# Promotion of LncRNA HOXA11-AS on the proliferation of hepatocellular carcinoma by regulating the expression of LATS1

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**Abstract. – OBJECTIVE:** To investigate the expression levels of lncRNA HOXA11-AS in HCC tissues and cells, and to explore its biological role in the development and progression of HCC.

**PATIENTS AND METHODS:** We detected the relative expression level of HOXA11-AS in 72 HCC tissues and cells by the real-time quantitative PCR (qRT-PCR) assay. After interference with HOXA11-AS expression in HCC cells, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), clone formation, flow cytometry and an established nude mice transplanted tumor model were used to detect the biological behavior of HCC cells. qRT-PCR and Western blotting assays were used to detect the expression level of large tumor suppressor kinases 1 (LATS1). The subcellular localization of HOXA11-AS in HCC was detected by separating nuclei from the cytoplasm. The molecular mechanism of HOXA11-AS was regulated by ribonucleoprotein immunoprecipitation-microarray (RIP-Chip) experiments.

**RESULTS:** qRT-PCR assays showed that HOXA11-AS was relatively highly expressed in HCC tissues and cells. In vivo and in vitro experiments showed that HOXA11-AS could inhibit the proliferation of HCC cells, promote their apoptosis and retard the cell cycle progression from G1 to G0 phase. qRT-PCR and Western blotting assays results showed that LATS1 genes were the downstream target genes of HOXA11-AS. RIP and CHIP experiments showed that HOXA11-AS inhibited the expression of LATS1 genes by binding enhancer of zeste homolog 2 (EZH2) proteins.

**CONCLUSIONS:** HOXA11-AS inhibited the malignant transcription of the LATS1 genes and promoted the malignant proliferation of HCC cells. Interactions among HOXA11-AS, PRC2, and LATS1 may provide a new target for the treatment of HCC.

Key Words:
lncRNA HOXA11-AS, Hepatocellular carcinoma, HCC, PRC2, LATS1, Proliferation.

## Introduction

Primary hepatocellular carcinoma (HCC) is the main type of primary liver cancers, accounting for 85%-90%[1]. The incidence rate of primary HCC ranks fifth in global malignant tumors; the mortality rate ranks second. The molecular mechanism of the occurrence and development of HCC has not yet been fully elucidated. Therefore, to explore regulatory factors of the occurrence and development of HCC as well as its detailed molecular mechanism provides a theoretical basis for screening new anti-tumor target spot, which is of great significance for the treatment of HCC.

lncRNA HOXA11-AS is a member of the homeobox (HOX) family on chromosome 7p15.2 with a total length of 3885bp[2]. It has been reported in the literature that abnormal expression of HOXA1-AS plays an important role in the changing process of tumor biological behavior. In gastric carcinoma tissues and cells, highly expressed lncRNA HOXA11-AS binds the apparent complex DNA (cytosine-5)-methyltransferase 1 (DNMT1) and lysine-specific histone demethylase 1 (LSD1) to mediate methylation and demethylation of target genes at promoter regions, thus promoting the development of gastric carcinoma[3]. Kim et al[4] found that high expression of HOXA11-AS in cervical carcinoma can promote tumor cell proliferation as well as the invasion and metastasis of cervical carcinoma cells by affecting EMT. Our research group, for the first time, found that HOXA11-AS was relatively highly expressed in HCC, and interfering with the expression of HOXA11-AS could inhibit the proliferation of HCC cells.

PRC2 (polycomb repressive complex 2) is an apparent modification complex in the polycomb family proteins, which contains three core subunits: EZH2, SUZ12 and embryonic ectoderm



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development (EED). The complex has histone methyltransferase activity and can inhibit the transcription of target genes by mediating the methylation of the histone H3K27 locus[5,6]. It is reported in the literature that lncRNA can regulate the formation of the malignant phenotype of tumors by carrying PRC2. As shown by Kong et al[7], highly expressed lncRNA PVT1 in gastric carcinoma can bind PRC2 to inhibit the transcription of downstream target substrates P15 and P16, thus promoting the proliferation of gastric carcinoma cells. In oral squamous cell carcinoma, lncRNA HOTAIR can mediate E-cadherin histone methylation at promoter regions through EZH2, thus promoting invasion and metastasis of tumor cells[8]. However, we found for the first time in our study that HOXA11-AS could bind PRC2's core subunits EZH2 and carry them to the promoter region of LATS1 to inhibit its transcription.

In this work, biological functions of HOXA11-AS on HCC were studied through *in vivo* and *in vitro* researches. *In vivo* and *in vitro* experimental results showed that in HCC cells, HOXA11-AS could inhibit LATS1 transcription by carrying the apparent complex PRC2, thus promoting HCC cell proliferation and inhibiting apoptosis.

## Materials and Methods

### Culture of Tissue Specimens and Cells

The patients untreated with chemotherapy, radiotherapy, and molecular targeted therapy were selected as the study subjects. This study was approved by the Ethics Committee of Fuzhou General Hospital. Signed written informed consents were obtained from all participants before the study. HCC and its adjacent tissue specimens excised from 72 patients by surgery in our hospital were collected. Those patients were pathologically diagnosed as HCC, and the specimens excised from the patients after surgery were quickly put into liquid nitrogen at -180°C for preservation. HCC cell lines HepG2, Hep3B, MHCC-97H, and normal liver epithelial cell line L02 were purchased from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. Cells were cultured in Dulbecco modified Eagle medium (DMEM) (Gibco/Brl, Grand Island, NY, USA) containing 10% fetal bovine serum (10% FBS), 100 U/mL double anti-penicillin and 100 mg/mL streptomycin (Invitrogen Co., Carlsbad, CA, USA). Then the cells were conventionally cultured in an incubator with 5% $CO_2$ at the constant temperature of 37°C. The medium was replaced with a fresh one every 3 days. When the cell fusion degree reached 80%-90%, cell passage would begin, and at that time, the well-functioning cells were selected for experiments.

### Synthesis of siRNA and qRT-PCR Premiers Interfering with HOXA11-AS

The effective interference sequence of HOXA11-AS was listed in Table I, the control sequence was si-NC: 5'-CCTATCTGGTCAACACGTATT-3'. GAPDH-F: 5'-ATAGCACAGCCTGGATAGC AACGTAC-3' and GAPDH-R: 5'-CACCTTC TACAATGAGCTGCGTGTG-3'. The above sequences were synthesized by Invitrogen Co. (Carlsbad, CA, USA).

### Detection of the Expression of HOXA11-AS by Using Real-time Fluorescence Quantitative PCR

Total RNA was extracted from HCC and corresponding adjacent tissues by Trizol kits, RNA concentration was measured by an ultraviolet spectrophotometer, and RNA quality was determined by agarose gel electrophoresis. cRNA was synthesized according to the described procedures of PrimeScript™ RT Master Mix (Perfect Real Time) kits for further real-time fluorescence quantitative PCR. The reaction system of qRT-PCR Mix (20 μL): 10 μL SYBR qPCR Mix, 0.8 μL (10 μmol/L) upstream and downstream primers, respectively, 2 μL cDNA products, 0.4 μL 50×ROX reference dye, and water without RNase was made up to 20 μL. Reaction conditions: after 1 min of pre-denaturation at 95°C, the cells were pre-denatured at 95°C for 30 s and at 60°C for 40 s, and the cycle was repeated for 40 times. Three parallel wells were designed in the experiment, and all samples were repeatedly tested for three times. The relative expression level of target genes was expressed by -ΔCt and $2^{-\Delta\Delta Ct}$ using the relative quantification method, and all the sample operations were carried out on ice.

### Detection of the Proliferation of HCC Cell Lines by MTT Assay and Clone Formation Assay

The cell lines were transfected into a 96-well plate at a cell density of $3\times10^3$/well and transfected by siRNA. 20 μL methyl thiazolyl tetrazolium (MTT) solution (1.55 g/L) was added to each well at 0 h, 24 h, 48 h, 72 h and 96 h, and after the incubation at 37°C for 4 h, 150 μL dimethyl sulfoxide

(DMSO) was added to each well. The absorbance values were read, and the cell growth curve was drawn. The cells in the experiment group and the treatment group were seeded in a six-well plate at 1000 cells per well. After 4 days, culture solution on the medium was changed, and the cells were subjected to formaldehyde fixation and crystal violet staining after 14 days.

### Detection of the Changes in Cycles and Apoptosis by Flow Cytometry

Concentrations of cells in the logarithmic growth phase were adjusted to $3\times10^5$/mL, and they were inoculated into a 6-well plate at 2 mL/well. After 48 h transfection, the mice were divided into the experiment group and the control group. The cells in the two groups were collected after another 48 h-culture. The cells were recollected after they were washed by phosphate buffered saline (PBS). Then, double staining was performed for cells with Annexin V/PI (propidium iodide) and they were kept in the dark place for 10 min. The apoptosis rates were measured by flow cytometry. For the other group, cells were collected by the same method and under the same conditions. These cells were resuspended with 75% precooling ethanol and were fixed at -20°C overnight. The cells were divided into G1/G0 phase, S phase, and G2/M phase, respectively, when the intracellular DNA contents were detected by flow cytometry and PI staining. The percentage of each phase could be calculated by special software.

### Detection of the Changes in the Expression Level of LATS1 by Western Blotting

Cells of the experiment group and the control group were collected, and the cell lysate was added for the extraction of total protein. Then, the total protein was quantified by Bradford protein assay. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed, and the proteins extracted by electrophoresis were transferred to polyvinylidene difluoride (PVDF) membranes and kept on a confined space with 5% skimmed milk. 1:1000 rabbit anti-human LATS1 antibodies were added, respectively, and these cells were kept overnight at 4°C. The membranes were washed with Tris-buffered saline Tween-20 (TBST-20) solution for 3 times for 10 min each time. Then horseradish peroxidase conjugated anti-mouse or anti-rabbit antibodies (1:2000 diluent) were added and incubated at 37°C for 1 h. The membranes were rinsed with TBST solution for 3 times for 10 min each time and, then, washed with Tris-buffered saline (TBS) for 10 min. The color developing was detected from luminous compressed tablets, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was regarded as the internal reference.

### Nude Mice Tumorigenesis Experiment

4-5-week-old male BALB/c mice were purchased from the Xiamen University model animal center, and HepG2 cells were cultured. The cells were transfected with sh-HOXA11-AS or were not transfected to form a blank control group, respectively. $1\times10^7$ cells of the experiment group and the control group were suspended in 0.1 mL serum-free DMEM, and subcutaneous injection was performed to establish a tumor formation model of nude mice for the observation of the tumor formation in mice *in vitro*.

### Experiments of Separating Nuclei from Cytoplasm

We used the mirVana™ PARIS™ Kits (Ambion, Shanghai) and strictly operated in accordance with the instructions and referred to the instructions for details.

### RNA Binding Protein Immunoprecipitation

We used RIP kits from Millipore (Billerica, MA, USA) and strictly operated in accordance with the instructions: 1. Lysis products and immunoprecipitated beads were prepared; 2. Target RNA and protein immunoprecipitation. 3. Purified RNA; 4. Immunoprecipitated RNA was analyzed.

### Chromatin Immunoprecipitation

We used EZChIP (chromatin immunoprecipitation) kits from Millipore (Billerica, MA, USA) and strictly operated in accordance with the instructions: 1. Formaldehyde cross-linked cells; 2. Ultrasonic chromatin fragmentation; 3. Target antibodies and chromatin co-precipitation; 4. DNA samples were recovered and tested.

### Immunohistochemical Method

Immunohistochemical method was used to detect the expression level of LATS1 in the specimens. Light brown or brown area was regarded as a positive result, and a standardization analysis was conducted for the detection of results by using imaging mass spectrometry (IMS) cell image analysis system and medical image analysis software[9].

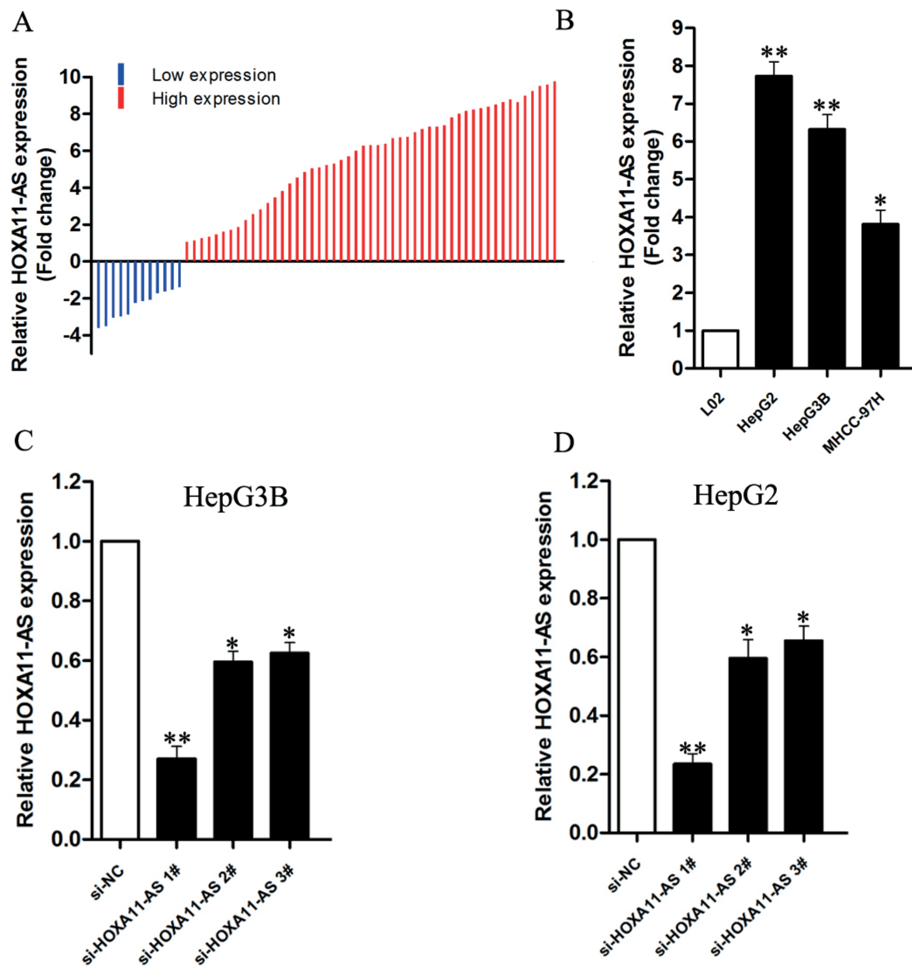


**Figure 1.** The expression level of lncRNA HOXA11-AS in HCC tissues and cells. ***A,*** Relative expression levels of 72 pairs of HCC and adjacent tissues are detected by qRT-PCR assays, in which the expression levels of 60 cases are up-regulated and those of 12 cases are down-regulated. ***B,*** qRT-PCR assays are used to detect the relative expression levels of HCC cells compared with normal liver cells. ***C*** and ***D,*** Specific interference sequences of HOXA11-AS are designed and synthesized, which transiently transfect into HCC cells. qRT-PCR assays are used to detect interference efficiency. ($^{**}$indicates $p<0.01$, and $^{*}$indicates $p<0.05$).

### *Statistical Analysis*

All experiments were repeated 3 times and were analyzed using Statistical Product and Service Solutions (SPSS Inc., Chicago, IL, USA) 15.0. Data were expressed as mean ± standard deviation, and tested using the *t*-test. $p<0.05$ represented that the difference was statistically significant.

## Results

### *The Expression Level of HOXA11-AS in HCC Tissues and Cells*

To detect the expression levels of HOXA11-AS in HCC and adjacent tissues, relative expression levels of HOXA11-AS in 72 pairs of HCC and adjacent tissues were detected by qRT-PCR assays. The results showed that HOXA11-AS expression level was significantly up-regulated in 75.32% (60 of 72, fold ≥1) HCC tissues compared with adjacent tissues (Figure 1A). The expression levels of HOXA11-AS in three HCC cell lines (HepG2, Hep3B, MHCC-97H) and normal liver cell line (L02) were detected by qRT-PCR assays. The results showed that the expression level of HOXA11-AS was significantly up-regulated in HCC cells (Figure 1B). We selected two cell lines with the most obvious up-regulation signs HepG2 and Hep3B as model cells. To study biological functions of HOXA11-AS, the specific interference sequences of HOXA11-AS were designed and synthesized. The interference sequences and control sequen-

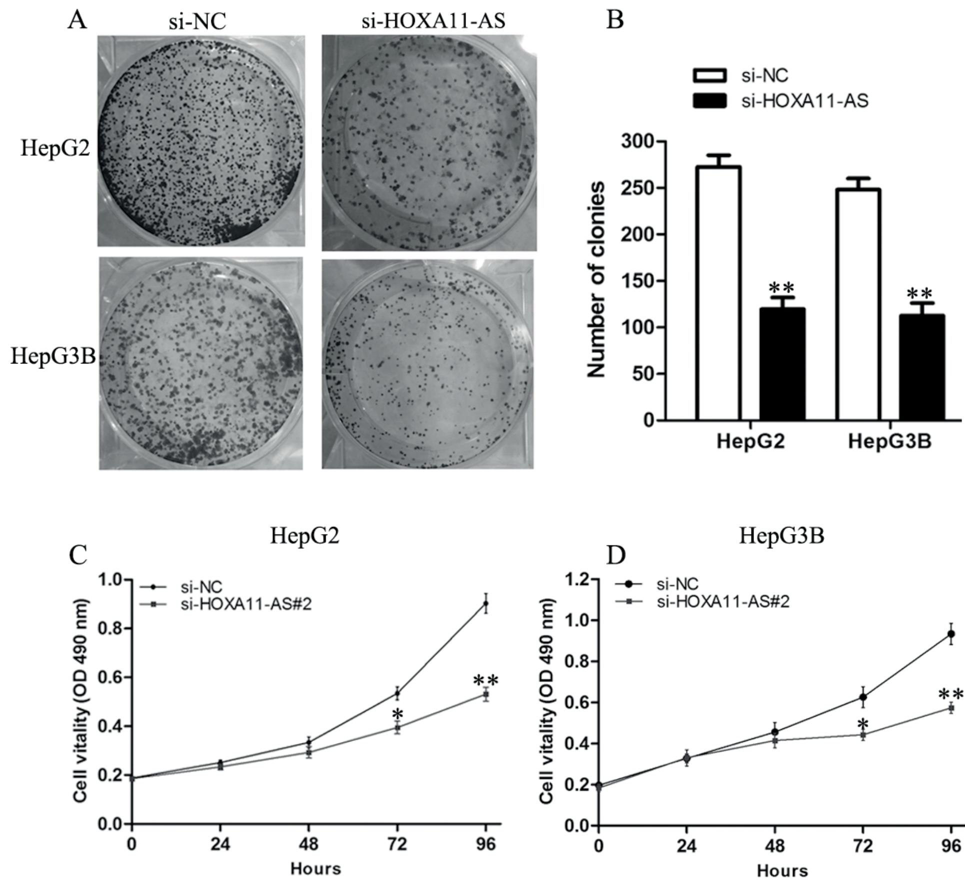


**Figure 2.** Effects of HOXA11-AS on proliferation ability of HCC cells. ***A*** and ***B,*** Clone formation assays are used to detect that in HCC cells, the cell proliferation ability is inhibited after the interference with HOXA11-AS expression. ***C*** and ***D,*** MTT assay results show that the cell viability is decreased after the interference with the expression of HOXA11-AS. (**indicates $p<0.01$, and *indicates $p<0.05$).

ces of target genes were transiently transfected into HCC cell lines by liposome lip2000. After 48 hours, we detected the interference rate of HOXA11-AS and selected 1# sequence for subsequent experiments (Figure 1C, D).

### Research on Biological Functions of HOXA11-AS Based on in vitro Experiments

First, we investigated effects of HOXA11-AS on the proliferation of HCC cells by MTT assay and clone formation experiments. MTT assay results showed that the proliferation activity of cells in the group transfected with HOXA11-AS was significantly inhibited compared with the control group (Figure 2C, D); and clone formation experiment results were consistent with MTT assay results (Figure 2A, B). To study whether promoting the proliferation of hepatocarcinoma cells influenced processes of cell cycles, interference sequences of HOXA11-AS were transiently transfected into HepG2 and Hep3B cells. Flow cytometry (FCM) results showed that compared with the transfected control group, cell cycle progressions of HepG2 and Hep3B cells of transfected HOXA11-AS interference sequences were retarded from G1 to G0 phase (Figure 3C, D). Then, we investigated the effects of HOXA11-AS on the apoptosis of HCC cells. These cells were treated with the same method, and changes in apoptosis were detected by FCM. Results showed that apoptosis rates of HepG2 and Hep3B cells were significantly increased after interference with HOXA11-AS compared with the control group (Figure 3A, B).

### Research on the effect of HOXA11-AS on tumor Formation capacity of HCC Based on in vivo Experiments

To investigate the role of HOXA11-AS *in vivo*, we selected the HepG2 cell line with high transfection efficiency. HepG2 cells were tran-

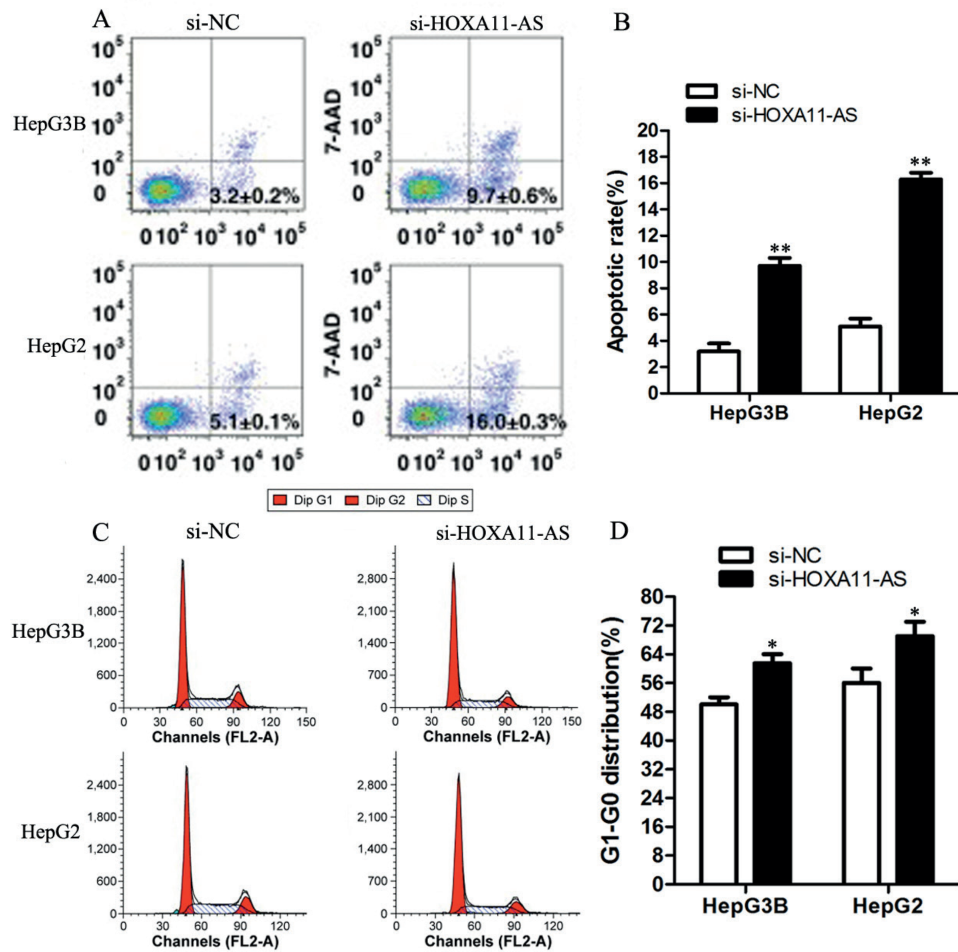


**Figure 3.** Effects of HOXA11-AS on the biological function of HCC cells. ***A*** and ***B*,** After the interference with the expression of HOXA11-AS in HCC cells, the apoptosis rate is detected to be increased by flow cytometry. ***C*** and ***D*,** After the interference with the expression of HOXA11-AS in HCC cells, cell cycle progression is detected to be retarded in G1-G0 phase by flow cytometry. (**indicates $p<0.01$, and *indicates $p<0.05$).

sfected with sh-HOXA11-AS or control sequences and, then, injected into the armpits of nude mice to establish a transplanted tumor model. 18 days after the subcutaneous injection, the nude mice were killed and the specimens of subcutaneous tumor tissues were taken. As shown in Figure 4A, the volume of transplanted tumor derived from transfected sh-HOXA11-AS cells was significantly reduced compared with the control group, and the weight of the tumor in the experiment group was significantly lower than that in the control group (Figure 4C). We extracted total RNA from part of tumor tissues and the relative expression levels of HOXA11-AS in tumor tissues by qRT-PCR assays. The results showed that the expression level of HOXA11-AS in the transplanted tumor from transfected sh-HOXA11-AS cells was significantly lower than that in the control group (Figure 4D). Then, we fixed the transplanted tumor, and the immunohistochemistry was conducted for slices. Afterwards, hematoxylin and eosin (HE) staining and proliferative cell-associated nuclear antigen staining were performed. HE staining results showed that a transplanted tumor model of nude mice was successfully constructed, and Ki67 staining results showed that the positive rate of Ki67 in transplanted tumors from transfected sh-HOXA11-AS cells was significantly lower than that in the control group (Figure 4B), which indicated that the tumorigenic ability of the cells in the experiment group was inhibited. Above *in vivo* experimental results confirmed that decreased HOXA11-AS could significantly inhibit tumor formation ability of HCC cells.

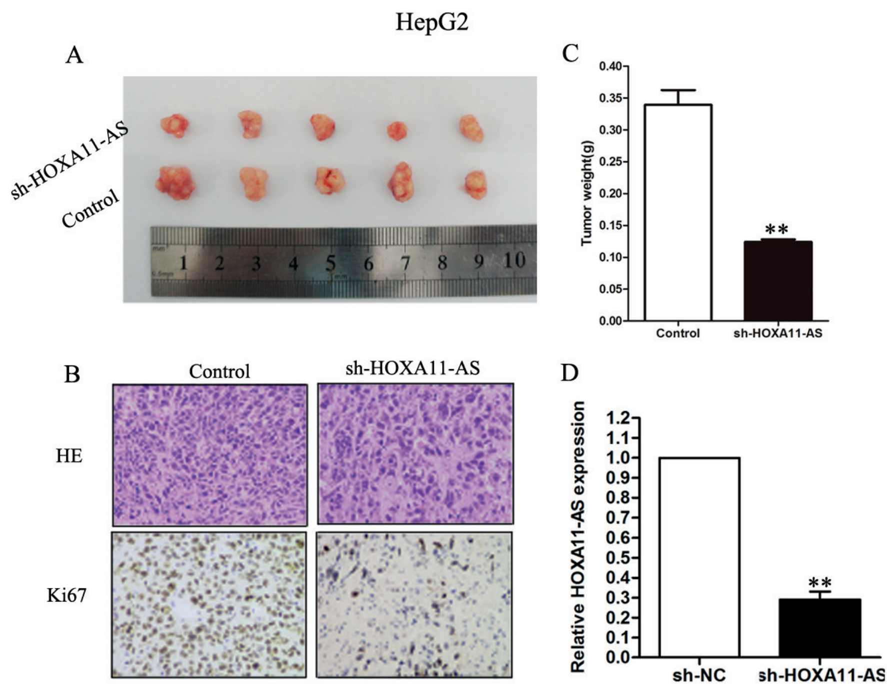


**Figure 4.** *In vivo* research on the effects of the interference with HOXA11-AS on tumor formation ability of tumor cells. ***A,*** Empty vectors and sh-HOXA11-AS are transfected into HepG2 cells and injected into male nude mice (n=6), respectively. 18 days after the transfection, we kill nude mice, remove their tumors and take pictures. ***B,*** We slice up tumor tissues and perform HE staining and Ki67 immunohistochemistry for them. ***C,*** We weigh the weight of transplanted tumors. ***D,*** qRT-PCR assays are used to detect the relative expression level of HOXA11-AS in transplanted tumors. (**indicates $p<0.01$, and *indicates $p<0.05$).

### *Proliferation Promotion of HCC cells Achieved by HOXA11-AS Based on the Regulation of LATS1*

After the interference with HepG2 and Hep3B with HOXA11-AS, we found from qRT-PCR and Western blot assays that the expression levels of LATS1 mRNA and proteins were significantly up-regulated (Figure 5A, B) and, then, we focused on the regulation of LATS1 molecular mechanism by HOXA11-AS. First, we conducted detection by separating nuclei from cytoplasm and found that subcellular localization of HOXA11-AS in HCC cells was predominantly located in the nucleus (Figure 5E). We detected the enrichment level of HOXA11-AS with EZH2 by RIP experiments. The results showed that HOXA11-AS could directly bind EZH2 in HCC cells (Figure 6A, B). We interfered with EZH2 in HepG2 cells, respectively, and qRT-PCR and Western blot assay results showed that the expression level of LATS1 was up-regulated (Figure 5C, D). However, does HOXA11-AS directly carry PRC2 to the gene promoter region of LATS1? Furthermore, we conducted chromatin immunoprecipitation (CHIP) experiments and found that HOXA11-AS could carry the methylation of histone H3K27 locus in the promoter region of EZH2 and LATS1 genes so as to inhibit the transcription of genes (Figure 6C, D).

## Discussion

The occurrence of HCC is a multivariate and multistage process, and typical studies[10,11] have shown that there are multiple genes and signaling pathways that have been developed or inhibited throughout the occurrence and development of HCC. It is believed that there exist proto oncogene activation and somatic mutations of tumor suppressor genes in HCC. However, with the deepening of the study of HCC, researchers[12] found that epigenetic changes also play an important role in the occurrence and development process of HCC, and changes in lncRNA-mediated epigenetics have been confirmed.

In recent years, high-throughput sequencing and molecular biology have been developed rapidly, and it has been found that lncRNA is abnormally expressed in tumors and plays a role similar

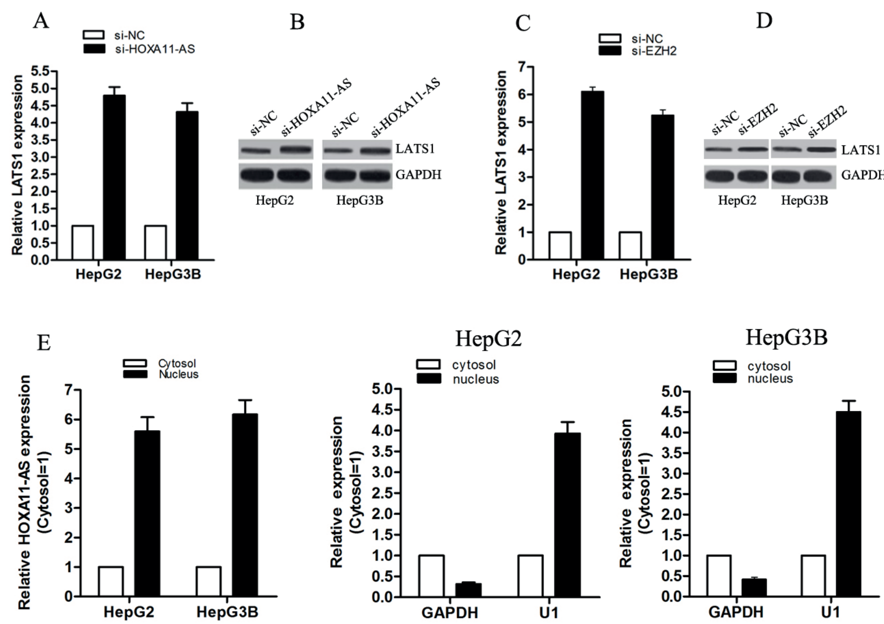


**Figure 5.** The ability of HOXA11-AS to inhibit LATS1 expression. ***A,*** qRT-PCR assays are used to detect LATS1 mRNA expression level after the interference with HOXA11-AS in HepG2 and HepG3B cells. ***B,*** The expression levels of LATS1 proteins in HepG2 and HepG3B cells are detected by Western blotting assays. ***C*** and ***D,*** The expression levels of LATS2 mRNA and proteins in HepG2 and HepG3B cells after the interference with EZH2 by qRT-PCR and Western blotting assays. ***E,*** The expression levels of PVT1 in A549 and PC-9 cells are detected by qRT-PCR assays. GAPDH is regarded as a reference in cytoplasm and U6 in nucleus. ($^{**}$indicates $p$<0.01, and $^{*}$indicates $p$<0.05).

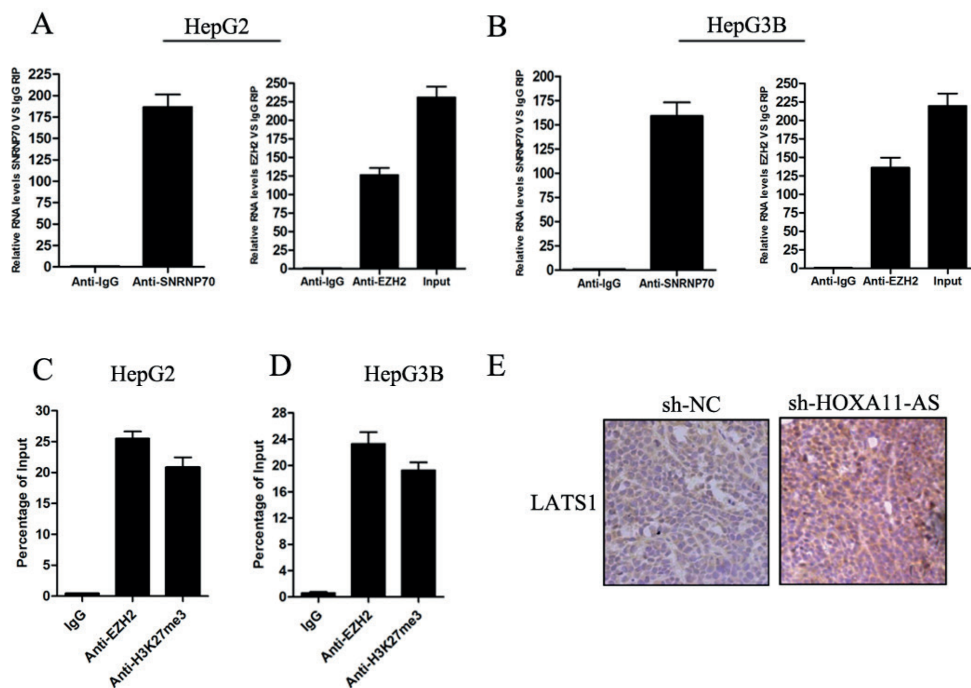


**Figure 6.** The ability of HOXA11-AS to raise PRC2 to LATS1 promoter regions to inhibit its transcription. ***A*** and ***B,*** Anti-EZH2 is extracted from HepG2 and HepG3B cell lines and immunoprecipitation is performed with IgG or 10% input RNA. qRT-PCR assays are used to detect RNA levels in coprecipitate. The level of EZH2-enriched HOXA11-AS is expressed as the multiple of IgG-enriched HOXA11-AS and the multiple of IgG-enriched U1 is taken as a positive reference for the SNRNP70-enriched U1 level. ***C*** and ***D,*** The abundance levels of EZH2 and H3K27-me3 at the LATS1 promoter regions of HepG2 and HepG3B cell lines are detected by Chip-qRT-PCR. ***E,*** Immunohistochemistry is performed for an obtained nude mice transplanted tumor model and the expression levels of LATS1 proteins in the tumor are detected.

to that of oncogene or tumor suppressor genes[13,14]. Biological functions of lncRNA draw wide concerns from researchers around the world. It has been reported in literature that lncRNA plays an important role in maintaining normal physiology of cells, and its expression imbalance can lead to many pathophysiological phenomena. lncRNA HIT000218960 can promote the transcription of thyroid papillary carcinoma by up-regulating the expression of HMGA2 genes[15]. Besides, lncRNA HOTAIR can promote the resistance of gastric carcinoma to cisplatin, and its underlying molecular mechanism is to activate PI3/AKT signaling pathways through the targeted regulation of miR-26[16]. In our work, HOXA11-AS was found to promote the proliferation of HCC cells and inhibit apoptosis by inhibiting the transcription of LATS1 for the first time.

LATS1 (Large tumor suppressor 1) is one of the core members of the Hippo signaling pathway, which regulates cell cycles and apoptosis[17]. LATS1 is lowly expressed in a variety of tumors, and over-expressed LATS1 can inhibit the growth of tumor cells. The downstream mechanism of LATS1 may be that LATS1 binds and phosphorylates the transcription factor yes-associated protein (YAP), thus inhibiting its own expression[18-20]. It has been reported in literature that LATS1 expression is also down-regulated in HCC, and its molecular mechanism of down-regulation in HCC has not been reported. The down-regulated expression of LATS1 is closely related to methylation at promoter regions in astrocytoma, breast carcinoma, and esophageal carcinoma, but the fact that lncRNA can mediate methylation at gene promoter regions has been confirmed[21-23]. Through bioinformatics and RIP experiments, we found that HOXA11-AS could be combined with histone apparent modified complex PRC2, and further CHIP experiments show that HOXA11-AS could carry PRC2 to LATS1 gene promoter regions to mediate and increase H3K27 transferase activity, thus inhibiting the transcription of LATS1.

## Conclusions

From this project, we found for the first time that HOXA11-AS was relatively highly expressed in HCC tissues and cells, and the inhibition of HOXA11-AS expression could inhibit the proliferation of HCC cell proliferation and promote the cell cycle to progress to G1-G0 phase as well as apoptosis. The potential molecular mechanism was that HOXA11-AS carried the apparent complex PRC2 to LATS1 promoter region to inhibit its transcription. Studying the interactions among HOXA11-AS, PRC2 and LATS1 provided an important theoretical basis for the clinical inhibition of HCC progression.

### Conflict of interest

The authors declare no conflicts of interest.

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