

# A Novelty of Long Non-coding RNA LOXL1-AS1: Suppresses Tumor Progression and Metastasis and an Independent Favorable Prognostic Factor in Hepatocellular Carcinoma

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## Research

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# Abstract

**Background:** Hepatocellular Carcinoma (HCC) is the second primary causes of cancer death globally, And the sixth mostly common malignant liver tumor with poor clinical results. The long term survival of HCC patients was effected and influenced by the low rate of early diagnosis and high risk of recurrence and metastasis in post operative .Although the survival of HCC patients had improved due to improved diagnosis, In Addition Increasing amount of long non-coding RNAs (lncRNAs) have been revealed to be implicated in the carcinogenesis and progression of HCC. The potential role of Loxl1-As1 in the progression and metastasis of HCC is still not clear and needs exploring and more researching.

**Methods:** By using a lncRNA microarray, we identified a novelty of lncRNA Loxl1-As1 .The expression of lncRNA high downregulated in metastatic HCC (Loxl1-As1) in cell lines and tissues was detected by quantitative real-time PCR (qRT-PCR) and in situ hybridization (ISH).and CCK-8, colony formation and flow cytometry were performed to investigate the role of Loxl1-As1 in HCC cell proliferation, cell cycle and apoptosis in vitro and migration were investigated in HCC cell lines bot in vitro, Western blot was used to detect the downstream of Loxl1-As1.

**Results:** Clinically investigation,Loxl1-As1 correlated with good and favorable prognosis of HCC patients. and Loxl1-As1 was down-regulated in HCC tissues and cell lines. The ISH assay revealed that Loxl1-As1 expression was significantly decreased in 177 paraffin-embedded samples from patients with HCC compared with Non-tumor tissues (adjacent tissues )and Loxl1-as1 expression directly correlated with patient prognosis. In vitro studies indicated that Loxl1-as1 promoted HCC cells' proliferation and clonogenicity, the expression of Loxl1-as1 suppressed the growth, migration, and metastasis of HCC cells in vitro .

**Conclusions:** Collectively, Our findings reveal a novelty Loxl1-As1for HCC progression and these study demonstrated that Loxl1-As1, overexpressed in HCC and associated with good prognosis , and it's an important role in the progression and metastasis of HCC. Finally we suggest that lncRNA Loxl1-As1 might be a potential biomarker and therapeutic target for HCC,

## Introduction

**Hepatocellular carcinoma (HCC)** is one of the deadliest malignancies worldwide, with an overall 5-year survival rate of 8%. The number of elderly people with malignancies of all types has been increasing along with longer life expectancy. It is expected that over 60% most of cancers will be exmined in elderly patients and that's remains a global challenge [1,2]. HCC is the most common primary liver cancer and has a high incidence of distal metastasis. It is the sixth most lethal malignancy and ranks as the third most common cause of cancer-related deaths worldwide [3]. High frequencies of recurrence and metastasis are the major causes for the poor clinical outcomes seen in HCC patients. Increasing evidence supports that mRNA dysregulation correlates with HCC progression [4,5]. Meanwhile, anti-inflammatory drugs have displayed certain anti-metastatic effects, although the mechanisms are unclear [6]. Besides,

no significant improvements in clinical outcomes have been observed in over a decade, due to metastasis and recurrence [17,18]. The molecular mechanisms of HCC still remain unclear. Long non-coding RNAs (lncRNAs) are a group of transcription molecules that are over 200 nucleotides long. They are not translated into proteins but are related to a number of biological processes. Many lncRNAs are uniquely expressed in different tissues or specific cancer types [7,8]. In the past few years a lot of lncRNAs have been reported to either promote tumors or be anti-oncogenes [19-20]. The role of lncRNAs in tumorigenesis and progression has been validated by accumulating experimental demonstrations [9–10]. One such lncRNA is LOXL1-AS1, which is encoded in the complementary strand of LOXL1 [11]. Recently, LOXL1-AS1 has been found to modulate the very aggressive phenotypes of glioblastoma, medulloblastoma, prostate cancer and cholangiocarcinoma [12,13]. It also antagonizes miR-708-5p to enhance tumorigenesis and stemness in gastric cancer. A previous study reported that LOXL1-AS1 down-regulation inhibited cell proliferation and arrested cell cycle progression in prostate cancer [11].

The lncRNA LOXL1 antisense RNA is located on human chromosome 15q24.1 and consists of 10,781 nucleotides and 5 exons. Some studies have validated the oncogenic role of LOXL1-AS1 in numerous human cancers [15].

However, the mechanism whereby LOXL1-AS1 functions in HCC cells needs to be clarified. We demonstrate that LOXL1-AS1 could potentially predict early HCC recurrence in patients who underwent curative surgery, that HCC patients with highly expressed LOXL1-AS1 had longer overall survival time and post-progression survival time, and that LOXL1-AS1 can suppress the proliferation of HCC cells.

To summarize, this study is to investigate the expression of LOXL1-AS1 and its clinical implications in HCC cells, as well as to determine its impact on more aggressive HCC cell types and its downstream mechanism and potential regulatory mechanism. Our results suggest Loxl1-as1 plays an important role in the progression and metastasis of liver cancer and which might bring novel ideas for treating patients with HCC patients...

## Materials And Methods

### Patient Sample

Between January 2011 and January 2016 data from 177 HCC patients were obtained from Zhejiang University School of Medicine Sir Run Run Shaw Hospital (Hangzhou, Zhejiang, China) for the study.

This research was conducted with the approval of the ethics committee from Zhejiang University School of Medicine Sir Run Run Shaw Hospital. All participants signed a written acknowledgment of informed consent. The clinical characteristics of patients were retrieved from medical records. None of the subjects in this study received any pre-treatment with chemotherapy or radiotherapy prior to surgical resection. HCC tissue and the adjacent normal tissues were surgically resected from each patient. After collection, all specimens were promptly frozen with liquid nitrogen and stored at -78 to -80 degrees Celsius.

## Cell Lines

The human HCC cell lines (Sk-hep1, Huh7, L02, Hep-G2, LM3, HA22T, JHH7) were provided by the American Tissue Culture Collection (Manassas, Virginia, USA). All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco) and supplemented with 10% fetal bovine serum (FBS) (Gibco), 10 U/mL penicillin and 10 mg/mL streptomycin. Cells were grown in a humidified atmosphere incubator at 37°C in 5% carbon dioxide. All cells were cultured according to the manufacturer's guidelines.

## Cell Transfection

Plasmids were used in this experiment. All transfections were done by utilizing Lipofectamine 2000 (Invitrogen, Carlsbad, California, USA). Sk-hep1 and Huh7 cells were seeded into 24-well plates (6 × 10<sup>4</sup> cells per well) 48 hours after successful transfection. They were then transfected with 50nm plasmids purchased from (Tsingke Biological Technology, Beijing, China). The plasmids served as negative controls to allow for high expression or down-regulation of the cell lines.

## Real-time Quantitative PCR (RT-qPCR)

Total RNA was isolated by using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Inc.). The quantity of total RNA was measured by using NanoDrop equipment (Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to the manufacturer's instructions.

Complementary DNA (cDNA) was synthesized using a cDNA synthesis kit (Yeasten, Shanghai, China). qPCR was performed using the SYBR Green PCR Master Mix (Yeasten, Shanghai, China). All target genes were normalized to the endogenous reference gene GAPDH by using an optimized comparative Ct (2<sup>-ΔΔCt</sup>) value method. qPCR assay was performed on the 7500 Fast qPCR system using a One-Step SYBR PrimeScript RT-PCR kit (Applied Biosystems, Foster City, California, USA). The specific primer sequences of LOXL1-AS1 and GAPDH were as follows:

LOXL1-AS1 forward primer: GGTGCCACGGCTTACCAA

LOXL1-AS1 reverse primer: TCCTATCCCTGCCATTCCCA

GAPDH forward primer: GAAGGTGAAGGTCGGAGTC

GAPDH reverse primer: GAAGATGGTGATGGGATTTC

The relative gene expression was quantified using the 2<sup>-ΔΔCt</sup> method.

## Fluorescence In Situ Hybridization (FISH)

The expression of LOXL1-AS1 was measured in paraffin-embedded tissue microarrays using an in situ hybridization detection probe. Digoxigenin-labeled sense and antisense LOXL1-AS1 probes were designed and made by Wuhan Servicebio Technology Co., Ltd. (China) for this purpose. The probe signals were

detected using an optimization FISH kit (Wuhan Servicebio Technology Co., Ltd., China). We then followed the steps below.

The slides were deparaffinized and rehydrated before incubation with Proteinase K 15 µg/ml at 37°C for 40 minutes at room temperature. Then, The slides were then washed three times with phosphate-buffered saline (PBS) for 15 minutes. Next, the slides were incubated with 5x saline-sodium citrate (SSC) solution at room temperature for 15 minutes. LOXL1-AS1 probes were added to the hybridization buffer for 1 hour at 50°C and then overnight at 4°C. The next day, the slides were washed with graded-diluted solutions at 50°C for 20 minutes and then placed in a blocking solution for 1 hour at room temperature. Finally, the slides were placed in a blocking solution containing alkaline phosphatase conjugated with anti-DIG Fab fragment overnight at 4°C.

The hybridization signals were visualized using NBT/BCIP (Thermo Fisher Scientific) according to the manufacturer's instructions. These slides were scored according to the staining intensity and number of positive cells. All images were acquired and scanned with an Eclipse Ci positive fluorescence microscope (Nikon Corporation). The laser-microscope was used to examine the results. The samples were divided into two groups, low expression and high expression.

Follow-up data were obtained via telephone contact. The end point was overall survival. Survival time was defined according to the dates between surgical resection and patient's death or last follow-up.

### **Cell Proliferation Assay (CCK-8 assay)**

Using the cell counting kit-8 (CCK-8) assay, transfected cells were incubated at a density of

$2 \times 10^3$  cells/well into 24-well plates and then cultivated for 0, 24, 48 or 72 hours. After incubation 20 µL of CCK-8 reagent (Yeasen, Shanghai) was added to each well and cultured for another hour at 37°C. The absorbance at 450 nm was recorded with a standard microplate reader (Multiskan MK3, Thermo Scientific). The absorbance on days 1 to 3 was normalized to the absorbance on day 0, which was used as a control (100%). Each experiment was performed independently three times

### **Colony Formation Assay**

HCC cells with concentrations of  $1 \times 10^3$ /mL were seeded in a 6-well plate. The culture medium was discarded after 2 weeks and the colonies were carefully washed with PBS two times. The colonies were fixed with 10% paraformaldehyde for 10 minutes and stained with 0.5% crystal violet for 20 minutes. After that, the formed colonies were counted and recorded.

### **Cell Migration Assay In Vitro**

Assay cells were incubated in a 24-well plate equipped with a transwell chamber using an 8-µm pore size polycarbonate membrane (Corning). After transfection, cells were resuspended in a serum-free medium and plated into the upper chamber. The bottom chamber was filled with DMEM containing 10% FBS. The

cells were incubated at 37°C and 24 hours after incubation the cells on the lower surface of the chamber were gently wiped clean with a cotton swab. The lower chamber was fixed using 95% ethanol for 20 minutes and then stained by 0.5% crystal violet for 10 minutes. The cells were then counted in five random fields under a microscope. Each experiment was independently performed three times.

### **Flow Cytometry and Apoptosis Analysis**

HCC cell cycle and cell apoptosis analyses were performed via flow cytometry using a FACScan (BD Biosciences, USA). For the cell cycle analysis, cells were seeded into 6-well plates, then harvested and fixed with 70% ethanol at 4°C overnight, after which the cells were collected and resuspended in a binding buffer. The cells were stained with propidium iodide (50 µg/ml) containing 100 µg/ml of RNase A for 15 minutes.

Cells were harvested for the apoptosis analysis then washed with PBS and incubated with Annexin V-FITC and propidium iodide (Beyotime, China), according to the manufacturer's instructions. The cells were then analyzed using a BD LSRFortessa cell analyzer (BD Biosciences, USA).

### **Western Blot Analysis**

Proteins from analyzed cells were extracted using the RIPA lysis buffer (Beyotime, Shanghai, China) with a protease inhibitor. Protein concentration was determined using a Bio-Rad protein assay system (Bio-Rad, Hercules, California, USA). All of the extracted protein was separated with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked in Tris-buffered saline (TBS) containing 5% non-fat milk at room temperature for 1 hour, followed by an overnight incubation at 4°C with the appropriate primary antibody. After incubating overnight, the membranes were washed by TBS with Tween 20 (TBST) three times and then the second corresponding antibody (Beyotime, Shanghai, China) was applied.

The following primary antibodies were used: CDK2 (1:1000, Cell Signaling Technology, Massachusetts, USA), CDK4 (1:2000, Abcam, Cell Signaling Technology, Massachusetts, USA) and Tubulin (1:20000, Abcam). Incubation with the secondary antibodies was done overnight at 4°C, then for another 2 hours at room temperature with a horseradish peroxidase-conjugated anti-rabbit antibody. After that, the proteins were visualized using the ECL Western blotting kit (Hangzhou Fude Biological Co., Ltd., China) to check for color reaction. The densities of the bands were detected using BioImaging Systems (Bio-Rad, Hercules, California, USA). All experiments were performed three times.

### **Statistical analysis**

All statistical analyses were implemented using SPSS 20.0 software (SPSS, Inc., Chicago, Illinois) and GraphPad Prism 6.0 (Graph-Pad Software, Inc., USA). The experimental data were presented as mean ± SD. The survival curves were calculated using the Kaplan-Meier method and the differences were assessed by a log-rank test. Statistical significance was tested using the Student's t-test or a Chi-square

test. The Pearson's test was used to analyze the relationship between LOXL1-AS1 expression and the clinicopathologic features of HCC. The Student's t-test was used to detect significant differences in data obtained from qPCR experiments and colony formation assays. A multi-way classification analysis of variance tests was performed to assess data obtained from the CCK-8 assays, tumor growth and correlations among LOXL1-AS1 expression. All data were presented as mean  $\pm$  SD. A p value less than 0.05 indicated statistical significance.

## Results

### **Loxl1-As1 Expression is downregulated in HCC cell lines and human HCC tissues and predicts poor prognosis.**

This study first identified the expression level of Loxl1-As1 in several HCC cell lines (Sk-hep1, Huh7, Hep-G2, LM3, HA22T, JHH7). Then assessed by qRT-PCR and compared it to the immortalized (normal) human liver cell line L02. Loxl1-As1 was expressed relatively lower in the above HCC cell lines (**Fig. 1A**). Subsequently, the expression of Loxl1-As1 in HCC cells was compared to adjacent liver tissues. The mean level of Loxl1-As1 was observed to be significantly lower in HCC tissues compared to adjacent tumor tissues (**Fig. 1C**). Further analysis showed Loxl1-As1 expression in five paired HCC and adjacent tissues of patients in the Liver Cancer Institute at SRRS Hospital, Zhejiang University. The mean LOXL1-AS1 expression level was also lower in HCC tissues than in adjacent liver tissues (**Fig. 1B**). FISH staining analysis was used to determine and detect the expression tissue microarray of Loxl1-As1 in 177 paraffin-embedded surgical specimens of HCC from SRRS Hospital. The expression of Loxl1-As1 was significantly down-regulated in the tumor tissues compared with the adjacent tissues (**Fig. 1D**). In addition, the results showed that LOXL1-AS1 expression was markedly decreased in tumor tissues as well as in patients in an advanced stage of TNM (**Fig. 1E**).

### **Overexpression of Loxl1-As1 is associated with Poor prognosis in HCC.**

The correlation between Loxl1-As1 expression and the clinicopathologic aspects of HCC was also investigated. The results of AFP levels showed no significant statistical difference between the two groups (high expression and low expression of Loxl1-As1). Only tumor stage and TNM stage classification showed statistical significance ( $P = 0.04$ ) (**Table: 1**). **Follow**, The correlation between Loxl1-As1 and HCC prognosis was initially analyzed using The Cancer Genome Atlas Program (TCGA) database. The results showed that patients with high LOXL1-AS1 expression exhibited significantly longer overall survival compared to patients with low expression ( $P = 0.18$  vs  $0.00037$ ) (**Fig. 2 A, B**). Kaplan-Meier survival

curves showed. Patients with high Loxl1-As1 expression showed longer recurrence-free survival than patients with low expression ( $P = 0.02$  vs  $0.87$ ) (**Fig. 2 C, D**). Moreover, We performed univariate analysis and identified TNM Stage ( $P < 0.001$ ), T Stage ( $P < 0.001$ ), T size ( $P = 0.046$ ), Tumor number (solitary/multiple) ( $P < 0.004$ ). So, The parameters including Tumor size, TNM stage and Tumor number were proved to be associated with overall survival as indicated by univariate analysis (**Table: 2**),

However, Age, gender, AFP, Hepatitis, Alcohol differentiation status and therapeutic strategy had no prognostic significance in this studied population. Furthermore, The Multivariate analysis showed TNM Stage (  $P= 0.0099$  ), and Tumor size (  $P= 0.031$  ) was an prognostic factors for HCC patients (**Table:2**). As a whole, these results indicate that Loxl1-As1 could be a predictor of recurrence among HCC patients.

### **Loxl1-As1 Promotes HCC cell proliferation, migration in vitro.**

To investigate the function, biological significance, and role of Loxl1-As1 in the progression of HCC, benefit-of-function and loss-of-function studies were performed in vitro. First, this study elucidated the expression level of Loxl1-As1 in HCC cell lines compared with normal liver cells (**Fig. 3 A**). To explore the biological concept of Loxl1-As1 in HCC progression, the cell proliferation assays and colony formation assays were analyzed using the CCK-8 colony assay. Results showed that over-expression of Loxl1-As1 significantly reduced the proliferative capacity of HCC cells, as well as cells that inhibited HCC growth and clone formation ability, in comparison with the control group (**Fig. 3 B, C**). Moreover, as seen from the transwell migration assay, Loxl1-As1 over-expression significantly inhibited the migration abilities of HCC cells. Results likewise revealed that the migratory capacity of HCC cells was evidently inhibited with the transfection of the control group (**Fig. 3 D**).

### **Loxl1-As1 promotes cell apoptosis in HCC.**

Apoptosis is an important cell process. Our study found that the over-expression of Loxl1-As1 significantly promoted apoptosis in Sk-Hep1 and Huh7 cell lines. The effects of Loxl1-As1 expression on cell survival were assessed (**Figure 4 A**). Compared with the OE and CON groups, CDK2 protein expression was significantly reduced. In addition, the CDK4 and tubulin protein expression rapidly increased in the CDK2 of Sk-Hep1 and Huh7 cells (**Fig. 4B**).

### **Expression of Loxl1-As1 induces cell cycle at the S Phase and leads to cell apoptosis in HCC cells.**

In the cell cycle assay, it was found that the expression of Loxl1-As1 induced cell cycle progression (**Fig 5A**). In particular, S phase cells were significantly similar approximately 20% in control Sk-hep1 cells and nearly 20% in Loxl1-As1-depleted Sk-hep1 cells. Accordingly, the cell proportion in the G2/M phase decreased by approximately 40% . Comparable cell cycle arrest at the S phase was also noted in Huh7 cells (**Fig.5B**). Finally, a schematic summary of the role of lncRNA Loxl1-As1 in the regulation of HCC progression was formed (**Fig. 6**).

## **Discussion**

A great deal of evidence has been found over the last few decades that suggest that some genes are of great clinical value for the early detection, curative observation and prognostic evaluation of human cancers. Some of these genes are down-regulated in HCC patients and act as tumor suppressors, whereas other genes are up-regulated and play oncogenic roles. All of these interactions are correlated with the carcinogenesis, progression and prognosis of HCC.

HCC is a leading cause of cancer-related death worldwide. Recurrence and metastasis contribute to the mortality of HCC patients and cause the main problems associated with the 5-year survival rate. HCC remains unfavorable for clinical outcomes. In these patients, high-serum vascular endothelial growth factor (VEGF) has been associated with tumor recurrence, metastasis and poor survival [21].

Recent studies have shown that LOXL1-AS1 activity, plays an important role in tumor invasion and metastasis. Our results were comparable and the functional experiments demonstrated that LOXL1-AS1 promoted cell proliferation, migration and tumor metastasis. The gene LOXL1-AS1 is extremely down-regulated in HCC tissues and cells. Additional, over expression of LOXL1-AS1 was closely correlated with poor prognosis of HCC. Nevertheless, the biological function of LOXL1-AS1 in Hepatocellular cancer is still unclear and more research is needed.

A Study by (Gao R, et al 2018) Showed that LOXL1-AS1 knockdown suppressed D283 and D341 cell colony formation and proliferation, partly through regulating the PI3K-AKT signal pathway [22]. Yet, LOXL1-AS1 functions as an oncogene in the progression of osteosarcoma [23]. The tumor promoting role of LOXL1-AS1 has been confirmed in many cancers including glioblastoma [35], gastric cancer [12,24], prostate cancer [14], osteosarcoma [16], cholangiocarcinoma [13] and breast cancer [26].

Wang et al. showed that LOXL1-AS1 knockdown attenuated glioblastoma mesenchymal characteristics by NF- $\kappa$ B pathway regulation [35]. Ming Li also reported that LOXL1-AS1 is up-regulated in gastric cancer tissue and cells and that its high expression was closely linked to adverse clinical features [24]. In the same manner, Tian Liang et al. demonstrated the expression of LOXL1-AS1 in doxorubicin-resistant prostate cancer. Their microarray analysis showed that both lncRNA LOXL1-AS1 and EGFR were down-regulated, while miR-let-7a-5p was up-regulated in doxorubicin-resistant prostate cancer DU-145 cells [25]. Dong et al reported that LOXL1-AS1's over-expression leads to an inhibition of miR-708-5p expression in breast cancer cells [26]. However, Xu et al. analyzed the genome-wide lncRNA expression profiles of breast cancer at The Cancer Genome Atlas (TCGA) database and found that there was no statistical difference for LOXL1-AS1 expression between breast cancer tissues and normal mammary tissue [31].

Many studies have indicated that lncRNAs could regulate cancer development by effecting cell proliferation, tumor suppression, anti-apoptosis, and metastasis. They are also intimately connected to the regulation of the Warburg effect to support growth and survival of cancer cells [27,29,30]. According to Wei, lncRNAs play an important role in the development of different cancers [28]. Braconi et al. showed that the lncRNA MEG3 was regulated by miR-29a in a methylation-dependent, tissue-specific manner and that it contributed to the growth of HCC [33]. Moreover, recent research indicates that proper control of mRNA expression is required for a balanced physiological condition, as these small molecules influence almost every genetic pathway from cell proliferation to apoptosis, with a wide range of target genes. As implicated, miR-195 functions as a tumor-suppressor by increasing cancer cell apoptosis [32]. Yang et al reported that histone deacetylase 3 (HDAC3) was involved in the suppression of HCC-related lncRNA LET [34].

It has been reported that LOX activity promotes tumor migration, invasion and metastasis via induction of the epithelial-mesenchymal transition (EMT) [23,14]. In this paper, the authors observed the regulatory mechanism of LOXL1-AS1. It was found that LOXL1-AS1 expression was overtly down-regulated in HCC tissues and cells. In addition, high LOXL1-AS1 expression closely correlated with good prognosis in HCC. The functional experiments demonstrated that LOXL1-AS1 promoted cell proliferation, migration and cell apoptosis. Microarrays were used to identify the expression of LOXL1-AS1 and showed that these were significantly less in the highly aggressive HCC cell line Sk-hep1 compared with Huh7 cells. By observing the qRT-PCR of a full-length transcript of LOXL1-AS1 we found that its expression was reduced in both tissue samples and HCC cell lines. Therefore, the decreased expression of LOXL1-AS1, especially in highly aggressive cell lines, indicates that this lncRNA may be a promising marker for HCC. Moreover, FISH results showed that the expression of LOXL-AS1 was significantly down-regulated in 177 paraffin-embedded HCC specimens and this decreased expression was correlated with poor survival in patients with HCC.

An analysis of clinical follow-up data and clinicopathological parameters demonstrated that low expression of LOXL1-AS1 was significantly correlated with T Stage ( $P=0.04$ ) and TNM classification ( $P = 0.04$ ) in patients with HCC, which predicted a poor prognosis.

The findings revealed the impact of LOXL1-AS1 on HCC and suggested its possible function as a tumor suppressor gene. The long non-coding gene LOXL1-AS1 can serve as a potential prognostic predictive marker for HCC patients in the future.

## Conclusion

In conclusion, our findings suggest that LOXL1-A1 and lncRNA is over-expressed in HCC cells and tissues. Furthermore, we present the first evidence that LOXL1-AS1 expression is down-regulated at both the mRNA and protein levels and that decreased expression is associated with a poor histological HCC grade. More importantly, over-expression of LOXL1-AS1 correlates significantly with favorable prognosis in HCC patients. Conversely, low LOXL1-AS1 expression is associated with aggressive tumor phenotypes and poor survival in HCC patients. Furthermore, over-expression can promote cell proliferation, migration, and apoptosis. Hence, its expression level could also be used to predict early cancer progression and metastasis in HCC patients.

Findings of this study suggest that LOXL1-AS1 could play a potentially critical role in the pathogenesis and progression of HCC. With more in-depth studies regarding LOXL1-AS1, it is a potentially promising biomarker for predicting clinical outcomes among HCC patients.

## Abbreviations

**ATCC:** American Type Culture Collection, **LncRNAs:** Long noncoding RNAs

**CCK-8:** Cell Counting Kit-8, **HCC:** Hepatocellular carcinoma ,**FISH:** Fluorescent in situ hybridization, **CDK2:** Cyclin-Dependent Kinase 2, **CDK4:** Cyclin-Dependent Kinase 4

**HCC:** Hepatocellular carcinoma, **qRT-PCR:** Quantitative Real time- , **SDS–PAGE:** Sodium dodecyl sulfate polyacrylamide gel electrophoresis, **GAPDH:** Glyceraldehyde-3-phosphate dehydrogenase

**ECL:** Enhanced Chemiluminescence, **TBST:** Tris-buffered saline with Tween 20

**NC:** Normal control, **OS:** Overall survival, **DFS:** Disease-free survival

## Declarations

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### *Authors contributions*

Cai XJ and Maher H, Pan Y, and Kwabena GO Conceived and Designed the Experiments; Maher H, Pan Y Performed the Experiments; Maher H,Pan Y, Kwabena GO and Zhang B Analyzed the Data; Cai XJ and Maher H and Pan Y and Lu JM and Wang YF wrote the paper. All authors discussed the results and revised the manuscript. All authors read and approved the final manuscript.

### *Availability of data and materials*

All data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### *Ethics approval and consent to participate*

Ethical approval was obtained from the Sir RunRun Shaw Hospital Research Ethics Committee, and written informed consent was obtained from each patient.

### *Consent for publication*

All authors have agreed to publish this manuscript.

### *Competing interests*

The authors declare that they have no competing interests..

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## Tables

**Table 1:** *Correlations between LOXL1-AS1 Expression and Clinicopathological Characteristics in HCC Patients.*

<i>Parameter</i>	<i>Low LOXL1-AS1(n=89)</i>	<i>High LOXL1-AS1(n=88)</i>	<i>P value</i>
Age(mean±SD,y)	65.88±1.43	62.93±1.11	0.11
Gender(M/F)	76/13	76/12	0.85
BMI(mean±SD,kg/m <sup>2</sup> )	23.31±0.33	23.45±0.36	0.77
Smoking history(Y/N)	33/56	45/43	0.06
Alcohol history(Y/N)	28/61	41/47	0.04*
Albumin	39.15±0.74	41.09±0.73	0.07
Viral hepatitis(Y/N)	69/20	72/16	0.48
Cirrhosis(Y/N)	64/25	66/22	0.64
AFP			0.3
≤200ng/ml	54	63	
>200ng/ml	33	24	
NA	2	1	
Tumor number (solitary/multiple)	85/4	85/4	1.00
Tumor size (mean±SD,cm)	4.96±0.37	4.33±0.29	0.18
T Stage			0.04*
I	58	68	
II	15	16	
III	1	0	
IV	15	4	
TNM Stage			0.04*
I	58	68	
II	16	16	
III	12	2	
IV	3	2	

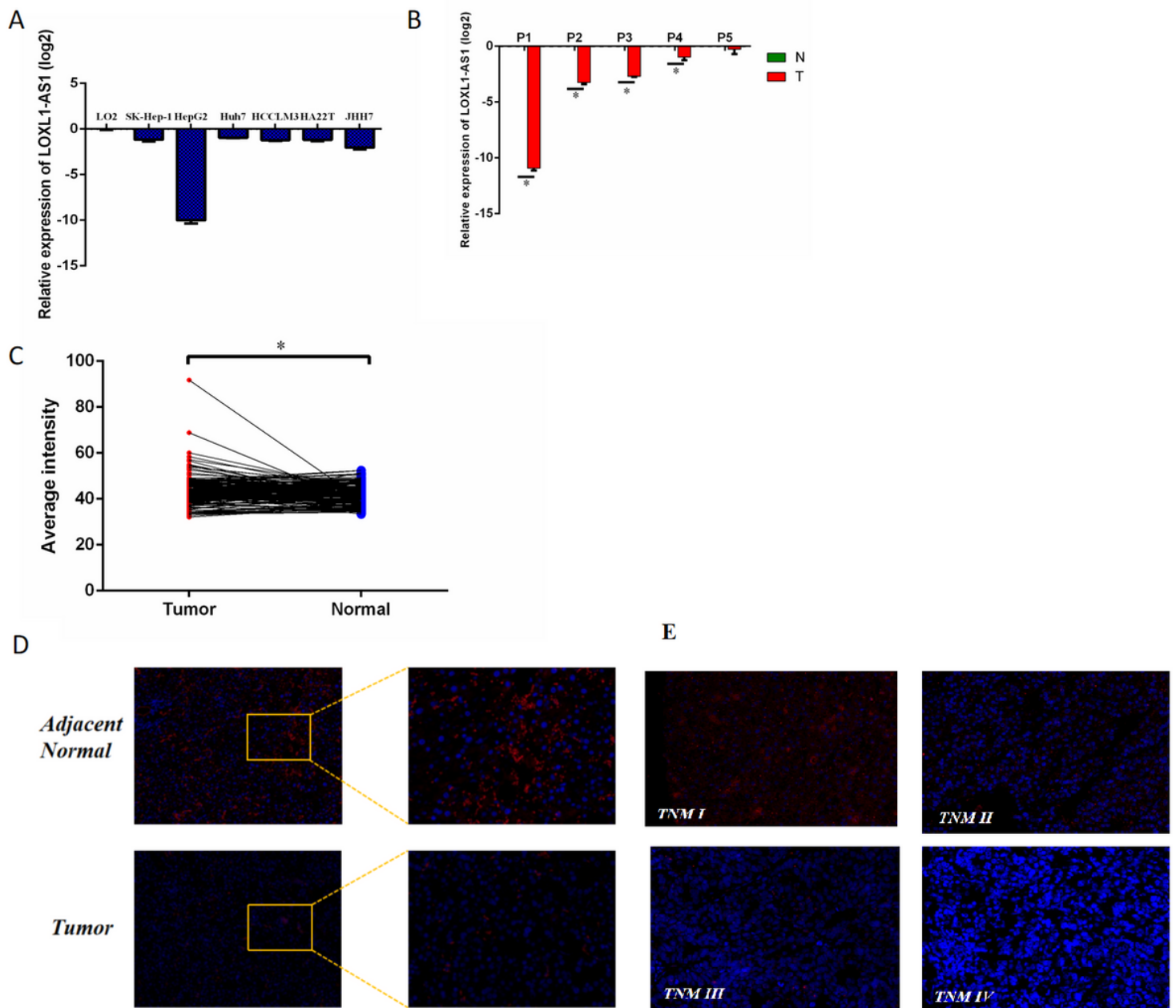
**Table 2:** Univariate and Multivariate analysis of various Potential Prognostic Factors in HCC Patients

<i>Parameter</i>	<i>Univariate analysis</i>			<i>Multivariate analysis</i>		
	<i>HR</i>	<i>95% (CI)</i>	<i>P-Value</i>	<i>HR</i>	<i>95% (CI)</i>	<i>P-Value</i>
Age (mean±SD,y)	0.99	0.97 - 1	0.19			
Gender (M/F)	1.9	0.88 - 4.2	0.1			
BMI (mean±SD,kg/m <sup>2</sup> )	0.99	0.92 - 1.1	0.83			
Smoking history (Y/N)	1.6	1 - 2.6	0.033*	1.5	0.91 - 2.4	0.12
Alcohol history (Y/N)	0.99	0.68 - 1.7	0.72			
Albumin (mean±SD)	0.96	0.93 - 1	0.066	0.97	0.93 - 1	0.16
Viral hepatitis (Y/N)	1.7	0.9 - 3.1	0.1	1.9	0.97 - 3.6	0.062
Cirrhosis (Y/N)	NA	NA	NA			
AFP (≤200ng/≤200ng/ml)	0.9	0.54 - 1.5	0.68			
Tumor number (solitary/multiple)	2.4	1 - 5.4	0.04*	0.74	0.23 - 2.4	0.62
Tumor size (mean±SD,cm)	1.1	1 - 1.2	0.046*	1.1	1 - 1.2	0.031*
T Stage ( I/II/III/IV)	1.5	1.2 - 1.8	<0.001*	0.44	0.18 - 1.1	0.78
TNM Stage ( I/II/III/IV)	1.9	1.4 - 2.4	< 0.001*	4.6	1.4 - 15	0.0099*

*\*P < 0.05. HR: Hazard ratio. CI: Confidence interval. AFP: alpha-fetoprotein*

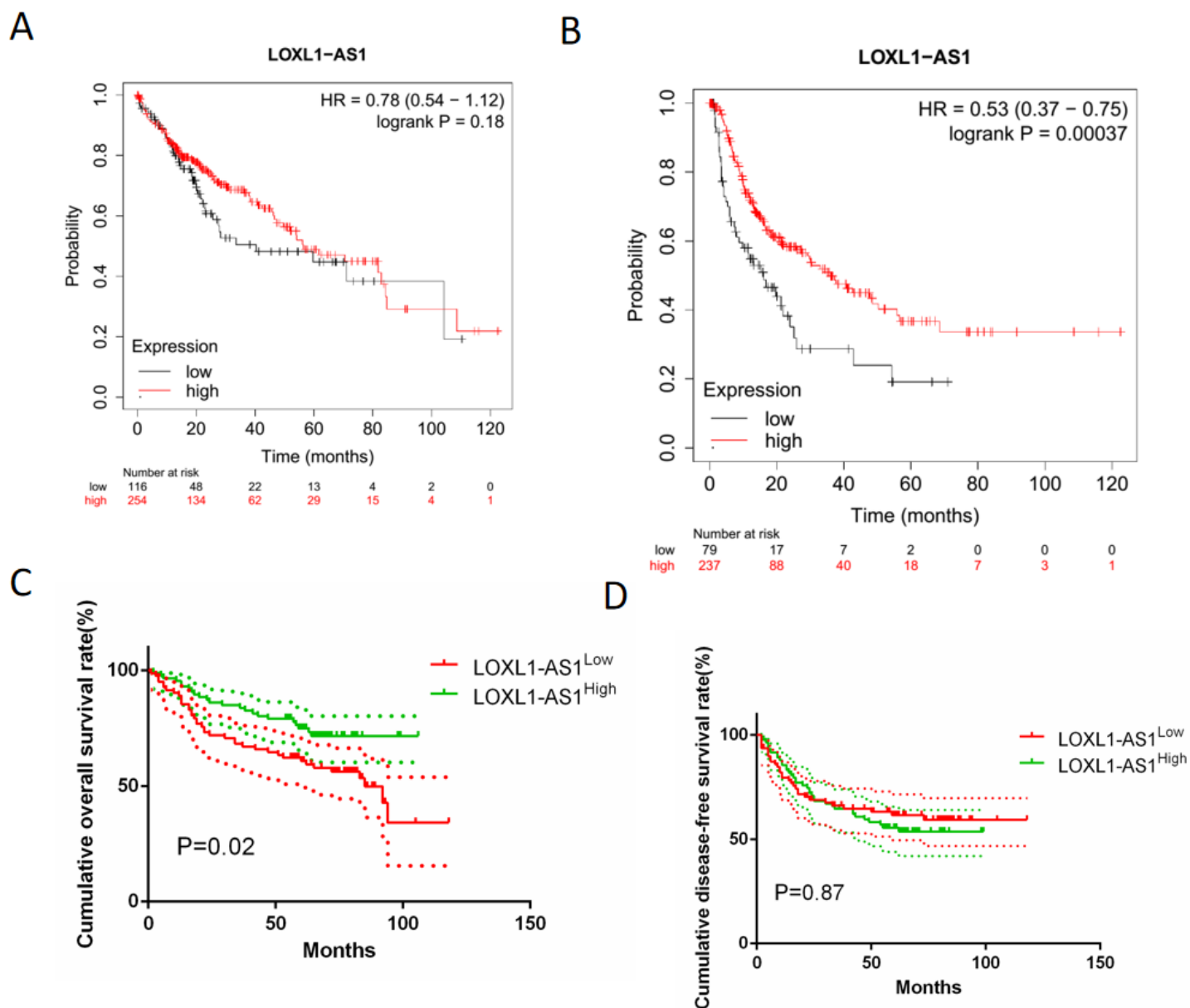
*TNM, T, tumor; N, lymph node; M, distant metastasis.*

## Figures



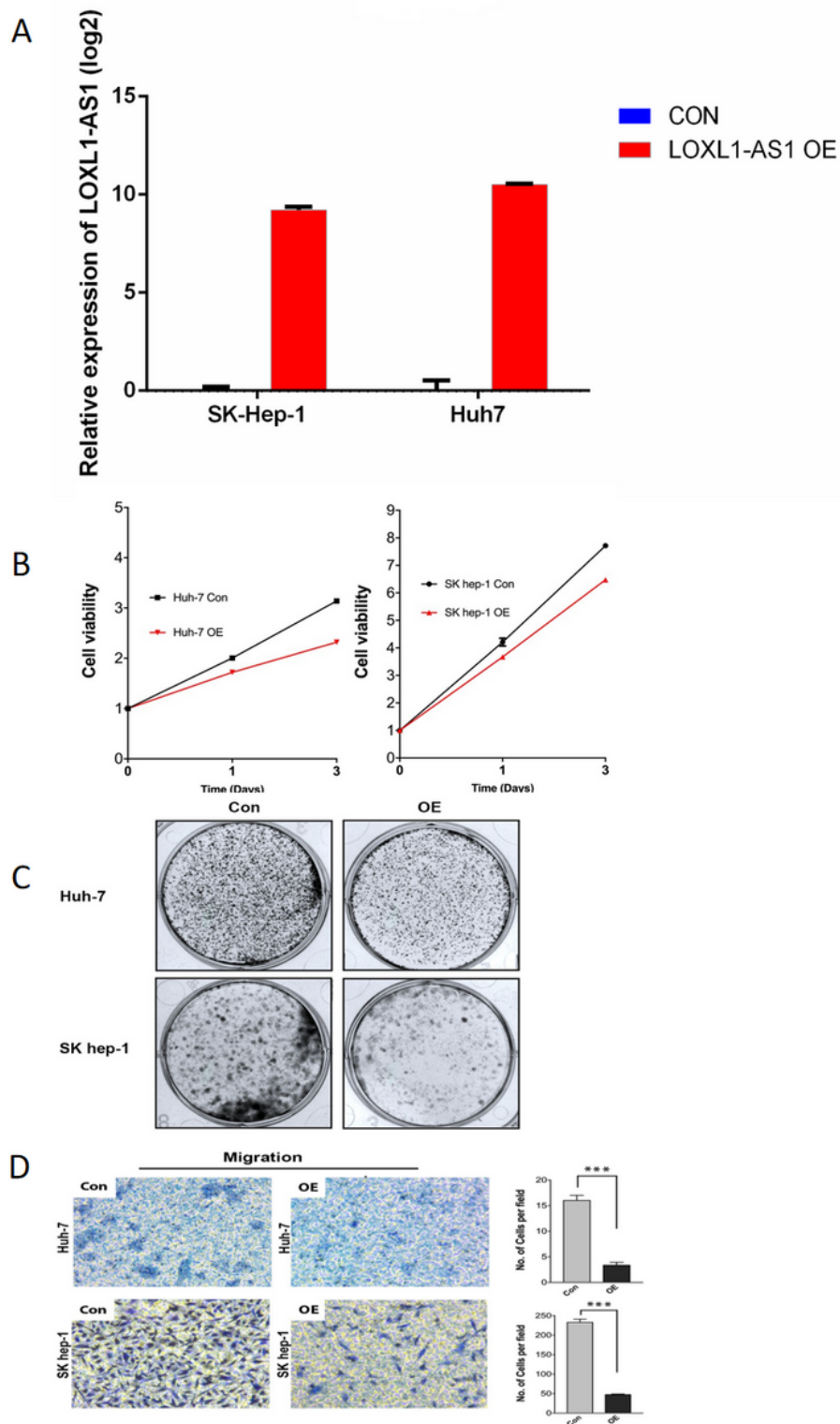
**Figure 1**

Aberrant expression of LOXL1-AS1 is Down-regulated in HCC tissues and predicts poor prognosis. (A) Relative LOXL1-AS1 expression in paired HCC tissues and adjacent tissues. (B) Relative expression of LOXL1-AS1 in different HCC cell lines. (C) LOXL1-AS1 expression levels in 90 paired HCC tissues and adjacent non-cancerous tissues was analyzed by qRT-PCR. (D) Representative FISH images of LOXL1-AS1 expression in HCC tumor tissues. (E) Different TNM stages (blue, DAPI; red, positive staining)



**Figure 2**

The prognostic significance of Loxl1-As1 in HCC patients. (A-B) The overall survival and post-progression survival of HCC patients with high and low expression levels of LOXL1-AS1 analyzed by LOXL1-AS1 based on TCGA database. (C) Kaplan-Meier analysis shows elevated expression of LOXL1-AS1 is associated with overall survival (OS) in HCC patients. (D) The relationship between expression of LOXL1-AS1 and recurrence-free survival of HCC patients in SRRS hospital. Kaplan-Meier analysis (\*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001) .

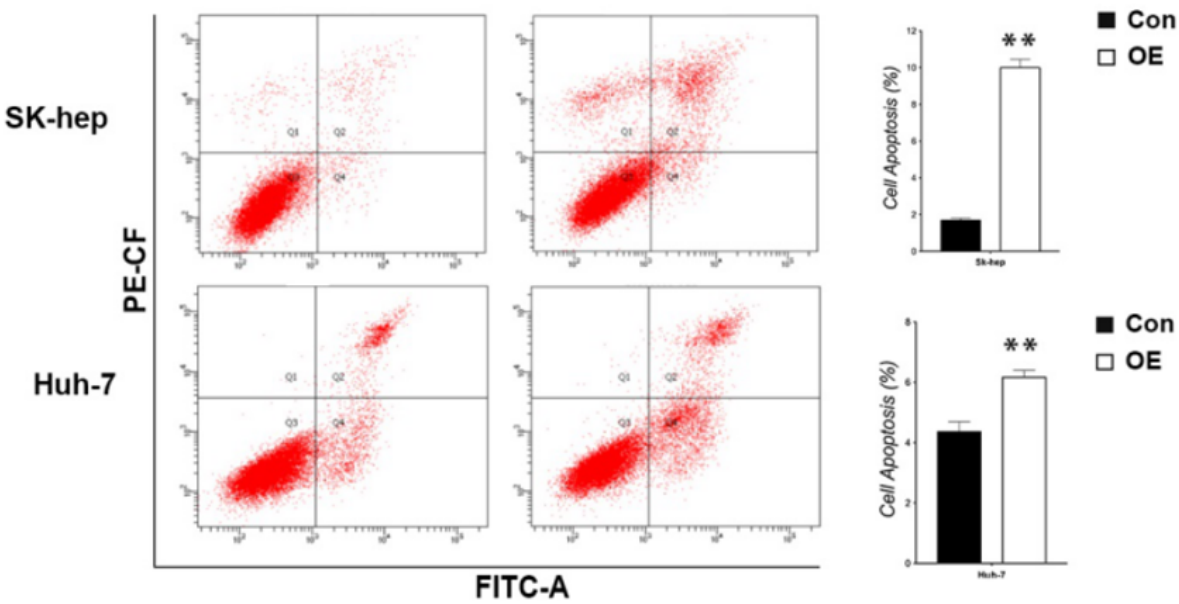


**Figure 3**

LOXL-AS1 Promotes the proliferation, migration, and cell apoptosis of HCC in vitro Transfection with LOXL1-AS1 significantly increases the expression of LOXL1-AS1. (A) Relative LOXL1-AS1 expression in HCC cells that overexpressed LOXL1-AS1 in Sk-Hep1 and Huh7 cells. (B) The proliferation of cells was detected, CCK-8 assays were used to examine the proliferation of Sk-Hep1 and Huh7 cells at 0, 24, 48, 72, hours post-transfection with LOXL1-AS1. (C) Colony formation assays. (D) Transwell assays showing

downregulated LOXL1-AS1 significantly decreased the migrated number of Sk-Hep1 and Huh7 cells through the membrane compared to the control group. (\*P<0.05, \*\*P < 0.01, \*\*\*P < 0.001).

A



B

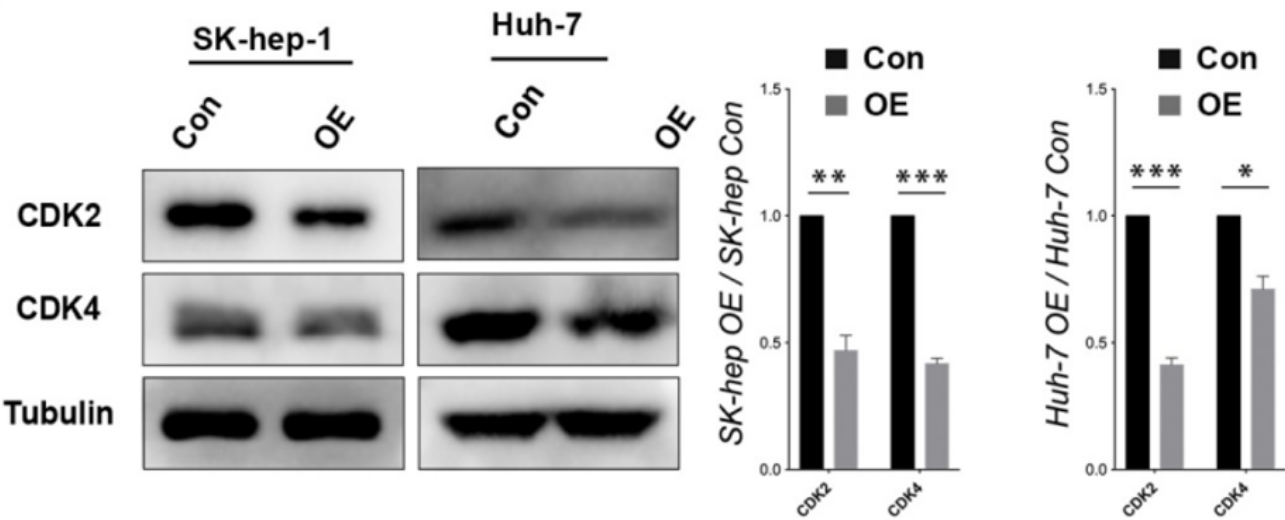
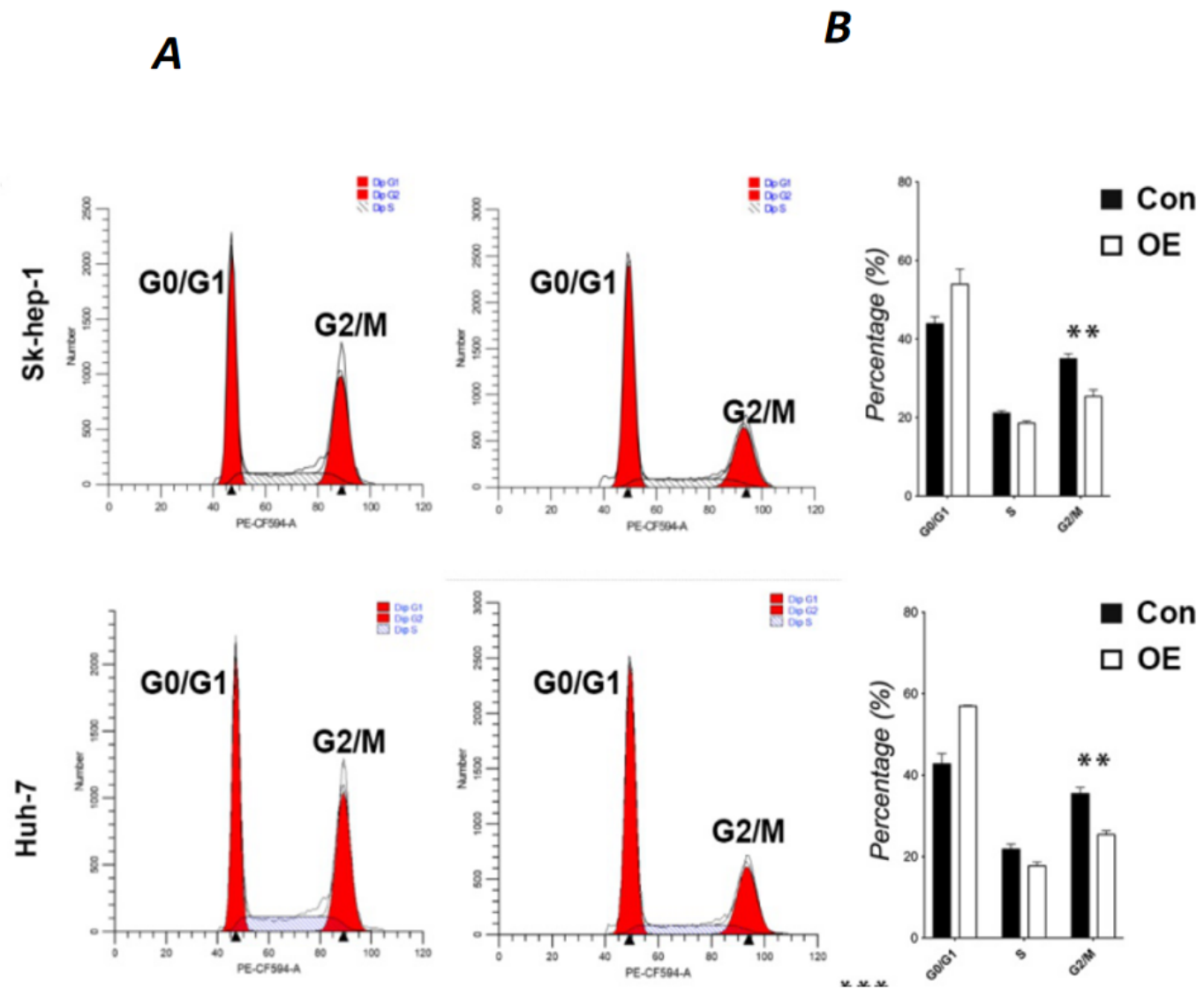


Figure 4

Silencing CDK2 promoted cell apoptosis in HCC cells. (A) Cell apoptosis in Sk-hep1 or Huh7 cells, with the group divided into control, OE group, CON group. Cell apoptosis in Sk-hep1 and Huh7 cells determined by flow cytometry.(B) Western blotting was applied to assess apoptosis- and autophagy-

related protein levels. All data are presented as presented as the mean  $\pm$  S.D.from two independent experiments. The p-values represent comparisons between groups \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .



**Figure 5**

LOXL1-AS1 arrests cell cycle at the S phase in HCC. (A) Cell cycle progression analyzed in both Sk-hep1 cells and Huh7 cells that were pretreated with or without specific control against LOXL1-AS1. (B) Cell proportions in the G0/G1, S, and G2/M phases were calculated in Sk-hep1 cells and Huh7 cells, Respectively. It was found that cells were proportionally accumulated in the S phase while cells in the G2/M phase and results were significantly decreased in both cell lines. (\*\* $P < 0.05$  ).

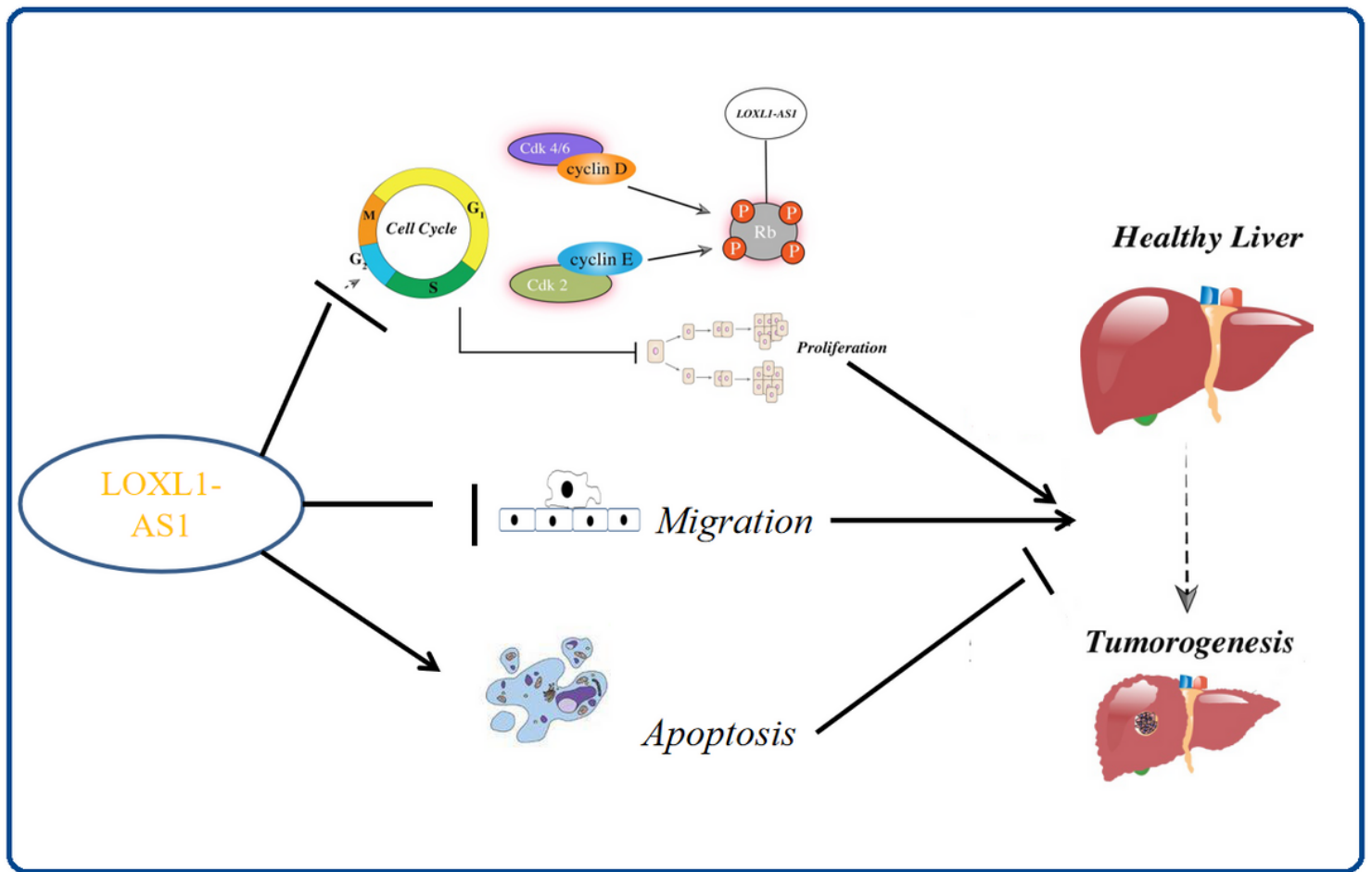


Figure 6

Schematic summary of the role of LncRNA LOXL1-AS1 in the regulation of HCC progression.