A Novel lncRNA loc339803 acts as CeRNA of miR-30a-5p to promote the migration and invasion of hepatocellular carcinoma cells

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Abstract

Background

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors, and has an unfavorable clinical outcome. Emerging evidences have demonstrated that long noncoding RNAs (lncRNAs) play an important role in the carcinogenesis and progression of HCC. However, the clinical significances, the biological roles of most lncRNAs in HCC remain poorly understood.

Methods

The expression levels of lncRNA loc339803 in HCC tissues and cell lines were determined by quantitative real-time polymerase chain reaction (qRT-PCR) assay. The cellular sublocalization of loc339803 were determined by fluorescence in situ hybridization and nuclear & cytoplasmic RNA isolation assay. Western blot, CCK-8, Edu, colony formation, migration and invasion assays were used to investigate the roles of loc339803 in progression of HCC in vitro. A mouse model for lung metastasis was constructed to evaluate the role of loc339803 in HCC development in vivo. The correlations among loc339803, miR-30a-5p and SNAIL1 were validated by qRT-PCR and a dual-luciferase reporter assay.

Results

The expression of loc339803 was upregulated in HCC tissues and cell lines, and positively correlated with tumor size, advanced tumor stage, higher serum AFP level and poor prognosis of HCC patients. loc339803 can promote the migration and invasion of HCC cells in vivo and in vitro. Further studies demonstrated the loc339803 functioned as a competing endogenous RNA (ceRNA) by directly binding to miR-30a-5p, thus up-regulating the expression of *snai1*, a target gene of miR-30a-5p. Moreover, miR-30a-5p upregulation blocked the enhancement of migration and invasion of HCC cells induced by loc339803 overexpression.

Conclusions

Loc339803 may be oncogenic in HCC and associated with poor clinical outcomes. LncRNA loc339803 might promote the invasion and migration of HCC cells through regulating miR-30a-5p/ SNAIL1 axis.

Introduction

According to 2019 Global Cancer Statistics, hepatocellular carcinoma is one of the most common malignant tumors with a fourth morbidity and a third mortality all over the world¹. Despite the use of advanced treatments, such as molecular targeted drugs, surgical resection, and radiofrequency ablation, the 5-year survival of HCC is still poor, usually due to the high rate of intrahepatic metastasis before

diagnosis establishment and recurrence after surgical resection²⁻⁶. Therefore, to explore the molecular mechanisms of HCC metastasis is taking on a fresh urgency.

Long non-coding RNA is a type of RNA that is not longer than 200 nt and has no protein-coding ability². According to its transcription sites on the gene, it can be divided into sense, antisense, intron, intergenic, and bidirectional lncRNA³. Numerous studies have showed that lncRNAs widely participate in the cell proliferation, invasion and apoptosis⁴⁻⁶. The lncRNAs located in the nuclear and cytoplasm may exhibit different functions ADDIN EN.CITE¹¹. As reported, lncRNAs located in the nucleus can function as scaffolds to bring proteins to form ribonucleoprotein complexes and as guides to recruit chromatin-modifying complexes to target genes⁶⁻⁸. The lncRNAs located in the cytoplasm can function as competing endogenous RNAs (ceRNAs) to modulate the depression of miRNAs targets through competitively binding miRNAs^{9,10}. For instance, the TCONS_00006195 located in the nucleus can represses the progression of liver cancer by repressing the activity of ENO1¹¹. lncRNA MIR31HG located in cytoplasm can suppress the development of HCC by binding to miR-575¹². However, most lncRNAs remain poorly understood in tumorigenesis.

In present study, we selected a lncRNA from GEO datasets and explored its role in HCC. Our finding may provide a new therapeutic target for the treatment of HCC.

Experimental Methods And Materials

Clinical samples

A total of 80 pairs of liver cancer and adjacent tissue samples were collected from Nanjing Drum Tower Hospital during the period from 2018-2020. None of the patients had used chemotherapy and other treatments before surgery. Tissue specimens were placed in liquid nitrogen immediately after being obtained, and stored at -80°C until use. The patients' pathological diagnoses were confirmed by two clinical pathologists. The basic clinical data and pathological grade of the patients are shown in Table 1. This study was approved by the Research Ethics Committee of Nanjing Drum Tower Hospital.

Cell culture

The human HCC cell lines Hep3B, HepG2, SMMC-7721, LM3,97H and LO2 were purchased from Cell Bank, Chinese Academy Sciences (Shanghai, China) and cultured in DMEM Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum, 100U/ml penicillin, and 100 µg/ml streptomycin. All the cell lines were cultured in a humidified incubator with 5% CO₂ at 37 °C.

Transfection of cell lines

Small interference RNA (siRNA) and control for loc339803 were designed and synthesized by Hanbio gene (Guangzhou, China). Loc339803 full length (pCDH-loc339803) plasmid and controls (pCDH, Cat: CD510B-1) were synthesized by Sangon Biotech (Shanghai, China). MiR-30a-5p mimics, miR-30a-5p inhibitor and the control were designed and synthesized by GenePharma (Shanghai, China). HCC cells were transfected with the siRNA and plasmid following the lip3000 (Invitrogen, USA) manufacturer's instruction. The target sequences of loc339803 were listed as follows: si-loc339803#1: GGTGCGGTCTAGTGATCTA, si-loc339803#2: GCAAAGGTTTCTTATTAA, and si-loc339803#3: GG TAGGAATCAAGGTTAGA. The sequence of siloc339803#2 was subcloned into the lentiviral shRNA expression vector plko.1(Addgene, USA). After a 48-h period of transfection, the cells were harvested for the subsequent experiments.

RNA extraction and RT-PCR detection

Total RNA from liver tissues and cells were extracted by using Trizol reagent (Invitrogen, Carlsbad, USA) following the introductions. Total RNA was reverse transcribed into cDNA with the using of First Strand cDNA Synthesis Kit (R232-01, Vazyme, Nanjing, China) and real-time PCR was carried out with the using of AceQ qPCR SYBR Green Master Mix (Q411-02, Vazyme, Nanjing, China). B-actin and U6 were used as the internal control. All the qPCR detections were performed using ABIQ6(ABI,USA) and statistics were analyzed with the $2^{-\Delta\Delta C_t}$ method. The primer used in this study are listed in table2.

WB detection analysis

Total cell protein extraction was lysed with RIPA lysate containing 0.1% protease inhibitor and phosphatase inhibitor, and quantified with BCA protein quantification kit. Subsequently, proteins were separated by SDS-PAGE and transferred to PVDF membranes. The above PVDF membranes were blocked with 5% milk in room temperature for 2 hours, then incubated with corresponding primary antibodies at 4°C overnight. The following antibodies were used for westernblot:E-cadherin(Cat:#3195T,CST),N-cadherin(Cat:#13116T,CST),Vimentin(Cat:#5741T,CST),MMP-9(Cat:#13667,CST),MMP2(Cat:#40994,CST),GAPDH(Cat:#5174,CST),SNAIL(Cat:#3879,CST). After incubated with a secondary antibody at room temperature for 2h, the membranes were luminescence imaged with ECL (Tanon, Shanghai, China) luminescent solution.

CCK8 and Edu proliferation assay

For proliferation experiment, 4,000 cells were planted in 96-well plates using 3 wells for technical replicates. 100ul DMEM of solution containing 10% CCK8 (Dojindo Laboratories, Kumamoto, Japan) were added into each well. After cultured for 2 hours in a humidified incubator. The absorbance was measured at 450nm wavelength. A Cell-Light Edu DNA cell proliferation kit (Ribobio, Guangzhou, China) was also used to evaluate the cell proliferation ability.

Clone formation experiment

For the colony formation assay, 600 cells were seeded in 6-well-plate and cultured with DMEM containing 10%FBS for 2 weeks. The colonies were fixed with 4% paraformaldehyde for 20 minutes, then stained with crystal violet for 5 minutes at room temperature.

Invasion and migration assays

The cell lines were harvested after transfection with siRNA or plasmids for 48h. 5×10^4 cells were resuspended in DMEM medium (200 μ L), and then seeded in the top chamber of transwell plates (8 μ m size, Corning, NY, USA). For invasion, the chamber was coated with 30ug Matrigel, while, for the migration assay, the insert was not coated with Matrigel. The lower chamber was supplemented with 1ml DMEM contain 10%FBS. Then the plates were cultured in a humidified incubator with 5% CO₂ at 37 °C for 12h. The cells having migrated and invaded were fixed with 4% paraformaldehyde and stained with crystal violet. The cells that had migrated and invaded were counted and pictures were taken by a 200 $\times$ microscope (Olympus, Japan).

In vitro experiments

For metastasis experiment in vivo, 5×10^6 cells Hep3B cells which had been infected with shRNA-loc339803 or empty vector were resuspended in 300 μ L PBS, and injected into 4-week-old BALC nude mice through the tail vein (5 mice per group). The experiment was terminated 8 weeks after the tail vein injection. The lungs were dissected, fixed with 4% paraformaldehyde, and prepared for histological examination

FISH experiment and nuclear-plasma separation

The Loc339803, U6 and 18S fish probes were designed and synthesized by Guangzhou Ribobio Co, Ltd. (Guangzhou, China). The fish excrement was carried out by using the fish kit according to the manufacturer's instructions. The images were acquired through confocal microscope (Leica)

Nuclear & cytoplasmic RNA isolation assay

The total, cytoplasm and nuclear RNA were isolated using a Cytoplasmic & Nuclear RNA Purification Kit according to the manufacture introduction (Norgen Biotek Canada). U6 acted as nuclear internal control, and 18s acted as cytoplasmic internal control. Data normalization was performed against total RNA: % of Input = $100 \times (2^{[Ct \text{ total RNA} - Ct \text{ RNA fraction}]})$.

Luciferase reporter gene experiment

3'-UTR of SNAIL or loc399803 was inserted into pGL3 luciferase reporter vector (Promega, Madison, WI, USA). 293 T cells were transfected with 0.5 ug reporter construct and 50 nmol siRNA (or miRNA mimic) per well using Lipofectamine 3000 (Invitrogen, Cat#L3000-015). After 12 h of transfection, we replaced the transfection medium with complete culture medium. After 48 h of culture, the cells were lysed with passive lysis buffer (Promega, Cat# E1910), and the reporter gene expression was assessed using a Dual

Luciferase reporter assay system (Promega, Cat# E1910). All transfection assays were carried out in triplicate.

Experimental Results

1. Loc33983 is up-regulated in liver cancer and positively correlate with the prognosis of the patient.

In order to discover abnormally expressed lncRNA in liver cancer tissues, we investigated the data in GEO database. We identified a large number of differentially expressed lncRNAs in GSE59747 dataset¹³. Among them, the lncRNA loc339803 which is upregulated 2.33-fold by the TGF- β caught our eyes (Table.s1). Then we investigated loc339803 by analyzing the TCGA data in Starbase 3.0¹⁴. The lncRNA loc339803 was highly expressed in HCC and had a positive correlation with the survival time of the patients (Figure1b.1c). We further examined 86 pair tumor and matched adjacent peritumoral tissues. The lncRNA loc339803 was upregulated in tumor tissues (Figure 1a). The loc339803 was also upregulated in HCC cell lines Hep3b, SMMC-7721, LM3, HEP2, compared with liver normal cell line LO2 (Figure 1f). Through analyzing the coding ability of loc339803 in CAPT and CPC2 databases, we found that loc339803 had a poor protein coding ability. (Figure 1d,1e)

Furthermore, to assess the clinical significance of loc339803 in HCC, we analyzed the correlation between the loc339803 expression level and the clinical outcome of HCC patients. The results showed that the patients with a higher expression of loc339803 had a higher degree of malignancy and a higher level of serum AFP. (Table 1)

2. loc339803 promotes the invasion and migration of liver cancer in vitro

In order to further investigate the function of loc339803 in the liver cancer, liver cancer cells Hep3B and LM3 were selected to perform loss- and gain-of function experiments. First, three siRNA oligos were transfected into Hep3B and LM3. The knockdown efficiency was showed in figure2a. The overexpression efficiency was showed in figure2b. The transwell assay showed the ability of migration and invasion of Hep3b and LM3 was reduced after loc339803 knockdown (Figure 2c,2d) and enhanced by loc339803 overexpression (Figure 2e,2f). The cck8, EDU and cloning formation assay showed the loc339803 had no effect on the proliferation of Hep3b and LM3 (Figure s1). The western blot results showed that loc339803 increased the expression N-cadherin, Vimentin, MMP-2, SNAIL1 and decreased the E-cadherin expression (Figure 2g).

3. loc339803 promotes the invasion and migration of liver cancer in vivo

In order to investigate the role of loc339803 in the tumor metastasis in vivo, the lung metastases model was established. The Hep3B cells stably transfected with sh-loc339803 or sh-nc were injected into nude mice through tail vein (each group 5 mice). Eight weeks later, the lung metastases foci were detected with the usage of a live imaging system. The bioluminescence show knockdown the expression of loc339803 can significantly inhibit the lung metastases (Figure 3a,3c). Then, histological examination of the lung

shows the mice injected with sh-loc339803 cells have fewer pulmonary metastatic nodules compared with the sh-nc group (Figure 3b ,3d).

4.loc339803 functions as ceRNA and sponges miR-30a-5p in HCC cell lines

Evidence has indicated that lncRNA can bind to protein in nuclear and sponge on miRNA to regulate the expression level of target gene in cytoplasm. To investigate the mechanism through which loc339803 regulates migration and invasion, we analyzed the location of loc339803. The lncRNA location database (lncLocator), the Fish-experiment and Nuclear & cytoplasmic RNA isolation assay exhibited that the loc339803 is mainly located in cytoplasm (Figure 4a,4b), indicating that loc339803 may function as ceRNA. Then, we investigated the miRNA that may bind to loc339803 in starbase v3.0, and miRNAs 30a/b/c/d/e were identified to bind to loc339803(Figure 4c). Thus, we examined the expression of miR-30a/b/c/d/e after loc339803 low- and over-expression. (figure s2) In the end, we found that the expression of miR-30a-5p was decreased the most significantly compare to miR-30b/c/d/e by loc339803 overexpression and increased by loc339803 knockdown (figure 4d). Furthermore, the luciferase reporter assay revealed that overexpression of miR-30a-5p reduced the luciferase activity of wild-type loc339803 vector in Hep3B and LM3(figure 4e). And the luciferase activity of wild-type loc339803 can be upregulated by inhibit the expression of miR-30a-5p.(Figure 4f)

5.SNAIL is a target of miR-30a-5p in HCC cell lines

By the analysis based on starbase v3.0, we found that SNAIL was a target of miR-30a-5p (figure 5a). The luciferase activity showed that the luciferase activation was reduced by miR-30a-5p mimics(figure5b). Furtherly, we found the expression level of SNAIL1 was downregulated when miR-30a-5p was highly expressed, and upregulated when miR-30a-5p was lowly expressed (figure 5c).

6.miR-30a-5p restoration attenuates the effect of loc339803 overexpression on HCC cells

After transfection of miR-30a-5p mimics with stably overexpressed loc339803, we found that the invasion of liver cancer that had been enhanced by loc339803 was blocked by miR-30a-5p (figure 6a). The upregulation of Snail1 induced by loc339803 was blocked by miR-30a-5p mimics. In loc339803 overexpression HCC cell lines, their enhanced migrative and invasive abilities were partly abolished by overexpression of miR-30a-5p (figure 6b).

Discussion

Emerging studies have shown that lncRNAs widely take part in the development of cancer^{15,16}. In this study, we identified that loc339803, a newly discovered lncRNA, was highly expressed in HCC tissue and positively associated with the grade of the HCC and the level of AFP in serum. Besides, HCC patients with a higher level loc339803 showed worse outcome and shorter survival time.

Transwell assay showed that the loc339803 enhanced the migration and invasion of HCC cell lines in vivo and in vitro, and loc339803 overexpression upregulated N-cadherin, Vimentin, SNAIL1 and downregulated E-cadherin, indicating that loc339803 may promote the EMT progress of HCC cell lines. EMT is a process of transformation of epithelial cells into mesenchymal cells, even malignant phenotype, which contributes to tumor metastasis^{17,18}. So far, lots of lncRNAs have been confirmed to be involved in EMT. For example, lncTCF7 and SNHG3 can promote the progress of cancer through enhancing EMT in HCC^{19,20}.

The function of lncRNA is decided by its location in the cell^{21,22}. One consensus is that lncRNA located in the cytoplasm can function as ceRNA by competitively binding to miRNA, thereby affecting the expression level of miRNA-targeted genes^{23,24}. In this study, loc339803 were first predicted locating in the cytoplasm by analysis the lncLocator database²⁵. And the follow experiments also verify the results. Thus, we believe that loc339803 may function as ceRNA to promote the migration and invasion of HCC cell lines. Then we used bioinformatics tools to predict the potential target miRNA of loc339803. The results showed that miR-30a-5p may be a target miRNA of loc339803, which was subsequently confirmed by the luciferase reporter gene tests. MiR-30a-5p can act as a suppressor gene to inhibit the migration and invasion in many cancers²⁶⁻²⁸. In this study, we found the migration and invasion of cells, which had been enhanced by loc339803 overexpression, was blocked by miR-30a-5p mimics. And the expression of miR-30a-5p can be suppressed by the overexpression of loc339803. So, we believe miR-30a-5p may be a target gene of loc339803.

Then bioinformatic assays were used to predict the target gene of miR-30a-5p. The results showed SNAIL1 may be a target gene of miR-30a-5p. In addition, SNAIL1 has been confirmed as a target of miR-30a-5p in lung cancer and stomach cancer, and a regulator in EMT.^{29,30}. In the following experiments, we found the SNAIL1 can be regulated by miR-30a-5p in HCC. The luciferase reporter gene tests also showed that miR-30a-5p could bind to SNAIL1. Taken together, loc339803 could positively regulate the expression of SNAIL1 via targeting miR-30a-5p in HCC cells.

Conclusion

In summary, loc339803 is highly expressed in HCC and has a positive correlation with the poor prognosis of patients. Loc339830 promotes the invasion and migration by sponging on miR-30a-5p. Our study provides news lights into the molecular mechanism of loc339803 in promoting HCC metastasis. Loc339803 may be a potential target for the treatment of liver cancer.

Declarations

Ethics approval and consent to participate

This study was approved by the Research Ethics Committee of Nanjing Drum Tower Hospital. This experiment was carried out in accordance with the World Medical Association Declaration of Helsinki. All

patients provided written informed consent.

Authors' contributions

XQ and CZ designed this study, CX wrote the manuscript. CX, DX and PG conducted all experiments. All authors analyzed experimental data and performed their contributions in this study. All the authors have read and approved the final manuscript.

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Availability of data and materials

All the datasets used during this research are included in this published article.

Conflicts of interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

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Not applicable.

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Tables

Table 1

characteristics	Loc339803 level		P value
	High expression	Low expression	
Case	43	42	
Age(years)			0.258
<60	25	30	
≥60	18	12	
Gender			
Man	31	29	0.815
Female	12	13	
Cirrhosis			
Yes	22	24	0.665
No	21	18	
Size			
<5	20	18	0.828
≥5	23	24	
TNM			
I-II	20	30	0.028*
III-IV	23	12	
AFP(ng/ml)			
<200	18	29	0.016*
≥200	25	13	

According to the mean value of loc339803 in the tumor tissues of 85HCC patients, we divide all the patients into two group: loc339803 high expression group (n = 43) and loc339803 low expression group (n = 42). *P < 0.05.

Table 2

Gene name	Primer sequences (5' to 3')	
hsa-miR-30a-5p	sense	ggggTGTAACATCCTCGACT
	anti-sense	CAGTGCGTGTCGTGGAGT
hsa-miR-30b-5p	sense	ggggTGTAACATCCTACACT
	anti-sense	CAGTGCGTGTCGTGGAGT
hsa-miR-30c-5p	Sense	ggggTGTAACATCCTACACTC
	anti-sense	CAGTGCGTGTCGTGGAGT
hsa-miR-30d-5p	sense	gggTGTAACATCCCCGACT
	anti-sense	CAGTGCGTGTCGTGGAGT
hsa-miR-30e-5p	sense	ggggTGTAACATCCTTGACT
	anti-sense	CAGTGCGTGTCGTGGAGT
LOC339803	sense	AGTGATTATGACCCGTGA
	anti-sense	TGAGTTGCTCCATCTTTC
RT- primer for miRNA		GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGAC
GAPDH	sense	CAGGAGGCATTGCTGATGAT GAAGGCTGGGGCTCATTT
	anti-sense	
U2	sense	ATACGTCCTCTATCCGAGGACA
	anti-sense	TGGAGGTACTGCAATACCAGGT
HPRT	sense	AGGCCATCACATTGTAGCCC
	anti-sense	CAACACTTCGTGGGGTCCTT

Figures

Figure 1

loc339803 is upregulated in HCC and have a significant correlation with the outcome of the patients. a. The expression of loc339803 in 85 pairs of HCC and matched precancerous tissues was measured by qRT-PCR. (P = 0.0002, N=85). b. TCGA dataset in starbase 3.0 indicated the loc339803 is high expressed in HCC compared to normal tissue. (P < 0.0001, N=86). c. Survival analysis of TCGA data from starbase 3.0 releval that the higher loc339803 expression indicated a poorer survival outcome. The median expression level of loc339803 was used as the cut-off. P = 0.0025. d.e. The coding ability of loc339803 was evaluated in CPAT and CPC2. f. loc339803 was widely overexpression in HCC cell lines.

Figure 2

loc339803 promote migration and invasion of HCC cell both in vitro. a. The interference effect of siRNA against loc339803 b. the loc339803 expression can be overexpressed by Lv-loc339803. c. Transwell analysis indicated knockdown loc339803 can inhibit the invasion and migration of HCC cells in hep3B and LM3. d.e. the migration and invasion ability of HCC cell can be enhanced by overexpressed loc339803. f. Western blot show loc339803 can promoted the expression of N-cadherin, Vimentin, MMP-2 and snail1.

Figure 3

loc339803 can promote the invasion and migration in vivo a.c.Sh-loc339803 or sh-nc hep3B cell were injected into nude mice through tail vein(N=5). the luciferase signal intensities of the group were examined at 8 weeks. (p=0.002) b.d. the Representative HE and number of lung metastatic tumor(p=0.003).

Figure 4

loc339803 functions as a molecular sponge of miR-30a-5p in HCC cells. a. Fish-experiment and Nuclear & cytoplasmic RNA isolation assay(b) exhibited that the loc339803 is mainly located in cytoplasm. c. bioinformatics predicts that miR-30a-5p can bind to the 3'UTR of loc339803. d. the overexpression of loc339803 can reduce the expression of miR30a-5p, while knockdown loc399803 can promote the expression of miR-30a-5p. e. Luciferase reporter gene showed that miR-30a-5p reduced luciferase activity of the wild type loc339803. f. Luciferase reporter gene showed that miR-30a-5p can not reduced luciferase activity of the mutation type loc339803

Figure 5

Snail1 is a target gene of miR-30a-5p. a. the bioinformatics prediction that miR-30a-5p can target the 3UTR region of snail1. b. luciferase reporter gene showed that miR-30a-5p could significantly reduce the luciferase activity of wild type snail1. c. western blot shows the expression of snail1 can be regulated by miR-30a-5p.

Figure 6

miR-30a-5p can block loc339803-induced tumor invasion and migration. a. loc339803 can promote the migration and invasion of HCC cell lines Hep3B and LM3, and overexpression of miR30a-5p can block the enhanced invasion and migration ability of cells induced by loc339803.(b.d). c.miR-30a-5p was able to restore the over-expression of snail1 which enhanced by loc339803.

Supplementary Files

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- [Tables1.xlsx](#)
- [S2.tif](#)
- [S1.tif](#)