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ORIGINAL ARTICLE

LINC01980 stimulates the progression of hepatocellular carcinoma *via* downregulating caspase 9

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Summary

Purpose: *To uncover the role of LINC01980 in aggravating the progression of hepatocellular carcinoma (HCC) via targeting caspase 9.*

Methods: *The expression levels of LINC01980 and caspase 9 in HCC tissues and paracancer tissues were determined by qRT-PCR. The prognostic potentials of LINC01980 and caspase 9 in HCC were assessed by Kaplan-Meier method. The regulatory effects of LINC01980 and caspase 9 on the viability, clonality and apoptosis of Huh7 and Hep3B cells were examined. Finally, the interaction between LINC01980 and caspase 9 was evaluated by performing dual-luciferase reporter gene assay and rescue experiments.*

Results: *LINC01980 was upregulated in HCC tissues and cells. High level of LINC01980 indicated worse prognosis in HCC patients. Knockdown of LINC01980 could attenuate viability and clonality, but induced apoptosis in Huh7 and Hep3B cells. Caspase 9 was downregulated in HCC, and its high level predicted a better prognosis in HCC patients. Overexpression of caspase 9 achieved the same regulatory effects as LINC01980 knockdown on HCC cells. Caspase 9 was the downstream target for LINC01980, and its level was negatively regulated by LINC01980. In HCC, LINC01980 regulated HCC cell behaviors by downregulating caspase 9.*

Conclusions: *Upregulation of LINC01980 in HCC predicts a poor prognosis. LINC01980 aggravates the progression of HCC via downregulating caspase 9.*

Key words: *LINC01980, caspase 9, hepatocellular carcinoma*

Introduction

Globally, the morbidity and mortality of hepatocellular carcinoma (HCC) rank sixth and third, respectively. About 700,000 people die of HCC every year [1-3]. Metastasis of HCC is mainly hematogeneous, lymphatic, and by adjacent organ infiltration [4,5]. Surgical resection, radiofrequency ablation, liver transplantation, radiotherapy, chemotherapy and particle implantation for HCC are being used. However, the 5-year survival of HCC is less than 12%, and has not been improved for decades [6,7]. HCC has an insidious onset, rapid progression, complicated treatment, and short survival time. Currently, there are no effective and reliable screening and prognostic methods in the early stage [7,8]. Therefore, it is necessary to uncover the pathogenesis of HCC and to develop sensitive hallmarks for diagnosis and treatment.

Molecular mechanisms underlying the occurrence, progression and metastasis of HCC are unclear. Multiple genes, factors and pathways are involved in the genesis of HCC [9-12]. With the advance in high-throughput analysis, sequencing

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and other technologies, the biological roles of long non-coding RNAs (lncRNAs) in tumors have been well concerned. LncRNAs are extensively distributed in eukaryotes, which are over 200 nt long. Although they cannot encode proteins, lncRNAs are capable of regulating gene expressions at epigenetic, transcriptional and post-transcriptional levels [13-15]. It is reported that lncRNAs are involved in tumor cell apoptosis, autophagy, tumor metastasis, chemotherapy resistance etc [16]. They serve as oncogenes or tumor-suppressor genes in tumor progression [17]. Their important biological functions in malignancies have attracted the attention of an increasing number of researchers [18,19].

The specific role of LINC01980 is rarely reported in tumor biology [20]. In this paper, we first identified the expression pattern and biological role of LINC01980 in HCC. LncRNAs can recognize target mRNA by incomplete base pairing from seed sequences of lncRNA 5'end to target mRNA 3'-UTR [21-23]. Through bioinformatics prediction and dual-luciferase reporter assay verification, caspase 9 was determined to be the target gene of LINC01980. Furthermore, we explored the interaction between LINC01980 and caspase 9 in influencing the progression of HCC.

Methods

Patients and samples

HCC tissues and matched adjacent normal tissues were surgically resected from 44 HCC patients. None of enrolled patients received preoperative anti-tumor therapies. Clinical indexes and follow-up data of them were collected for further analyses. Patients and their families in this study have been fully informed. This study was approved by Ethics Committee of Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital. All patients provided written informed consent and the study was conducted in accordance with the Declaration of Helsinki.

Cell culture

Normal hepacytes LO2 and lung cancer cells SMMC-7221, MHCC88H, Bel-7402, HepG2, Hep3B and Huh7 were purchased from ATCC, USA. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) and maintained in a 37°C, 5% CO_2 incubator. At 80-90% confluence, cell passage was conducted using 1×trypsin + ethylene diamine tetraacetic acid (EDTA).

Transfection

Cells were pre-seeded in the 6-well plates and transfected with the plasmids constructed by GenePharma, Shanghai, using Lipofactamine 2000 (Invitrogen, Carlsbad, CA, USA) at 60% confluence. At 48 h, cells were harvested for verification of transfection efficacy and subsequent experiments.

Cell counting kit (CCK-8)

Cells were seeded in the 96-well plates with 2×10^3 cells per well. At the appointed time points, absorbance value at 450 nm of each sample was recorded with a microplate reader using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) for plotting the viability curves.

Colony formation assay

Cells were seeded in a 6-well plates with 200 cells per well and incubated for 10-14 days. Colonies were washed with phosphate buffered saline (PBS) twice, fixed

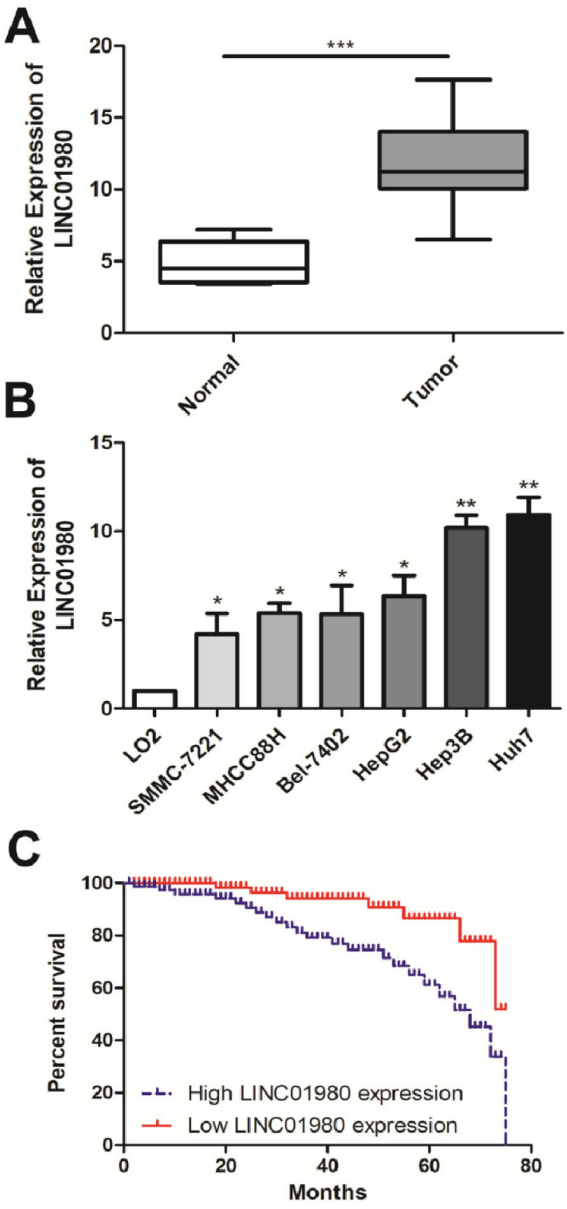

Figure 1. LINC01980 was highly expressed in HCC. **A:** LINC01980 level in HCC tissues and adjacent normal ones (***p<0.001). **B:** LINC01980 level in normal hepatocytes LO2 and lung cancer cells SMMC-7221, MHCC88H, Bel-7402, HepG2, Hep3B and Huh7 (*p<0.05, **p<0.01). **C:** Kaplan-Meier curves performed for survival in HCC patients with high and low level of LINC01980 (p<0.05).

in 4% paraformaldehyde and dyed with Giemsa solution for 30 min. Colonies containing over 50 cells were counted.

Flow cytometry

Cells were washed with PBS, digested, and suspended in binding buffer. They reacted with 5 μL of AnnexinV-FITC and 5 μL of propidium iodide (PI) in the dark for 15 min. Finally, cell apoptosis was determined by flow cytometry (Partec AG, Arlesheim, Switzerland).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), purified by DNase I treatment, and reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using Primescript RT Reagent (TaKaRa, Otsu, Japan). The obtained cDNA was subjected to qRT-PCR using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Japan). Glyceraldheyde 3-phosphate dehydrogenase (GAPDH) and U6 were used as internal references. Each sample was performed in triplicate, and the relative level was calculated by $2^{-\Delta\Delta Ct}$. Primer 5.0 was used for designing qRT-PCR primers.

Dual-luciferase reporter gene assay

Cells were co-transfected with pmirGLO-LINC01980-WT/pmirGLO-LINC01980-MUT/pmirGLO and NC/pcDNA-caspase 9 using Lipofectamine 2000. Twenty-four h later, co-transfected cells were harvested for determining luciferase activity using a dual-luciferase reporter assay system (Promega, Madison, WI, USA).

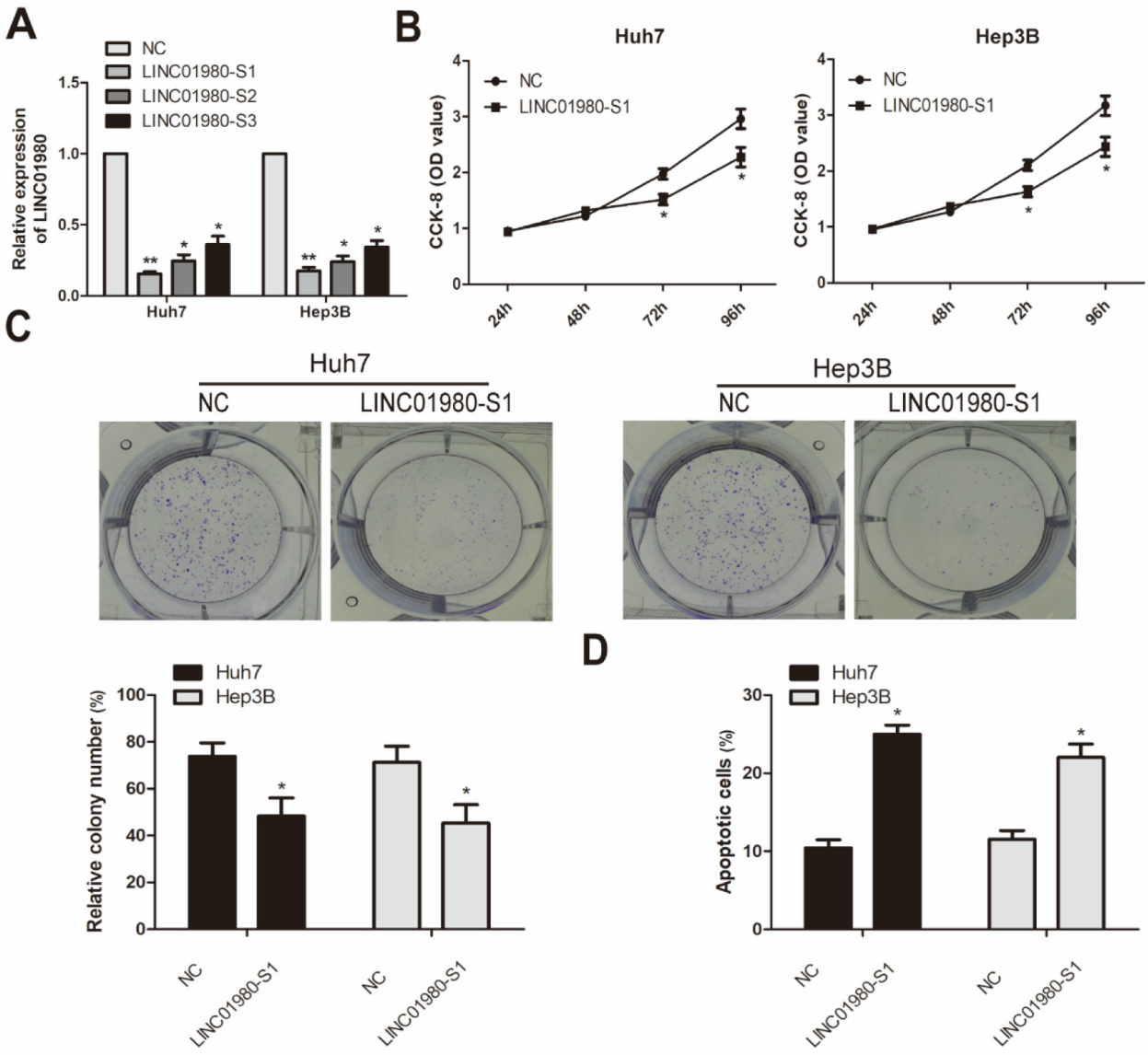

Figure 2. LINC01980 influenced viability, clonality and apoptosis in HCC. **A:** Transfection efficacies of LINC01980-S1, LINC01980-S2 and LINC01980-S3 in Huh7 and Hep3B cells (*p<0.05, **p<0.01). **B:** Viability in Huh7 and Hep3B cells transfected with NC or LINC01980-S1 (*p<0.05). **C:** Relative colony number in Huh7 and Hep3B cells transfected with NC or LINC01980-S1. The Figure shows that the relative colony number was reduced after transfection of LINC01980-S1 in Huh7 and Hep3B cells. **D:** Apoptotic cell ratio in Huh7 and Hep3B cells transfected with NC or LINC01980-S1 (*p<0.05).

Western blot

Total protein from cells was extracted using radioimmunoprecipitation assay (RIPA) (Beyotime, Shanghai, China), quantified by bicinchoninic acid (BCA) method (Beyotime, Shanghai, China) and loaded for electrophoresis. After transferring on a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA), it was blocked in 5% skim milk for 2 h, incubated with primary antibodies at 4°C overnight and secondary antibodies on the other day for 2 h. Bands were exposed by electrochemiluminescence (ECL) (Pierce, Rockford, IL, USA) and analyzed by Image Software (NIH, Bethesda, MD, USA).

Statistics

GraphPad Prism 5 V5.01 (La Jolla, CA, USA) was used for data analysis. Data were expressed as mean ± standard deviation. Intergroup differences were analyzed by the t-test. Kaplan-Meier curves were performed for survival analysis and log-rank test assessed the intergroup differences. Chi-square test was performed to evaluate the relationship between two genes. $P<0.05$ was considered as statistically significant.

Results

LINC01980 was highly expressed in HCC

First, the LINC01980 level in HCC and paracancer tissues was determined. LINC01980 was upregulated in HCC tissues relative to adjacent normal ones (Figure 1A). Identically, LINC01980 was highly expressed in lung carcinoma cells than in normal hepatocytes (Figure 1B). Follow-up data of enrolled HCC patients were collected for survival analysis. Kaplan-Meier curves revealed that HCC patients expressing high level of LINC01980 had worse prognosis than those with low level (Figure 1C).

LINC01980 influenced viability, clonality and apoptosis in HCC

A total of three LINC01980 siRNAs were constructed. Transfection of LINC01980-S1, LINC01980-S2 or LINC01980-S3 all could markedly downregulate LINC01980 in Huh7 and Hep3B

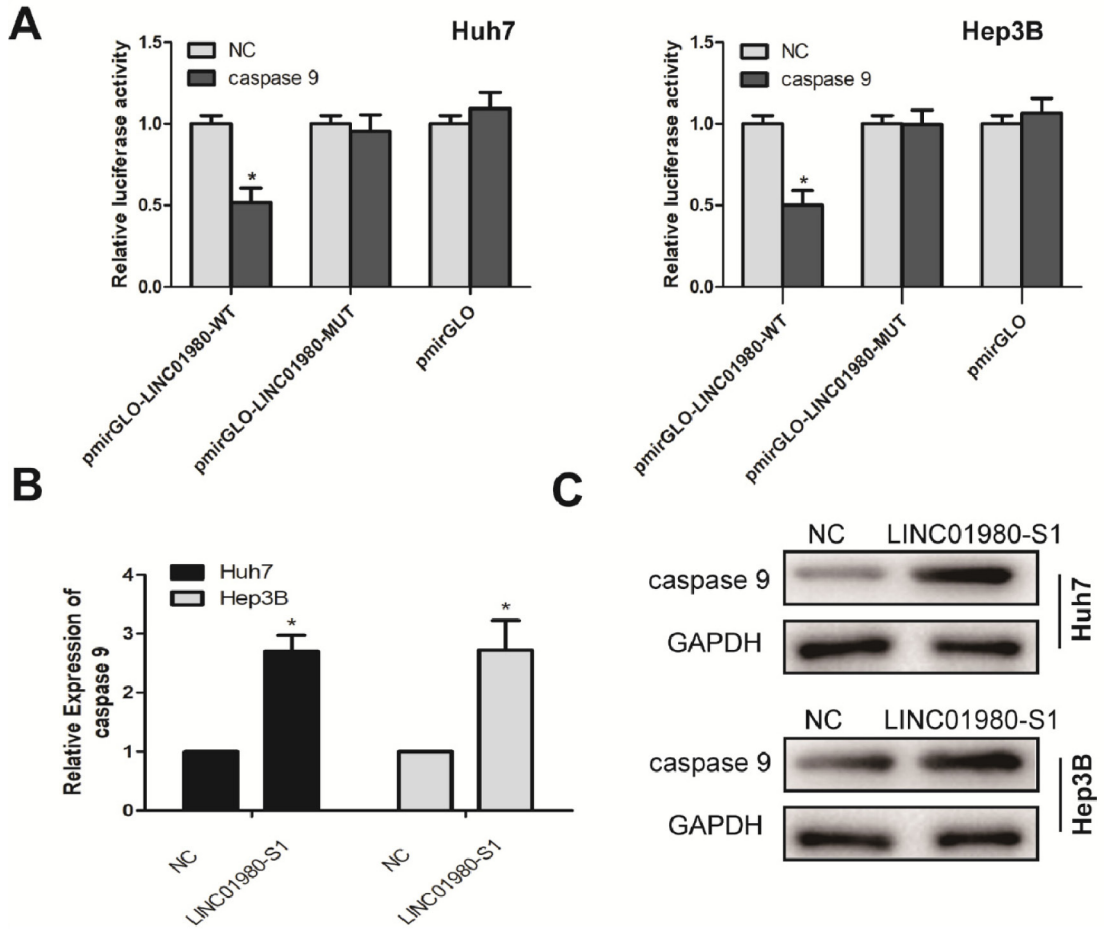

Figure 3. LINC01980 bound to caspase 9. **A:** Luciferase activity in Huh7 and Hep3B cells co-transfected with pmirGLO-LINC01980-WT/pmirGLO-LINC01980-MUT/pmirGLO and NC/pcDNA-caspase 9 (*$p<0.05$). **B:** The mRNA level of caspase 9 in Huh7 and Hep3B cells transfected with NC or LINC01980-S1 (*$p<0.05$). **C:** The protein level of caspase 9 in Huh7 and Hep3B cells transfected with NC or LINC01980-S1. The Figure shows that both mRNA and protein levels of caspase 9 were markedly upregulated in Huh7 and Hep3B cells transfected with NC or LINC01980-S1.

cells (Figure 2A). In the following experiments, LINC01980-S1, the most effective one, was used for transfection. CCK-8 assay showed that the viability was lower in Huh7 and Hep3B cells transfected with LINC01980-S1 compared with those transfected with NC (Figure 2B). The relative colony number was reduced after transfection of LINC01980-S1 in Huh7 and Hep3B cells, suggesting the attenuated clonality (Figure 2C). Meanwhile, the apoptotic cell ratio was remarkably elevated after silencing LINC01980 (Figure 2D).

LINC01980 bound to caspase 9

Through bioinformatics prediction, caspase 9 was the potential target of LINC01980. Our results showed a decline in luciferase activity after co-transfection of pmirGLO-LINC01980-WT and pcDNA-caspase 9 in HCC cells, verifying LINC01980 can be targeted by caspase 9 through their binding sites in the promoter region (Figure 3A). In Huh7 and Hep3B cells transfected with LINC01980-S1, both mRNA and protein levels of caspase 9 were markedly upregulated (Figure 3B,3C).

Caspase 9 was lowly expressed in HCC

Subsequently, we focused on the expression pattern and biological role of caspase 9 in HCC. Caspase 9 was downregulated in HCC tissues and cell lines (Figure 4A,4B). In 44 selected HCC tissues, caspase 9 level was negatively correlated with that of LINC01980 (Figure 4C). Kaplan-Meier curves revealed worse prognosis in HCC patients expressing low level of caspase 9 (Figure 4D). Therefore, caspase 9 was downregulated in HCC and predicted poor prognosis in HCC patients.

Overexpression of caspase 9 inhibited viability, clonality and induced apoptosis in HCC

Furthermore, pcDNA-caspase 9 was constructed. Transfection of pcDNA-caspase 9 remarkably upregulated caspase 9 in Huh7 and Hep3B cells (Figure 5A). In HCC cells overexpressing caspase 9, their viability was markedly reduced than in controls (Figure 5B). Similarly, clonality was attenuated by overexpression of caspase 9 in HCC (Figure 5C). Apoptotic rate was remarkably elevated after transfection of pcDNA-caspase 9 (Figure 5D).

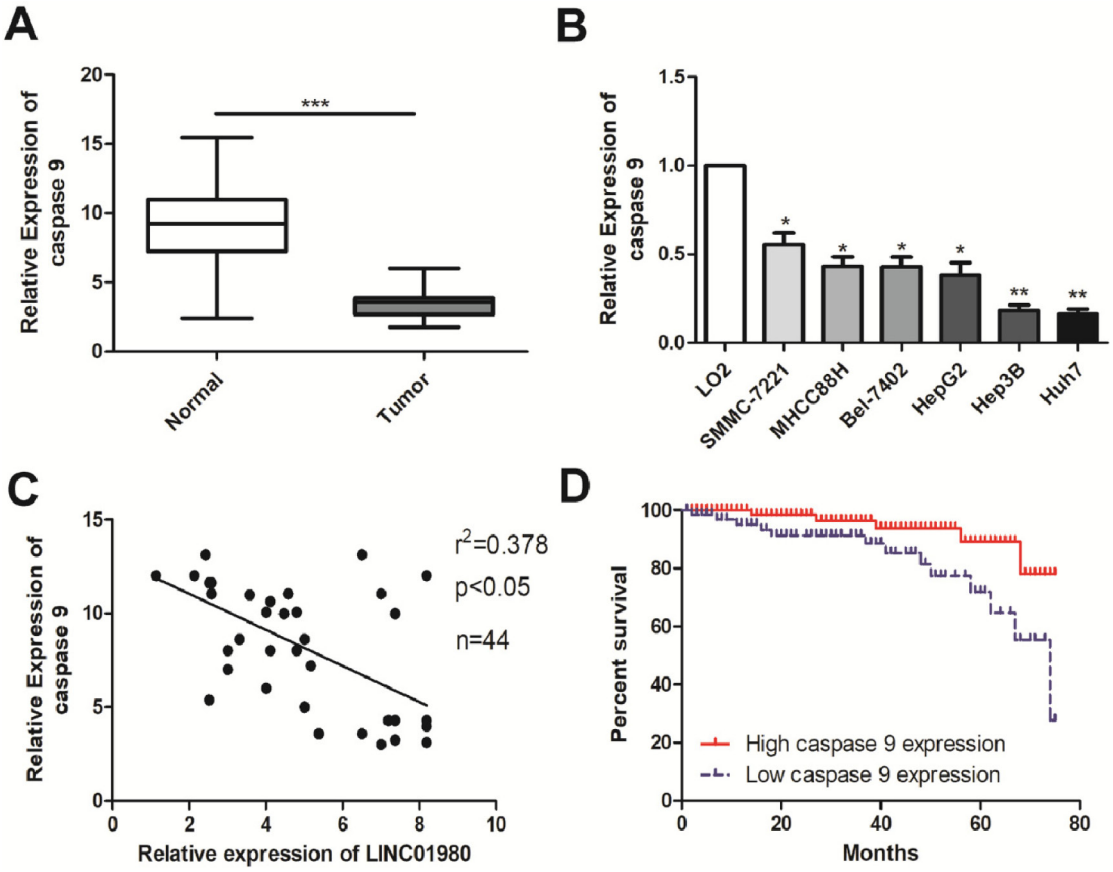

Figure 4. Caspase 9 was lowly expressed in HCC. **A:** Caspase 9 level in HCC tissues and adjacent normal ones (***p<0.001). **B:** Caspase 9 level in normal hepatocytes LO2 and lung cancer cells SMMC-7221, MHCC88H, Bel-7402, HepG2, Hep3B and Huh7 (*p<0.05, **p<0.01). **C:** A negative correlation between expression levels of LINC01980 and caspase 9 in HCC tissues. **D:** Kaplan-Meier curves performed for survival in HCC patients with high and low level of caspase 9 (p<0.05).

LINC01980 regulated HCC performance by downregulating caspase 9

Both LINC01980 and caspase 9 were involved in the progression of HCC, and caspase 9 was the target of LINC01980. Hence, we speculated that LINC01980 may influence HCC cell performances by regulating caspase 9 level. LINC01980 level was found to be partially upregulated in HCC cells co-transfected with LINC01980-S1 and si-caspase 9 (Figure 6A). Interestingly, silence of LINC01980 decreased colony number, which was reversed by silencing caspase 9 in HCC (Figure 6B). The enhanced apoptotic rate in HCC cells transfected with LINC01980-S1 was further reduced by co-transfection of si-caspase 9 (Figure 6C). Collectively, caspase 9 could abolish the regulatory effects of LINC01980 on the proliferative and apoptotic potentials in HCC.

Discussion

The occurrence and progression of HCC are complex processes involving both environmental and genetic factors [4-7]. Abnormally expressed genes and dysregulated signaling pathways are of significance in the progression of HCC [8-10]. Generally speaking, oncogene activation and tumor-suppressor gene inactivation are the molec-

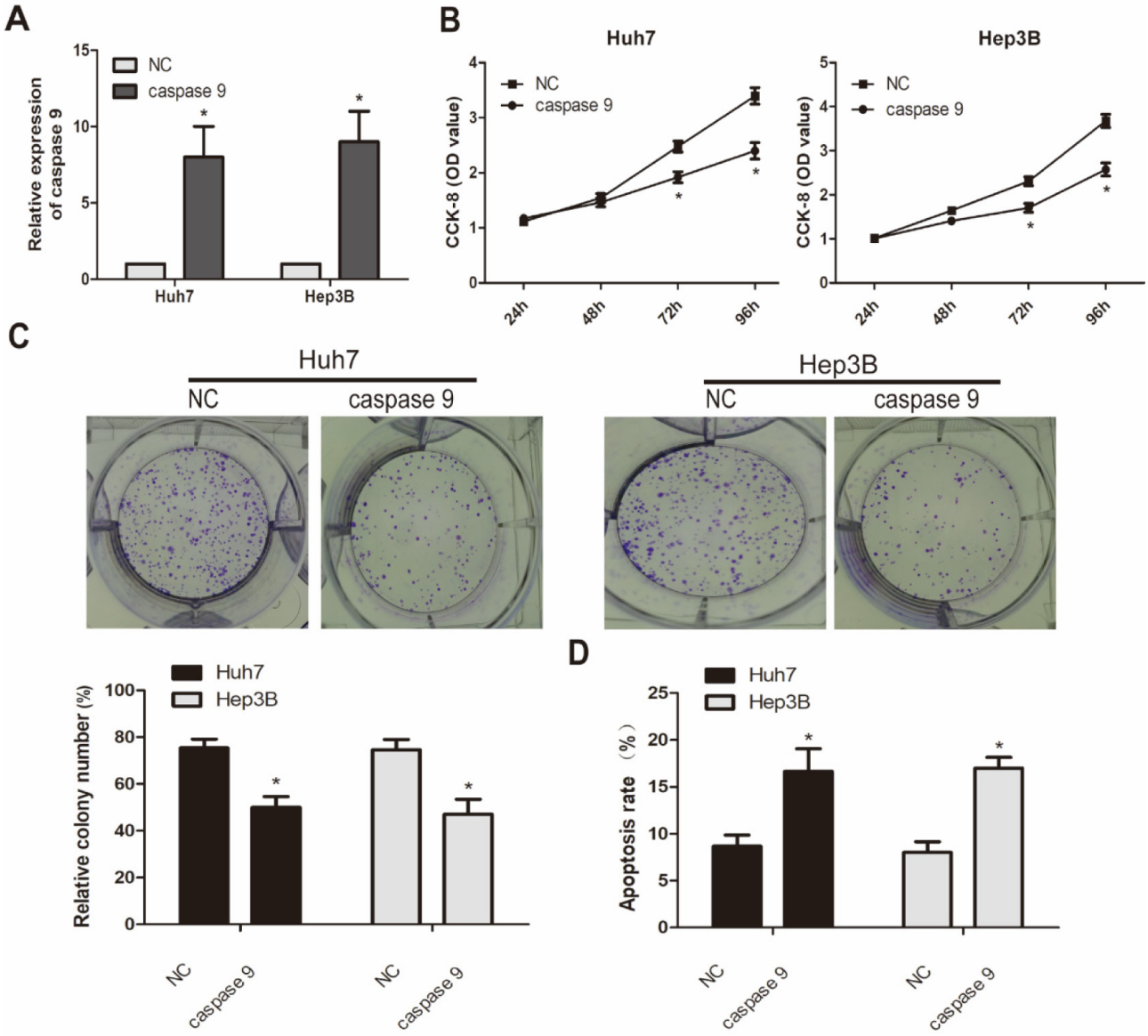

Figure 5. Overexpression of caspase 9 inhibited viability, clonality and induced apoptosis in HCC. **A:** Transfection efficacy of pcDNA-caspase 9 in Huh7 and Hep3B cells (*p<0.05). **B:** Viability in Huh7 and Hep3B cells transfected with NC or pcDNA-caspase 9 (*p<0.05). **C:** Relative colony number in Huh7 and Hep3B cells transfected with NC or pcDNA-caspase 9. The Figure shows that clonality was attenuated by overexpression of caspase in HCC. **D:** Apoptotic cell ratio in Huh7 and Hep3B cells transfected with NC or pcDNA-caspase 9 (*p<0.05).

ular basis for the tumorigenesis of HCC [11,12].

Differential expression profiles of lncRNAs in tumors are closely related to their occurrence, development and metastasis, which broaden our understanding of the pathogenesis and treatment options of malignant tumors. These lncRNAs may be utilized for early-stage intervention and effective treatment of HCC [13-16]. LINC01980 is a newly discovered lncRNA. Upregulation of LINC01980 could predict the poor prognosis of esophageal cancer [17-20]. In this experiment, LINC01980 was upregulated in HCC tissues and cells. High level of LINC01980 indicated worse prognosis in HCC patients. A series of *in vitro* experiments identified that silencing LINC01980 could attenuate the viability and clonality, but induced apoptosis in Huh7 and Hep3B cells. It is suggested that LINC01980 may play a carcinogenic role during the progression of HCC.

Caspase activation is a process where the protective sequences of procaspases are hydrolyzed, followed by the formation of an active tetramer

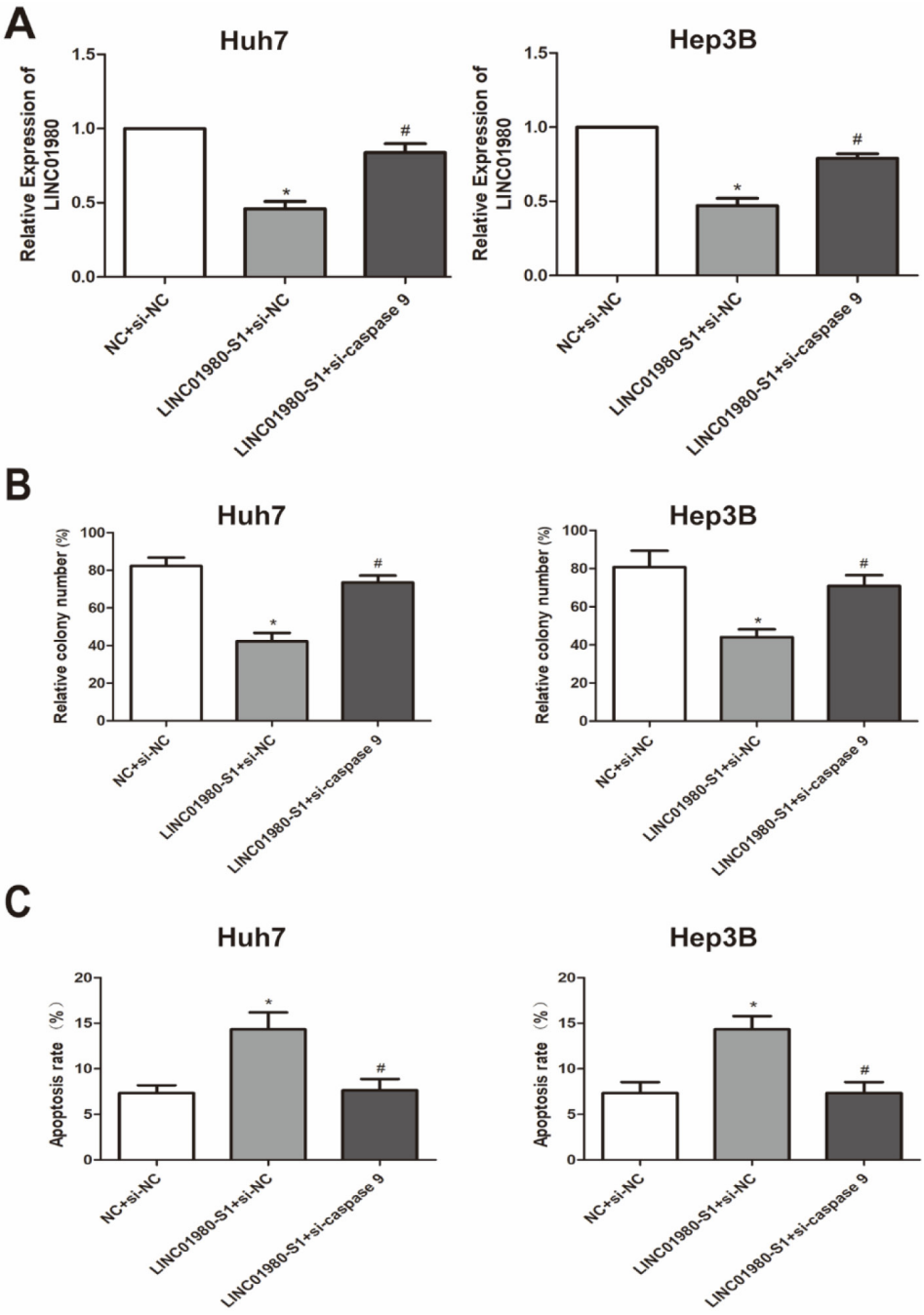

Figure 6. LINC01980 regulated HCC performance by downregulating caspase 9. Huh7 and Hep3B cells were transfected with NC+si-NC, LINC01980-S1+si-NC or LINC01980-S1+si-caspase 9. **A:** LINC01980 level (*p<0.05, #p<0.05). **B:** Relative colony number and **C:** Apoptotic cell ratio (*p<0.05, #p<0.05).

by the released subunits [20-25]. Caspase 9 is the upstream of the caspase cascade, which is a key factor initiating the mitochondrial apoptotic pathway [26,27]. Through bioinformatics prediction, caspase 9 was identified to be the downstream gene of LINC01980, which was further verified by dual-luciferase reporter gene assay. LINC01980 could negatively regulate caspase 9 level in HCC cells. Caspase 9 was lowly expressed in HCC tissues and cells. In addition, caspase 9 overexpression was proved to attenuate the viability and clonality, but induced apoptosis in HCC cells. Interestingly, silencing caspase 9 could abolish the attenuated viability and clonality, as well as the stimulated apoptosis in HCC cells with LINC01980 knockdown. Collectively, LINC01980 aggravated the progression of HCC *via* downregulating caspase 9.

Conclusions

Upregulation of LINC01980 in HCC predicts a poor prognosis. LINC01980 aggravates the progression of HCC *via* downregulating caspase 9.

Conflict of interests

The authors declare no conflict of interests.

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