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lncRNA OSER1-AS1 acts as a ceRNA to promote tumorigenesis in hepatocellular carcinoma by regulating miR-372-3p/Rab23 axis

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ABSTRACT

Long non-coding RNAs (lncRNAs) are crucial regulators of tumorigenesis and progression in human cancer, including hepatocellular carcinoma (HCC). However, the role of most lncRNAs that are dysregulated in HCC remains to be elucidated. Here, we investigated the role of OSER1-AS1 in the progression of HCC. The results of database and qRT-PCR analysis demonstrated that OSER1-AS1 was highly expressed in HCC tissues and the high expression of OSER1-AS1 was closely associated with larger tumor size, advanced tumor stages, lower disease free survival and overall survival of HCC patients. OSER1-AS1 knockdown significantly inhibited the proliferation, invasion and migration of HCC cells, and induced the apoptosis. In addition, the dual luciferase reporter assay directly demonstrated that OSER1-AS1 functioned as a molecular sponge for miR-372-3p to promote Rab23 expression. Moreover, the results of immunohistochemistry and western blot analysis showed that Rab23 was highly expressed in HCC tissues, and the high expression of Rab23 was closely associated with the poor overall survival of HCC patients. Immunofluorescence assay also found the subcellular localization of Rab23 in HCC cells. Rab23 was obviously downregulated in cells that were transfected with miR-372-3p mimics. MiR-372-3p mimics significantly inhibited the proliferation and invasion of HCC cells). Rab23 restoration partially reversed miR-372-3p-induced tumor suppressive effects on HCC cells. In conclusion, we found that OSER1-AS1 acted as a ceRNA to sponge miR-372-3p, thereby positively regulating the Rab23 expression and ultimately acting as a tumor suppressor gene in HCC progression.

1. Introduction

Hepatocellular carcinoma (HCC) is the prevalent malignancy, recognized as the fourth leading cause of incidence and the second leading cause of mortality among all types of human cancers in China [1]. Although continued improvement in HCC diagnosis and therapy has been made, the 5-year survival rate of HCC patients remains dismal [2]. The high frequency of recurrence and metastasis following surgery lead to poor prognosis of HCC [3]. Therefore, it is vital to further understand the molecular mechanism of HCC progression.

Long non-coding RNAs (lncRNAs) are transcripts containing 200 nucleotides and possess no or limited protein coding function [4]. Increasing studies demonstrate that lncRNAs are crucial regulators of tumorigenesis and progression in human cancer [4]. LncRNAs could serve as a competitive endogenous RNA (ceRNA) to sponge microRNA (miRNA), indirectly regulating target genes. MiRNAs could silence gene expression through targeting 3′ untranslated regions (3′UTR) of mRNAs [5]. For example, in HCC, lncRNA SOX9-AS1 drove progression and metastasis by regulating miR-5590-3p/SOX9 axis [6]. lncRNA EIF3J-AS1 regulated CTNND2 by targeting miR-122-5p to promote HCC [7].

In present study, we confirmed the high expression of lncRNA OSER1-AS1 in HCC tissues, and examined the effect of OSER1-AS1 knockdown on HCC cell proliferation, apoptosis, migration and invasion. Furthermore, we found that OSER1-AS1 contributed to HCC progression by targeting miR-372-3p/Rab23 axis.

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2. Materials and methods

2.1. Clinical samples

Paired HCC and corresponding adjacent normal tissues were acquired from 34 cases of HCC patients who underwent pneumonectomy in Qilu Hospital. The pathological type of each tumor sample was confirmed by experienced pathologists. Fresh samples were immediately frozen in the liquid nitrogen. None of the patients had received any adjuvant chemotherapy or radiotherapy before surgery. The study was approved by the Ethical Committee of Qilu Hospital, and each patient signed the informed written consent.

2.2. Cell culture

Human HCC cell lines including HepG2 and Hep3b were obtained from American Type Culture Collection (ATCC). Both cell lines were cultured in DMEM containing 10% fetal bovine serum (FBS) (Gibco, NY), 100 U/mL penicillin, and 100 mg/mL streptomycin (Invitrogen, CA) in an incubator at 37 °C with 5% CO_2.

2.3. Cell transfection

ShRNAs against OSER1-AS1 (OSER1-KD) and non-targeting shRNA (NC) were synthetised by Geneseed Biotech (Guanghzou, China). MiR-372-3p mimics and matched negative control (miNC) were obtained from RiboBio (Guangzhou, China). The full length sequences of Rab23 was integrated into pcDNA3.1 vector (Invitrogen, Carlsbad, Calif, USA) to generate pcDNA3.1/Rab23 (termed Rab23). Cell transfection was carried out by using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions.

2.4. RNA isolation and RT-PCR analysis

Total RNA was extracted from tissues and cells using Trizol reagent (Invitrogen), then was reversely transcribed to cDNAs using the Reverse Transcription System Kit (Invitrogen). RT-PCR was carried out following the instruction of SYBR®Premix EX Taq kit (TaKaRa, Japan). OSER1-AS1 and Rab23 mRNA expression was normalized to GAPDH expression and miR-372-3p to U6, respectively. The relative expression of target RNA was calculated by $2^{-\Delta\Delta Ct}$ method.

2.5. CCK8 assay

1×10^3 cells were plated in each well of a 96-well plate, and 10 μl CCK-8 reagent was added when HCC cells were cultured for 0, 24, 48, 72 and 96 h. Then cells were further incubated for 2 h at 37 °C. Then, the OD value at 450 nm was determined by a microplate reader (Model 550; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

2.6. Colony formation assay

Approximately 500 cells were cultured in each well of six-well plates for 2 weeks. Then the colonies were fixed by 4% methanol, stained with Giemsa for 15 min. The number of colonies was counted under an optical microscope.

2.7. Flow cytometry

After 48 h of transfection, cells were collected by Annexin V binding buffer. Subsequently, cells were double stained with 5 μl V-FITC and 5 μl propidium iodide (PI) (Sigma-Aldrich, St. Louis, MO) and detected by FACS alibur flow cytometer (Becton-Dickinson, CA). Data were analyzed using FlowJo software.

2.8. Western blot

Proteins were lysed from tissues and cells using RIPA buffer (Beyotime, Shanghai, China) and the protein concentrations were determined by the BCA reagent kit (Beyotime, Jiangsu, China). Proteins in samples were separated using 10% SDS-PAGE. The separated proteins were electro-transferred onto PVDF membranes, and subsequently incubated with nonfat milk for 2 h at the room temperature. The PVDF membranes were incubated with the primary antibodies at 4 °C overnight. The intensity of protein bands was quantified using ImageJ software (NIH, USA). Bcl2 (ab92498), Bax (ab184917), Actived-caspase3 (ab2302), N-cad (ab18203), Vimentin (ab8978), Rab23 (ab230200), and GAPDH (ab181602) primary antibodies were purchased from Abcam (Cambridge, MA, USA).

2.9. Transwell assay

To examine cell invasion, 1×10^5 cells in serum-free medium were seeded into the upper chamber of the Transwell inserts (BD Biosciences, San Jose, CA, USA) with 8 μm-pore membranes pre-coated with Matrigel (BD Biosciences). Medium containing 10% FBS were added to the bottom chambers. Cells were then cultured for 24 h at 37 °C. Cells on the upper surface of the membrane were removed and invaded cells were fixed with 4% methanol for 15 min and stained by crystal violet for 15 min. Invasive cells were observed under an optical microscope and the number of invaded cells was counted from 5 randomly chosen fields. To examine cell invasion, the experimental steps were identical except Matrigel pre-coat.

2.10. Wound healing assay

Cells were grown to confluence in 6-well plates. The "wound" was created by a sterile plastic tip on the confluent cell monolayer. Then, the wound at 0 and 24 h was imaged using a microscope (Olympus, Tokyo, Japan). The average of five random widths of each wound was measured for quantification.

2.11. Dual luciferase reporter assay

OSER1-AS1 and 3′UTR of Rab23 containing the putative binding sequence or a mutated binding sequence for miR-372-3p were synthesized and cloned into pGL3 reporter vector (Promega, Madison, WI, USA). HepG2 cells were co-transfected with reconstructed vectors and miR-372-3p mimics or negative control (miNC). The fluorescence intensity was measured by using the Dual-Luciferase Reporter Assay System (Promega).

2.12. Immunohistochemical analysis

Each sample was fixed in formalin for 12 h and then embedded in paraffin. The paraffin-embedded tissues were cut into 4 μm sections, and were deparaffinized. Sodium citrate buffer (pH 6.0) was used for antigen retrieval. After blocked with 5% sheep serum at room temperature for 1 h, slides were exposed to anti-Rab23 (1:100) for 2 h at room temperature, and then was incubated with secondary antibody. Detection was conducted by the enhanced DAB chromogenic kit. Hematoxylin was used for counterstaining. Image was performed by a microscope.

2.13. Immunofluorescence (IF)

HCC cells seeded on glass coverslips in a 6-well plates were fixed in 4% formaldehyde. After treated with pepsin, cells were

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dehydrated by ethanol. Then, it was permeabilizated in Triton X100 (Sigma-Aldrich Co., St Louis, MO, USA), and blocked with goat serum. Following incubation with anti-Rab23 (Abcam, Cambridge, MA) overnight at 4 °C, cells were washed with PBS and incubated with appropriate secondary antibody for 1 h. Then, the nuclei were stained with DAPI (Cell Signaling Technology). Visualization was performed by a fluorescence microscope (DMI4000B, Leica).

2.14. Statistical analysis

Statistical analyses were performed by SPSS22.0 (IBM, USA). The data were shown as mean ± standard deviation (x±s). The Student's t-test was used to analyze the significance of the difference between any two groups, whereas one-way ANOVA was used among more than two groups. Differences were regarded statistically significant if $P < 0.05$.

3. Results

3.1. OSER1-AS1 is highly expressed in HCC

We firstly quantified the expression of OSER1-AS1 between HCC and adjacent non-tumor tissues via database analysis and qRT-PCR. The data from GEPIA [8] revealed the higher levels of OSER1-AS1 in HCC tissues compared to normal liver tissues (Fig. 1A). In additional, it demonstrated that the high expression of OSER1-AS1 was closely associated with lower disease free survival and overall survival of HCC patients ($P < 0.05$, Fig. 1B and C). We also clinically collected 34 paired HCC and adjacent normal tissues, and assessed the expression of OSER1-AS1 by qRT-PCR. The results revealed that the expression of OSER1-AS1 in HCC was significantly higher than that in adjacent normal tissues ($P < 0.001$, Fig. 1D). Furthermore, OSER1-AS1 expression was obviously higher in HCC tissues with tumor size >1.5 cm and advanced tumor stages compared to matched controls ($P < 0.05$, Fig. 1E and F). These results suggested that OSER1-AS1 might be a prognostic biomarker for HCC.

3.2. Knockdown of OSER1-AS1 attenuates HCC cell proliferation, invasion and migration

To expose the role of OSER1-AS1 in HCC cells, OSER1-AS1 level was significantly down-regulated by shRNA in HepG2 and Hep3b cells ($P < 0.05$, Fig. 1G). The results of CCK8 assay revealed that knockdown of OSER1-AS1 significantly inhibited the viability of

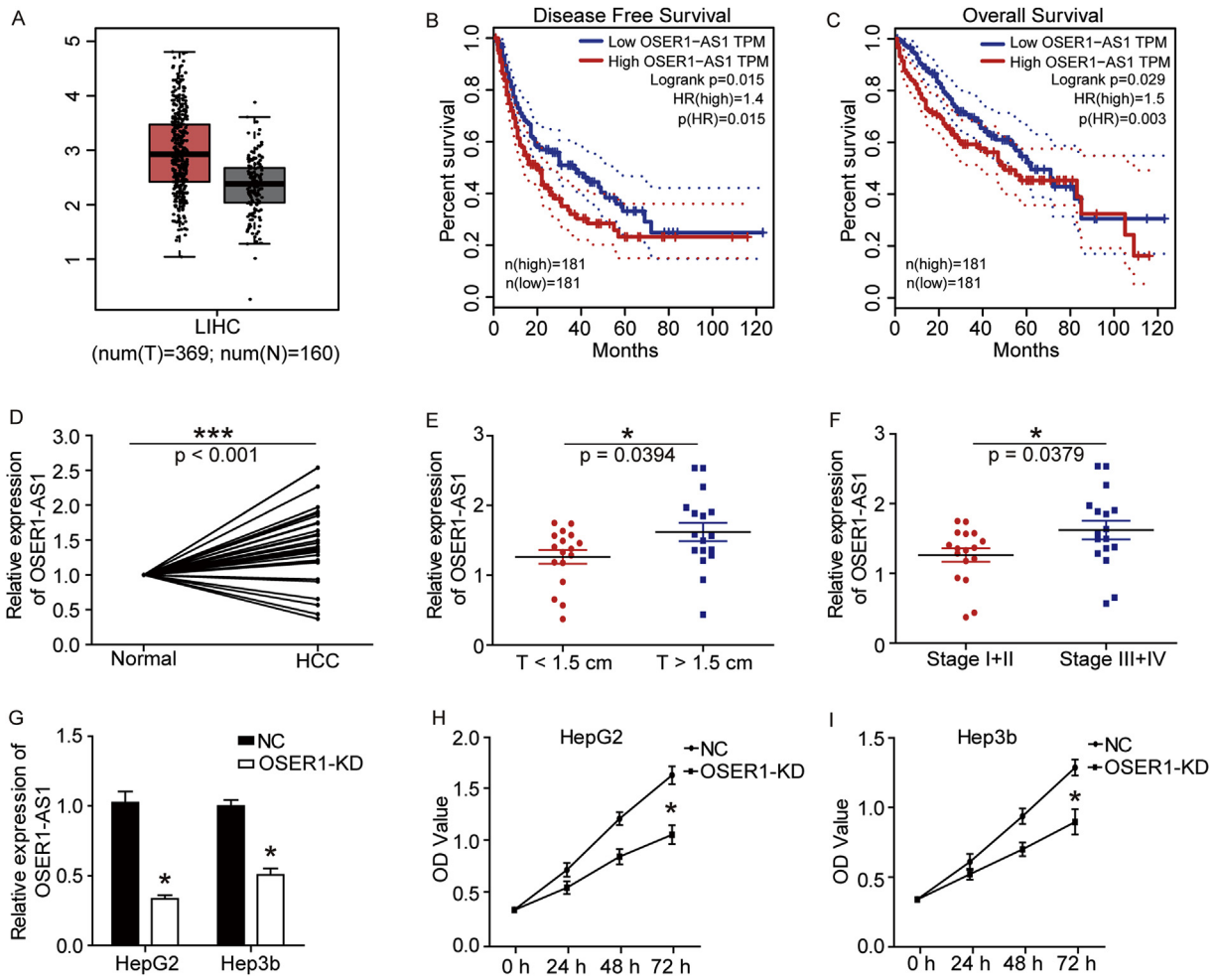

Fig. 1. OSER1-AS1 is highly expressed in HCC and OSER1-AS1 knockdown attenuates HCC cell proliferation. (A)The boxplot of OSER1-AS1 expression level. Red and gray boxes represent liver hepatocellular carcinoma (LIHC) tissue and normal liver tissue, respectively. The data came from the GEPIA database. (B and C) The prognostic value of OSER1-AS1 in LIHC patients. Red and blue represent high and low expression of OSER1-AS1, respectively. The data came from TCGA database. (D) OSER1-AS1 levels in hepatocellular carcinoma (HCC) tissues and adjacent normal ovarian tissues (n = 34) were evaluated via qRT-PCR. (E) Relative expression of OSER1-AS1 in T < 1.5 cm group and T ≥ 1.5 cm group. (F) Relative expression of OSER1-AS1 in stage I + II group and stage III + IV group. (G) OSER1-AS1 levels in HepG2 and Hep3b cells transfected with shRNA targeting OSER1-AS1 were evaluated via qRT-PCR. *$P < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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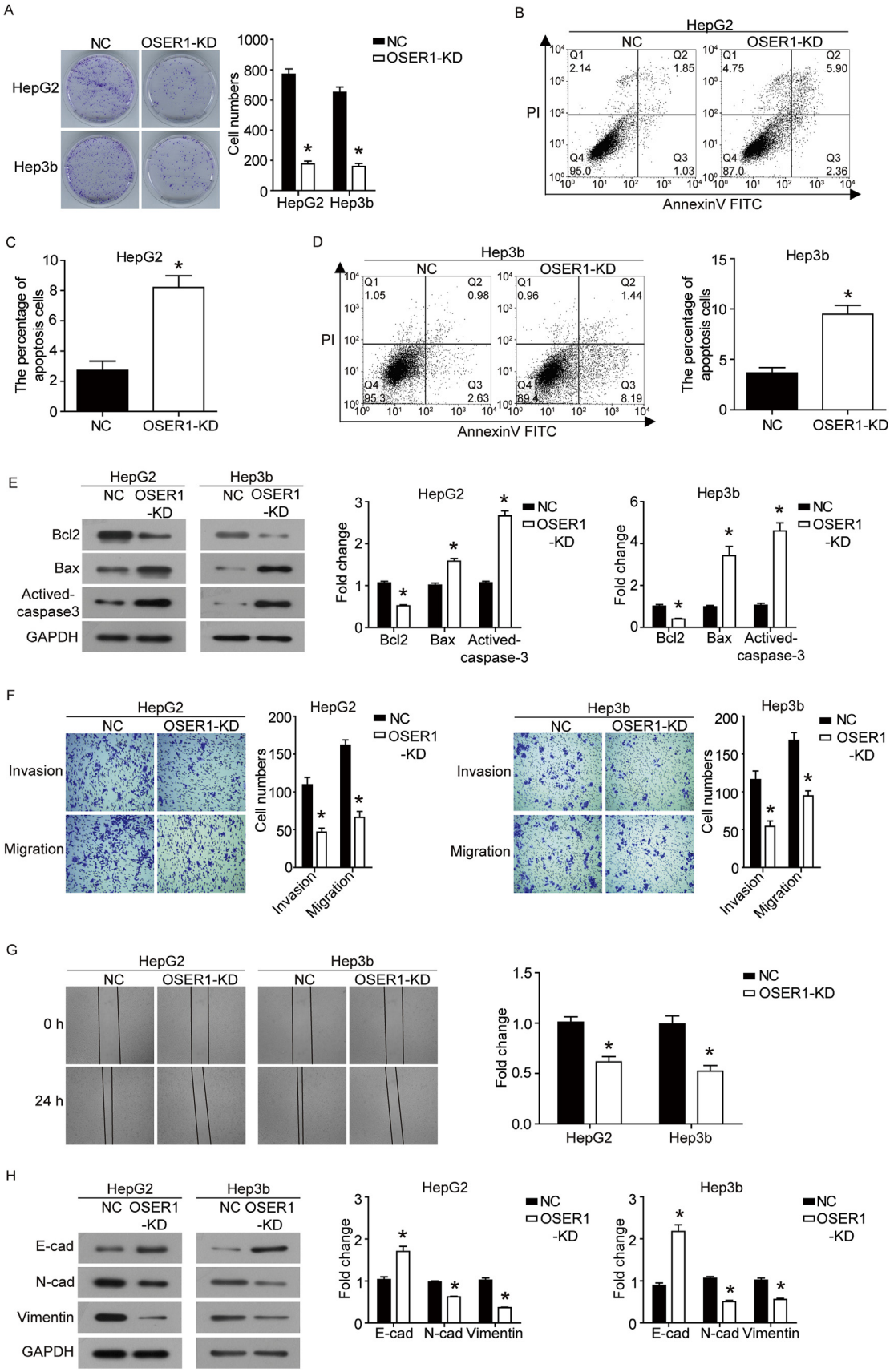

Fig. 2. Knockdown of OSER1-AS1 attenuates HCC cell proliferation, invasion and migration. (A) The clonogenicity of HepG2 and Hep3b cells transfected with shRNA targeting OSER1-AS1 was detected by clone formation assay. The apoptosis of HepG2 (B and C) and Hep3b (D) cells was detected and quantified by flow cytometry. (E) The expression of apoptosis-related proteins was detected and quantified by western blot. (F) The migration and invasion of HepG2 and Hep3b cells were determined and quantified by transwell assay. (G) The migration of HepG2 and Hep3b cells were determined and quantified by wound healing assay. (H) The expression of EMT-related proteins was detected and quantified by western blot. $^{*}P < 0.05$.

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HepG2 and Hep3b cells ($P < 0.05$, Fig. 1H and I). Furthermore, the results of the colony formation assay also showed the same conclusion (Fig. 2A). In addition, the percentage of apoptosis cells was prominently increased by OSER1-AS1 silencing ($P < 0.05$, Fig. 2B–D). The expression of apoptosis-related proteins also was altered with OSER1-AS1 silencing ($P < 0.05$, Fig. 2E). Moreover, the migration and invasion of HepG2 and Hep3b cells were markedly decreased by OSER1-AS1 knockdown as suggested by Transwell and wound healing assays ($P < 0.05$, Fig. 2F and G). Furthermore, the expression of EMT-related proteins also was altered with OSER1-AS1 silencing ($P < 0.05$, Fig. 2H). Thus, OSER1-AS1 might play an oncogenic role in HCC.

3.3. OSER1-AS1 functions as a molecular sponge for miR-372-3p to promote Rab23 expression

MiR-372-3p containing binding sequences for OSER1-AS1 was predicted (Fig. 3A). Furthermore, miR-372-3p over-expression obviously decreased the luciferase activity of vector carrying WT OSER1-AS1 but not Mut OSER1-AS1 ($P < 0.05$, Fig. 3B). We also found the significantly decreased miR-372-3p level in HCC tissues compared to adjacent normal tissues with qRT-PCR ($P < 0.05$, Fig. 3C). A correlation analysis showed a significant negative correlation between miR-372-3p and OSER1-AS1 level in HCC tissues ($P < 0.001$, Fig. 3D).

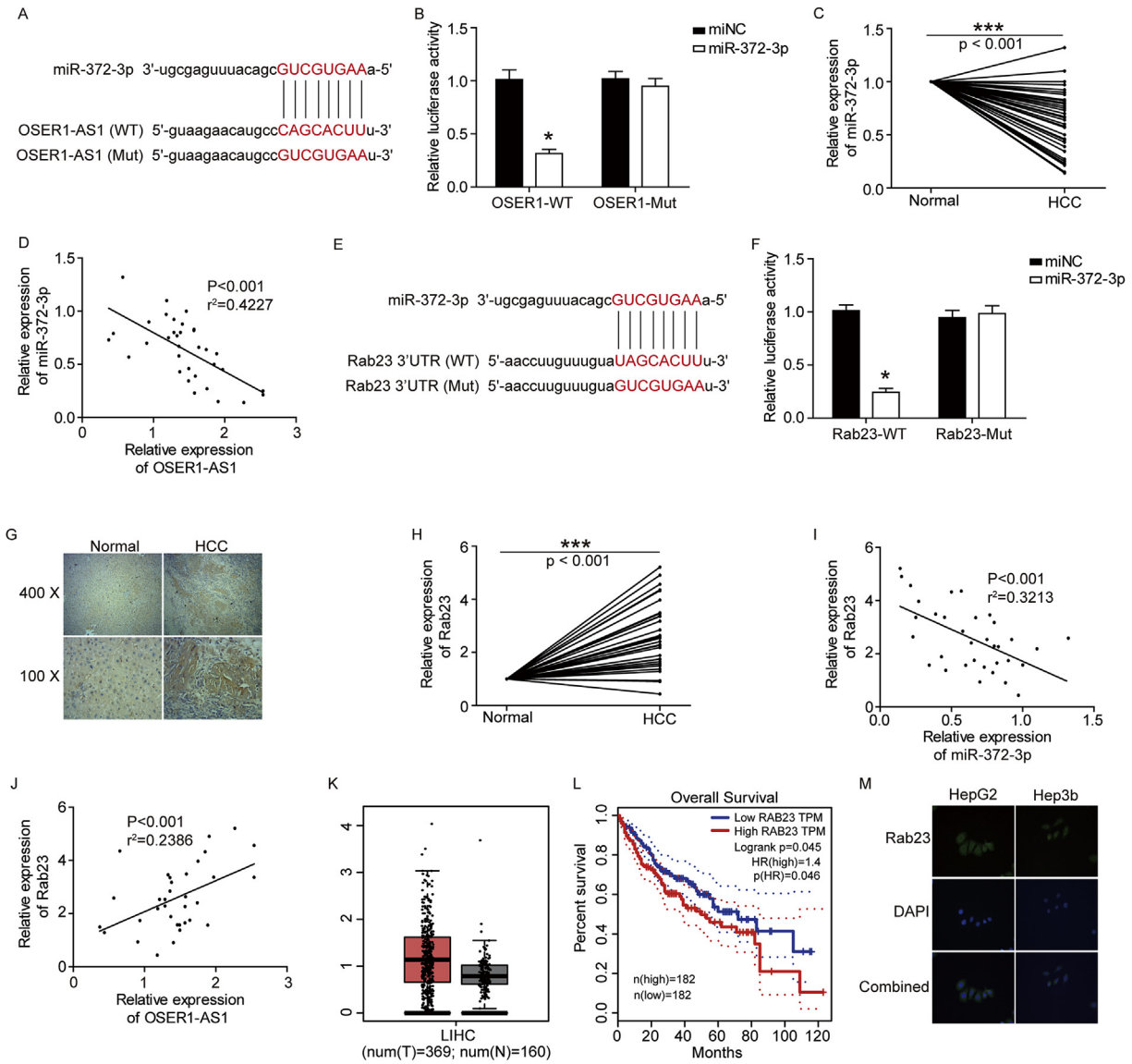

Fig. 3. OSER1-AS1 functions as a molecular sponge for miR-372-3p to promote Rab23 expression. (A) The sequences of miR-372-3p, wide type of OSER1-AS1 (WT) and mutated OSER1-AS1 (Mut). (B) The expression levels of luciferase of HepG2 cells transfected with wild-type (WT) or mutated (Mut) OSER1-AS1 reporters plus miR-372-3p mimic or miNC were determined. (C) MiR-372-3p levels in HCC tissues and adjacent normal ovarian tissues (n = 34) were evaluated via qRT-PCR. (D) Correlation analysis between OSER1-AS1 and miR-372-3p. (E) The sequences of miR-372-3p, wide type of Rab23 (WT) and mutated Rab23 (Mut). (F) The expression levels of luciferase of HepG2 cells transfected with wild-type (WT) or mutated (Mut) Rab23 reporters plus miR-372-3p mimic or miNC were determined. (G) Rab23 levels in HCC tissues and adjacent normal ovarian tissues (n = 34) were evaluated via immunohistochemistry. (H) Rab23 levels in HCC tissues and adjacent normal ovarian tissues (n = 34) were evaluated via western blot. (I) Correlation analysis between Rab23 and miR-372-3p. (J) Correlation analysis between Rab23 and OSER1-AS1. (K) The boxplot of Rab23 expression level. Red and gray boxes represent liver hepatocellular carcinoma (LIHC) tissue and normal liver tissue, respectively. The data came from the GEPIA database. (L) The prognostic value of Rab23 in LIHC patients. Red and blue represent high and low expression of Rab23, respectively. The data came from TCGA database. (M) The subcellular localization of Rab23 in HepG2 and Hep3b cells was detected via Immunofluorescence. *$P < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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Next, Rab23 was predicted as a candidate target of miR-372-3p (Fig. 3E), and ectopic expression of miR-372-3p significantly reduced the luciferase activity of vector carrying WT Rab23 3′UTR rather than Mut Rab23 3′UTR ($P < 0.05$, Fig. 3F). Rab23 was highly expressed in HCC tissues compared into normal liver tissues by immunohistochemistry and western blot analysis ($P < 0.05$, Fig. 3G and H). Furthermore, the expression of Rab23 was negatively correlated with miR-372-3p expression, and positively correlated with OSER1-AS1 expression ($P < 0.05$, Fig. 3I and J). GEPIA data also indicated that Rab23 mRNA was overexpressed in HCC tissues (Fig. 3K). In additional, high expression of Rab23 was closely associated with the poor overall survival of HCC patients ($P < 0.05$, Fig. 3L). Moreover, we also found the subcellular localization of Rab23 in HCC cells (Fig. 3M). Collectively, OSER1-AS1 promoted Rab23 expression via sponging miR-372-3p.

3.4. miR-372-3p attenuates HCC progression by inhibiting Rab23

To further verify the function of miR-372-3p, we quantified the expression of Rab23 protein in HepG2 and Hep3b cells transected with miR-372-3p mimics by western blot. The results indicated that Rab23 was obviously downregulated in cells that were transfected with miR-372-3p mimics ($P < 0.05$, Fig. 4A and B). And the co-transfection of Rab23 over-expression plasmid neutralized the effect of miR-372-3p mimics (Fig. 4A and B). MiR-372-3p mimics significantly inhibited the proliferation and invasion of HepG2 and Hep3b cells ($P < 0.05$, Fig. 4C–E). Furthermore, Rab23 restoration partially reversed miR-372-3p-induced tumor suppressive effects on HepG2 and Hep3b cells ($P < 0.05$, Fig. 4C–E). Taken together, OSER1-AS1 contributed to HCC progression via miR-372-3p/Rab23 axis.

4. Discussion

HCC which accounts for about 90% of primary liver cancers witnesses low 5-year survival rate. Identifying new biomarkers for HCC diagnosis, prognosis and therapy are necessary to improve its status. LncRNAs play a key role in this process. For example, H19 is

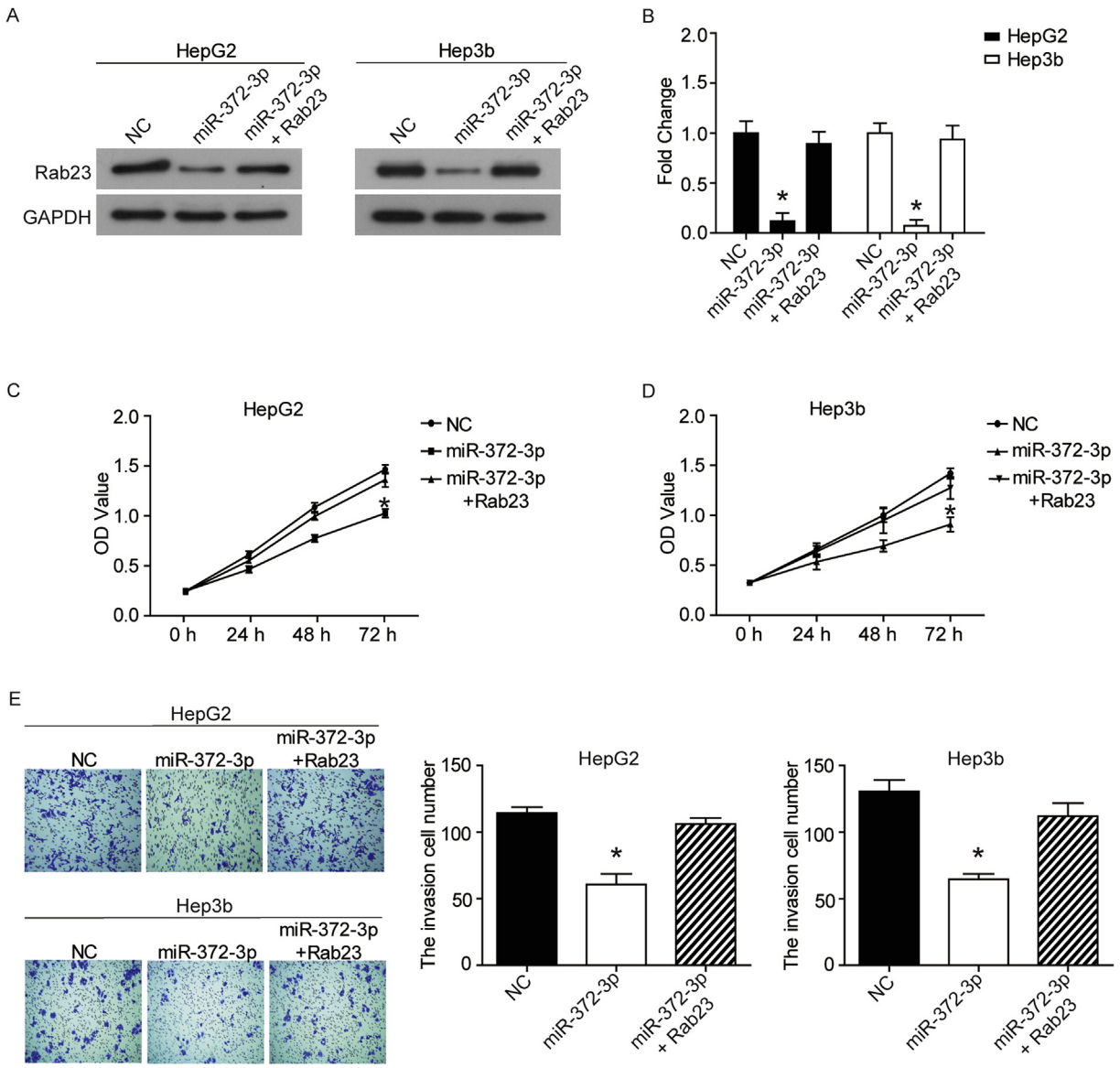

Fig. 4. miR-372-3p attenuates HCC progression by inhibiting Rab23. (A and B) Rab23 levels in HepG2 and Hep3b cells were determined via western blot. (B) The proliferation of HepG2 (C) and Hep3b (D) cells was determined via CCK8 assay. (E) The invasion of HepG2 and Hep3b cells were determined and quantified by transwell assay. *$P < 0.05$.

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the first non-coding RNA found in HCC [9], and contributes to carcinogenesis [10]. HOTAIR also is overexpressed in HCC and is closely related to tumor progression and recurrence [11,12]. In fact, only a small fraction of lncRNAs in our transcriptome have been studied, and the functional role of most lncRNAs that are dysregulated in HCC remains to be elucidated. Here, we investigated the role of OSER1-AS1 in the progression of HCC.

In this study, we firstly found that OSER1-AS1 might play an oncogenic role in HCC. The results of database and qRT-PCR analysis demonstrated that OSER1-AS1 was highly expressed in HCC tissues and the high expression of OSER1-AS1 was closely associated with larger tumor size, advanced tumor stages, lower disease free survival and overall survival of HCC patients. To further investigate the role of OSER1-AS1 in HCC cells, OSER1-AS1 was knocked down by shRNA in HepG2 and Hep3b cells. OSER1-AS1 knockdown significantly inhibited the proliferation, invasion and migration of HCC cells, and induced the apoptosis. These data showed that OSER1-AS1 might be a prognostic and therapeutic biomarker for HCC.

In addition, we found that OSER1-AS1 functioned as a molecular sponge for miR-372-3p. The dual luciferase reporter assay directly demonstrated the physical interaction between the two. MicroRNAs are single-stranded, small RNA molecules. They regulate the expression of approximately a third of human genes, and thereby influence important cellular functions [13]. MicroRNA expression profiles are altered in many diseases including HCC. Mi-372-3p is one of the powerful miRNAs and has been found to promote tumor formation in testicular germ cell tumor (TCGT), lung cancer [14], parathyroid carcinoma [15] and osteosarcoma [16]. Some researches report that the serum level of miR-372-3p is overexpressed in all malignant TGCT, and highly sensitive and specific for the diagnosis of malignant TGCT [17,18]. MiR-372-3p promotes growth and tumorigenesis of TGCT via neutralization of p53-mediated CDK inhibition and activation of the Wnt/β-catenin signaling pathway [19,20]. In this study, miR-372-3p was identified to be under-expressed in HCC tissues and negatively correlated with the level of OSER1-AS1. Unlike the overexpression of miR-372-3p in TCGT and lung squamous cell carcinoma (LSCC), and the promotion of miR-372-3p in cell proliferation and invasion, miR-372-3p inhibits the growth and metastasis of HCC and osteosarcoma cells [16]. This may depend on the specificity of the tumor type.

Rab23, a member of the Ras-associated small GTPase family, plays a role in vesicle trafficking and protein trafficking in eukaryotic cells and is an important negative regulator of the Sonic hedgehog (Shh) signaling pathway [21]. In addition, RAB23 is upregulated in many types of cancer, including HCC [22,23]. Here, we found that miR-372-3p attenuated HCC progression by inhibiting Rab23. Dual luciferase reporter experiments demonstrated that Rab23 was a target for miR-372-3p. Furthermore, the expression of Rab23 was negatively correlated with miR-372-3p expression, and positively correlated with OSER1-AS1 expression in HCC tissues. Moreover, the results of immunohistochemistry and western blot analysis showed that Rab23 was highly expressed in HCC tissues, and the high expression of Rab23 was closely associated with the poor overall survival of HCC patients. IF assay also found the subcellular localization of Rab23 in HCC cells. These results were consistent with previous studies. Liu et al. find that Rab23 is not expressed in normal liver tissues, but is expressed in HCC tissues and cells [24]. Sun et al. also confirm the subcellular localization of Rab23 in HepG2 and Hpe3b cells [25].

In summary, we found the expression patterns and roles of OSER1-AS1, miR-372-3p, and Rab23 in HCC. These results help to better understand the pathogenesis and development of HCC, as well as better diagnosis and treatment of HCC.

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