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LOXL1-AS1 promotes cell proliferation in hepatocellular carcinoma through miR-1224-5p/ITPRIPL2/AKT axis

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ABSTRACT

LncRNA has been validated to be related to different cancers, whereas its regulation mechanism in hepatocellular carcinoma (HCC) is poorly known. In this study, LOXL1-AS1 was overexpressed in HCC cell lines, and LOXL1-AS1 knockdown repressed cell proliferation and stimulated apoptosis in HCC. Besides, the activating role of LOXL1-AS1 in the AKT pathway was also confirmed. Further, miR-1224-5p was sponged by LOXL1-AS1, and overexpression exerted inhibitory function in HCC. Moreover, ITPRIPL2 as amiR-1224-5ptarget gene. Meanwhile, ITPRIPL2 deficiency suppressed HCC cell proliferation. Finally, miR-1224-5p inhibitor reversed the hindering role of LOXL1-AS1 depletion in HCC cell proliferation and AKT pathway, and this rescuing effect was offset by ITPRIPL2 silencing. In summary, LOXL1-AS1 induced cell proliferation and suppresses cell apoptosis in primary HCCvia activating AKT pathway, sponging miR-1224-5p and upregulating ITPRIPL2, which may provide some fresh thoughts for researches about the molecular regulation mechanism of lncRNA in HCC.

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Introduction

By referring to Global Cancer Statistics in 2018, around 850,000 new liver cancer cases were diagnosed, and China alone occupies about half of the total number of these two aspects (1,2). Hepatocellular carcinoma (HCC) takes about 76-81% proportion of all liver cancer cases (1). Although the incidence and death rate for HCC has significantly decreased in China, a giant population base and rapid population growth have increased the number of newly diagnosed HCC cases (3). Therefore, it is worthy of better comprehending the molecular mechanism relating to HCC occurrence and development.

Long non-coding RNAs (lncRNAs) play a vital role in cancer initiation and development, including HCC. It has been shown that lnc-SNHG16 promotes bladder cancer by modulating miR-98 (4). Lnc-LOWEG expression is downregulated in gastric cancer (GC) and suppresses cell invasion (5). By activating the Wnt pathway, lncRNA FEZF1-AS1 contributes to tumorigenesis among GC patients with poor prognoses (6). Lncfacilitatesthe lung cancer (LUC) development by upregulating the MTDH by sponging miR-145-5p (7).

The tumor-promoter role of lncRNA LOXL1-AS1 has been reported in recent researches, for example, it promotes tumorigenesis and strengthens the stemness of GC by modulating miR-708-5p/USF1 axis (8). LOXL1-AS1 affects the prostate cancer (PCA)doxorubicin resistance via miR-let-7a-5p/EGFR pathway (9) and serves as a competing endogenous RNA (ceRNA) for miR-324-3p to accelerate cholangiocarcinoma progression through regulating ABCA1 (10), it is also associated with poor prognosis for osteosarcoma patients and contributes to osteosarcoma progression (11), influences cell proliferation and cell cycle in PCAby regulating the expression of miR-541-3p and CCND1 (12), promotes cell metastasis in medulloblastoma via stimulating the PI3K/AKT pathway (13). But the LOXL1-AS1 mechanism in HCC has not been reported yet.

Furthermore, the AKT pathway is constitutively activated HCC, and results in cancer initiation and progression. For illustration, lncRNA FAL1 motivates the EMT process in LUC via PTEN/AKT signaling pathway (14). LncRNA PlncRNA-1 accelerates the development of CRC by modulating the PI3K/Akt signaling pathway (15). SP1-inducedlncRNA UCA1 expedites GC cell proliferation by recruiting EZH2 and stimulating the AKT pathway (16). MEG2 suppresses cell growth and metastasis in HCC via repressing the AKT pathway (17). LncRNA AFAP1-AS1 facilitates cell proliferation in GC through the PTEN/p-AKT pathway (18). Besides, the interactions between LOXL1-AS1 and AKT pathway have been validated in medulloblastoma (13) and osteosarcoma (11).

Therefore, we aimed to explore whether LOXL1-AS1 affected HCC proliferation by regulating the AKT pathway.

Materials and Methods

Cell culture

The four human PHCC cell lines (MHCC97H, HCCLM3, SK-Hep-1and Huh7) and one immortalized nor-

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mal hepatocyte line (THLE-2) were all available from the ATCC (Rockville, Maryland). All of the cell lines were propagated in DMEM (Gibco, Carlsbad, CA) supplied with 10% FBS (Gibco) in a 5% CO_2 humidified incubator at 37°C.

RNA extraction, cDNA synthesis and qRT-PCR

TRIzol (Invitrogen, Carlsbad, CA) was acquired for total RNA extraction to synthesize cDNA as per the standard method of the Reverse Transcription Kit (Takara, Shiga, Japan). As a control, we detected gene expression by qRT-PCR using PCR Master Mix (Invitrogen) and processed the results using a two-color cycle technique used to compare GAPDH and U6 expression.

Cell transfection

The shRNAs specifically targeted to LOXL1-AS1 or ITPRIPL2 were synthesized from GenePharma (Shanghai, China) for gene silencing using Lipofectamine2000 (Invitrogen). The miR-1224-5p mimics/inhibitor (all, GenePharma) were used for overexpression or silencing in MHCC97H and HCCLM3 cells.

Colony formation

For the 14-day culture of MHCC97H and HCCLM3, 500 cells per well were prepared in the 6-well plates. A 4% paraformaldehyde fixation was followed by a 0.1% crystal violet staining and manual counting of cell samples.

EdU incorporation assay

Cells at 5×10^4 were placed in 96-well plates for incubation with EdU medium, then washed in PBS. EdU incorporation assay was performed using RiboBio Co. Ltd.'s EdU kit (Guangzhou, China). After culturing in the DAPI solution, cells were observed under the fluorescence microscope.

JC-1 assay

We cultured cells overnight in 96-well plates, centrifuged them for 5 minutes, and then loaded them with JC-1 dye to determine mitochondrial transmembrane potential ($\Delta\Psi m$). 30 min later, a fluorescence microscope was used.

TUNEL assay

Fixed cells were permeated for 2 min on ice, suspended in PBS, and mixed with 50 µl of TUNEL reaction mixture in accordance with the instruction of the TUNEL assay kit. After DAPI staining, positively-stained cells by TUNEL kit were observed with a fluorescence microscope.

Flow cytometer assay

Cultured cells were fixed for 1 h on ice for dual-staining in the dark with Annexin V-FITC for 15 min. Flow cytometry was used to determine the apoptosis rate of cells using FACSCalibur (BD Biosciences, NJ).

Trypan blue staining assay

5×10^4 cultured cells were placed in each well of a 24-well plate for 48 h at 37°C. After washing, cells were trypsinized before dying in trypan blue dye (Beyotime Biotechnology, Shanghai, China).

Western blot

Proteins were extracted from MHCC97H and HCCLM3 cells before separation on 12% SDS-PAGE. After transferring to PVDF membranes, samples were mixed with 5% skim milk before incubation with primary antibodies (1: 2000; Abcam, Cambridge, MA) against loading control GAPDH and p-PI3K, PI3K, p-AKT, AKT. Samples were then washed in TBST for probing with diluted secondary antibody (1:5000; Abcam). In order to analyze proteins, we used Amersham's enhanced chemiluminescence test kit (Amersham, Arlington Heights, IL).

Subcellular fractionation

MHCC97H and HCCLM3 cells were severally isolated using PARIS™ Kit (Invitrogen) method. The extracted RNAs (U6, GADPH and LOXL1-AS1) were subjected to qRT-PCR.

FISH

Cell samples were incubated in a hybridization solution with the FISH probe targeting LOXL1-AS1 (Ribobio) in line with the user guide. After samples were rinsed thrice in PBS and counterstained with Hoechst solution, a fluorescence microscope was applied for imaging.

RNA pull-down

Using Pierce Magnetic RNA-Protein Pull-Down Kit (Thermo Fisher Scientific, Waltham, MA) method, mixed protein extracts with the magnetic beads-bound LOXL1-AS1 biotin probe or LOXL1-AS1 non-biotin probe for 1 h. The relative miRNA enrichment was monitored.

Luciferase reporter assay

The fragments of LOXL1-AS1 or ITPRIPL2 3'UTR covering the miR-1224-5p wild-type and mutated binding sites were obtained and cloned to luciferase reporter pmirGLO (Promega, Madison, WI). The constructed vectors LOXL1-AS1-WT/Mut and ITPRIPL2 3'UTR-WT/Mut were co-transfected with miR-1224-5p mimics or NC mimics for 48 h, following analysis of Luciferase Reporter Assay System (Promega).

RNA immunoprecipitation (RIP)

For one hour, magnetic beads were pre-cultured with anti-Ago2 antibody or control anti-IgG antibody (Abcam). Cell lysates were then immunoprecipitated with beads in RIP buffer. Purified RNA was processed by qRT-PCR.

Statistical analyses

Results from independent bio-triplicates were exhibited as the mean ± standard deviation (SD). Data analysis was progressed by Student's t-test or one-way ANOVA employing GraphPad Prism V5.0, with $p<0.05$ indicating statistical significance.

Results

LOXL1-AS1 promotes cell proliferation and activates the AKT pathway in HCC

To understand LOXL1-AS1role in HCC cells, qRT-PCR detected that LOXL1-AS1expression was significantly upregulated in HCC cell lines (MHCC97H, HCCLM3, SK-Hep-1 and Huh7) when comparing with hepatocyte cell line THLE-2, MHCC97H and HCCLM3 cells contained the higher level of LOXL1-AS1than the other two HCC cells lines (Figure 1A). And the OXL1-AS1

expression was remarkably declined (Figure 1B). The following loss-of-function experiments formation tested that HCC cell colony number was decreased (Figure 1C), and EdU positive cells percentage was also decreased when knockdown LOXL1-AS1 (Figure 1D). JC-1 assay detected that the JC-1 ratio was reduced (Figure 1E). Afterward, the TUNEL assay detected that cell TUNEL-positive cell percentage was lowered through downregulating LOXL1-AS1 (Figure 1F). Flow cytometry more visually exhibited that LOXL1-AS1 downregulation decelerated cell apoptosis rate (Figure 1G). These two experiments explained that LOXL1-AS1 knockdown contributes to HCC cell apoptosis. Furthermore, trypan blue staining demonstrated that LOXL1-AS1 inhibition gradually decreased cell viability (Figure 1H). Additionally, since it has validated that LOXL1-AS1 could promote medulloblastoma (13) and osteosarcoma (11) via activating the AKT pathway, it's worth for us to confirm whether LOXL1-AS1 also accelerated cell proliferation in HCC via stimulating AKT pathway. Here, western blot assay detected that expression of p-PI3K, PI3K, p-AKT and AKT in sh-LOXL1-AS1#1/#2 transfected HCC cells, and results displayed that protein expression of p-PI3K and p-AKT were suppressed. Further, SC79 treatment remedied the inhibitory effects of sh-LOXL1-AS1#1/#2. Meanwhile, the protein level of PI3K and AKT wasn't affected (Figure 1I). In a word, LOXL1-AS1induced cell proliferation and repressed cell apoptosis also could activate the AKT pathway in HCC.

MiR-1224-5p is sponged by LOXL1-AS1 in HCC

To make LOXL1-AS1 regulatory mechanism in HCC clear, FISH and subcellular fraction assays measured that LOXL1-AS1 expression mainly distributed in the cytoplasm (Figure 2A-2B), indicating LOXL1-AS1 might serve as a ceRNA in regulating HCC progression. In addition, lncRNA usually competitively bind with miRNA to modulate target gene in cancers (19). Accordingly, in our research, fifty-eight miRNAs that might bind with LOXL1-AS1 were obtained through starBase. Then miR-1224-5p, miR-556-3p, miR-3139, miR-520a-5p and miR-382-3p were downregulated in HCC cell lines were obtained (Figure 2C). Subsequently, miR-1224-5penrichment was the most in the LOXL1-AS1 biotin probe group among the mentioned miRNAs (Figure 2D). As well, starBase predicted a binding site between LOXL1-AS1 and miR-1224-5p (Figure 2E). After verifying the effectiveness of the binding site (Figure 2F), the luciferase reporter assay delineated that the luciferase activity of LOXL1-AS1-WT was inhibited by miR-1224-5p mimics, and that of LOXL1-AS1-

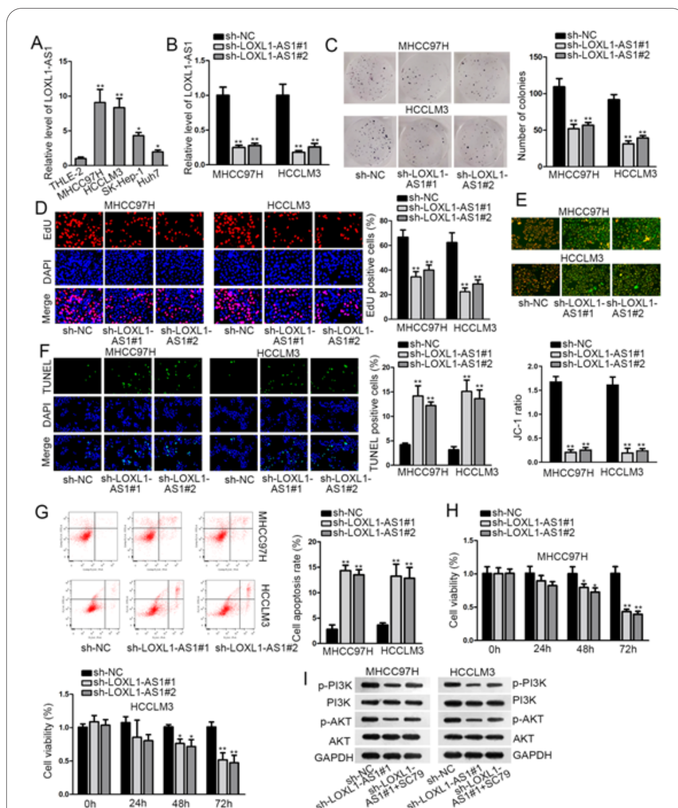

Figure 1. LOXL1-AS1 promotes cell proliferation and activates the AKT pathway in HCC. (A) QRT-PCR measured the expression of LOXL1-AS1 in HCC cell lines and immortalized normal hepatocyte lineTHLE-2. (B) QRT-PCR detected the knockdown efficiency of LOXL1-AS1 in MHCC97H and HCCLM3 cells. (C-D) HCC cell proliferation ability was tested by colony formation and EdU assays. (E-G) HCC cell apoptosis ability was measured by JC-1, TUNEL and flow cytometry assays. (H) HCC cell viability was measured by the trypan blue staining method. (I) The expression of AKT-related proteins was tested by western blot assay. *P<0.05, **P<0.01.

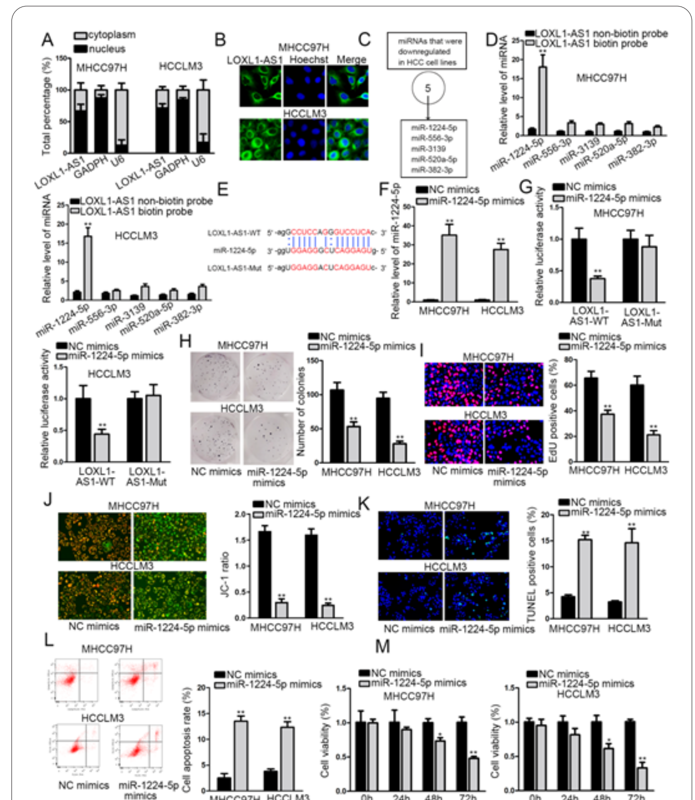

Figure 2. MiR-1224-5p is sponged by LOXL1-AS1 in HCC. (A-B) Subcellular fraction and FISH assays measured that LOXL1-AS1 expression was mainly distributed in the cytoplasm. (C) Five miRNA downregulating HCC cells were screened out through starBase and qRT-PCR. (D) RNA pull-down assay detected the expression of these five miRNAs in the LOXL1-AS1 biotin probe group and the LOXL1-AS1 non-biotin probe group. (E) The binding site of LOXL1-AS1 and miR-1224-5p was predicted by starBase. (F) QRT-PCR measured the overexpression efficiency of miR-1224-5p. (G) Luciferase reporter assay detected the luciferase activity of LOXL1-AS1-WT and LOXL1-AS1-Mut. (H-I) HCC cell proliferation ability was tested by colony formation and EdU assays. (J-L) HCC cell apoptosis ability was measured by JC-1, TUNEL and flow cytometry assays. (M) HCC cell viability was measured by the trypan blue staining method. *P<0.05, **P<0.01.

Mut wasn't influenced, suggesting the binding relation of LOXL1-AS1 and miR-1224-5p in HCC cells (Figure 2G). Similarly, related gain-of-function assays were carried out. On one hand, colony formation and EdU assays measured that miR-1224-5p upregulation suppressed cell proliferation (Figure 2H-2I). Cell apoptosis was encouraged by miR-1224-5p overexpression (Figure 2J-2L). In addition, cell viability was decreased when upregulating miR-1224-5p expression (Figure 2M). In summary, miR-1224-5p is sponged by LOXL1-AS1 in HCC, and miR-1224-5p upregulation inhibited HCC cell proliferation.

ITPRIPL2 is the targeted gene of miR-1224-5p in HCC

To find out the downstream target gene of miR-1224-5p in HCC, we utilizedstarBase to screen out three prequalified messenger RNAs (mRNAs), including ITPRIPL2, PPP1R9BandIPO11 (Figure 3A). In HCC cells, ITPRIPL2 was aberrantly expressed compared to PPP1R9B and IPO11 (Figure 3B). Further, RIP assay detected the expression of LOXL1-AS1, miR-1224-5p and ITPRIPL2 largely enriched in the Anti-Ago2 group, validating these three genes coexisted in an RISC (RNA-induced silencing complex) (Figure 3C). Subsequently, the binding site

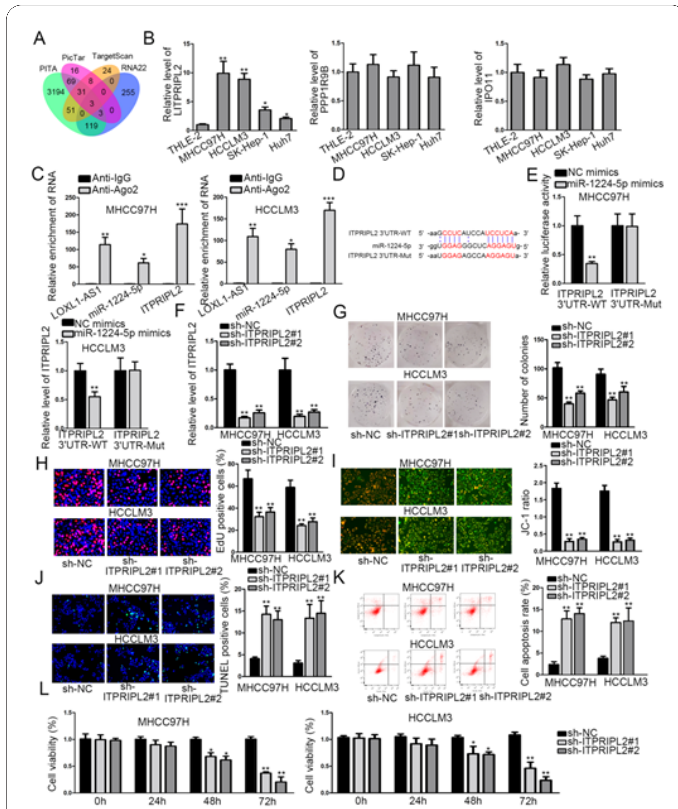

Figure 3. ITPRIPL2 is targeted by miR-1224-5p in HCC**.** (A) Three mRNAs that may bind with miR-1224-5p were screened out by the utilization of starBase. (B) QRT-PCR measured the expression of these three mRNAs in HCC cell lines and immortalized normal hepatocyte lineTHLE-2. (C) RIP assay detected the relative enrichment of LOXL1-AS1, miR-1224-5p and ITPRIPL2. (D) The binding site of ITPRIPL2 and miR-1224-5p was predicted by starBase. (E) Luciferase reporter assay detected the luciferase activity of ITPRIPL2 3'UTR-WT and ITPRIPL2 3'UTR-Mut. (F) QRT-PCR measured the knockdown efficiency of ITPRIPL2. (G-H) HCC cell proliferation ability was tested by colony formation and EdU assays. (I-K) HCC cell apoptosis ability was measured by JC-1, TUNEL and flow cytometry assays. (L) HCC cell viability was measured by the trypan blue staining method. *P<0.05, **P<0.01, ***P<0.001.

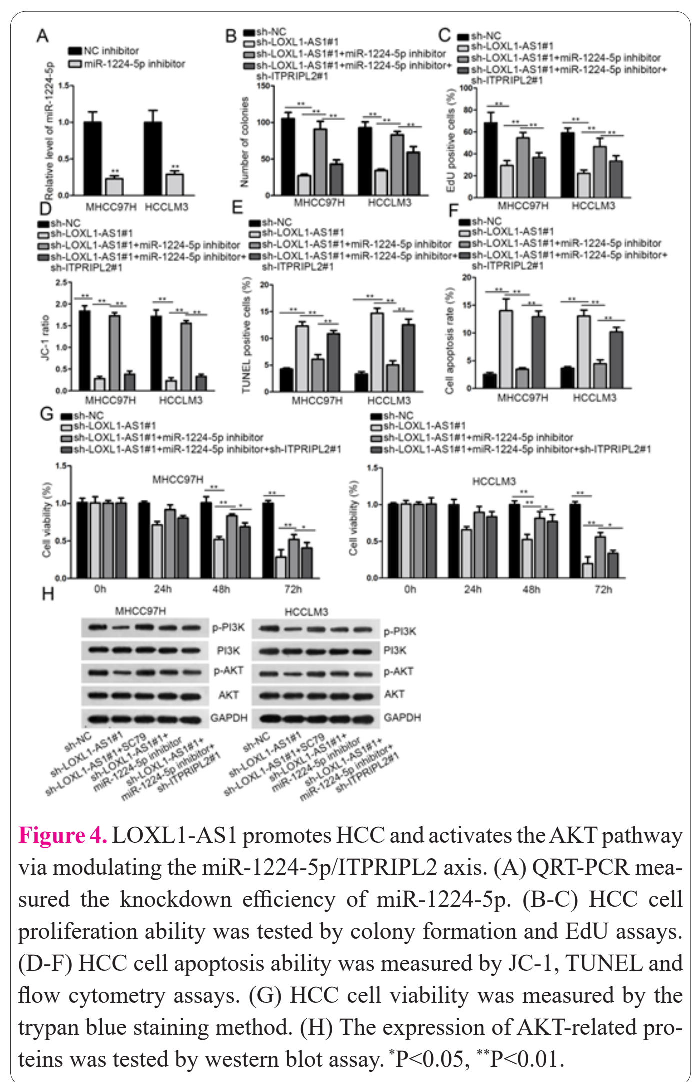

Figure 4. LOXL1-AS1 promotes HCC and activates the AKT pathway via modulating the miR-1224-5p/ITPRIPL2 axis. (A) QRT-PCR measured the knockdown efficiency of miR-1224-5p. (B-C) HCC cell proliferation ability was tested by colony formation and EdU assays. (D-F) HCC cell apoptosis ability was measured by JC-1, TUNEL and flow cytometry assays. (G) HCC cell viability was measured by the trypan blue staining method. (H) The expression of AKT-related proteins was tested by western blot assay. *P<0.05, **P<0.01.

between miR-1224-5p and ITPRIPL2 was predicted by starBase (Figure 3D). The luciferase activity of ITPRIPL2 3'UTR-WT rather that of ITPRIPL2 3'UTR-Mut was obstructed by miR-1224-5p mimics (Figure 3E). In a similar way, the loss-of-function assays related to ITPRIPL2 were exercised. QRT-PCR measured that ITPRIPL2 expression was significantly downregulated when knocking down ITPRIPL2 (Figure 3F). On one hand, cell migration ability was weakened when depleting ITPRIPL2 expression (Figure 3G-3H). On the other, cell apoptosis ability was strengthened after silencingITPRIPL2 (Figure 3I-3K). Besides, cell viability was stimulated by ITPRIPL2 deficiency (Figure 3L). In a word, ITPRIPL2 is targeted by miR-1224-5p in HCC, and the knockdown of ITPRIPL2 suppressed cell proliferation in HCC.

LOXL1-AS1 promotes HCC and activates the AKT pathway via modulating the miR-1224-5p/ITPRIPL2 axis

To confirm whether LOXL1-AS1 affects HCC progression via regulating miR-1224-5p and ITPRIPL2, rescuing experiments were performed. In addition, the knockdown efficiency was checked using q-PCR (Figure 4A). The results indicated that miR-1224-5p downregulation offset the inhibitory effects of LOXL1-AS1 knockdown, and next ITPRIPL2 suppression counteracted the rescuing role of miR-1224-5p downregulation (Figure 4B-4C). Further, LOXL1-AS1 depletion promotion-mediated cell apoptosis was countervailed by miR-1224-5p inhibitor, howe-

ver, the alleviative function of miR-1224-5p inhibitor was neutralized by ITPRIPL2 downregulation (Figure 4D-4F). A downregulation of miR-1224-5p might also reverse the impeditive effect of sh-LOXL1-AS1 on cell viability, then knocking down ITPRIPL2canceled the reversed role of downregulating miR-1224-5p (Figure 4G). According to previous findings that LOXL1-AS1 could activate the AKT pathway, we hypothesized that LOXL1-AS1 could promote the AKT pathway by regulating miR-1224-5p and ITPRIPL2, thus promoting HCC development. Therefore, the final western blot assay measured p-PI3K, PI3K, p-AKTand AKT expression in following transfected conditions as shown in Figure 4H, except that protein level of PI3Kand AKT didn't change, whereas that of p-PI3K and p-AKT was originally was decreased by sh-LOXL1-AS1, then was recovered by SC79 treatment or miR-1224-5p inhibitor, lastly sh-ITPRIPL2 offset the rescuing effects of miR-1224-5p inhibitor. On the whole, LOXL1-AS1 promotes HCC and activates the AKT pathway via the miR-1224-5p/ITPRIPL2 axis.

Discussion

LncRNAs contain more than 200 nucleotides and lack significant protein-coding ability. Nevertheless, they exert vital functions in physiological processes. More and more pieces of evidence have elucidated that various lncRNAs usually possess aberrant expressions in cancers, exhibiting spatial and temporal regulation expression modes. These differentially expressed lncRNAs play roles of oncogene or tumor inhibitor genes (20,21). Accordingly, it's necessary to make more efforts to further comprehend the mechanisms of lncRNAs in cancers.

LOXL1-AS1 oncogene role has been reported in GC (8), PCA (9,12), cholangiocarcinoma (10), osteosarcoma (11) and medulloblastoma (13). In addition, LOXL1-AS1 was upregulated in HCC cells, accordingly, exerted the same cancer-promoting function in HCC. In this study, LOXL1-AS1 knockdown inhibited cell function in HCC, suggesting that LOXL1-AS1 serves as an oncogene in HCC. Furthermore, it has been reported that LOXL1-AS1 promotes cancer by activating the AKT pathway (11,13). Besides, AKT signaling drives cellular proliferation (22). Accordingly, we detected the LOXL1-AS1 downregulation effects on p-PI3K, PI3K, p-AKTand AKT, and concluded that LOXL1-AS1 activated AKT in HCC cells.

Interestingly, lncRNAs can act as sponges or inhibitors for miRNAs, thereby modulating miRNA target genes' expression (23-25). For example, lncRNA 00152 plays a role of ceRNA to modulate NRP1 via sponging miRNA-206 in CRC (26). Lnc DLX6-AS1 enhances glioma development via competitively endogenously binding with miR-197-5p to upregulate the expression of E2F1 (27). Lnc MAGI2-AS3 modulates CCDC19 expression by sequestering miR-15b-5p and suppressing the development of bladder cancer (28). In our study, a group of downregulated miRNAs that may bind with LOXL1-AS1 was screened out, and the enrichment degree of miR-1224-5p in the LOXL1-AS1 biotin probe was the most obvious. Further, the binding relation between LOXL1-AS1 and miR-1224-5p was verified in HCC cells. Additionally, miR-1224-5p has been reported in the latest researches, such as osteosarcoma (29), melanoma (30), keloids (31), and glioma (32). Nevertheless, miR-1224-5p in HCC is still poorly comprehended. Here, miR-1224-5p overexpression induced cell proliferation and accelerated apoptosis in HCC. Moreover, through starBase, a series of mRNAs that possibly bind with miR-1224-5p was screened out. And qRT-PCR measured that ITPRIPL2 exhibited significant differences in the HCC cell line and immortalized normal hepatocyte lineTHLE-2. Then it also confirmed that LOXL1-AS1, miR-1224-5p and ITPRIPL2 coexisted in one RISC. AndITPRIPL2 was a target gene of miR-1224-5p in HCC. In addition, ITPRIPL2 downregulation also hindered cell proliferation in HCC. Subsequently, to confirm the LOXL1-AS1/miR-1224-5p/ITPRIPL2 axis function in HCC via activating AKT pathway, rescuing experiments indicated that LOXL1-AS1-downregulation inhibition-mediated cell proliferation and AKT pathway was remedied by miR-1224-5p inhibitor, then ITPRIPL2 knockdown counteracted this redemptive effect.

In conclusion, LOXL1-AS1 promotes cell proliferation in primary HCC by sponging miR-1224-5p and upregulating ITPRIPL2. which may contribute to finding more HCC-related molecular regulatory mechanisms.

Acknowledgement

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Conflicts of interest

The authors declare no conflicts of interest in this study.

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