Cell J
Cell Journal (Yakhteh)
Homepage: https://www.celljournal.org/
Original Article
Vol 25, No 12, December 2023, Pages: 829-838

Deciphering Role of lncRNA 91H in Liver Cancer: Impact on Tumorigenesis

Zhiyuan Mo, Ph.D., Zhuangqiang Wang, Ph.D.*

Department of Hepatobiliary Surgery, Shanxi Bethune Hospital, Shanxi Academy of Medical Sciences, Tongji Shanxi Hospital, Third Hospital of Shanxi Medical University, Taiyuan, China

Abstract

Objective: This study aimed to investigate functional role of long ncRNA (lncRNA) 91H in liver cancer tumorigenesis, focusing on its effect on cell proliferation, apoptosis, cell cycle progression, migration, invasion, epithelial-mesenchymal transition (EMT) and *in vivo* tumor growth.

Materials and Methods: In this experimental study, liver cancer tissues and cell lines were analyzed for lncRNA 91H expression using quantitative reverse transcription polymerase chain reaction (qRT-PCR). By employing si-RNA to silence 91H, we aimed to gain a more in-depth understanding of its specific contributions and effects within these cells. Cell proliferation was assessed through the CCK-8 assay, while apoptosis and cell cycle progression were quantified using Annexin V-FITC staining and flow cytometry, respectively. Migration and invasion capabilities of liver cancer cells were assessed through transwell assay. EMT was assessed by analyzing protein expression levels of EMT-associated markers through western blotting. *In vivo* effect of 91H was assessed through xenograft experiments.

Results: Significantly higher levels of lncRNA 91H were observed in the liver cancer tissues and cell lines, than the normal cells. Silencing 91H in liver cancer cells led to a notable reduction of cell proliferation by inducing apoptosis and arresting the cell cycle. Liver cancer cells with decreased 91H expression exhibited diminished migration and invasion abilities, suggesting a role for 91H in promoting these processes. Furthermore, 91H knockdown weakened EMT in liver cancer cells, indicating its involvement in modulating this critical cellular transition. Furthermore, growth of subcutaneous xenograft tumors and weight was effectively suppressed by sh-lncRNA 91H.

Conclusion: Our study strongly supports lncRNA 91H's role in liver cancer progression by enhancing proliferation, migration, invasion, and EMT. Targeting 91H reduced in vivo tumor growth, highlighting its potential as a therapeutic liver cancer target. These findings suggest 91H's pivotal role in liver cancer aggressiveness, opening doors for future therapeutic approaches.

Keywords: Apoptosis, Epithelial-Mesenchymal Transition, Liver Cancer, lncRNA 91H, Tumorigenesis

Citation: Mo Zh, Wang Zh. Deciphering role of lncRNA 91H in liver cancer: impact on tumorigenesis. Cell J. 2023; 25(12): 829-838. doi: 10.22074/CELLJ.2023.2010456.1395

Introduction

In the recent years, liver cancer has become one of the most overwhelming and highly challenging human health disorders. Neoplastic human malignancies are grouped with the top five human cancers, both in terms of disease prevalence and associated mortality at the global level (1, 2). Liver cancer ranks as the fourth leading global cause of mortality, and the overall incidence of liver cancer has increased over the last two to three decades (3). Liver cancer incidence is anticipated to surge by 55.0%, with a corresponding 56.4% rise in the number of fatalities expected between 2020 and 2040. Moreover, this disease occurs at a comparatively high frequency in East Asian countries (4). However, the incidence of liver cancer has slightly declined in China and Japan (5). Chronic liver infections, such as hepatitis B and C, fatty liver disease, alcohol consumption, and obesity are among some of the commonly reported risk factors for liver cancer (6). While surgery and liver transplantation are the first-line treatment strategies employed for liver cancer, fewer than 20% of patients qualify for therapy (7). In the rest of cases, localized or systematic chemo- or radiotherapeutic approaches showed limited clinical success and often exhibited metastasis because of the heterogeneous nature of cancer cells (8). In addition, advanced stages of liver cancer are associated with a high risk of recurrence (9). Hence, exploration of liver cancer pathogenesis at the molecular level is necessary for better understanding of its progression and allocation of more effective therapeutic targets.

Long non-coding RNAs (lncRNAs) have gained immense attention with development of the human genome project, and they have received considerable research attention. Classified as the RNA transcripts lacking protein coding potential and comprising more

Received: 30/August/2023, Revised: 19/October/2023, Accepted: 03/December/2023

***Corresponding Address: Department of Hepatobiliary Surgery, Shanxi Bethune Hospital, Shanxi Academy of Medical Sciences, Tongji Shanxi Hospital, Third Hospital of Shanxi Medical University, Taiyuan, China**
Email: zhuangqiangwang@126.com

Royan Institute
Cell Journal (Yakhteh)

than 200 nucleotides, lncRNAs have been demonstrated to affect several physiological and pathological aspects of the human body including the growth, progression, and metastasis of cancer (10, 11).

lncRNAs have garnered substantial attention in the recent years, due to their pivotal roles in regulating various cancer pathways and their potential as valuable biomarkers for diagnosing diseases. What was once considered an enigmatic facet of molecular biology has now evolved into a significant area of study, shedding light on the intricate involvement of lncRNAs in virtually all of the fundamental characteristics associated with cancer. Various studies showed that lncRNAs exhibited significant dysregulation in human cancers, including liver cancer, while they were implicated to serve as key prognostic and therapeutic targets (12, 13). Traditionally, cancer research revolved around several well-defined hallmarks of the disease, including sustained proliferation, replicative immortality, evasion of growth suppressors, induction of angiogenesis, resistance to programmed cell death, and the formidable process of metastasis. Notably, lncRNAs have become instrumental in understanding and manipulating these hallmark features. They are now recognized as key orchestrators, exerting a profound influence on each of these facets of cancer progression. For instance, lncRNAs have been found to govern the uncontrolled cell division observed in the sustained proliferation, thereby playing a central role in driving tumor growth. Moreover, they contributed to the cancer cell ability to evade the normal mechanisms that would otherwise halt their relentless growth, thus ensuring replicative immortality. lncRNAs have also been implicated in promoting formation of the new blood vessels, a process known as angiogenesis, which is crucial for the nutrient supplied to the growing tumor. Additionally, they are closely linked to thwarting the programmed cell death or apoptosis, allowing cancer cells to persist and proliferate unchecked. Perhaps one of the most concerning hallmarks of cancer is metastasis, the spread of cancer cells to distant organs and tissues. lncRNAs have been identified as key facilitators in this perilous journey, aiding cancer cells in their escape from the primary tumor and enabling their successful establishment in new sites.

lncRNAs affect diverse signaling pathways in liver cancer cells to regulate their malignant behavior and promote tumorigenesis (14). Significantly, lncRNAs have a pivotal role in modulating the immune response, facilitating liver regeneration, and regulating redox signaling. These functions are of paramount importance in shaping the liver microenvironment and influencing the course of chronic liver diseases. When lncRNAs become dysregulated within these processes, it can trigger chronic hepatitis, abnormal liver growth, and oxidative stress, ultimately paving the way for the onset and advancement of hepatocellular carcinoma (HCC). Certainly, high-throughput technologies like RNA-sequencing and microarrays have unveiled distinct expression profiles of lncRNAs in HCC tissues compared to noncancerous liver tissues. These findings strongly suggested that biogenesis of the specific lncRNAs underwent dysregulation during development of HCC. Aberrations in lncRNA biogenesis encompassed various processes, including the epigenetic silencing or activation of lncRNAs with tumor-suppressive or promoting roles, transcriptional activation or repression of lncRNAs by specific oncogenic or tumor-suppressive transcription factors, distinct processing patterns that imbue lncRNAs with oncogenic properties, and interactions between lncRNAs, microRNAs or proteins that have an impact on lncRNA stability.

lncRNA 91H exhibited expression patterns during embryonic development and it is typically suppressed after birth, except in the particular tissues, like the mammary gland and uterus. Imprinting modifications, leading to aberrant H19 expression, are associated with developmental disorders. lncRNA 91H is known for its tumor-promoting regulatory role in colorectal cancer (15). Overexpression of 91H has been shown to be associated with the growth and metastasis of hepatocellular, in addition to affecting survival via positive regulation of IGF2 (16). Involvement of H19 in tumorigenesis has been extensively documented, with H19 being consistently overexpressed in the various solid tumors such as prostate, bladder, and breast cancers. In fact, it has been demonstrated that H19 is overexpressed in 73% of the breast cancer tissues compared to the healthy tissues. This study aimed to investigate functional role of 91H in liver cancer. The results indicated that 91H acted as an oncogene in liver cancer and suggested its possible prognostic and therapeutic utility in the future.

Materials and Methods

Patient samples

The current experimental study involved a total of 25 patients (15 male and 10 female, age range of 32-84 years old) diagnosed with liver cancer after obtaining written informed consent. Tumor and adjacent non-tumor liver tissue samples (verified by hospital pathologists) were collected once the patients underwent surgery at the Shanxi Bethune Hospital, Taiyuan, China, but prior to the application of radiotherapy or chemotherapy. The tissues were snap-frozen in liquid nitrogen and stored at -80°C until use. Diagnosis of pathological and metastatic/nonmetastatic stages was performed by three pathologists of the Shanxi Bethune Hospital, Taiyuan, China, who were blinded to the study. This study was approved by the Shanxi Bethune Hospital Ethics Committee (No. 2022521).

Culture and transfection of cell lines

A normal liver epithelial cell line (THLE-3) and four liver tumor cell lines (CSQT-2, Hep3B, Huh-7, and PLC) were obtained from the Chinese Academy of Sciences (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM, HyClone; GE Healthcare Life Sciences, USA) containing 10% fetal bovine serum (FBS, Thermo Fisher Scientific, USA) supplemented with 100 U/ml and 100

mg/ml concentrations of penicillin and streptomycin, respectively (both from Gibco Inc., USA), was used for the culture and maintenance of the cell lines at 37°C with 5% CO_2 in a humidified incubator.

Synthetic small-interfering si-91H (45 nM) and negative control si-NC (45 nM) oligos were acquired from GenePharma Co., Ltd (Shanghai, China). For transfection, the cells were cultured in six-well plates until reaching more than 80% confluence, at which point they were stably transfected with suitable oligos using Lipofectamine® 3,000 reagent (Invitrogen, Thermo Fisher Scientific Inc., USA) according to the manufacturer's guidelines. The cells were harvested 48 hours after transfection.

RNA isolation and quantitative reverse transcription polymerase chain reaction

Total RNA was extracted from the tissues and cell lines using TRIzol reagent (Thermo Fisher Scientific Inc., USA) according to the manufacturer's protocol. Following quantification, using NanoDrop, RNA was reverse-transcribed into cDNA using the RevertAid First Strand cDNA synthesis kit (Thermo Fisher Scientific Inc., USA). To detect relative transcript levels of 91H, quantitative reverse transcription PCR (qRT-PCR) was performed on a QuantStudio 5.0 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific Inc., USA) using SYBRTM Green Master Mix (Thermo Fisher Scientific Inc., USA). Relative mRNA expression levels were quantified using 2-ΔΔCt method and normalized to that of *β-actin*. The primer sequences used were as follows:

91H-

F: 5′-GCTTGTCAGTAGAGTGCGCC-3′
R: 5′-CATCCAGTTGACCGAGCTTG-3′

β-actin-

F: 5′-CAAGATCATTGCTCCTCCTGA-3′
R: 5′-AGTCCGCCTAGAAGCATTTG-3′.

CCK-8 cell proliferation assay

Proliferation of the transfected cancer cells was determined using Cell counting kit-8 (CCK-8, Beyotime Inst. of Biotech., China) according to the manufacturer recommendations. Briefly, 2×10^5 cells were seeded per well in a 96-well plate. After culturing the cells for 0, 24, 48, or 72 hour(s) at 37°C, 15 µl of CCK-8 reagent was added to each well, followed by incubation for 2 hours at 37°C. Finally, the optical density (OD) at 450 nm (OD450) of each well was determined using a spectrophotometer and plotted to generate proliferation curve.

Migration and invasion assays

A 24-well BD Matrigel Transwell chamber (BD Biosciences, USA) coated with or without matrigel was used to analyze migration and invasion of the transfected cancer cells. Briefly, 2×10^4 transfected cells were suspended in serum-free DMEM (Gibco, USA) and added to the upper part of the transwell chamber, whereas 500 µl of DMEM with 10% FBS was placed in the lower portion of the chamber. After 24 hours of incubation at 37°C, the non-migrating or invading cells were carefully removed with the help of cotton swabs, while those migrating or invading the lower part of the chamber were fixed with methanol (Thermo Scientific, USA) and stained with 0.1% crystal violet (Thermo Scientific, USA). Finally, the cells were visualized and counted manually under a light microscope (100×, Olympus, Japan).

Western blotting

To extract total proteins from the cells, we initiated the process by lysing the cells using the radioimmunoprecipitation assay (RIPA) lysis and extraction buffer (Thermo Fisher Scientific, USA), complemented with 1% protease inhibitors (Sigma-Aldrich, USA) to ensure integrity of the proteins during extraction. Following the lysis step, the cell lysates underwent centrifugation to separate cellular components. To quantify presence of the total proteins in the suspension, we employed Bradford's assay, a widely-used method for protein concentration determination. This step allowed us to precisely measure protein content and ensure accuracy in our subsequent analyses.

For separation of proteins, we utilized a 12% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were then transferred onto polyvinylidene fluoride membranes (Millipore, USA). These membranes were subsequently blocked with 5% skimmed milk for 1.5 hours, serving as a means to prevent non-specific binding during the antibody incubation process. Membranes were probed with the specific primary antibodies like anti-E-cadherin (Sigma-Aldrich, USA) anti-α-catenin, (Thermo Fisher Scientific, USA), anti-N-cadherin (Cell signaling technology, USA), anti-fibronectin (Abcam, USA) and anti-GAPDH (Abcam, USA) overnight at 4°C. The membranes were then incubated with HRP-conjugated secondary antibody (Abcam, USA) and specific protein signals were detected using an Enhanced Chemiluminescence kit (Thermo Fisher Scientific, USA). The acquired signals were quantified with Image J software, and data normalization was achieved by utilizing GAPDH protein.

Flow cytometry

Hep3B and Huh-7 cancer cells, transfected with si-91H or si-NC, were seeded into six-well plates at a density of 5×10^3 cells/well and cultured for 24 hours at 37°C. Afterward, the cells were collected by centrifugation, mixed with 0.025% Triton X-100, and treated with 50 ng/ml-1 propidium iodide (PI, Thermo Fisher Scientific, Fisher) for 25 minutes. A FACSCalibur flow cytometer (BD Biosciences, USA) was used to study cell cycle of the transfected cells.

To study apoptosis, the transfected cells were analyzed using an Annexin V-FITC Apoptosis Detection Kit (Dojindo, China) at room temperature following the manufacturer's instructions. Finally, a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) was used to assess apoptosis.

Animal experiment description

A total of 20 male BALB/c nude mice, aged 6-8 weeks and weighting 18 ± 2 g, were procured from Shanxi Academy of Medical Sciences, China. The mice were divided randomly into two groups. In the first group, either Hep3B cells (2×10^6 cells/100 µl) or Huh-7 cells (2×10^6 cells/100 µl) were injected subcutaneously (100 µl/mouse) to establish the tumor xenograft model. Each group consisted of 10 mice. Subsequently, each group was further divided randomly into two subgroups, each containing five mice.

For the purpose of gene silencing, lentivirus carrying a short hairpin RNA (shRNA) targeting lncRNA 91H (sh-lncRNA 91H) were obtained from GenePharma company (China). The lentivirus had a titer of 108 TU/ml and it was administered at a dose of 20 µl per mouse. As a control, a negative control shRNA (sh-NC), provided by Invitrogen, was also injected. This treatment was carried out weekly for a total of three consecutive weeks. In conclusion of the 28-days period, all nude mice were humanely euthanized. The subcutaneous tumors were then removed, photographed, and weighed for further analysis. All of the experimental procedures adhered to the established guidelines and received approval from the Ethics Committee of Third Hospital of Shanxi Medical University.

Statistical analysis

Graphpad prism 7.0 (GraphPad Software, Inc., San Diego, CA, USA) offline software was used for performing the statistical analysis. The experiments were performed using three replicates and the values given are the representative of mean ± standard deviation. Student's t test (unpaired, two-tailed) was performed for analyzing the differences between two treatments and $P<0.05$ difference was considered statistically significant.

Results

91H is overexpressed in liver cancer

qRT-PCR was performed to analyze expression of lncRNA 91H in liver cancer and normal adjacent tissues. The results showed that 91H expression level was significantly higher ($P<0.05$) in the liver cancer samples than the normal tissue samples (Fig.1A). Interestingly, metastatic liver cancer tissues showed markedly higher ($P<0.05$) transcript levels of 91H than the matched nonmetastatic cancer tissues (Fig.1B). Moreover, 91H expression level was significantly lower ($P<0.05$) in tumor tissues corresponding to the stages I and II than that in the stages III and IV of liver cancer (Fig.1C). Expression analysis was also performed in liver cancer cell lines (CSQT-2, Hep3B, Huh-7 and PLC) using THLE-3 and normal liver epithelial cells, as a reference. The cancer cell lines showed significant overexpression ($P<0.05$) of 91H compared to the normal liver epithelial cells (Fig.1D). The Hep3B and Huh-7 cancer cell lines were shown to express comparatively higher 91H levels among the cancer cell lines, chosen for further studies.

Knockdown of 91H inhibited growth of liver cancer cells

To characterize functional role of 91H in liver cancer, Hep3B and Huh-7 cancer cells were transfected with si-91H to induce 91H knockdown. Using si-NC-transfected cancer cells as a negative control, 91H expressions were downregulated 6.5 and 7.2-fold in si-91H transfected Hep3B and Huh-7 cancer cells, respectively (Fig.2A). Relative proliferation of the 91H downregulating liver cancer cells was studied with respect to the corresponding negative control cells using the CCK-8 assay. Both Hep3B and Huh-7 cancer cells with downregulated 91H showed significantly lower ($P<0.05$) proliferation than the respective negative control cells (Fig.2B, C).

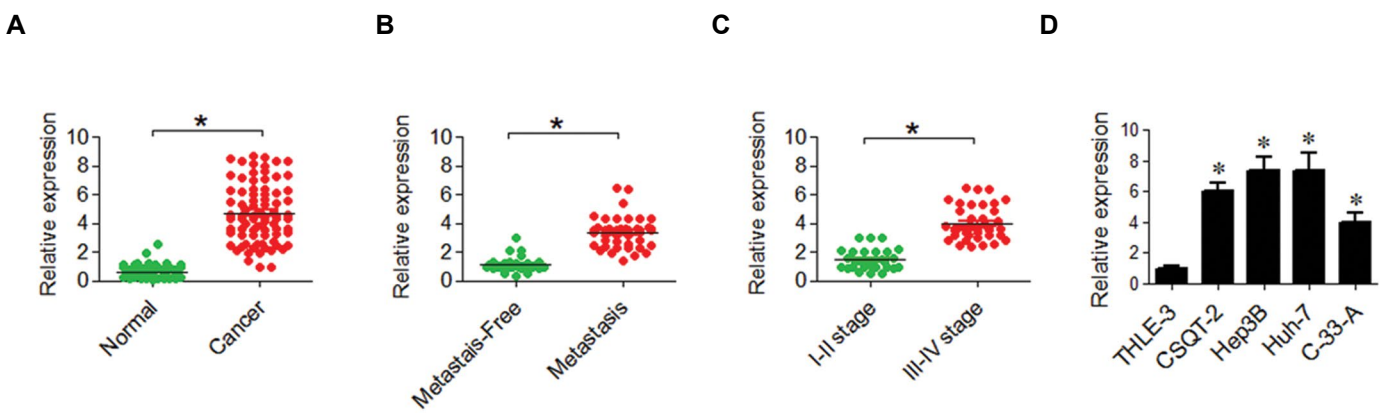

Fig.1: Expression analysis of lncRNA 91H in liver cancer tissues and cell lines. **A**. Comparison of lncRNA 91H expression levels between liver cancer tissues and normal adjacent tissues. Liver cancer tissues exhibited significantly higher expression of 91H compared to normal tissues. **B.** Analysis of 91H expression in metastatic and nonmetastatic liver cancer tissues. Metastatic liver cancer tissues showed markedly higher expression of 91H compared to nonmetastatic tissues. **C.** Assessment of 91H expression in different stages of liver cancer. Tumor tissues corresponding to stage III and IV liver cancer exhibited significantly higher expression of 91H compared to stage I and II tissues. **D.** Evaluation of 91H expression in liver cancer cell lines (CSQT-2, Hep3B, Huh-7, and PLC) compared to THLE-3 normal liver epithelial cells. The cancer cell lines showed significant overexpression of 91H compared to normal liver epithelial cells. Hep3B and Huh-7 cell lines were selected for further study due to their comparatively higher levels of 91H expression. *; $P<0.05$.

Knockdown of 91H inhibited migration and invasion of liver cancer cells

Transwell assay results demonstrated that knockdown of 91H in the both Hep3B and Huh-7 cells significantly inhibited their migration and invasion abilities, compared to the respective negative control cells. In the invasion assay, knockdown of 91H led to a significant decrease in the number of invasive cells compared to the negative control group in both cell lines (P<0.05, Fig.3A, B). Similarly, in the migration assay, number of the migratory cells was dramatically reduced in the 91H knockdown group compared to that in the negative control group for the both Hep3B and Huh-7 cells (P<0.05, Fig.3C, D).

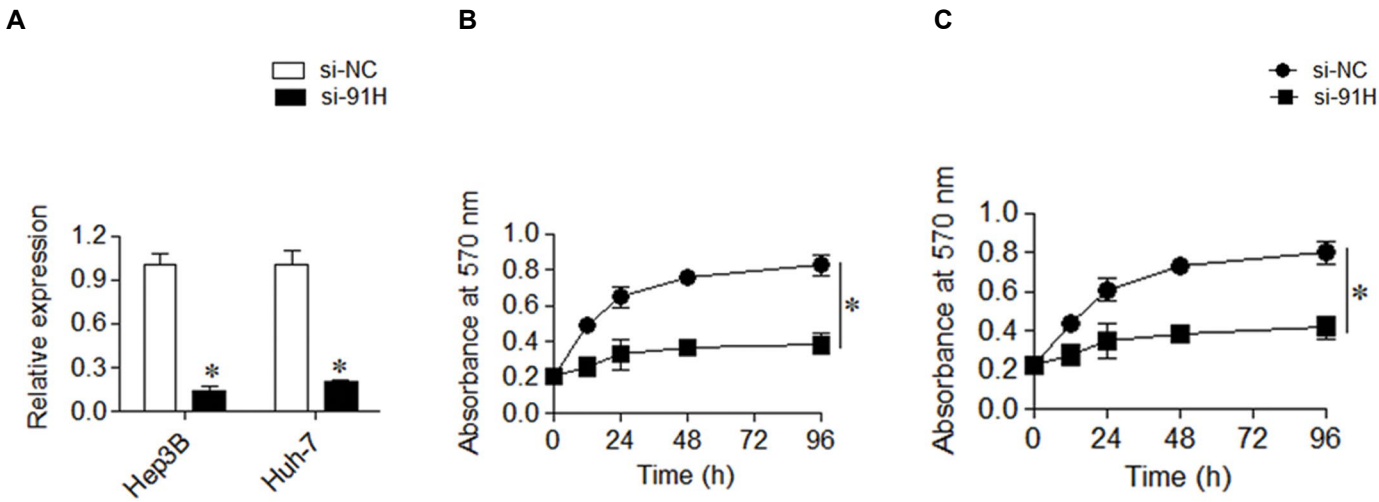

Fig.2: Functional characterization of lncRNA 91H knockdown in liver cancer cells. **A.** Efficiency of lncRNA 91H knock-down in Hep3B and Huh-7 cancer cells. Transfection of si-91H oligos led to a significant downregulation of 91H expression in both Hep3B and Huh-7 cells compared to si-NC-transfected cells, with a fold change of 6.5 and 7.2, respectively. Relative proliferation of liver cancer cells after knockdown of 91H. CCK-8 assays demonstrated that **B.** Hep3B and **C.** Huh-7 cells with downregulated 91H exhibited significantly lower proliferation compared to the respective negative control cells. *; P<0.05 and h; Hours.

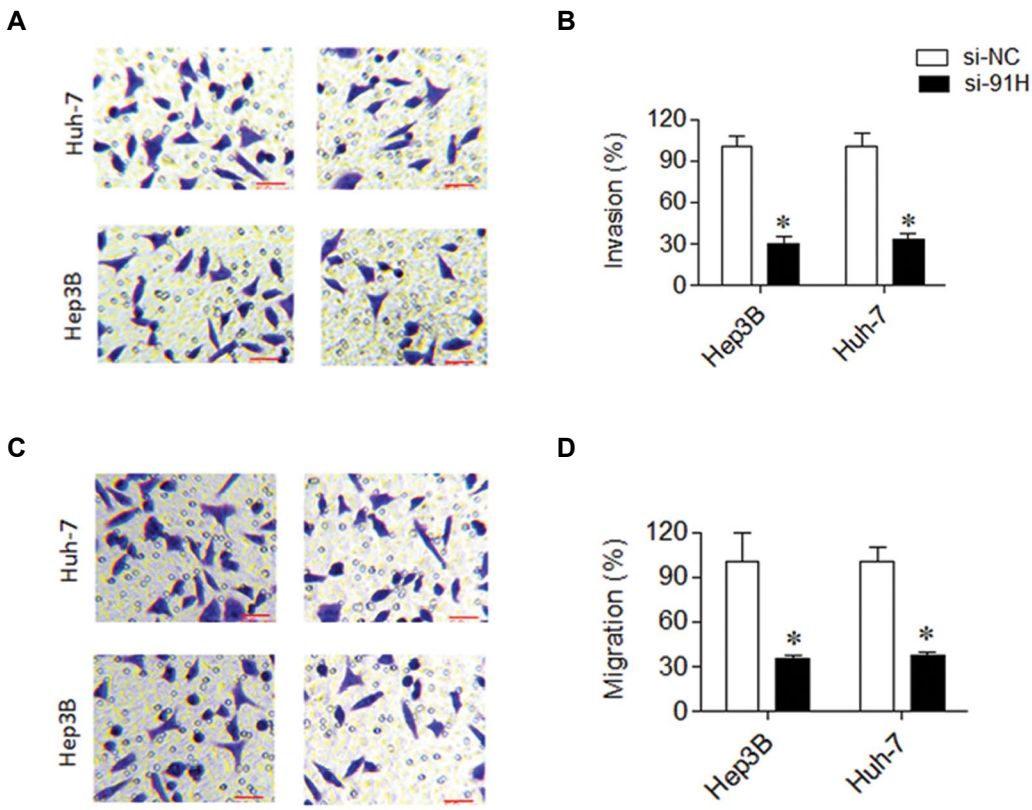

Fig.3: Migration and invasion assay results upon 91H knockdown in Hep3B and Huh-7 cancer cells. **A, B.** Representative images of invasion assays indicating the impact of 91H knockdown on Hep3B and Huh-7 cell invasion. 91H downregulation led to a significant decrease in cell invasion compared to the negative control cells (*; P<0.05). **C, D.** Representative images of migration assays showing the effect of 91H knockdown on Hep3B and Huh-7 cell migration. Knockdown of 91H resulted in a marked reduction in cell migration compared to the negative control cells (*; P<0.05, scale bar: 50 µm).

Knockdown of 91H weakened the epithelial to mesenchymal transition of liver cancer cells

Our results demonstrated that 91H knockdown in the both Hep3B and Huh-7 cells had a significant impact on the expression of epithelial-to-mesenchymal transition (EMT)-related marker proteins. Downregulation of 91H in Hep3B and Huh-7 cancer cells significantly enhanced ($P<0.05$) expression of epithelial marker proteins (E-cadherin and α-catenin), while it significantly suppressed ($P<0.05$) the intracellular protein levels of mesenchymal markers (N-cadherin and fibronectin), indicating the inhibitory effect of 91H silencing on the EMT of liver cancer cells (Fig.4A-E).

91H silencing induced cell cycle arrest and apoptosis in the host liver cancer cells

To determine mechanism underlying the inhibitory effects of 91H silencing on the growth, migration, invasion and EMT of liver cancer cells, cell cycle and apoptosis of the Hep3B and Huh-7 cancer cells with downregulated 91H levels were studied with reference to the respective negative control cells, using flow cytometry. The results showed that knockdown of 91H significantly increased relative percentage of the both Hep3B and Huh-7 cancer cells at the G0/G1 transition phase ($P<0.05$), while it significantly decreased ($P<0.05$) relative percentage of the host cells in the S-phase, indicative of G0/G1 cell cycle arrest (Fig.5A, B). Furthermore, relative percentage of apoptotic Hep3B and Huh-7 cancer cells was significantly increased by 91H silencing (Fig.5C, D). The results revealed that knockdown of 91H induced cell cycle arrest and promoted apoptosis to exert tumor-suppressive effects against liver cancer cells.

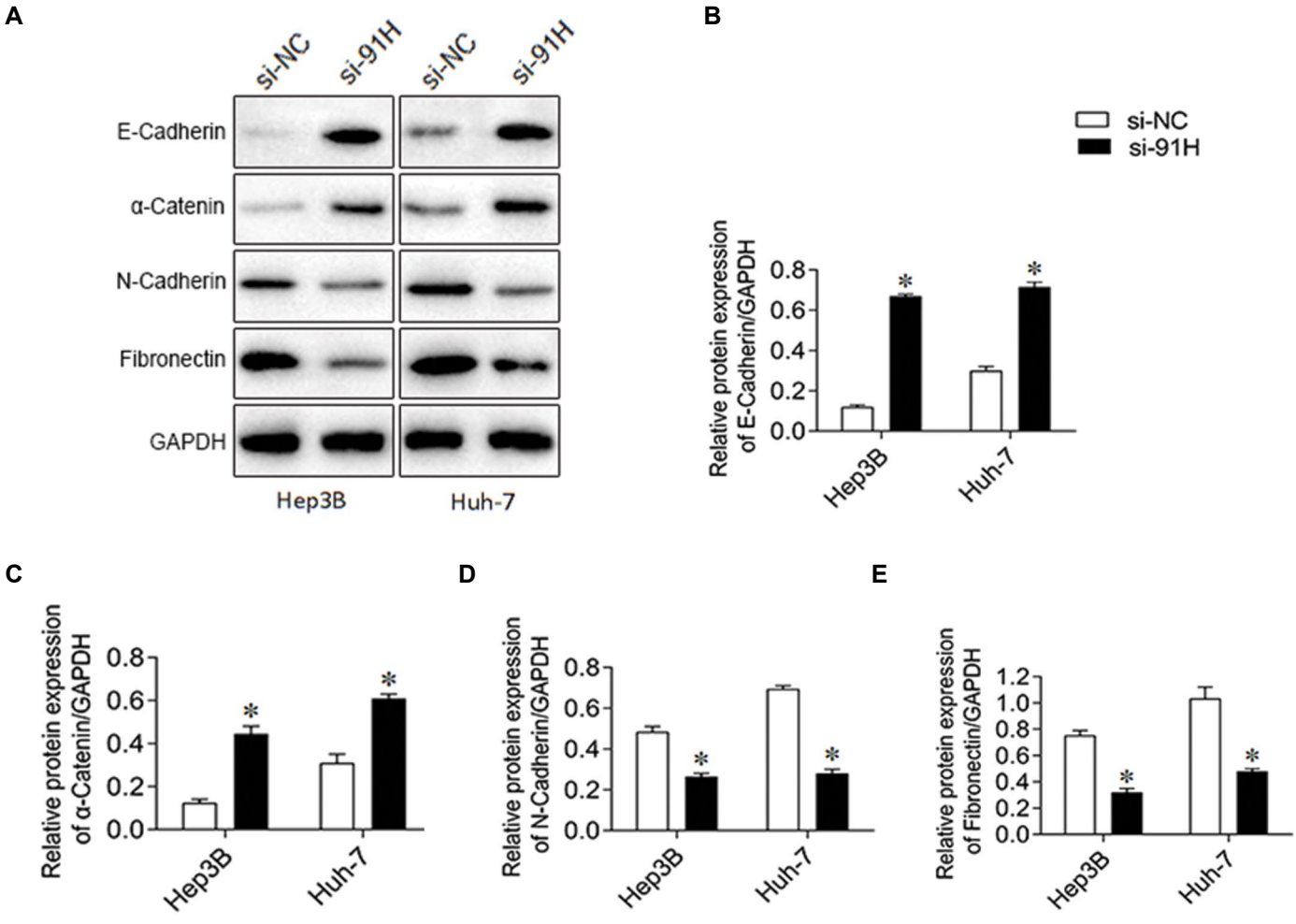

Fig.4: Western blot analysis of epithelial and mesenchymal markers in 91H downregulating Hep3B and Huh-7 cells. **A.** Representative Western blot images showing the protein levels of epithelial markers (E-cadherin and α-catenin) and mesenchymal markers (N-cadherin and fibronectin) in Hep3B and Huh-7 cells with 91H knockdown. Silencing of 91H increased the protein levels of epithelial markers and decreased the levels of mesenchymal markers, suggesting inhibition of the epithelial-to-mesenchymal transition (EMT) in liver cancer cells. **B.** Densitometry analysis of the Western blot for E-cadherin in Hep3B and Huh-7 cells. The protein level of E-cadherin significantly increased upon 91H knockdown compared to the negative control cells ($P<0.05$). **C.** Densitometry analysis of the Western blot for α-catenin in Hep3B and Huh-7 cells. The protein level of α-catenin significantly increased upon 91H knockdown compared to the negative control cells. **D.** Densitometry analysis of the Western blot for N-cadherin in Hep3B and Huh-7 cells. The protein level of N-cadherin significantly decreased upon 91H knockdown compared to the negative control cells. **E.** Densitometry analysis of the Western blot for fibronectin in Hep3B and Huh-7 cells. The protein level of fibronectin significantly decreased upon 91H knockdown compared to the negative control cells. *; $P<0.05$.

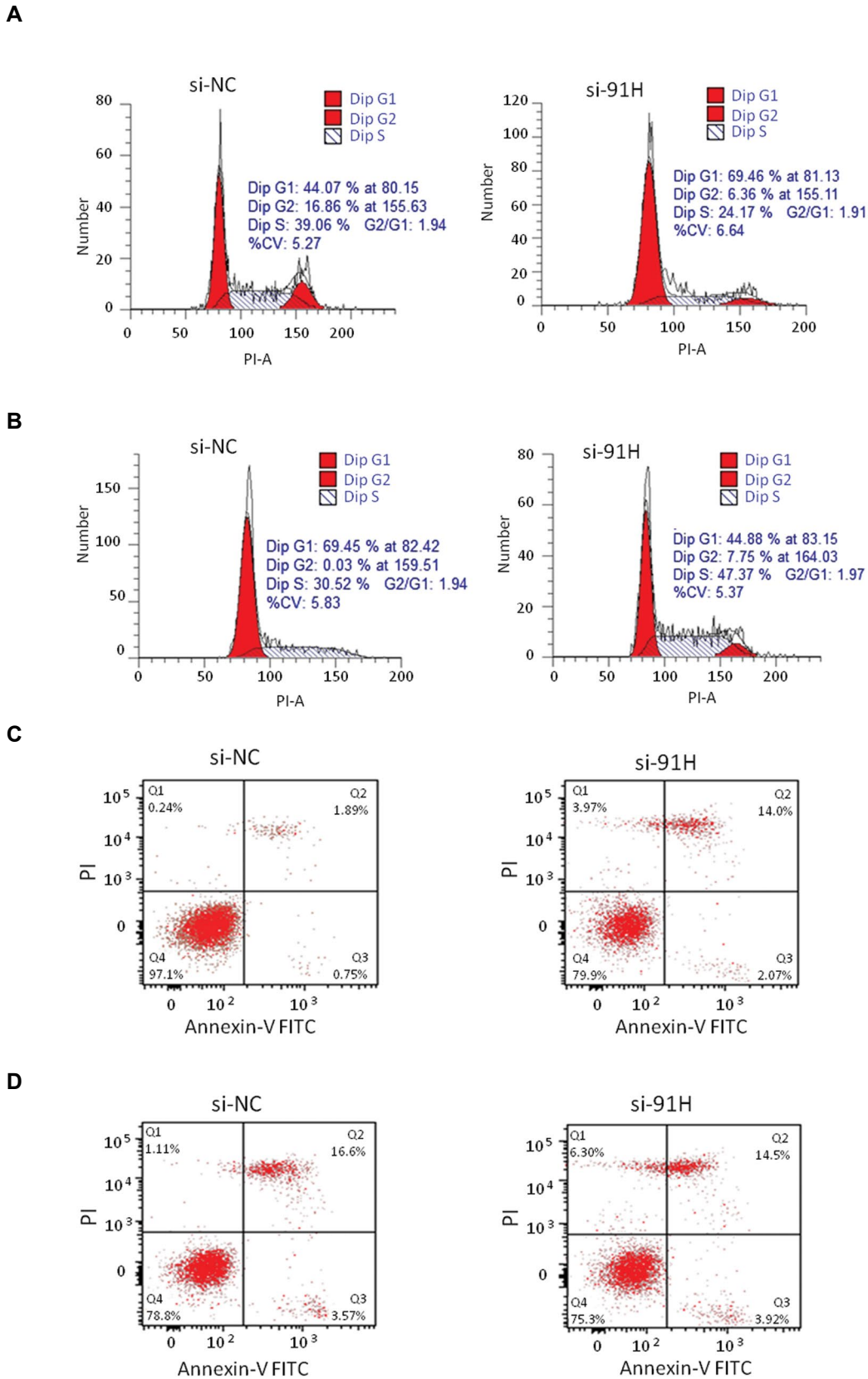

Fig.5: Effects of lncRNA 91H knockdown on cell cycle progression and apoptosis in Hep3B and Huh-7 cancer cells. **A.** Cell cycle analysis of Hep3B cells after 91H knockdown. Flow cytometry revealed a significant increase in the relative percentage of cells at the G0/G1 phase and a decrease in the percentage of cells at the S-phase, indicating G0/G1 cell cycle arrest in 91H downregulating cells compared to the negative control cells. **B.** Cell cycle analysis of Huh-7 cells after 91H knockdown. Flow cytometry revealed increase in the relative percentage of cells at the G0/G1 phase and a decrease in the percentage of cells at the S-phase, indicating G0/G1 cell cycle arrest in 91H downregulating cells compared to the negative control cells. **C.** Representative flow cytometry plots illustrating the apoptotic populations in Hep3B cells after 91H knockdown. Annexin V-FITC/PI staining was used to detect apoptotic cells. The relative percentage of apoptotic cells significantly increased in 91H downregulating cells compared to the negative control cells. **D.** Representative flow cytometry plots illustrating the apoptotic/necrotic populations in Huh-7 cells after 91H knockdown. Annexin V-FITC/PI staining was used to detect apoptotic/necrotic cells. The relative percentage of necrotic cells increased in 91H downregulating cells compared to the negative control cells.

Inhibition of liver cancer growth *in vivo* by silencing lncRNA 91H

To thoroughly investigate impact of lncRNA 91H on liver cancer growth in a live organism context, we utilized nude mouse models, as a platform for our study. In these models, we meticulously measured volume and weight of the formed tumors. Through the implementation of shRNA targeted at lncRNA 91H, we observed a substantial hindrance in tumor development. This intervention led to a remarkable reduction in the both tumor volume and weight, a trend that became evident starting from the 10th day post-treatment in the case of the tumor xenograft mice injected with Hep3B cells, as compared to the mice treated with sh-NC.

Similarly, in the xenograft mice injected with Huh-7 cells, suppression of lncRNA 91H through shRNA yielded parallel results. Following the 10th day of treatment, a discernible decrease in tumor volume and weight was observed, further highlighting the influence of lncRNA 91H silencing on curbing tumor growth compared to the sh-NC-treated mice. This experimental insight underscored the significant role of lncRNA 91H in promoting liver cancer growth *in vivo* and supported the potential therapeutic avenue of targeting this molecule to impede tumor progression (Fig.6).

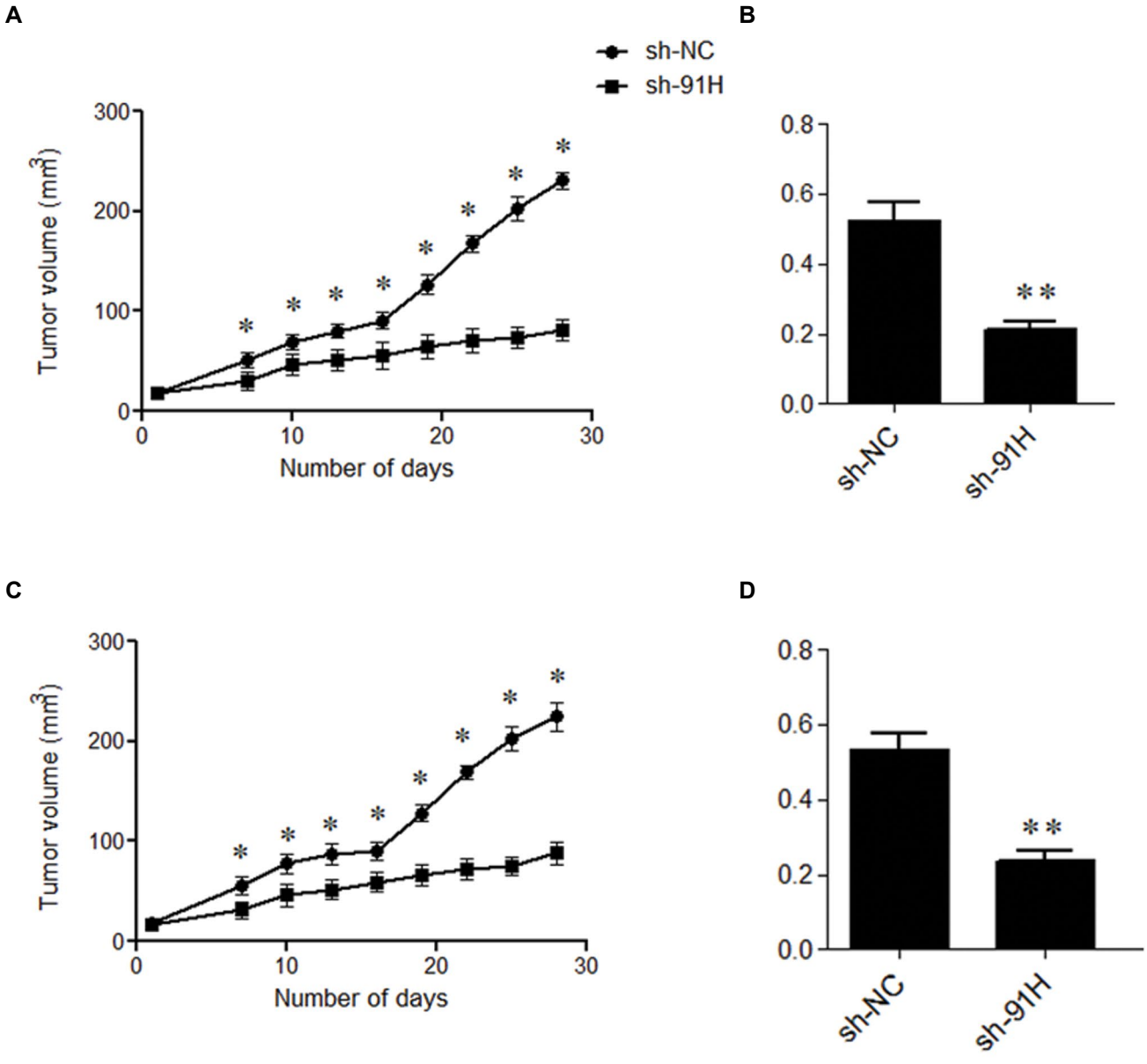

Fig.6: Inhibition of growth by silencing lncRNA 91H. Nude mouse models were utilized to explore the effects of silencing lncRNA 91H on growth. Hep3B cells or Huh-7 cells were subcutaneously injected, followed by treatment with either sh-NC or sh-lncRNA 91H. The subcutaneous tumor volume and tumor weight of **A.** Hep3B cells mouse model and **B.** Huh-7 cells mouse model were plotted. *; P<0.05 and **; P<0.01.

Discussion

In the recent years, there has been an exponential surge in research centered on lncRNAs. The growing body of evidence suggested that lncRNAs exerted a profound influence on various facets of animal cell biology, orchestrating critical processes like cell proliferation, differentiation and apoptosis (17, 18). Perturbed expression of lncRNAs has been closely associated with development and advancement of human cancers, including liver cancer (12, 13). These lncRNAs can function as either oncogenes or tumor suppressors, actively shaping the course of liver cancer tumorigenesis (19, 20). Investigation of the specific regulatory targets of lncRNAs in liver cancer holds the promise of enhancing our understanding of the disease pathogenesis, potentially paving the way for more effective therapeutic strategies against this formidable malignancy.

Previous reports have indicated that ncRNA 91H was upregulated in the various human cancers, with hepatocellular carcinoma, the most prevalent form of liver cancer, being no exception. Furthermore, this upregulation has been linked to the promotion of cancer growth and progression (15, 16). The present study contributes further insight into the functional role of 91H in liver cancer.

Our study revealed that both liver cancer tissues and cells exhibited significantly elevated levels of 91H expression. Importantly, this expression was found to be increased in parallel with the progression of liver cancer and establishment of metastatic lesions. These findings lent support to the prognostic potential of 91H and suggested that it may serve as a predictive marker for liver cancer survival, as previously reported (16, 21). Notably, knockdown of 91H in liver cancer cells resulted in a substantial reduction in their growth, migration and invasion, underscoring its direct involvement in modulating the aggressiveness and malignant behavior of these cancer cells (22).

An analysis of the expression of EMT molecular markers unveiled that downregulation of 91H inhibited EMT in liver cancer cells *in vitro*. EMT is a dynamic cellular process characterized by cytoskeletal rearrangements that underlie transition from an epithelial to a mesenchymal phenotype, enhancing cell motility and, consequently, promoting migration and invasion (23). EMT in cancer cells is characterized by distinct features and plays a pivotal role in fostering characteristics such as stemness, drug resistance and metastasis (24). Therefore, suppression of cancer cell EMT due to 91H knockdown hints at the therapeutic potential of targeting 91H in the context of liver cancer.

Furthermore, our study revealed that inhibition of 91H resulted in cell cycle arrest and induction of apoptosis in liver cancer cells. Similar findings have been reported in relation to silencing H19 (the sense RNA of 91H) in breast cancer cells (25). In summation, this study established that H19, an lncRNA, was upregulated in the both liver cancer tissues and cell lines and it may represent one of the underlying molecular factors propelling growth, progression, and metastasis of liver cancer.

In our quest to unveil the oncogenic role of lncRNA 91H in liver cancer and its implications for proliferation, migration, invasion, EMT and *in vivo* tumor growth, it is essential to consider the potential molecular mechanisms underlying these effects. While this study primarily focused on the functional outcomes of 91H dysregulation, it is plausible that specific pathways and molecular interactions are at play. One key avenue for investigation involves the potential targets of lncRNA 91H. Identifying downstream genes or proteins regulated by 91H may shed light on its influence on cell proliferation, as well as its role in promoting metastasis and EMT. For instance, prior studies demonstrated modulation of the key EMT-related genes by lncRNAs, and it would be valuable to explore whether 91H similarly impacts these genes in liver cancer cells. Moreover, examining the interaction between 91H and other non-coding RNAs or proteins is of interest. It is conceivable that 91H may act in concert with other molecules to drive its oncogenic effects. Elucidating these interactions can provide insights into the complex regulatory networks that underlie liver cancer progression. The study observation of cell cycle arrest and apoptosis induction following 91H knockdown suggests potential involvement in cell cycle regulation and anti-apoptotic pathways. Investigating these pathways, including cyclins, cyclin-dependent kinases and anti-apoptotic proteins, may provide mechanistic insights into how 91H influences cell proliferation and survival. Furthermore, a deeper exploration of signal transduction pathways, such as PI3K/AKT and MAPK/ERK, which are known to play crucial roles in cancer progression, could reveal whether 91H is connected to these pathways. It is possible that 91H indirectly influences these signaling cascades, impacting cell migration, invasion and EMT in liver cancer.

Limitations of this study include the exclusive reliance on *in vitro* experiments, the study limited clinical sample size, omission of the exploration of potential variations in 91H expression among different populations, absence of long-term patient follow-up, and need for further research to understand 91H molecular mechanisms and translate findings into clinical therapies. Additionally, the study exclusive focus on 91H does not consider other lncRNAs implicated in liver cancer, warranting further comparative research.

Conclusion

The lncRNA 91H was significantly upregulated in liver cancer and its expression was increased with disease progression and metastasis. Silencing 91H expression in liver cancer cells inhibited their growth, migration, invasion and EMT *in vitro*. These inhibitory effects were deduced to result from induction of cell cycle arrest and apoptosis in liver cancer cells. Future investigations should prioritize gaining a deeper understanding of its underlying mechanisms, validate its clinical relevance through larger

patient cohorts and trials, develop therapeutic strategies, explore combination therapies, incorporate it into broader biomarker panels, utilize *in vivo* models and conduct long-term patient follow-up studies. These avenues hold promise for advancing liver cancer research and improving clinical outcomes.

Acknowledgements

This study was supported by Shanxi Province "136 Revitalization Medical Project Construction Funds" from Science and research fund of Shanxi Health Commission (Grant No: 2022043). The authors declare no conflict of interest.

Authors' Contributions

Z.M., Z.W.; Conceptualization and Data curation. Z.M; Investigation. Z.M; Wrote the original draft. Z.W; Wrote, Reviewed, and Edited the manuscript. All authors read and approved the final manuscript.

References

1. Anwanwan D, Singh SK, Singh S, Saikam V, Singh R. Challenges in liver cancer and possible treatment approaches. Biochim Bi phys Acta Rev Cancer. 2020; 1873(1): 188314.
2. Wang G, Wang Q, Liang N, Xue H, Yang T, Chen X, et al. Oncogenic driver genes and tumor microenvironment determine the type of liver cancer. Cell Death Dis. 2020; 11(5): 313.
3. Valery PC, Laversanne M, Clark PJ, Petrick JL, McGlynn KA, Bray F. Projections of primary liver cancer to 2030 in 30 countries worldwide. Hepatology. 2018; 67(2): 600-611.
4. Liu Z, Jiang Y, Yuan H, Fang Q, Cai N, Suo C, et al. The trends in incidence of primary liver cancer caused by specific etiologies: results from the global burden of disease study 2016 and implications for liver cancer prevention. J Hepatol. 2019; 70(4): 674-683.
5. Wu J, Yang S, Xu K, Ding C, Zhou Y, Fu X, et al. Patterns and trends of liver cancer incidence rates in Eastern and Southeastern Asian Countries (1983-2007) and Predictions to 2030. Gastroenterology. 2018; 154(6): 1719-1728. e5.
6. Huang J, Lok V, Ngai CH, Chu C, Patel HK, Thoguluva Chandraseka V, et al. Disease burden, risk factors, and recent trends of liver cancer: a global country-level analysis. Liver Cancer. 2021; 10(4): 330-345.
7. Strobel O, Neoptolemos J, Jager D, Buchler MW. Optimizing the outcomes of pancreatic cancer surgery. Nat Rev Clin Oncol. 2019; 16(1): 11-26.
8. Castelli G, Pelosi E, Testa U. Liver cancer: molecular characterization, clonal evolution and cancer stem cells. Cancers (Basel). 2017; 9(9): 127.
9. von Felden J, Schulze K, Krech T, Ewald F, Nashan B, Pantel K, et al. Circulating tumor cells as liquid biomarker for high HCC recurrence risk after curative liver resection. Oncotarget. 2017; 8(52): 89978-89987.
10. Zhang Y, Tang L. The application of lncRNAs in cancer treatment and diagnosis. Recent Pat Anticancer Drug Discov. 2018; 13(3): 292-301.
11. Nandwani A, Rathore S, Datta M. LncRNAs in cancer: regulatory and therapeutic implications. Cancer Lett. 2021; 501: 162-171.
12. Wang Y, Zhu P, Luo J, Wang J, Liu Z, Wu W, et al. LncRNA HAND2-AS1 promotes liver cancer stem cell self-renewal via BMP signaling. Embo J. 2019; 38(17): e101110.
13. Jiao Y, Li Y, Ji B, Cai H, Liu Y. Clinical value of lncRNA LUCAT1 expression in liver cancer and its potential pathways. J Gastrointestin Liver Dis. 2019; 28(4): 439-447.
14. Hu XY, Hou PF, Li TT, Quan HY, Li ML, Lin T, et al. The roles of Wnt/beta-catenin signaling pathway related lncRNAs in cancer. Int J Biol Sci. 2018; 14(14): 2003-2011.
15. Gao T, Liu X, He B, Nie Z, Zhu C, Zhang P, et al. Exosomal lncRNA 91H is associated with poor development in colorectal cancer by modifying HNRNPK expression. Cancer Cell Int. 2018; 18: 11.
16. Yi T, Wang T, Shi Y, Peng X, Tang S, Zhong L, et al. Long noncoding RNA 91H overexpression contributes to the growth and metastasis of HCC by epigenetically positively regulating IGF2 expression. Liver Int. 2020; 40(2): 456-467.
17. Liu X, She Y, Wu H, Zhong D, Zhang J. Long non-coding RNA Gas5 regulates proliferation and apoptosis in HCS-2/8 cells and growth plate chondrocytes by controlling FGF1 expression via miR-21 regulation. J Biomed Sci. 2018; 25(1): 18.
18. Li J, Tian H, Yang J, Gong Z. Long noncoding RNAs regulate cell growth, proliferation, and apoptosis. DNA Cell Biol. 2016; 35(9): 459-470.
19. Huo X, Han S, Wu G, Latchoumanin O, Zhou G, Hebbard L, et al. Dysregulated long noncoding RNAs (lncRNAs) in hepatocellular carcinoma: implications for tumorigenesis, disease progression, and liver cancer stem cells. Mol Cancer. 2017; 16(1): 165.
20. Zhao J, Fu Y, Wu J, Li J, Huang G, Qin L. The diverse mechanisms of mirnas and lncrnas in the maintenance of liver cancer stem cells. Biomed Res Int. 2018; 2018: 8686027.
21. Deng Q, He B, Gao T, Pan Y, Sun H, Xu Y, et al. Up-regulation of 91H promotes tumor metastasis and predicts poor prognosis for patients with colorectal cancer. PLoS One. 2014; 9(7): e103022.
22. Vennin C, Spruyt N, Robin YM, Chassat T, Le Bourhis X, Adriaenssens E. The long non-coding RNA 91H increases aggressive phenotype of breast cancer cells and up-regulates H19/IGF2 expression through epigenetic modifications. Cancer Lett. 2017; 385: 198-206.
23. Yang J, Antin P, Berx G, Blanpain C, Brabletz T, Bronner M, et al. Guidelines and definitions for research on epithelial-mesenchymal transition. Nat Rev Mol Cell Biol. 2020; 21(6): 341-352.
24. Zhang Y, Weinberg RA. Epithelial-to-mesenchymal transition in cancer: complexity and opportunities. Front Med. 2018; 12(4): 361-373.
25. Su J, Zhang E, Han L, Yin D, Liu Z, He X, et al. Long noncoding RNA BLACAT1 indicates a poor prognosis of colorectal cancer and affects cell proliferation by epigenetically silencing of p15. Cell Death Dis. 2017; 8(3): e2665.