Original Article

LncASAP1-IT1 promotes hepatocellular carcinoma progression through the regulation of the miR-1294/TGFBR1 pathway *in vitro* and *in vivo*

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Background: Hepatocellular carcinoma (HCC) is the most common tumor with severe morbidity and high mortality. The lncRNA ASAP1-IT1 [the intronic transcript 1 (IT-1) of ArfGAP with SH3 domain, ankyrin repeat and PH domain 1 (ASAP1)] have been shown to promote tumor formation in a variety of cancers. This study sought to investigate the effects of dysregulated ASAP1-IT1 on the biological processes of HCC.

Methods: The expression levels of ASAP1-IT1 in 30 paired HCC and adjacent non-tumor tissues were measured by real-time-quantitative polymerase chain reaction (RT-qPCR). Several functional tests were performed to investigate the molecular mechanism of ASAP1-IT1 in HCC progression.

Results: Our study showed that ASAP1-IT1 was highly expressed in the HCC tissues and cell lines. The knockdown of ASAP1-IT1 inhibited cell proliferation, migration, invasion, and epithelial-mesenchymal transition (EMT) progression and enhanced the sorafenib sensitivity of the HCC cells. Further investigations revealed that ASAP1-IT1 served as a sponge of microRNA-1294 (miR-1294) to promote transforming growth factor beta receptor 1 (TGFBR1) expression. In addition, the tumor-promoting effect of ASAP1-IT1 was blocked by inhibiting miR-1294/TGFBR1. Tumorigenic assays in nude mice demonstrated that the inhibition of ASAP1-IT1 inhibited the growth of HCC *in vivo*.

Conclusions: These results suggest that lncASAP1-IT1 promotes HCC development by targeting TGFBR1 through miR-1294, which provides a potential target for HCC diagnosis and treatment.

Keywords: LncASAP1-IT1; transforming growth factor beta receptor 1 (TGFBR1); hepatocellular carcinoma (HCC); microRNA-1294 (miR-1294)

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Introduction

Hepatocellular carcinoma (HCC) is the most prevalent pathological subtype of primary liver cancer (75–85%) with a high mortality rate worldwide (1). The pathogenesis of liver cancer is commonly thought to be complicated by various genetic and environmental factors (2-4). In recent years, long non-coding RNAs (lncRNAs) have been shown to be critical in regulating a wide range of biological and pathological processes, including the formation of tumors (5). LncASAP1-IT1 has been reported to regulate the development and the progression of various types of cancer, including lung, bladder, and ovarian cancer (6-8). Fu *et al.* reported that lncASAP1-IT1 was highly expressed in early-stage epithelial ovarian cancer and low-grade tumors (6).

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Yang *et al.*'s findings suggest that lncASAP1-IT1 promotes the stemness of bladder cancer and is associated with a poor prognosis (7). In non-small cell lung cancer, Zhang *et al.* found that lncASAP1-IT1 induced cytotoxic behaviors in cancer cells, such as proliferation, invasion, and metastasis, by activating the PTEN/AKT pathway (8). LncASAP1-IT1 was reported to be upregulated in 3 different HCC cell lines in contrast to normal human hepatocytes, evidenced by searching lncRNA expression profile (GSE25859). However, the role of lncASAP1-IT1 in HCC development remains to be investigated.

LncRNAs bind with micro RNAs (miRNAs) bearing complementary sequences and serve a "sponge" function to re-activate target messenger RNA, and some lncRNAs bind with multiple miRNAs. This competitive endogenous RNA (ceRNA) pathway has been linked to various illnesses, including cancer. Our bioinformatic analysis indicated that miR-1294 may bind with lncASAP1-IT1. miR-1294 has been reported to be involved in the regulation of multiple types of cancer, such as gastric cancer, breast cancer, ovarian cancer, osteosarcoma, pancreatic ductal adenocarcinoma, and HCC (9-11). A study has reported that ceRNA networks exhibit significant potential in the diagnosis and targeted therapy of cancer. However, the function of lncASAP1-IT1/miR-1294 in HCC remains incompletely defined (12).

In the present study, we hypothesized that lncASAP1-IT1 controls the miR-1294/transforming growth factor beta receptor 1 (TGFBR1) signaling axis during the development of HCC. Thus, we investigated the possible significance of lncASAP1-IT1 in HCC and its molecular mechanism. We present this article in accordance with the ARRIVE and MDAR reporting checklists (available at https://jgo.amegroups.com/article/view/10.21037/jgo-23-327/rc).

Highlight box

Key findings

- We found that the long non-coding RNA ASAP1-IT1 regulated cell proliferation, invasion, migration, and epithelial-mesenchymal transition (EMT) progression and enhanced sensitivity to sorafenib in hepatocellular carcinoma (HCC) by targeting microRNA-1294 (miR-1294)/transforming growth factor beta receptor 1 (TGFBR1) and identified a new regulatory mechanism in HCC.

What is known and what is new?

- ASAP1-IT1 is upregulated in HCC tissues and cells.
- The knockdown of ASAP1-IT1 impeded cell proliferation, migration, invasion, and EMT progression and enhanced sensitivity to sorafenib in HCC through the miR-1294/TGFBR1 axis.

What is the implication, and what should change now?

- We identified a new biomarker and potential therapeutic target for the prognosis and treatment of HCC.

Methods

Human liver cancer tumor samples and cell lines

In total, 30 paired human HCC and adjacent histologically normal tissues (>2 cm from the tumors' edges) were collected from HCC patients (20 male and 10 female; age range: 40–73 years) who underwent surgical treatment at The Fourth Hospital of Hebei Medical University. During the surgery, samples were taken from patients, and the diagnosis was verified by skilled pathologists. Patients were excluded from the study if they had previously undergone radiation or chemotherapy, had a history of malignancy, or had not provided informed consent. Real-time-quantitative polymerase chain reaction (RT-qPCR) was used to determine the expression levels of lncASAP1-IT1 in the liver tissues. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by ethics board of the Fourth Hospital of Hebei Medical University (No. 2022KS025) and informed consent was taken from all the patients.

The Shanghai Cell Bank provided the THLE-2 normal human cell line and the Bel-7405, Huh7, Hep3B, and Sk-Hep1 malignant cell lines.

Bioinformatics prediction

The interaction between miR-1294 and lncASAP1-IT1 was predicted by DIANA TOOLS (http://diana.imis.athena-innovation.gr) and LncBook (https://ngdc.cncb.ac.cn/lncbook/interaction). MiRDB (http://www.mirdb.org/), Targetscan (http://www.targetscan.org/), and Starbase (http://starbase.sysu.edu.cn/) predicted the potential target gene TGFBR1 that could be targeted by miR-1294.

Luciferase reporter assays

The Dual-Luciferase Reporter Assay System (Promega, Shanghai) was used to quantify Firefly and Renilla luciferase activity (13).

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Cell Counting Kit 8 (CCK-8) assays

The cell suspensions of each group were collected, and 100 μL of cells (1×10^6 cells/well) were inoculated into 96-well plates; each group comprised 3 replicate wells. After 48 hours of growth, 10 μL of CCK-8 reagent (Shanghai Biyuntian Co., Ltd.) was added to each well and incubated at 37 ℃ for 1 h. The absorbance was read at 490 nm with a microplate reader (14).

Colony formation assays

100 cells were counted and seeded in triplicate in a 6-well plate, and cultured continuously for 14 days. Culture was stopped when visible clones appeared in the petri dish. After discarding the supernatant, the cells were washed with phosphate buffered saline. The cells were fixed with 4% paraformaldehyde for 15 min, stained with 0.1% crystal violet for 10 min, and air-dried. The number of effective clones with at least 15 cells was counted under low magnification (15).

Transwell assays

Transwell chambers coated (invasion) or uncoated (migration) with Matrigel (BD, USA) were placed in a 24-well cell plate, and cell solution containing 10^5 cells was added to the upper chamber. Dulbecco's Modified Eagle Medium (500 μL) containing 10% fetal bovine serum was then added to the lower chamber. After 24 h of incubation, the chamber was removed. After washing the cells with phosphate buffered saline, the remaining cells on the filter membrane were carefully wiped off with a cotton swab, and 4% paraformaldehyde and 0.5% crystal violet were added for fixation and staining, respectively, for 15 min. We then randomly selected 3 visual fields under the microscope to observe the number of transmembrane cells (16).

Nude mouse tumorigenicity assays

A total of 10 female BALB/c nude mice (aged 4–6 weeks old and weighing 18–20 g) were acquired from the Vital River Laboratory Animal Center (Beijing, China). The nude mice were randomly allocated to two groups (the NC and shASAP1-IT1 groups), of 5 mice per group, based on their body weight, and reared in a specific pathogen-free environment. Next, 2×10^6 cells were implanted subcutaneously into the nude mice to form tumors. When the maximum diameter of the tumors reached about 1 cm, the nude mice underwent uniform euthanasia, and the tumor tissues were stripped, photographed, and weighed. Formalin-fixed and paraffin-embedded tissue sections were incubated with TGFBR1 primary antibody (dilution 1:200; Cell Signaling Technology), Ki67 antibody (dilution 1:200; Proteintech), and CD34 (dilution 1:200; Abcam) overnight at 4 ℃ and the Horseradish peroxidase (HRP)-labeled secondary antibodies (dilution 1:2,000; Abcam) were then incubated. Animal experiments were performed under a project license (No. 2019033) granted by the ethics committee of the Fourth Hospital of Hebei Medical University, in compliance with national guidelines for the care and use of animals. A protocol was prepared before the study without registration.

RT-qPCR

Complementary DNA was generated by reverse transcription using RNA as the template, according to the instructions of the PrimeScript RT Reagent Kit (Invitrogen, Thermo Fisher Scientific). RT-qPCR was performed on an ABI 7500 PCR System using SYBR-Green Master Mix (Invitrogen, Thermo Fisher Scientific). The following primer sequences were used:

- ASAP1-IT1: forward: 5'-AAACATCATCCCCAGAGTGG-3';
- ASAP1-IT1: reverse: 5'-GCCTTGCTCACCTCTGAAAC-3';
- miR-1294: forward: 5'-CTCACGAGAGAGGAAGGCA-3';
- miR-1294: reverse: 5'-ACCTCAAGAACAGTATTTCCAGG-3';
- U6: forward: 5'-CGCTTCACGAATTTGCGTGTCAT-3';
- U6: reverse: 5'-CGCTTCACGAATTTGCGTGTCAT-3';
- TGFBR1: forward: 5'-TGCCATAACCGCACTGTCA-3';
- TGFBR1: reverse: 5'-AATGAAAGGGCGATCTAGTGATG-3';
- GAPDH: forward: 5'-CGCTCTCTGCTCCTCCTGTTC-3';
- GAPDH: reverse: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

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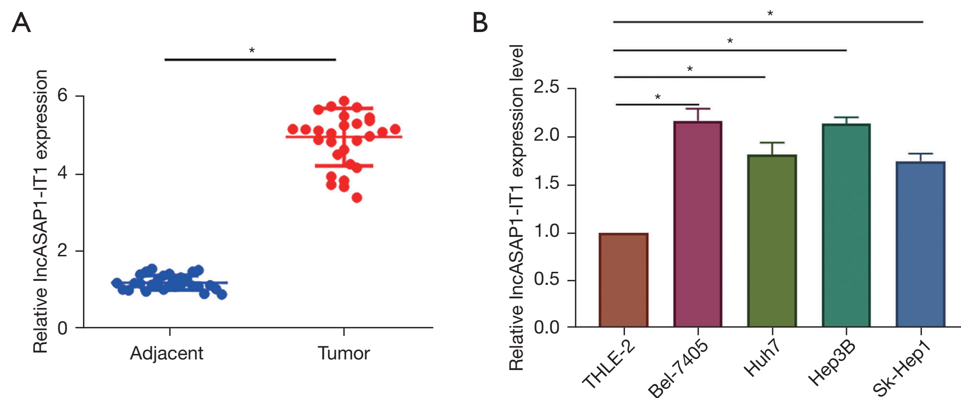

Figure 1 LncRNA ASAP1-IT1 expression in hepatocellular carcinoma. (A) RT-qPCR was used to determine the expression levels of ASAP1-IT1 in the HCC tissues; (B) RT-qPCR was used to determine the expression levels of ASAP1-IT1 in the HCC cell lines. *, P<0.05. LncRNA, long non-coding RNA; RT-qPCR, real-time-quantitative polymerase chain reaction; HCC, hepatocellular carcinoma.

Western blot

The cells were collected from each group during the logarithmic growth phase, and radio immunoprecipitation assay (RIPA) lysis buffer lysate was added for lysis and centrifugation to extract the total cell proteins. The cell protein concentration was determined using the bicinchoninic acid (BCA) method. Protein samples of 40 µg were thoroughly mixed with loading buffer, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) then transferred on polyvinylidene fluoride (PVDF) membranes. The membranes were incubated overnight at 4 ℃ with primary antibodies against E-cadherin, N-cadherin, TGFBR1, β-actin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (all 1:1,000, from Cell Signaling Technology) were added, and at 4 ℃ overnight. The membrane was then washed with Tris-buffered saline-Tween-20 buffer (TBST), secondary antibodies (Abcam, 1:10,000) for 2 h at room temperature, and TBST washed the membrane. Protein bands were developed by a standard enhanced chemiluminescence (ECL) kit (Solarbio), and observed via a gel image analysis system (Bio-Rad).

Statistical analysis

The data were analyzed using GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA, USA). An analysis of variance or a Student's *t*-test was used. A two-sided P value <0.05 was considered statistically significant.

Results

LncASAP1-IT1 is overexpressed in liver cancer tissue and liver cancer cell lines

The expression of lncASAP1-IT1 was initially assessed by RT-qPCR to examine its involvement in liver cancer pathogenesis and pathogenesis. We discovered that lncASAP1-IT1 was significantly more highly expressed in the liver cancer tissues than the healthy liver tissues. Further, the expression level of lncASAP1-IT1 was significantly higher in the HCC lines (i.e., Bel-7405, Huh7, Hep3B, and Sk-Hep1) than the normal human cell line (i.e., THLE-2) (*Figure 1*).

Knocking down lncASAP1-IT1 significantly suppresses the cytotoxic behaviors of liver cancer cells

RT-qPCR revealed that compared to the control group, the transfection of sh-lncASAP1-IT1-1 and sh-lncASAP1-IT1-2 significantly decreased the level of lncASAP1-IT1-1 in the Bel-7405 and Hep-3B cells (*Figure 2A*). The knockdown of lncASAP1-IT1 inhibited the survival rates of the Bel-7405 and Hep-3B cells (*Figure 2B*). The nuclear condensation in the shASAP1-IT1 groups that underwent Giemsa staining by microscopic observation is shown in *Figure 2C*. A colony formation experiment was also conducted to investigate the effect of lncASAP1-IT1 on the proliferative abilities of the Bel-7405 and Hep-3B cells. Our results showed that lncASAP1-IT1 knockdown

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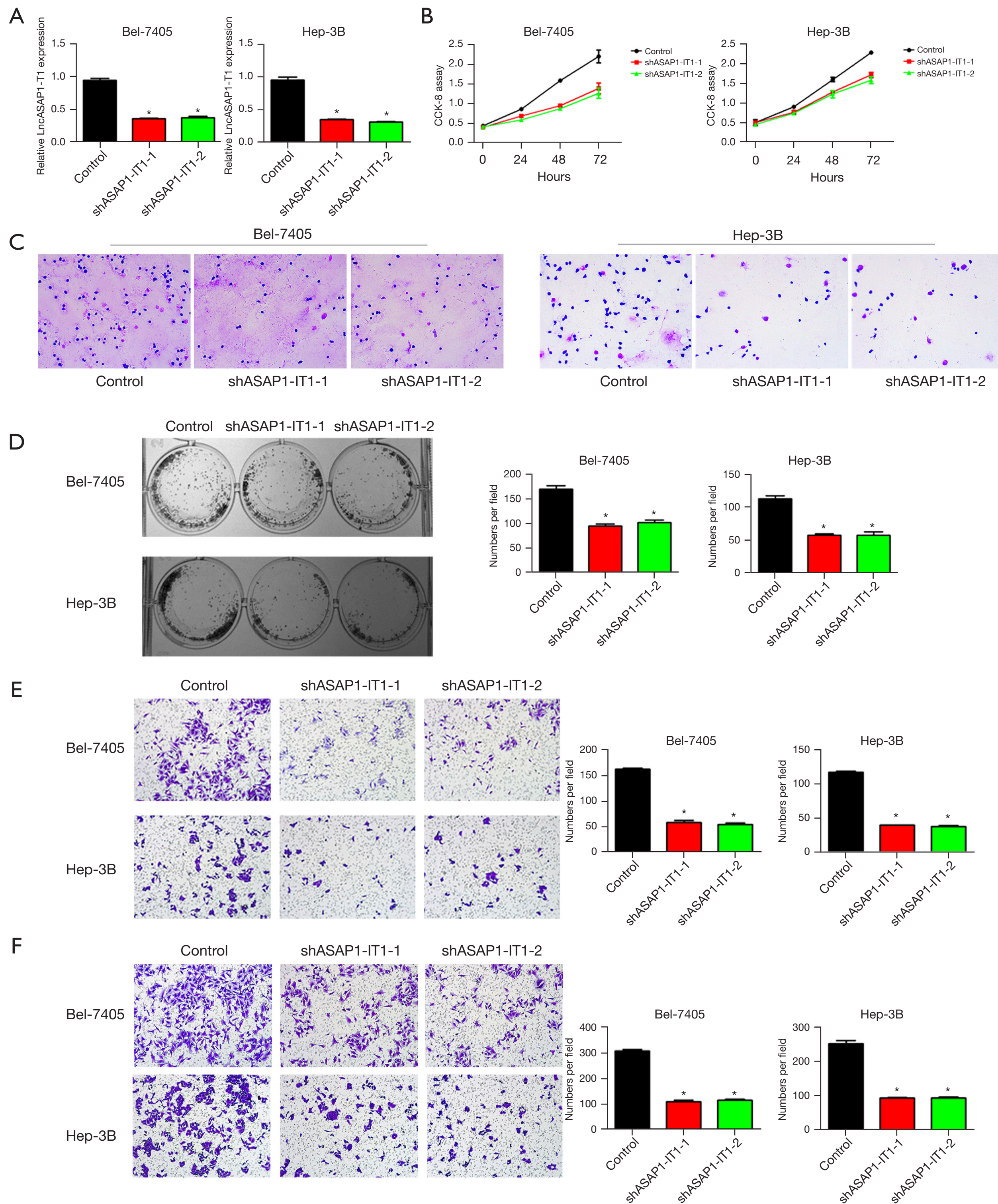

Figure 2 The HCC cells were transfected with shASAP1-IT1. (A) The level of ASAP1-IT1 in HCC cells was detected by RT-qPCR; (B) cell viability was determined by CCK-8 assays; (C) the morphological changes of the cells were detected by Giemsa staining (×200); (D) colony formation assays. The biological role of shASAP1-IT1 on cell invasion (E) and (F) migration was assessed by transwell assays (200× magnification; crystal violet staining). *, P<0.05. CCK-8, Cell Counting Kit 8; HCC, hepatocellular carcinoma; RT-qPCR, real-time-quantitative polymerase chain reaction.

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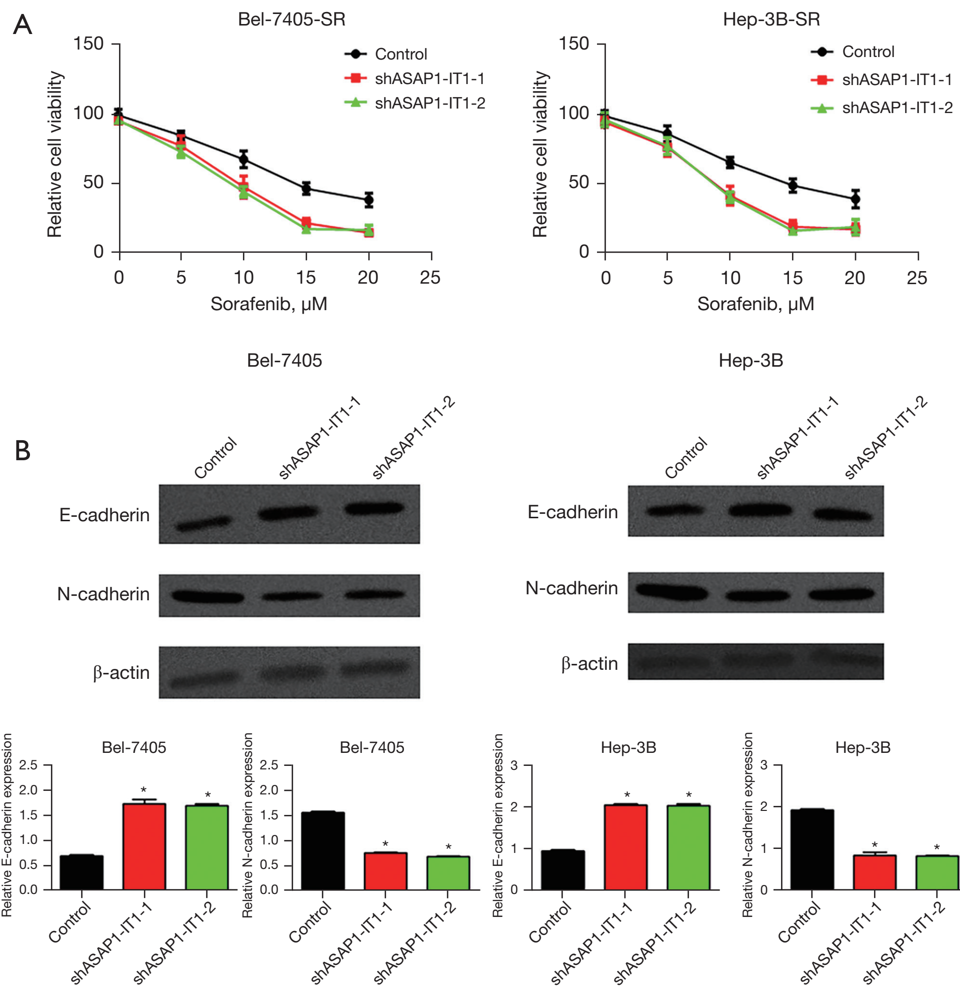

Figure 3 Effect of ASAP1-IT1 expression on the sensitivity of the hepatic carcinoma cells against sorafenib. (A) CCK-8 was used to detect cell viability; (B) the levels of EMT markers (E-cadherin and N-cadherin) were determined by western blotting. *, P<0.05. SR, sorafenib resistant; CCK-8, Cell Counting Kit 8; EMT, epithelial-mesenchymal transition.

significantly decreased the colony numbers in both the Bel-7405 and Hep-3B cells (*Figure 2D*). The transwell assay results showed that lncASAP1-IT1 knockdown reduced Bel-7405 and Hep-3B cell migration and invasion capacities (*Figure 2E,2F*). These findings indicated that lncASAP1-IT1 knockdown decreased HCC cell growth and migration.

LncASAP1-IT1 contributes to HCC cell lines sorafenib resistance and ETM

To better understand lncASAP1-IT1 function in chemoresistance, we generated sorafenib-resistant HCC lines (using Bel-7405-SR Hep3B-SR) and evaluated drug resistance using the CCK-8 test. As seen in *Figure 3A*, we found that silencing lncASAP1-IT1 enhanced the sorafenib sensitivity of the HCC cells. We then sought to decipher how ASAP1-IT1 contributes to epithelial-mesenchymal transition (EMT). The Western blot analysis revealed that compared to the control, interfering with ASAP1-IT1 increased E-cadherin expression but decreased N-cadherin expression (*Figure 3B*).

LncASAP1-IT1 acts as a sponge for miR-1294

As predicted by the DIANA and LncBase tools, we found that lncASAP1-IT1 directly binds to miR-1294 (*Figure 4A*). We then used RNA mimics to overexpress miR-1294 and miR-1294 inhibitors to downregulate its expression in the

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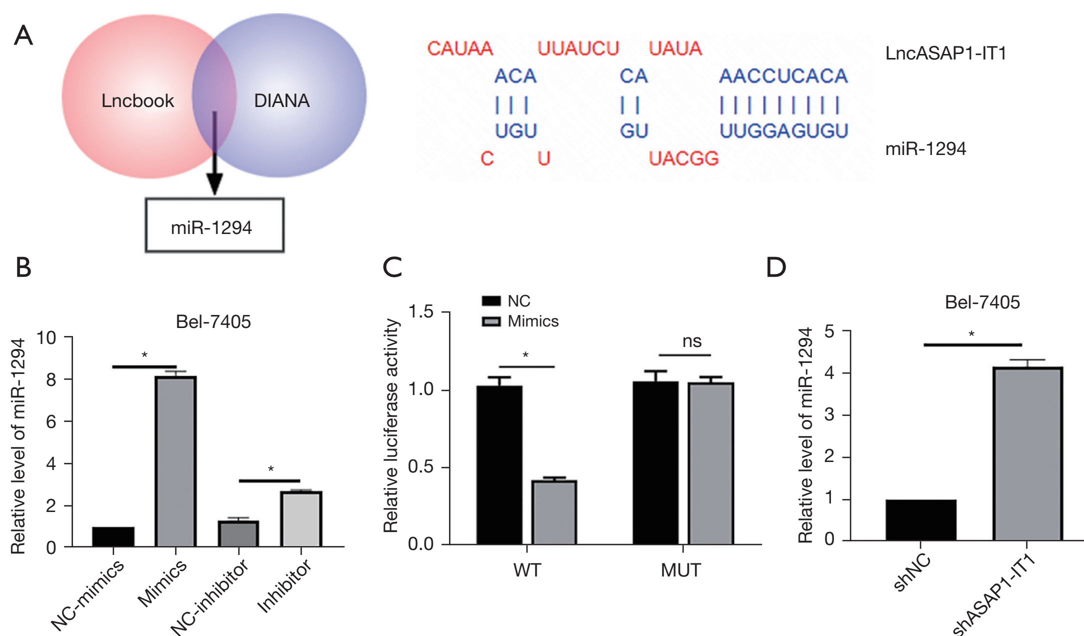

Figure 4 MiR-1294 binds to ASAP1-IT1. (A) DIANA and LncBase were used to estimate the binding sites between miR-1294 and ASAP1-IT1; (B) RT-qPCR was used to assess relative miR-1294 expression following transfection with a miR-1294 mimic or inhibitor; (C) the relative luciferase activity was determined using luciferase reporter assays; (D) after transfection with sh-ASAP1-IT1, the relative miR-1294 expression was assessed by RT-qPCR. *, P<0.05. NC, negative control; WT, wild type; MUT, mutant type; ns, not significant; shNC, negative control shRNA; ns, not significant; RT-qPCR, real-time-quantitative polymerase chain reaction.

Bel-7405 cells (*Figure 4B*). To establish the binding of miR-1294 to lncASAP1-IT1, we used luciferase assays. Our findings indicated that lncASAP1-IT1 binds directly to miR-1294 (*Figure 4C*). Conversely, silencing lncASAP1-IT1 resulted in a reduction in miR-1294 expression in comparison to the control group (*Figure 4D*).

TGFBR1 is the downstream target of miR-1294

The data indicated that miR-1294 might target TGFBR1 (*Figure 5A*). Compared to the control groups, the miR-1294 mimics significantly inhibited TGFBR1 expression, and the miR-1294 inhibitors significantly increased TGFBR1 expression (*Figure 5B*). These findings provided further evidence of the effect of miR-1294 on TGFBR1 expression. The regulation of miR-1294 on TGFBR1 was subsequently confirmed by a luciferase experiment. The results indicated that the miR-1294 mimic significantly reduced the luciferase activity of wild-type TGFBR1 (*Figure 5C*). Additionally, silencing lncASAP1-IT1 lowered TGFBR1 expression compared to the control group, but silencing miR-1294 reversed the decrease in TGFBR1 expression induced by shASAP1-IT1 (*Figure 5D*). In terms of cytotoxic behavior modulation, we assessed the migratory ability of the liver cancer cells using the transwell test. The overexpression of miR-1294, silencing of lncASAP1-IT1, and silencing of TGFBR1 all resulted in a substantial reduction in Bel-7405 cell movement compared to the control. Additionally, the treatment with the miR-1294 inhibitors restored the decreased migratory abilities of the Bel-7405 cells caused by the TGFBR1 knockdown, while the overexpression of TGFBR1 restored the reduced migration abilities of the Bel-7405 cells induced by the shASAP1-IT1 (*Figure 5E*).

LncASAP1-IT1 suppressed the liver cancer growth of the xenograft tumors in vivo

Bel-7405 cells expressing shASAP1-IT1 or sh-NC were implanted into the subcutaneous region of the necks of the nude mice. After dissection, the tumor volume was determined, and the shASAP1-IT1 group had a larger tumor volume than the control group (*Figure 6A-6C*). The immunohistochemistry staining showed the lower expression of TGFBR1 and the proliferation and

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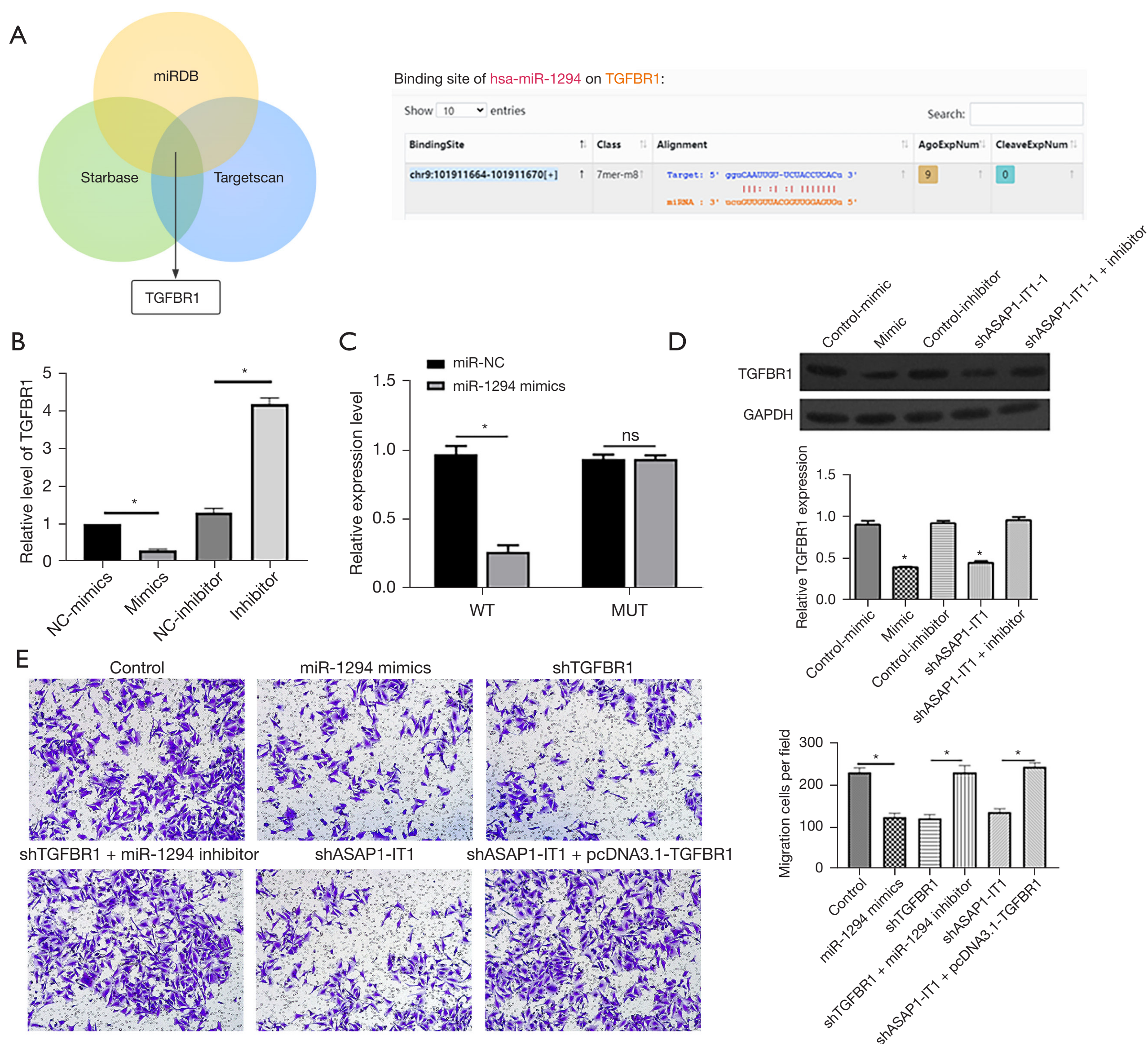

Figure 5 MiR-1294 negatively regulated TGFBR1 expression in HCC cells. (A) Bioinformatics website prediction of miR-1294 and TGFBR1; (B) RT-qPCR assays were conducted to determine the relative levels of TGFBR1 after transfection with the miR-1294 mimics or miR-1294 inhibitor; (C) dual-luciferase reporter assays were performed in the Bel-7405 cells; (D) the levels of TGFBR1 were determined by western blotting; (E) the migration cell number of different groups was determined by transwell assays (200× magnification; crystal violet staining). *, P<0.05. NC, negative control; WT, wild type; MUT, mutant type; ns, not significant; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCC, hepatocellular carcinoma; RT-qPCR, real-time-quantitative polymerase chain reaction; TGFBR1, transforming growth factor beta receptor 1.

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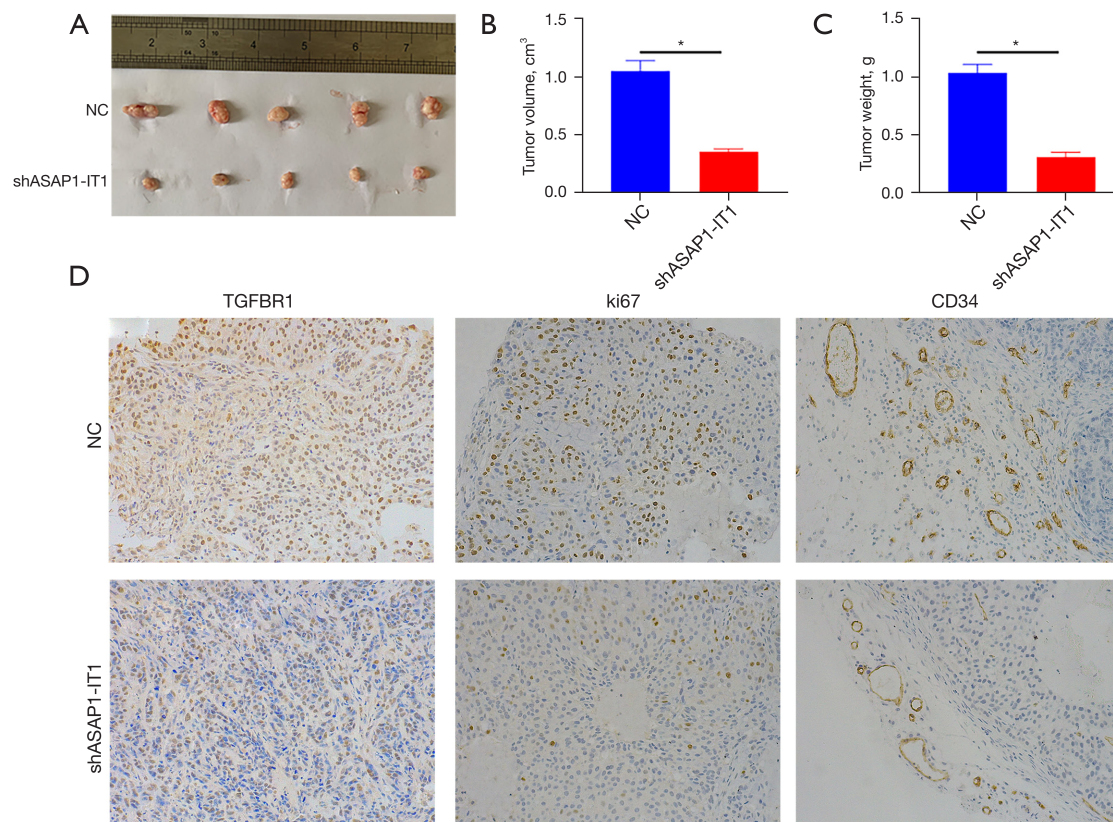

Figure 6 The knockdown of ASAP1-IT1 inhibited tumor growth in a xenograft mouse model. (A) Representative images of tumors in the shASAP-IT1 and the NC groups; (B) tumor volume and (C) weight changed in the mice injected with Bel-7405 cells after shASAP-IT1; (D) IHC detected the expression of TGFBR1, ki67, and CD34 in tumor tissues (magnification, ×200). *, P<0.05. NC, negative control; TGFBR1, transforming growth factor beta receptor 1; IHC, immunohistochemistry.

angiogenesis markers (Ki-67 and CD34) in the shASAP1-IT1 group compared with the NC group (*Figure 6D*).

Discussion

HCC is a prevalent liver cancer globally. Despite breakthroughs in clinical intervention tactics, high rates of recurrence and metastasis impede the therapeutic efficacy and result in poor outcomes (2,4,17). Thus, knowledge of the process driving carcinogenesis is required to diagnose and treat HCC early.

LncRNAs have been reported to be critical non-coding RNAs that regulate liver cancer progression in various aspects of the cellular functions of liver cancer cells, including proliferation, apoptosis, migration, and invasion (18,19). For example, Xu *et al.* showed that lncRNA RP11-386G11.10 was overexpressed in HCC and positively correlated with tumor size, tumor node metastasis stage, and a poor prognosis in HCC patients (20). Moreover, linc Hes family bHLH transcription factor 6 (lncSHRG) has been shown to accelerate HCC cell progression by targeting miR26a/SKP2 axis (21). The current study found that lncASAP1-IT1 expression was significantly elevated in the HCC tissues compared to the adjacent healthy tissues. Experiments on the cytotoxic behaviors of HCC cells suggest that lncASAP1-IT1 regulates the proliferation, migration, invasion, and sorafenib sensitivity of the Bel-7405 and Hep-3B cells.

In the present study, we also demonstrated that lncASAP1-IT1 regulates the target gene TGFBR1 by binding with miR-1294 by the ceRNA mechanism. Many studies have indicated that miR-1294 is a suppressor in the pathogenesis of HCC (22,23). We found that lncASAP1-IT1 regulates HCC tumorigeneses by facilitating miR-1294. TGFBR1 has been reported to be related to the development of colorectal cancer, enhance the migration

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and invasion of breast cancer cells and participate in bladder cancer and non-small cell lung cancer (24-27). However, further research needs to be conducted to determine if TGFBR1 regulates liver cancer cells. Our results suggest that the upregulation of TGFBR1 through miR-1294 promotes the migration abilities of liver cancer cells and thus suggests that TGFBR1 plays a role in promoting liver cancer progression. In addition, there are some problems and challenges that need to be overcome in the clinical application of lncRNA, such as delivery, specificity and tolerability.

Conclusions

Our findings suggest that lncASAP1-IT1 is highly expressed in HCC and supports its progression through the miR-1294/TGFBR1 axis. The inhibition of ASAP1-IT1 impedes cell proliferation, migration, invasion, and EMT progression, and enhances sensitivity of HCC cells to sorafenib in HCC. Taken together, our results provide new insight into the role of lncRNAs in the development of HCC and support the notion that lncASAP1-IT1 may serve as a prognostic biomarker and a potential therapeutic molecular target for the treatment of HCC.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE and MDAR reporting checklists. Available at https://jgo.amegroups.com/article/view/10.21037/jgo-23-327/rc

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://jgo.amegroups.com/article/view/10.21037/jgo-23-327/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Animal experiments were performed under a project license (No. 2019033) granted by the ethics committee of the Fourth Hospital of Hebei Medical University, in compliance with national guidelines for the care and use of animals. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by ethics board of the Fourth Hospital of Hebei Medical University (No. 2022KS025) and informed consent was taken from all the patients.

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