-well plates were cultured to 50-70% confluence, and transfected with shLINC00355 or shNC (negative control/NC) using polybrene. If the infection rate was greater than 60%, puromycin (2 μg/mL) was used aiming to screen the infected cells. Transfection of miR-217-5p inhibitor or NC inhibitor was conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Transfection efficacy was determined at 48 h by quantitative real-time polymerase chain reaction (qRT-PCR).

Cell proliferation assay

2.5×10^3 cells were implanted in each of 6-well plates and cultured for 1, 2, 3, or 4 days, where 10 μL of cell counting kit-8 (CCK-8) solution was added (TaKaRa, Dalian, China). After 1-h culturing in the dark, the optical density at 490 nm was measured using a microplate reader.

Colony formation assay

Cells were inoculated in 6-well plates (200 cells per well) and cultured for 2 weeks. Visible colonies were fixed in 70% ethanol for 2 h and stained using 0.5% crystal violet for 1 h. The number of visible colonies was counted.

Flow cytometry analysis of cell apoptosis

Cell suspension was prepared at a density of 1×10^6 cells/mL. Cells were re-suspended in 0.5 mL of pre-cooled 1×binding buffer and 1.25 μL of Annexin V-FITC (fluorescein isothiocyanate) in the dark for 15 min. After centrifugation at 1000×g for 5 min, the precipitant was re-suspended in 0.5 mL of pre-cooled 1×binding buffer and 10 μL of propidium iodide (PI) in the dark, followed by flow cytometry detection of cell apoptosis.

QRT-PCR

TRIzol (Invitrogen, Carlsbad, CA, USA) was used for isolating total RNAs, which were reversely transcribed. qRT-PCR was prepared using the complementary deoxyribose nucleic acid (cDNA) as the template and it was conducted using the SYBR®Premix Ex Taq™ (TaKaRa, Dalian, China) using the StepOne Plus Real-time PCR system. Three independent records were calculated by $2^{-\Delta\Delta Ct}$ method, and data were analyzed by the ABI Step One software (Applied Biosystems, Foster City, CA, USA). The following primers were used: LINC00355: Forward: 5'-GTTGGTGCCTGCTTTTCCAC-3', Reverse: 5'-GGCGTGATACAACTGTCTGC-3. glyceraldheyde 3-phosphate dehydrogenase (GAPDH): Forward: 5'-GCTGCCCAGAACATCATCC-3', Reverse: 5'-GTCAGATCCACGACGGACAC-3'. miR-217-5p: Forward: 5'-TACTGCATCAGGAACTGATTGGA-3', Reverse: 5'-CATCAGTTCCTAATGCATTGCCT-3'. U6: Forward: 5'-GCTTCGGCAGCACATATACTAAAAT-3', Reverse: 5'-CGCTTCACGAATTTGCGTGCAT-3'.

Western blot

Cells were lysed with radioimmunoprecipitation assay (RIPA) (Beyotime, Shanghai, China) and the concentration of isolated protein was measured by the Bradford method. Protein samples were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and loaded on polyvinylidene fluoride (PVDF) membranes (Roche, Basel, Switzerland). Subsequently, the membranes were soaked in 5% skim milk for 2 h. Primary antibodies were applied for overnight incubation at 4°C. On the next day, horse radish peroxidase (HRP)-labeled secondary antibodies were used for 2-h incubation. Band exposure was achieved by electrochemiluminescence (ECL) with GAPDH as the internal reference.

Dual-luciferase reporter assay

HEK293T cells were inoculated in 24-well plates and were co-transfected with miR-217-5p mimic/NC mimic and wild-type/mutant-type LINC00355 vector, respectively. Luciferase activity (Promega, Madison, WI, USA) was measured at 48 h based on standard procedures.

Statistics

GraphPad Prism 5 V5.01 software (La Jolla, CA, USA) was used for statistical analyses. Student's t-test was used to analyze the statistical differences between groups and each experiment was repeated in triplicate. Data were expressed as mean ± standard deviation. Significant difference was set at $p<0.05$.

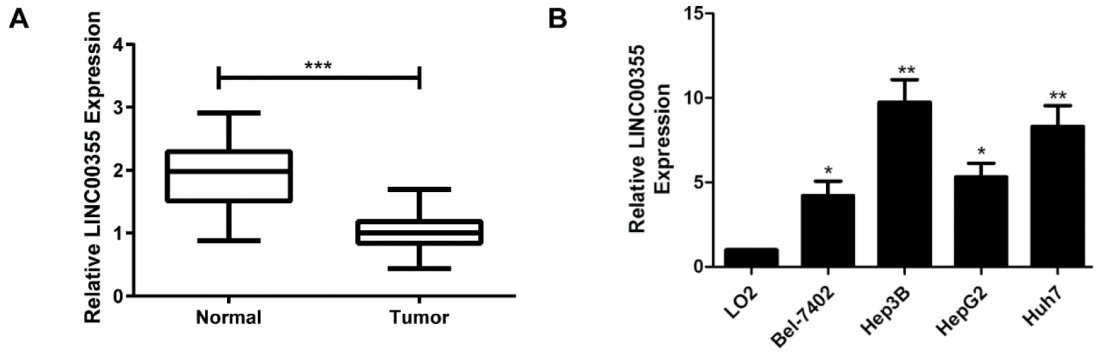

Figure 1. LINC00355 was up-regulated in HCC. **A:** LINC00355 expressions in HCC and paracancerous tissues. **B:** LINC00355 expressions in HCC cell lines. Data are expressed as mean±SD. *p<0.05, **p<0.01, ***p<0.001.

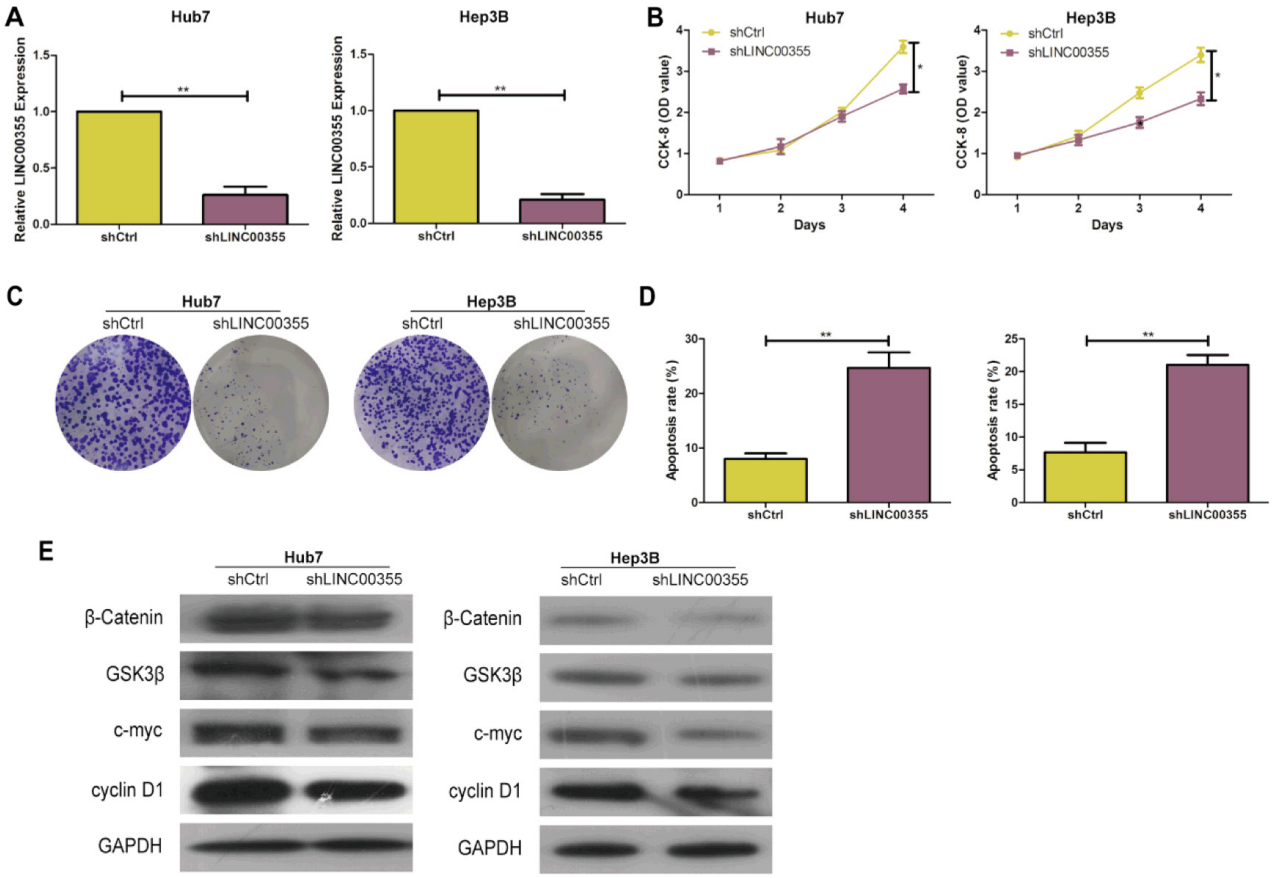

Figure 2. Influence of LINC00355 on proliferative and apoptotic potentials in HCC. **A:** Transfection efficacy of shLINC00355 in Hub7 and Hep3B cells. **B:** Viability in Hub7 and Hep3B cells after transfection of shLINC00355. **C:** Colony number in Hub7 and Hep3B cells after transfection of shLINC00355. **D:** Apoptotic rate in Hub7 and Hep3B cells after transfection of shLINC00355. **E:** Protein levels of β-catenin, GSK3β, c-myc and cyclin D1 in Hub7 and Hep3B cells after transfection of shLINC00355. Data were expressed as mean±SD. *p<0.05, **p<0.01.

Results

LINC00355 was upregulated in HCC

We detected differential expressions of LINC00355 in HCC and paracancerous tissues which was upregulated in HCC tissues (Figure 1A). As expected, LINC00355 was highly expressed in HCC cell lines (Figure 1B).

Influence of LINC00355 on proliferative and apoptotic potentials in HCC

Transfection of shLINC00355 in Hub7 and Hep3B cells was largely downregulated (Figure 2A). CCK-8 assay showed that viability markedly declined in Hub7 and Hep3B cells transfected with shLINC00355 on days 3 and 4 (Figure 2B). Similarly, colony number decreased after knockdown of LINC00355, suggesting attenuated proliferative ability (Figure 2C). On the contrary, flow cytometry demonstrated that knockdown of LINC00355 enhanced the apoptotic rate in Hub7 and Hep3B cells (Figure 2D). Protein levels of β-catenin, GSK3β, c-myc and cyclin D1 were downregulated by transfection of shLINC00355 in HCC cells (Figure 2E).

Influence of miR-217-5p on proliferative and apoptotic potentials in HCC

Bioinformatic prediction suggested that miR-217-5p, miR-3192-5p and miR-568 were potential candidates binding LINC00355. After knockdown of LINC00355, the expression change of miR-217-5p was most pronounced among the three miRNAs (Figure 3A). Subsequently, we constructed miR-217-5p inhibitor and examined its transfection efficacy in the Hub7 and Hep3B cells (Figure 3B). Viability markedly increased after knockdown of miR-217-5p (Figure 3C). Furthermore, the apoptotic rate was reduced by transfection of miR-217-5p inhibitor in HCC cells (Figure 3D). Western blot analyses showed upregulated β-catenin, GSK3β, c-myc and cyclin D1 in the Hub7 and Hep3B cells transfected with miR-217-5p inhibitor (Figure 3E).

A negative interaction between LINC00355 and miR-217-5p

Overexpression of miR-217-5p decreased luciferase activity in the wild-type LINC00355 vector. However, no significant difference in luciferase activity was found between HEK293T cells trans-

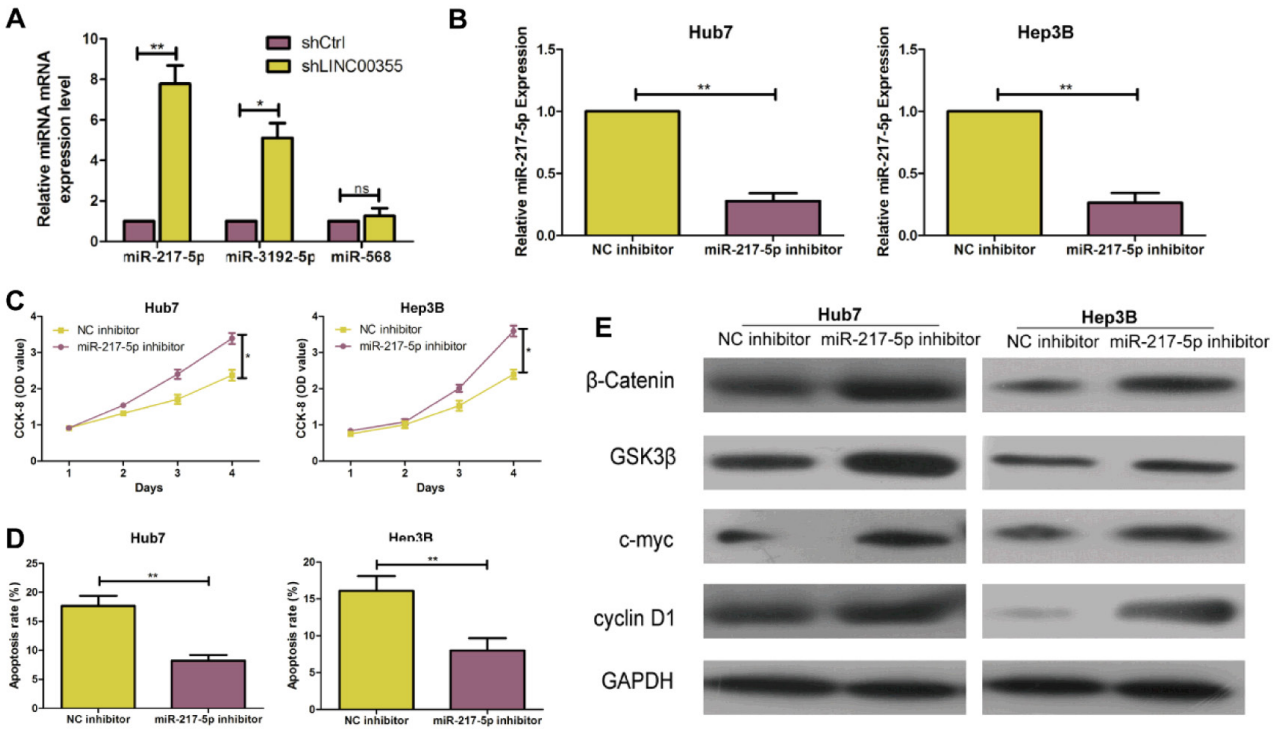

Figure 3. Influence of miR-217-5p on the proliferative and apoptotic potentials in HCC. **A:** Relative levels of miR-217-5p, miR-3192-5p and miR-568 after transfection of shLINC00355. **B:** Transfection efficacy of miR-217-5p inhibitor in Hub7 and Hep3B cells. **C:** Viability in Hub7 and Hep3B cells after transfection of miR-217-5p inhibitor. **D:** Apoptotic rate in Hub7 and Hep3B cells after transfection of miR-217-5p inhibitor. **E:** Protein levels of β-catenin, GSK3β, c-myc and cyclin D1 in Hub7 and Hep3B cells after transfection of miR-217-5p inhibitor. Data are expressed as mean±SD. *p<0.05, **p<0.01.

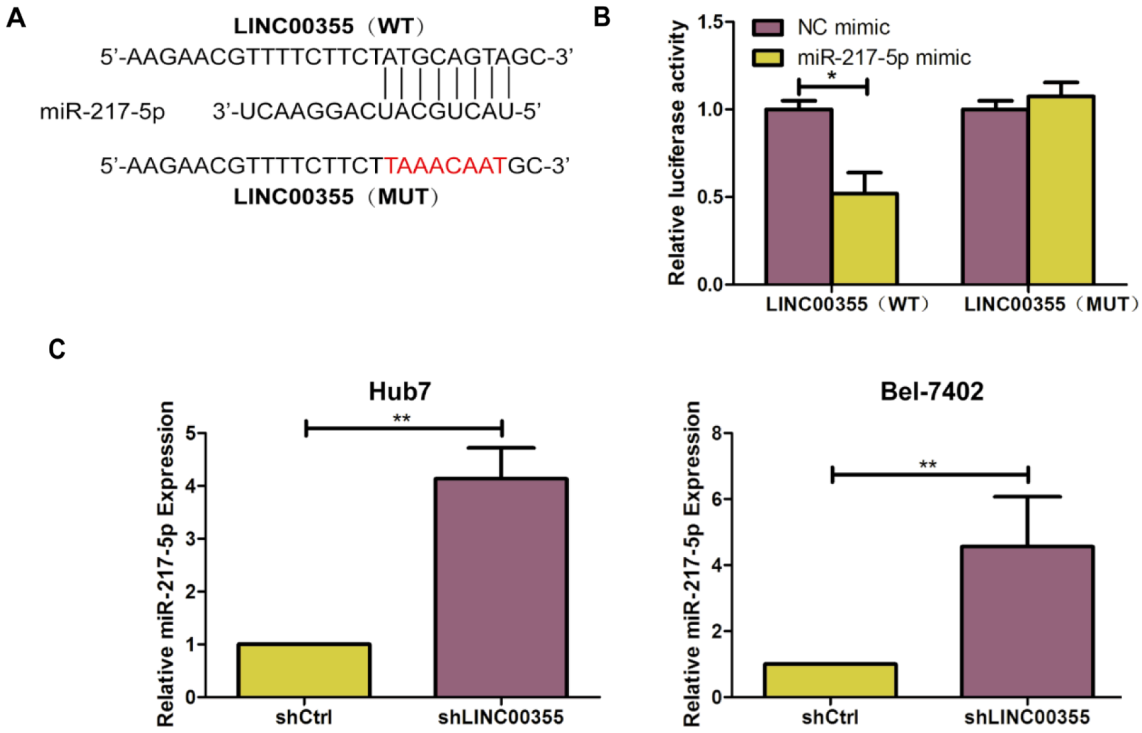

Figure 4. A negative interaction between LINC00355 and miR-217-5p. **A and B:** Luciferase activity in wild-type and mutant-type LINC00355 vectors after transfection of miR-217-5p mimic. **C:** MiR-217-5p expressions in Hub7 and Hep3B cells after transfection of shLINC00355. Data are expressed as mean±SD. *p<0.05, **p<0.01.

fected with NC mimic and those transfected with miR-217-5p mimic (Figures 4A and 4B). Consistently, miR-217-5p was upregulated in HCC cells with LINC00355 knockdown (Figure 4C).

Discussion

Primary liver cancer is a highly malignant tumor, posing a heavy burden on global health management. The mortality of primary liver cancer is up to 8.2% [1-4] and HCC is the major histological subtype [2-4]. As new mechanisms for the development of HCC have been revealed, its therapeutic strategies are also constantly updated [5,6]. However, despite the many options available for HCC patients, its overall survival has not been greatly improved [6,7]. HCC is a highly heterogeneous tumor with complicated pathogenesis [7]. Therefore, individualized treatment based on clinical features and molecular characteristics of HCC patients is required [8-10].

LncRNAs constitute the most abundant part of the transcribed genome [13-15]. They can alleviate the inhibitory effect of miRNAs on their target genes by acting as sponges, and even serve as precursors of some miRNAs [15]. Additionally, lncRNAs are specifically expressed in different types of cells and tissues, and different phases of development [13,14]. Identification of differentially expressed lncRNAs and exploration of their functions are conductive to develop therapeutic targets [13-15]. Recently, the carcinogenic role of LINC00355 has been demonstrated [16-18]. In this study, we collected clinical cases of HCC and compared with paracancerous ones, LINC00355 was upregulated in HCC tissues. We thereafter explored the *in vitro* functions of LINC00355 in regulating HCC cell phenotypes. Knockdown of LINC00355 attenuated the proliferative ability and induced apoptosis in Hub7 and Hep3B cells. In addition, knockdown of LINC00355 markedly inactivated the Wnt signaling.

LncRNAs can serve as competing endogenous RNAs, which exert mRNA sponge effect to influence the expression of target mRNAs and thus cell functions [15]. Through bioinformatic prediction, LINC00355 could sponge miR-217-5p. Here, miR-217-5p was downregulated in HCC cells and it was able to inhibit the proliferative rate and increase the apoptotic rate in Hub7 and Hep3B cells, and also to inactivate the Wnt signaling as a tumor suppressor. We have confirmed the binding between LINC00355 and miR-217-5p by dual-luciferase reporter assay. To sum up, LINC00355 aggravated

the malignant progression of HCC through negatively regulating miR-217-5p and activating the Wnt/β-catenin signaling.

Conclusions

LINC00355 is upregulated in HCC specimens, triggers the proliferative rate and inhibits apoptosis in HCC cells by negatively regulating miR-217-5p and thus activating the Wnt/β-catenin signaling.

Funding acknowledgements

Shanghai Science and Technology Commission - Natural Science Foundation Project, No.: 20ZR473100; Shanghai Municipal Science and Technology Commission Number: 19511121303.

Conflict of interests

The authors declare no conflict of interests.

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