A Novel Long Non-coding RNA, ENSG00000236199, Inhibits Proliferation and Metastasis through NRIP1 by Sponging miR-576-5p in Hepatocellular Carcinoma

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Abstract

Background: Hepatocellular carcinoma (HCC) is a common malignancy of the digestive system. A novel long non-coding RNA, ENSG00000236199 (lncRNA-199) , whose role in tumors has not been reported, especially in HCC.

Methods: The expression of lncRNA-199 and miR-576-5p were detected by Real-time quantitative polymerase chain reaction (RT-qPCR), NRIP1 was measured by Western blotting. HCC cell proliferation and metastasis of HCC were examined using functional tests. Luciferase reporter assay was performed to confirm the relationship between lncRNA-199 and miR-576-5p, between miR-576-5p and NRIP1.

Results: LncRNA-199 was frequently down-regulated in HCC and this down-regulation was negatively correlated with prognosis. Overexpression of lncRNA-199 could inhibit the growth and metastasis of HCC, while knockdown lncRNA-199 had the opposite effect. Down-regulated lncRNA-199 also could induce epithelial-mesenchymal transformation (EMT). Luciferase reporter assay demonstrated lncRNA-199 could modulate NRIP1 by sponge miR-576-5p.

Conclusion: LncRNA-199 inhibited growth and metastasis through miR-576-5p/NRIP1 axis in HCC. This study revealed a novel unknown lncRNA function in malignant tumors, especially in HCC. At the same time, the regulatory mechanism of lncRNA-199 in HCC was clarified.

Background

Hepatocellular carcinoma (HCC) is a common malignant tumor of the digestive system, which has the characteristics of insidious onset and insensitivity to radiotherapy and chemotherapy (Goh et al. 2015). HCC accounts for the majority of primary liver cancer, ranks as the sixth most common cancer, and was the fourth leading cause of cancer death worldwide in 2018 (Bray et al. 2018). The main pathogenic factors of HCC are viral hepatitis infection (HBV/HCV), ingestion of aflatoxin, diabetes, tobacco intake, and heavy alcohol intake (Singal et al. 2015). There are many treatments for HCC and surgical treatment is the most effective, but the cure rate and survival time of patients have not been improved effectively (Khemlina et al. 2017). Therefore, we need to further study the pathogenesis of HCC, which is one of the most urgent tasks at present.

Long non-coding RNAs (lncRNAs) are a kind of RNA whose transcript length over 200nt and has no translation function (Li et al. 2014). Current studies have demonstrated that lncRNAs play an important role in the maintenance of life, such as gene expression (Khorkova et al. 2015; Wu et al. 2014; Song et al. 2014), and a large number of studies have demonstrated that lncRNAs can regulate tumor cell signaling at transcriptional, post-transcriptional, and apparent level (Xie et al. 2018). All of those make it particularly important to study the role of lncRNAs in HCC.

MicroRNAs (miRNAs) are a type of RNA with a transcriptional length of 18-24nt (Kim et al. 2019). In the past, miRNAs were also considered as non-functional RNA, but more and more studies have shown that

miRNAs are involved in various biological functions of cells (Tarallo et al. 2019; Xu et al. 2020; Vignard et al. 2020). Many studies have shown that miRNA-576-5p plays an important role in a variety of cancers, such as esophageal cancer (Zhang et al. 2019), colorectal cancer (Makondi et al. 2019) and melanoma (Kordaß et al. 2018).

Nuclear receptor-interacting protein 1 (NRIP1) expressed in multiple cancer types and served as a critical role in tumorigenesis, treatment response, and prognosis (Docquier et al. 2010; Rosell et al. 2014). One study has shown that NRIP1 is the target gene of miRNA-576-5p (Ni et al. 2018), and NRIP1 is low expressed in HCC (Zhang et al. 2015). However, the association between miR-576-5p and NRIP1 in HCC remains unknown.

According to previous results from microarray (Yang et al. 2016), we screened out a new lncRNA. Ensembl version is ENSG00000236199, which was a long non-coding RNA. The information about this lncRNA was accessible at Ensembl website (www.ensembl.org). There has not been any report for this new lncRNA in current study, especially in HCC. Therefore, in this paper, we will explore the role of this novel lncRNA in HCC. We did not name this novel lncRNA, for the convenience of description we refer to it as lncRNA-199 temporarily.

Methods

HCC tissue specimens

HCC tissue samples were taken from patients who had undergone liver resection and signed informed consent at the First Affiliated Hospital of Harbin Medical University between 2010 and 2014. Only paired HCC tissues and adjacent normal tissues confirmed to be HCC pathologically were included in this study. The Research Ethics Committee has already approved this study. Detailed pathological features of the patients were summarized in Table 1.

Table 1
Relationship between lncRNA-199 expression and clinicopathological parameters of HCC patients (n = 80)

Features	lncRNA-199 expression		*P* Value
	Low(n = 52)	High (n = 28)	
Age			0.5548
≤ 60	24	11	
> 60	28	17	
Gender			0.7934
Male	30	17	
Female	22	11	
AFP (μg/L)			0.2945
≤ 20	21	8	
> 20	31	20	
HBV infection			0.6551
Yes	27	16	
No	25	12	
Tumor diameter (cm)			**0.0244**
≤ 5	32	24	
> 5	20	4	
metastasis			**0.0444**
Yes	16	3	
No	36	25	
TNM stage			**0.0293**
I-II	28	22	
III-IV	24	6	

HCC cells and transfection

The normal human liver cell Chang and human HCC cell lines BEL-7402, Huh7, HepG2, HCCLM3, and SK-Hep-1 were purchased from the Chinese Academy of Science (Shanghai, China). All cell lines were

cultured in Dulbecco's Modified Eagle Medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 100U/ml penicillin and 100μg/ml streptomycin. Cells stably transfected with lncRNA-199 plasmid and NRIP1 plasmid (GeneChem, Shanghai, China) using Lipo-2000 (Invitrogen, USA). Following transfection (4h), cells were cultured in complete medium and un-transfected cells were screened with G418 for 7-14 days. Si-lncRNA-199 and Si-NRIP1 (GenePharma, Shanghai, China) were used to silence the expression of lncRNA-199 and NRIP1. The sequences of siRNA were showed as following:

Si-lncRNA-199: sense, 5’-AGAACAAACUGCUUAGGAATT-3’

antisense, 5’-UUCCUAAGCAGUUUGUUCUTT-3’.

Si-NRIP1: sense, 5’-GAGGAUCAGAACUUUAACATT-3’

antisense, 5’-UGUUAAAGUUCUGAUCCUCTT-3’.

MiR-576-5p mimics (miR-576-5p) and its matched control (miR-NC), miR-576-5p inhibitor (anti-miR-576-5p) and its matched control (anti-miR-NC) were obtained from GenePharma (Shanghai, China).

Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated from tissues and cells with RNA Extraction Kit (Thermo, USA), and cDNA was synthesized using a High Capacity RT Kit (Applied Biosystems, Carlsbad, USA). RT-qPCR was performed using SYBR Green (Roche, Indianapolis, USA) on a 7500 Fast PCR System. The primer sequences were shown as following:

lncRNA-199: forward, 5’- TGCCATGAAGGAGCCAGTA-3’;

reverse, 5’-ATGTGCCATTCTCAGTGATAAGTC-3’.

GAPDH: forward, 5’-GCACCGTCAAGGCTGAGAAC-3’;

reverse, 5’-TGGTGAAGACGCCAGTGGA-3’.

MiR-576-5p: forward, 5’-TTGGGTCAAGAGTCAGAAGTTT-3’;

reverse, 5’- TGGCTTCTACTTGTCCTTTCC-3’.

U6: forward, 5’-CTCGCTTCGGCAGCACA-3’.

reverse, 5’-AACGCTTCACGAATTTGCGT-3’.

NRIP1: forward, 5’-GAGCACTCCACCTTTACTTACAT-3’;

reverse, 5’ -CAATCATACCTATCGGTTTATCTG-3’.

Western blotting analysis

Western blot was performed as previously described (Cui et al. 2019). GAPDH antibody was purchased from ZSGB-BIO (Beijing, China). Ki-67, E-cadherin, N-cadherin and Vimentin antibodies were purchased from Abcam (Cambridge, USA).

Cell Counting Kit-8 (CCK-8) experiments

Stably transfected cells were inoculated in 96-well plates and cultured overnight for attachment. The following day, the culture solution was replaced with a solution containing the CCK-8 reagent and cultured for 2h in the dark. Then, the absorbance at 450nm was measured for each well.

Colony formation experiments

Stably transfected cells in the logarithmic growth phase were seeded in 6-well plates for two weeks. The colonies were then fixed and stained for easy observation, and photographs were taken.

Wound-healing assays

Stably transfected cells were inoculated in 6-well plates and cultured until cell fusion occurred. A straight cut was made at the bottom of the plate with a 10-μl pipette tip. The floating cells were washed away and the wound closure was photographed at 0 and 24 h.

Transwell migration and invasion assay

Matrigel-coated (BD Biosciences, Franklin Lakes, NJ) or non-Matrigel-coated Transwells were used to examine the invasion and migration ability of cells. Stably transfected cells were inoculated in the upper chamber of the transwells and serum-free media was added. Normal media was injected into the plate wells. After a 24 h incubation period, the cells in the upper layer filter were removed and the cells in the bottom layer were fixed, stained and counted.

Luciferase reporter assay

Cells were inoculated in 24-well plates, and the wild-type or mutated 3'-UTR sequence of lncRNA-199 and NRIP1 were co-transfected with pRL-TK Renilla, respectively. After incubation for 48h, luciferase activities were measured by the dual luciferase reporter assay kit.

Animal model

Male BALB/c nude mice (4–6 weeks old) were purchased from the Charles river (Beijing, China). Subcutaneous xenograft tumors were established as follows: 5×10^6 cells were dissolved in 0.15 ml phosphate buffered saline, then subcutaneously injected into the flanks of the mice. The size of the hypodermic ectopic neoplasms was observed weekly. After 6 weeks the nude mice were sacrificed and the xenograft tumor was excised. The volume of the tumor was measured by the following calculation: V=W×L×H/2.

The excised subcutaneous xenograft tumor was divided into 1 mm^3 cubes and transplanted into the livers (left lobes) of mice from the same line to establish the orthotopic xenograft nude mice model. The mice were sacrificed after 6 weeks, after which the tumors were resected.

The pulmonary metastases nude mice model was established as follows: 4×10^6 cells dissolved in 0.15 mL phosphate buffered saline were injected into the tail veins of each mouse. After 6 weeks, the mice were sacrificed and their lungs were collected. Animal experiments were approved by the Animal Ethics Committee of Harbin Medical University, and each step was carried out in accordance with animal care and use standards.

Results

Low expression of lncRNA-199 is undesirable in HCC

To verify the microarray results, lncRNA-199 expression was reduced in HCC, RT-qPCR were performed. The results showed that the expression level of lncRNA-199 in HCC tissues was significantly lower than that in adjacent normal tissues with an average fold change of 2.07 ($p = 0.0042$; Fig. 1a). Based on the mean of fold changes, we divided the patients into high expression group and low expression group, and evaluated the relationship between lncRNA-199 and clinicopathological parameters. Notably, decreased lncRNA-199 expression in HCC was positively correlated to tumor diameter ($p = 0.0244$), metastasis ($p = 0.0444$) and TNM stage ($p = 0.0293$; Table 1). However, all other pathological parameters, such as age, gender, AFP and HBV infection, were not associated with lncRNA-199. In addition, Kaplan–Meier survival analysis showed that patients with low lncRNA-199 expression had poorer overall survival than patients with high lncRNA-199 expression (Fig. 1b). In the available database sites, our survival analysis results are supported (http://gepia.cancer-pku.cn/; Fig. 1c). Univariate and multivariate Cox regression analysis indicated that low level of lncRNA-199, metastasis and III-IV TNM stages were independent risk factors for prognosis of HCC patients (Table 2).

Table 2
Univariate and multivariate Cox regression analysis for overall survival in patients with HCC

	Univariate			Multivariate		
	HR	95% CI	*p* value	HR	95% CI	*p* value
Age ≥/<50	0.922	0.577–1.472	0.733			
Gender M/F	1.184	0.739–1.898	0.482			
AFP ≥/<20	1.482	0.905–2.429	0.118			
HBV +/−	1.310	0.820–2.094	0.258			
Tumor diameter ≥/<5	3.125	1.771–5.517	0.000	0.750	0.281–2.005	0.567
metastasis +/−	5.859	3.021–11.362	0.000	2.389	1.017–5.507	0.046
TNM stage I-II/III-IV	4.429	2.460–7.975	0.000	3.479	1.021–11.858	0.046
LncRNA-199	2.144	1.290–3.564	0.003	1.750	1.030–2.947	0.038

Furthermore, RT-qPCR was performed to verify the expression of lncRNA-199 in the HCC cell lines, and it was also found that the expression of lncRNA-199 in the HCC cell lines were lower than that in normal liver cell, and the expression of lncRNA-199 gradually decreased with the increase of cell malignancy (Huh7, $p = 0.0145$; HepG2, $p = 0.0088$; BEL-7402, $p = 0.0041$; SK-Hep-1, $p = 0.0027$; HCCLM3, $p = 0.0013$; Fig. 1d). Huh7 cells with relatively high expression and HCCLM3 cells with relatively low expression were selected for further study. Huh7 and HCCLM3 cells were silenced and overexpressed by siRNA and plasmid transfection, respectively. Transfection efficiency is shown in Fig. 1E and Fig. 1F. The above results indicated that the abnormally low expression of lncRNA-199 is a key factor affecting the occurrence and development of HCC, and can affect the overall survival of patients, while its role in HCC is still unknown.

LncRNA-199 can inhibit the HCC growth

Low expression of lncRNA-199 is associated with HCC tumor size, so we investigated the effect of lncRNA-199 on the proliferation ability of HCC cells. CCK-8 assays showed that HCCLM3 cell growth was restrained in lncRNA-199 overexpression group compared with control group, and Huh7 cell growth was enhanced in lncRNA-199 silencing group compared with corresponding control group (Fig. 2A). Similar results were obtained in colony formation assays, that is, overexpression of lncRNA-199 in HCCLM3 cell could reduce the number and size of clones, while silencing expression of lncRNA-199 in Huh7 cell could form more and larger clones (Fig. 2B). To further prove that lncRNA-199 inhibits the growth of HCC *in vivo*, we constructed subcutaneous and orthotopic xenograft models. Smaller subcutaneous and orthotopic xenograft tumors were observed in HCCLM3-lncRNA-199 group when compared to control group (Fig. 2C and D). Subcutaneous tumors were sectioned and IHC analysis showed that lower levels of Ki-67 were observed in HCCLM3-lncRNA-199 group when compared to control group (Fig. 2E). In short,

our results demonstrated that lncRNA-199 can inhibit the growth of HCC both *in vitro* and *in vivo*, and that overexpression of lncRNA-199 can alleviate the activity and proliferation of HCC.

Down-regulated lncRNA-199 promoted HCC metastasis and induced EMT

We then investigated the role of lncRNA-199 in metastasis of HCC. Wound-healing assays showed that overexpression of lncRNA-199 inhibited migration in HCCLM3 cell lines, which slowed the area of scratch growth. The silent expression of lncRNA-199 had opposite results in Huh7 cells (Fig. 2F). Consist with these data, transwell invasion and migration assays showed that the invasive and migratory abilities of HCC cells were decreased and increased by lncRNA-199 overexpression and silencing (Fig. 2G). In the pulmonary metastases nude mice model, HCCLM3 cells could form less and smaller lung metastatic foci after overexpression of lncRNA-199 (Fig. 2H).

Numerous studies have demonstrated that EMT plays a crucial role in the invasion and migration of tumor cells, a process that loosens tight structures between cells and makes them prone to metastasis (Barriga et al. 2019). Therefore, we hypothesized that lncRNA-199 inhibits HCC metastasis by regulating EMT. We evaluated the EMT markers by western blotting. When lncRNA-199 was overexpressed, the expression of E-cadherin was increased, while the expression of N-cadherin and vimentin were decreased (Fig. 2I). On the contrary, silent expression of lncRNA would have opposite results in Huh7 cells. Similar results can be obtained in RT-qPCR, that is, the corresponding mRNA content also changes with the change of lncRNA-199 (Fig. 2J). The above results indicated that down-regulated lncRNA-199 can promote the metastasis of HCC both *in vivo* and *in vitro*, and can induce the EMT process of HCC.

LncRNA-199 regulates HCC through miR-576-5p/NRIP1 axis

LncRNA-199 can regulate the growth and metastasis of HCC cells, but the underlying mechanism remains unclear. The mechanism of action of lncRNAs is so complex that it is not fully understood (Nair et al. 2020). The most classic mechanism is the sponge mechanism. Multiple miRNAs that can interact with lncRNA-199 were queried through open database analysis, including DIANA tools (http://carolina.imis.athena-innovation.gr/) and starbase (http://starbase.sysu.edu.cn/). Based on the screening indicators such as score, combined stability and high expression in HCC, we finally focused on miR-576-5p. Then we detected the expression of miR-576-5p in HCC tissues and cell lines by RT-qPCR. The results showed that the expression level of miR-576-5p was higher in HCC tissues than that in adjacent normal tissues (Fig. 3A), and similarly, the expression level of miR-576-5p was higher in HCC cell lines than in normal liver cells (Fig. 3B). Luciferase activity reports indicated that miR-576-5p can attenuate the activities of wild-type, but has no effect on the mutant-type in HCCLM3 cells (Fig. 3C). Moreover, overexpression of lncRNA-199 can reduce the expression of miR-576-5p, while silenced

expression of lncRNA-199 can increase the expression of miR-576-5p (Fig. 3D). In order to further clarify the regulatory mechanism of lncRNA-199 in HCC, we analyzed the downstream target genes of miR-576-5p through the available database, TargetScan (http://www.targetscan.org/vert_72/), miRDB (http://mirdb.org/) and miTarBase (http://mirtarbase.mbc.nctu.edu.tw/php/index.php). We also selected based on score and combined stability, finally, we focused on NRIP1 because previous studies have shown that miR-576-5p can target NRIP1 in esophageal squamous cell carcinoma (Ni et al. 2018). More importantly, the results from the TCGA database indicated that NRIP1 was low-expressed in HCC (Fig.3E). The binding sites of miR-576-5p within NRIP1 were shown in Fig. 3F. NRIP1 could be regulated by miR-576-5p mimic and inhibitor (Fig.3G and 3H). Pearson correlation analysis showed that there is a negative correlation between lncRNA-199 and miR-576-5p, and miR-576-5p was also negatively correlated with NRIP1, lncRNA-199 was positively correlated with NRIP1 (Fig. 3I-K). The above results indicated that lncRNA-199 can regulate the expression of NRIP1 through sponge miR-576-5p.

NRIP1 is crucial in the regulation of HCC by lncRNA-199

Since we have demonstrated that lncRNA-199 can regulate NRIP1 through sponge miR-576-5p, the detailed mechanism by which lncRNA-199 regulates HCC remains unclear. Huh7 cells co-transfected with Si-lncRNA-199 and NRIP1, and HCCLM3 cells co-transfected with lncRNA-199 and Si-NRIP1 were constructed. We used western blotting to detect the protein expression of NRIP1 (Fig. 4A). The CCK8 assays showed that the cell activity originally enhanced by silencing lncRNA-199 could be alleviated by overexpression of NRIP1 in Huh7 cells. On the contrary, silencing NRIP1 can partially block the role of overexpressed lncRNA-199 in HCCLM3 cell lines (Fig. 4B). Similarly, the colony formation assays produced similar results to the CCK8 assays (Fig. 4C). Meanwhile, wound-healing assays also showed that overexpression of NRIP1 in Huh7 cell lines could slow down the healing rate of Huh7 cells transfected with Si-lncRNA-199. In HCCLM3 cell lines, silent expression of NRIP1 could once again accelerate the healing rate slowed down by overexpression of lncRNA-199 (Fig. 4D). Similar results were also obtained in transwell migration and invasion assays, namely, silenced or overexpressed NRIP1, which could block the effects of overexpressed or silenced lncRNA-199 in HCCLM3 and Huh7 cell lines, respectively (Fig. 4E). On the other hand, NRIP1 can also reverse the EMT process induced by lncRNA-199. The expression of NRIP1 and E-cadherin were decreased after lncRNA-199 silencing, while the expression of N-cadherin and Vimentin were increased. NRIP1 overexpression can partially restore this effect in Huh7 cell line (Fig. 4A). Overexpression of lncRNA-199 in HCCLM3 cells had the opposite effect and these effects could be reversed by silent expression of NRIP1. These outcomes illustrate that NRIP1 is an important hub in the molecular mechanism of the regulation of lncRNA-199 on HCC.

Discussion

The incidence and mortality of HCC are critical to human health. Although great progress has been made in the diagnosis and treatment for HCC, these achievements were far from improving the survival time and cure rate of HCC patients. More and more studies had shown that lncRNAs play an important role in

various biological processes of human body (Wang et al. 2020; Qu et al. 2020; Lu et al. 2020). In order to understand the development, diagnosis and treatment of the disease we need to explore the regulation mode of human gene deeply and immediately. In this study, we screened the microarray and focused on this new lncRNA-199. There was no any report for this lncRNA in available data. We are the first one to report the function of lncRNA-199, especially in HCC.

In our study, we first confirmed that the expression of lncRNA-199 in HCC tissues was lower than that in adjacent normal tissues by RT-qPCR. Then, chi-square test indicated that lncRNA-199 was closely related to tumor diameter ($p = 0.0244$), metastasis ($p = 0.0444$) and TNM stage ($p = 0.0293$). Univariate and multivariate Cox regression analysis indicated that low expression of lncrRNA-199 was an independent risk factor for evaluating the prognosis of patients. Kaplan–Meier survival analysis also showed that the survival curve of patients with low lncRNA-199 expression was lower than that of patients with high lncRNA-199 expression. These results proved that the expression of lncRNA-199 was different in HCC tissues and adjacent normal tissues, and this difference was the key to the carcinogenesis of HCC.

Based on these results, we boldly hypothesized that down-regulated lncRNA-199 could induce the growth and metastasis of HCC. And it is necessary to further explore its biological functions in these aspects. Just as our expectations, the cell activity and clonogenesis were promoted by the down-regulated lncRNA-199 and inhibited by the up-regulated lncRNA-199 in HCCLM3 and Huh7 cells. In addition, overexpression of lncRNA-199 can moderate the size and weight of HCCLM3 cell subcutaneous and orthotopic xenograft tumors formation. Moreover, we also found that the expression of Ki-67 was affected by lncRNA-199 through IHC analysis. Ki-67 is a proliferative cell associated antigen, whose function is closely related to mitosis and is indispensable in cell proliferation (Li et al. 2014; Andre et al. 2015). Tumor cells have vigorous proliferative activity, and Ki-67 can be used as an indicator to evaluate the proliferative state of cells. Therefore, researchers conducted studies on Ki-67 in many tumors, involving the correlation of Ki-67 with tumor development, grading, staging, radiotherapy sensitivity, prognosis, cause of death, tumor markers and other aspects (Hammarsten et al. 2019; Zurrida et al. 2013; Adisa et al. 2015).

Moreover, we also examined the effects of overexpressed and silenced lncRNA-199 expression on the migration and invasion of HCC cells by transwell invasion and migration assays and wound-healing assays. The results showed that the healing ability of HCCLM3 cells was significantly impaired with overexpression of lncRNA-199, and the number of cells that passed through the membrane was also reduced. In contrast, after silencing lncRNA-199 expression, Huh7 cells exhibited improved healing ability and movement across the membrane. In addition, lncRNA-199 can also induce the EMT process of HCC. EMT is an important mechanism of tumor cell metastasis, when the cell EMT process occurs, the tight junctions between epithelial cells begin to become loose and the cell polarity disappears (Yang et al. 2008). As a result, epithelial cells become mesenchymal and thus more susceptible to migration and invasion (Thiery et al. 2006).

In order to further understand the mechanism of lncRNA-199 in HCC, we conducted predictive analysis through the open database and found that there were potential binding sites between miR-576-5p and

lncRNA-199. The luciferase reports confirmed the results. Therefore our next research focus is on miR-576-5p. Fortunately, the results showed that the downstream target genes of miR-576-5p, NRIP1, which was co-expressed genes with lncRNA-199. A series of subsequent remedial experiments confirmed the link between lncRNA-199 and miR-576-5p and the relationship between miR-576-5p and NRIP1. The results showed that the proliferation, migration and invasion abilities of the Huh7 cells that had been enhanced by silencing lncRNA-199 were restored by overexpression of NRIP1, and the results were consistent in HCCLM3 cells. NRIP1 was first discovered in cancer cells in 1995 (Cavaillès et al. 1995), but it has not been studied in detail in HCC. For the first time, we have discovered the mechanism by which the lncRNA-199/miR-576-5p/NRIP1 axis regulates HCC.

To sum up, our research is the first to confirm that this new lncRNA-199 is a functional non-coding RNA and to explore its role and mechanism in HCC. However, there are still many deficiencies in this study, and the potential mechanism has not been further explored. It is well known that many signaling pathways, such as MAPK, PI3K/AKT and IL6/STAT3 (Burotto et al. 2014; Fang et al. 2012; Park et al. 2010), play a significant role in the occurrence and development of HCC. The exact signaling pathway involved in the process of lncRNA-199/miR-576-5p/NRIP1-induced hepatocellular carcinoma should be further studied.

Conclusions

In conclusion, our outcomes indicate that lncRNA-199 is down-regulated in HCC and HCC cells. Low expression of lncRNA-199 seriously threatens the prognosis of HCC patients. In addition, lncRNa-199 regulates HCC mainly through the miR-576-5P/NRIP1 axis. Our study is the first to elucidate the role of lncRNA-199 in cancer, especially in HCC, and we also provide a new evidence for the regulatory network of HCC, which is expected to be a new target for the treatment of HCC in future in-depth studies.

Abbreviations

HCC: Hepatocellular carcinoma; RT-qPCR: real-time quantitative polymerase chain reaction; EMT: epithelial-mesenchymal transition; NRIP1: nuclear receptor interacting protein 1

Declarations

Ethics approval and consent to participate

The use of patient tissues and the use of animals are approved by ethics committees

Consent for publication

All listed authors agree to publish it.

Availability of data and materials

All data related to this study are included in this paper and its supplementary information files.

Competing interests

The authors declare that there are no competing interests.

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Authors' contributions

Zhaoyang Lu and Linqiang Li designed this research. Yifeng Cui, Han Lin, Yunzheng Zhao and Jiaming Liu contributed equally to this work. Yifeng Cui drafted this manuscript and participated in all the experiments. Han Lin helped write and participated in most of the experiments. Yunzheng Zhao was responsible for western blotting and immunohistochemistry. Jiaming Liu was responsible for data collation and calculation. Chengpeng Zhang and Fukai Wen assisted with animal experiments. Xin Wang and Yu Zhang assisted with cell experiments and assays. All the authors have confirmed the final manuscript.

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Figures

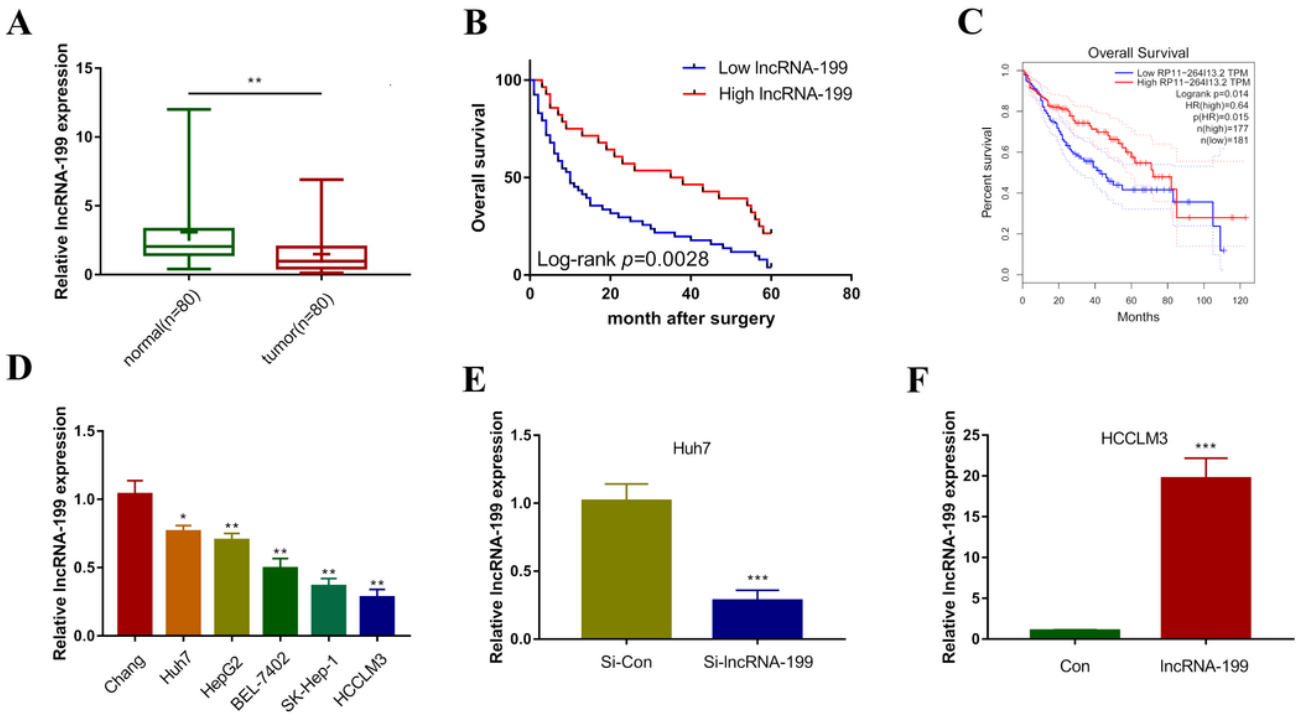

Figure 1

LncRNA-199 is reduced in human HCC and predicted poor prognosis. (a) The expression levels of lncRNA-199 were analyzed in 80 HCC tissue samples and compared them with adjacent normal samples. (b) Kaplan-meier analysis of the effect of lncRNA-199 on the overall survival (OS) of HCC patients. (c) Survival analysis of lncRNA-199 in the GEPIA database. (d) The expression levels of lncRNA-199 in Chang and 5 HCC cells were analyzed by RT-qPCR. (e and f) LncRNA-199 expression levels were detected by RT-qPCR after silencing and overexpression. Data are means ± SD of three independent experiments. *p < 0.05, **p < 0.01, ***P < 0.001.

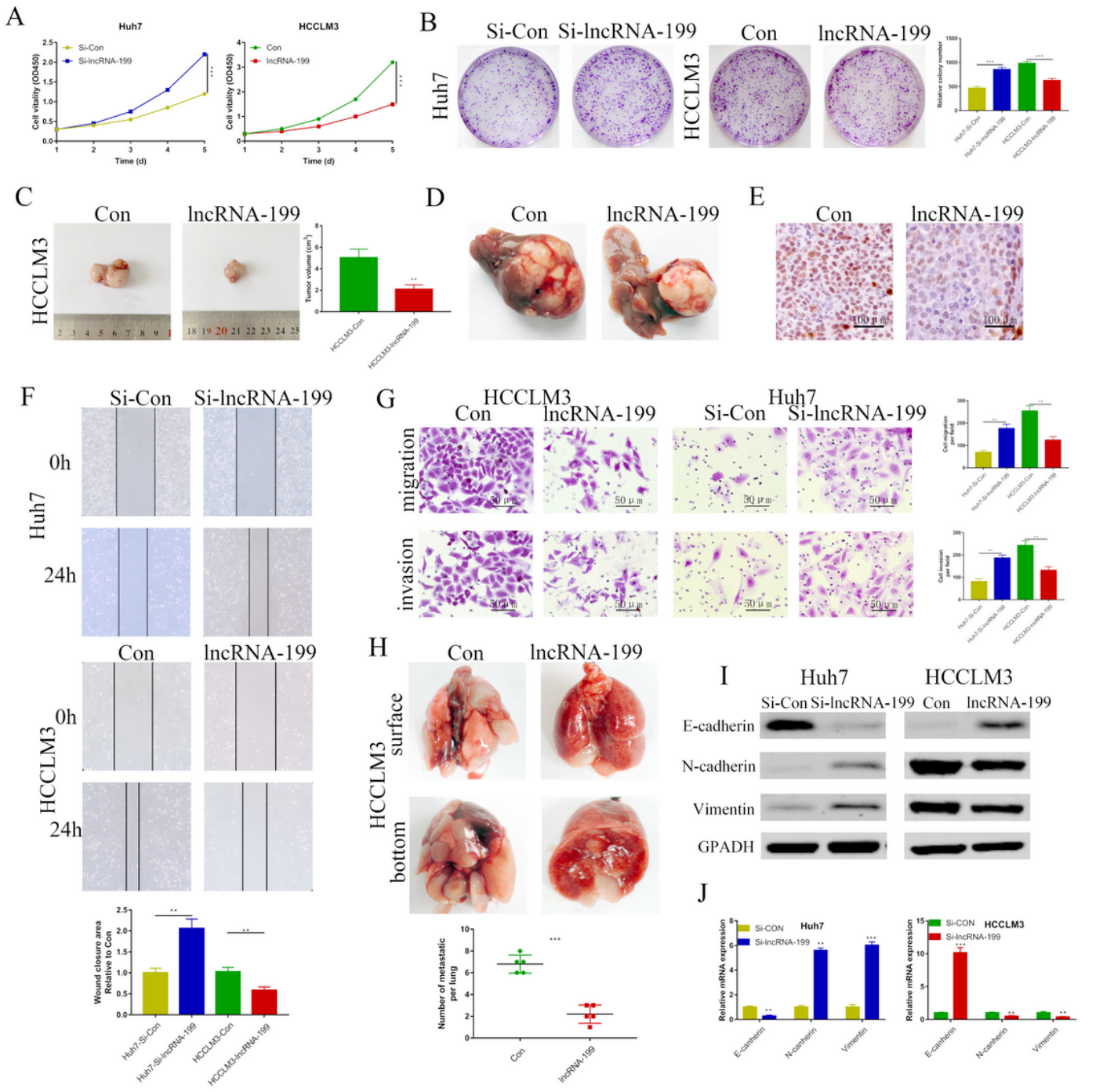

Figure 2

LncRNA-199 suppresses HCC cell proliferation and metastasis in vitro and in vivo. (a) Proliferation rate was analyzed by a CCK-8 assay of indicated HCC cells. (b) Representative images of colony formation assays are shown on the left; the number of foci was counted as shown on the right. (c) Overexpression of lncRNA-199 increased HCCLM3 cell subcutaneous xenograft growth in nude mice. Tumor volume is shown on the right (n = 5/group). (d) Overexpression of lncRNA-199 increased HCCLM3 cell orthotopic xenograft growth in nude mice. (e) Immunohistochemical detection of Ki-67 protein levels in xenograft

tissues, Scale bars: 100× = 100 μm. (f) Representative images of the wound-healing assay. (g) Transwell migration and invasion assays for indicated cell lines are shown on the left, Scale bars: 200× = 50 μm; counts of migrated and invaded HCC cells are shown on the right. (h) Representative photographs of lung tumors from indicated groups are shown on the upper; the number of lung metastatic nodules in the indicated groups are shown on the below. (i and j) Protein levels and mRNA levels of EMT markers were measured by western blotting and RT-qPCR. Data are means ± SD of three independent experiments. *p < 0.05, **p < 0.01, ***P < 0.001.

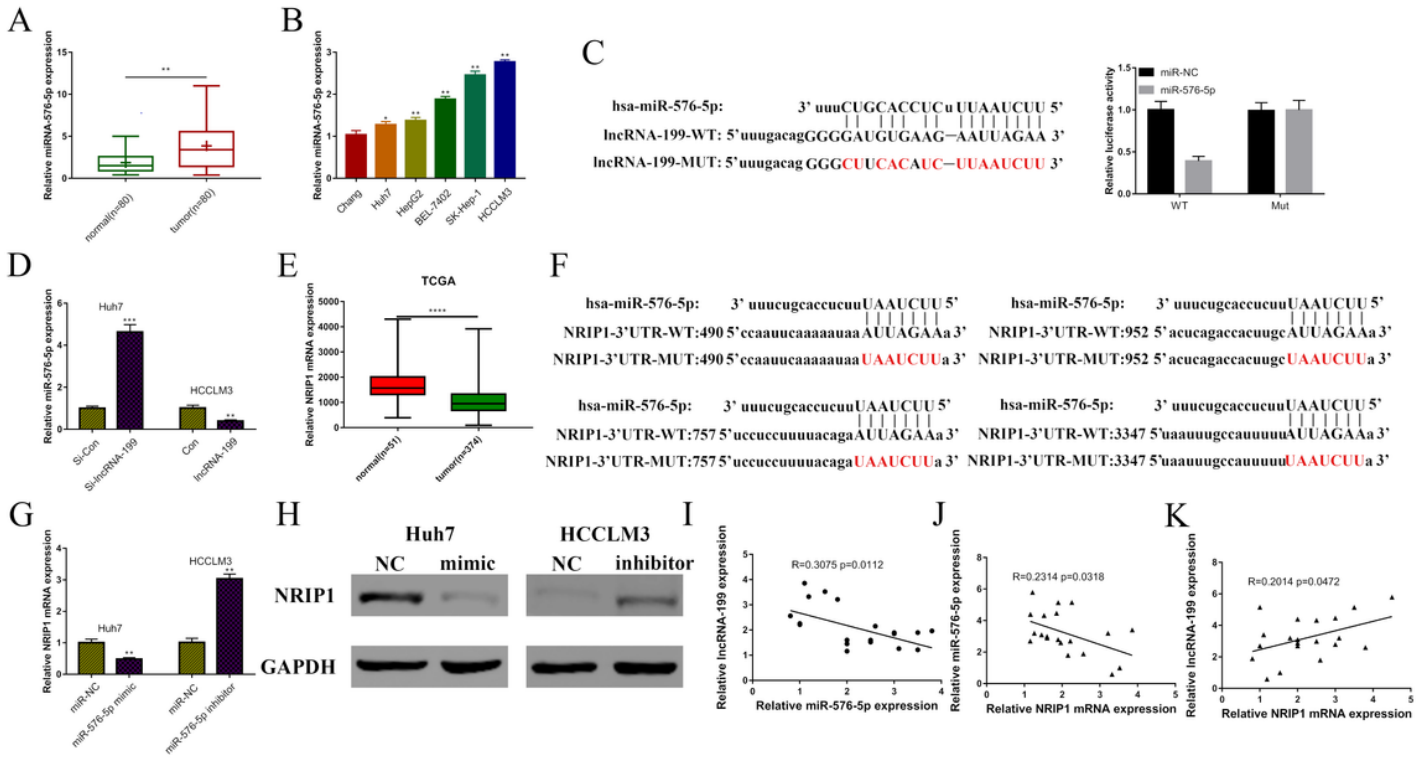

Figure 3

LncRNA-199 regulates NRIP1 through miR-576-5p. (a) The expression levels of miR-576-5p were analyzed in 80 HCC tissue samples and compared them with adjacent normal samples. (b) The expression levels of miR-576-5p in Chang and 5 HCC cells were analyzed by RT-qPCR. (c) Diagrammatic sketch of the binding sites for miR-576-5p in lncRNA-199 as shown on the left. The Luciferase reporter assay showed luciferase activity of HCCLM3 transfected with WT 3'-UTR was inhibited by miR-576-5p overexpression. (d) The expression of miR-576-5p was detected by RT-qPCR after overexpression and silencing of lncRNA-199. (e) Relative NRIP1 expression levels in 374 HCC and 51 normal samples from The Cancer Genome Atlas database. (f) Diagrammatic sketch of the binding sites for miR-576-5p in NRIP1. (g and h) Protein levels and mRNA levels of NRIP1 were measured by western blotting and RT-qPCR. (i) Correlation analysis of lncRNA-199 and miR-576-5p in HCC tissues. (j) Correlation analysis of miR-576-5p and NRIP1 in HCC tissues. (k) Correlation analysis of lncRNA-199 and NRIP1 in HCC tissues. Data are means ± SD of three independent experiments. *p < 0.05, **p < 0.01, ***P < 0.001.

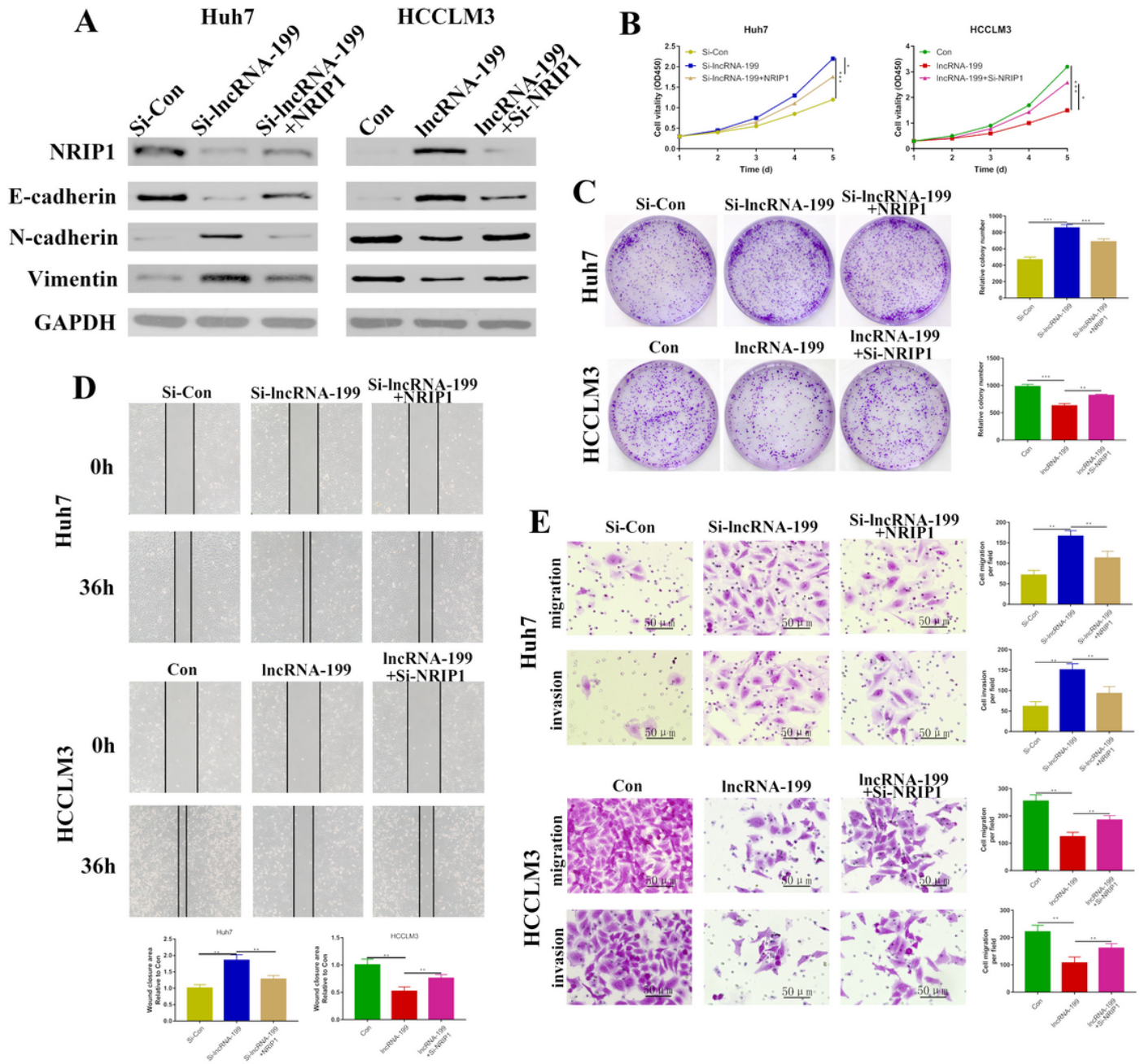

Figure 4

NRIP1 is crucial in the regulation of HCC by lncRNA-199. (a) Western blotting analysis of NRIP1 and EMT markers in indicated cells. (b) Proliferation rate was analyzed by CCK-8 assay of indicated HCC cells. (c) Representative images of colony formation assays are shown on the left; the number of foci counted are shown on the right. (d) Wound-healing assay for indicated cell lines. (e) Transwell migration and invasion assays for indicated cell lines are shown on the left, Scale bars: 200× = 50 μm; counts of migrated and

invaded HCC cells are shown on the right. Data are means ± SD of three independent experiments. *p < 0.05, **p < 0.01, ***P < 0.001.