lncRNA HAND2-AS1 mediates the downregulation of ROCK2 in hepatocellular carcinoma and inhibits cancer cell proliferation, migration and invasion

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Abstract. Long noncoding (lnc)RNA HAND2-AS1 inhibits the development of several human malignancies. The role of HAND2-AS1 was investigated in hepatocellular carcinoma (HCC). It was found that levels of HAND2-AS1 in serum were significantly lower, while serum levels of Rho-associated protein kinase 2 (ROCK2) in HCC patients were significantly increased compared with hepatitis B (HB) patients and healthy controls. Decreased HAND2-AS1 levels distinguished HCC patients but not HB patients from healthy controls. A significant negative correlation between HAND2-AS1 and ROCK2 was found in HCC patients but not in HB patients or healthy controls. HAND2-AS1 overexpression inhibited, while ROCK2 overexpression promoted HCC cell migration, proliferation and invasion. HAND2-AS1 overexpression led to downregulated ROCK2 expression. ROCK2 overexpression did not significantly affect ROCK2 expression but attenuated the inhibitory effects of HAND2-AS1 overexpression. It was therefore concluded that HAND2-AS1 might mediate the downregulation of ROCK2 in HCC to inhibit cancer cell migration, proliferation and invasion.

Introduction

Liver cancer causes more than 70,000 deaths every year worldwide (1,2). The incidence of liver cancer is quite different among different regions. In China, the high infection rate of hepatitis B and C leads to the high prevalence of liver cancer (3). Hepatocellular carcinoma (HCC) accounts for the majority of liver cancer and death among patients with cirrhosis is usually caused by HCC, which is mainly caused by chronic hepatitis C virus (HCV), and hepatitis B virus (HBV) infection (4). Survival of patients with early stages of HCC is generally satisfactory (5). However, once metastasis occurs, treatment outcomes will be extremely poor (6).

Rho associated coiled-coil containing protein kinase 2 (ROCK2) plays pivotal roles in regulating smooth muscle contraction, cytokinesis and the formation of focal adhesions as well as actin stress fibers (7). Previous studies have shown that ROCK kinases including ROCK2 also have critical functions in the development of human cancers including HCC (8,9). ROCK2 inhibition provides new insights to the treatment of certain malignancies (10,11). HAND2-AS1 suppresses several types of human cancers (12-15), but its functions in HCC are unknown. The present study performed deep sequencing-based transcriptome analysis and observed the inverse correlation between HAND2-AS1 and ROCK2 (data not shown). The present study revealed that HAND2-AS1 might mediate the downregulation of ROCK2 in HCC to inhibit cancer cell behaviors.

Materials and methods

Patients and serum specimens. A total of 44 HCC patients (44-68 years) and 38 hepatitis B (HB, 44-68 years) patients who were treated in Zhongshan Hospital, Shanghai were enrolled from January 2017 to May 2018. HBV infections were determined by sensitive PCR. Inclusion criteria are: i) Patients diagnosed in the Zhongshan hospital; ii) patients received treatment for the first time; iii) HCC patients at AJCC stage IA-IIIA. Exclusion criteria include: i) Patients complicated with other severe diseases or liver diseases; ii) patients who were treated within 3 months before this study; iii) patients have difficulties in understanding the experimental protocol. During the same time period, 32 healthy volunteers were also included to be control group of the present study. Blood (5 ml) was extracted from the elbow vein of each participant before breakfast. Blood samples were used to prepare serum using

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conventional methods. Patient characteristics are presented in Table I. Clinical factors were compared among groups using a chi-squared test. No significant differences in age (P=0.63), gender (P=0.52), smoking habit (P=0.36) and drinking habit (P=0.34) were found among groups. The present study received approval from The Ethics Committee of Zhongshan Hospital, Shanghai before patient admission. Informed consent was signed by all participants.

ELISA. Serum levels of ROCK2 were measured through an ELISA using a kit (MBS705283, MyBioSource). The detection sensitivity of the kit was 156 pg/ml and the detection range was 625-40,000 pg/ml.

Reverse transcription-quantitative (RT-q)PCR. RNAzol® RT (Sigma-Aldrich; Merck KGaA) was mixed with serum and cells to extract RNAs. Following reverse transcription (25˚C for 5 min, 55˚C for 30 min and 75˚C for 10 min), PCR reaction systems were prepared using SuperScript III Platinum One-Step RT-qPCR kit (Thermo Fisher Scientific, Inc.). Thermocycling conditions: 95˚C for 57 sec and then 40 cycles of 95˚C for 16 sec and 56.5˚C for 33 sec. Sequences of primers: 5'-GGGTGTTTACGTAGACCAGAACC-3' (forward) and 5'-CTTCCAAAAGCCTTCTGCCTTAG-3' (reverse) for HAND2-AS1; 5'-GACCTCTATGCCAACACAG-3' (forward) and 5'-AGTACTTGCGCTCAGGAGG-3' (reverse) for β-actin. All Cq values were processed through $2^{-\Delta\Delta Cq}$ method (16).

Cell lines, cell culture and cell transfection. SNU-398 human HCC cell line American Type Culture Collection (ATCC) and THLE-3 human normal liver epithelial cell line (ATCC) were included. HAND2-AS1 and ROCK2 expression pIRSE2 vectors as well as empty vectors were bought from GeneCopoeia, Inc. HAND2-AS1 and ROCK2 vectors (10 nM) or empty vectors (10 nM; negative control) were transfected into cells using lipofectamine 2000 (11668-019; Invitrogen; Thermo Fisher Scientific, Inc.). The control group included untransfected cells. Before the following experiments, overexpression of HAND2-AS1 and ROCK2 (overexpression rate: 200-240%) was confirmed by RT-qPCR. The following experiments were performed at 24 h post-transfection.

Cell proliferation analysis. Cell proliferation analysis was performed by Cell Counting Kit-8 (CCK-8) assay at 24 h post-transfection. Briefly, cells were harvested to prepare cell suspensions ($5x10^4$/ml). Cells were cultivated in a 96-well plate (0.1 ml per well) and 10 μl CCK-8 solution was added 4 h before the end of cell culture. Following the addition of 10 μl DMSO, optical density values at 450 nm were measured.

Cell migration and invasion analysis. Cell migration and invasion were analyzed at 24 h post-transfection following the same procedure except that Matrigel (356234; EMD Millipore) was used to coat the upper chamber before invasion assay. Transwell inserts (8 μm Dojindo Molecular Technologies, Inc.) were used. Cell suspensions ($5x10^4$/ml) were prepared using serum free RPMI 1640 medium (ATCC). A total of 0.1 ml cell suspension was transferred to the upper chamber and culture medium containing 20% fetal bovine serum (Sigma-Aldrich, Merck KGaA) was used to fill the lower chamber. Membranes were collected 24 h later, being followed by staining with 0.5% crystal violet (Sigma-Aldrich, Merck KGaA) at room temperature for 15 min. Under a light microscope, migrating cells and invading cells were observed.

Table I. General information of 3 groups of participants.

	HCC patients	HB patients	Controls
Cases	44	38	32
Sex			
Male	24	20	17
Female	20	18	15
Habits			
Smoking	22 (50.0%)	17 (44.7%)	15 (46.9%)
Drinking	24 (54.5%)	18 (47.4%)	19 (59.4%)

HCC, hepatocellular carcinoma; HB, hepatitis B.

Western-blotting. Following total protein extraction using RIPA solution, BCA assay (both from Sigma-Aldrich, Merck KGaA) was performed to measure protein concentrations. Electrophoresis was carried out using 12% SDS-PAGE gel with 30 μg denatured protein per well. After protein transfer to PVDF membranes, blocking in 5% non-fat milk was performed for 2 h at room temperature. Then membranes were incubated with primary antibodies of rabbit anti-human ROCK2 (1:1,300; cat. no. ab66320; Abcam) and GAPDH (1:1,300; cat. no. ab8245; Abcam) overnight at 4˚C. Followed by incubation with IgG-HRP goat anti-rabbit secondary antibody (1:800; cat. no. MBS435036; MyBioSource) at room temperature for 2 h. ECL Detection Reagent (Sigma-Aldrich; Merck KGaA) was used to develop signals. Image J v1.6 software (National Institute of Health) was used for signal normalization.

Statistical analysis. Experiments were performed in sets of 3 repeats and mean ± standard error of the mean values were calculated. Differences among groups were explored using analysis of variance (one-way) by Tukey test. Correlation analysis was performed by Pearson correlation coefficient. Diagnostic analyses were performed using receiver operating characteristic (ROC) curve. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of HAND2-AS1 and ROCK2 are altered only in HCC patients. The present study first detected the expression of HAND2-AS1 and ROCK2 in the serum of all 3 groups of participants. Results showed that serum levels of HAND2-AS1 were significantly downregulated (P<0.05; Fig. 1A), while serum levels of ROCK2 were significantly increased (P<0.05; Fig. 1B) in HCC patients compared with in HB patients and healthy controls. However, no significant differences in serum levels of HAND2-AS1 and ROCK2 were found between HB patients and healthy controls. It is worth noting that, HAND2-AS1

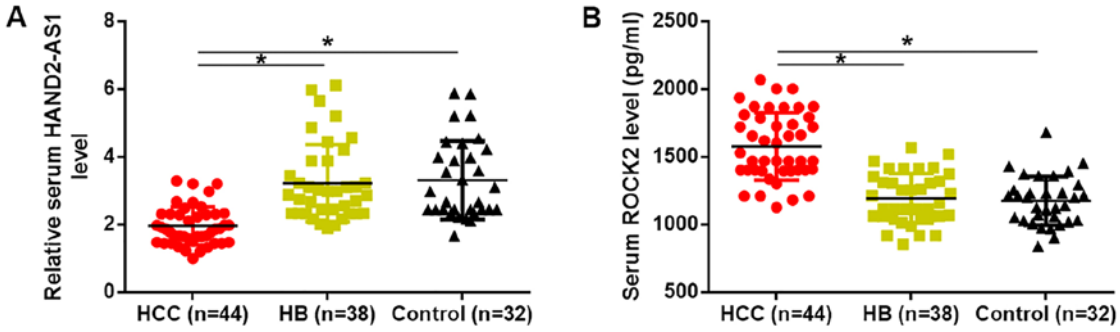

Figure 1. Expression of HAND2-AS1 and ROCK2 is altered only in HCC patients. (A) Serum levels of HAND2-AS1 were significantly downregulated, while serum levels of (B) ROCK2 were significantly increased in HCC patients compared with in HB patients and healthy controls. *P<0.05. HCC, hepatocellular carcinoma; HB, hepatitis B; ROCK2, Rho-associated protein kinase 2.

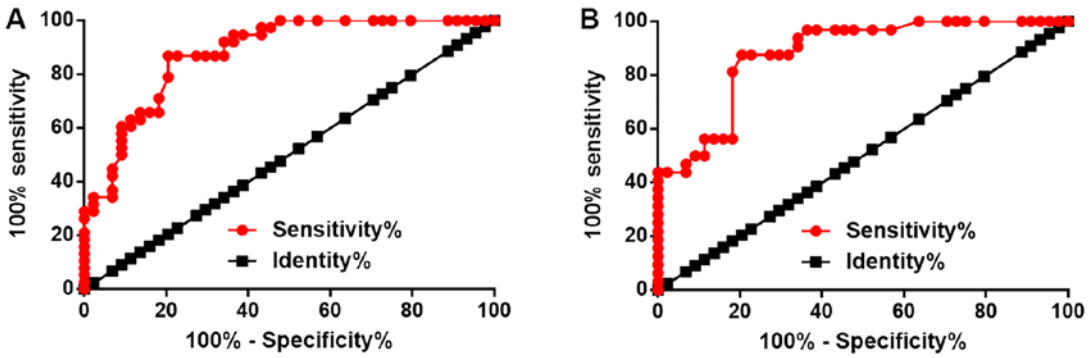

Figure 2. Downregulation of HAND2-AS1 distinguishes HCC patients from HB patients and healthy controls. Receiver operating characteristic curve analysis revealed that downregulation of HAND2-AS1 distinguished HCC patients from (A) HB patients and (B) healthy controls. HCC, hepatocellular carcinoma; HB, hepatitis B.

expression levels decreased, while ROCK2 expression levels increased with the increase of clinical stages, however the changes were not statistically significant (data not shown).

Decreased HAND2-AS1 levels distinguishes HCC patients from HB patients and healthy controls. ROC curve analysis was performed with HCC patients as true positive subjects and HB patients or healthy controls as true negative subjects (Fig. 2). With HB patients as references, the area under the curve (AUC) was 0.8792 (standard error: 0.03662; 95% confidence interval: 0.8074-0.9510). With healthy controls as references, the AUC was 0.8800 (standard error: 0.03795; 95% confidence interval: 0.8056-0.9544).

HAND2-AS1 and ROCK2 are negatively correlated in HCC patients. Pearson correlation analysis was performed to investigate the correlations between serum levels of HAND2-AS1 and ROCK2. Significant negative correlation between serum levels of HAND2-AS1 and ROCK2 was observed in HCC patients (P<0.0001; Fig. 3A). However, the correlation between serum levels of HAND2-AS1 and ROCK2 was not strong in HB patients (Fig. 3B) and healthy controls (Fig. 3C).

HAND2-AS1 overexpression leads to inhibited ROCK2 expression in HCC cells but not in normal liver epithelial cells. In this experiment, the control (C) group was the untransfected cells and the negative control (NC) group was the cells transfected with empty vectors. Compared with C and NC, HAND2-AS1 overexpression led to significantly inhibited ROCK2 expression in cells of SNU-398 human HCC cell line (P<0.05) but not in cells of THLE-3 human normal liver epithelial cell line (Fig. 4A). In contrast, ROCK2 overexpression did not significantly affect HAND2-AS1 expression in cells of those two cell lines (Fig. 4B).

HAND2-AS1 and ROCK2 play opposite roles in regulating HCC cell behaviors. In this experiment, the control group was the untransfected cells and NC group was the cells transfected with empty vectors. Compared with C (untransfected cells) and NC (empty vector transfection), HAND2-AS1 overexpression significantly inhibited, while ROCK2 overexpression significantly promoted the proliferation (P<0.05; Fig. 5A), migration (P<0.05; Fig. 5B) and invasion (P<0.05; Fig. 5C) of SNU-398 human HCC cell line, but not cells of THLE-3 human normal liver epithelial cell line. In addition, compared with SNU-398 cells with HAND2-AS1 overexpression only, SNU-398 cells with both HAND2-AS1 and ROCK2 overexpression showed significantly promoted proliferation (P<0.05; Fig. 5A), migration (P<0.05; Fig. 5B) and invasion (P<0.05; Fig. 5C).

Discussion

HAND2-AS1 as a tumor suppressor has been reported in several human malignancies (12-15), its role in liver cancer is

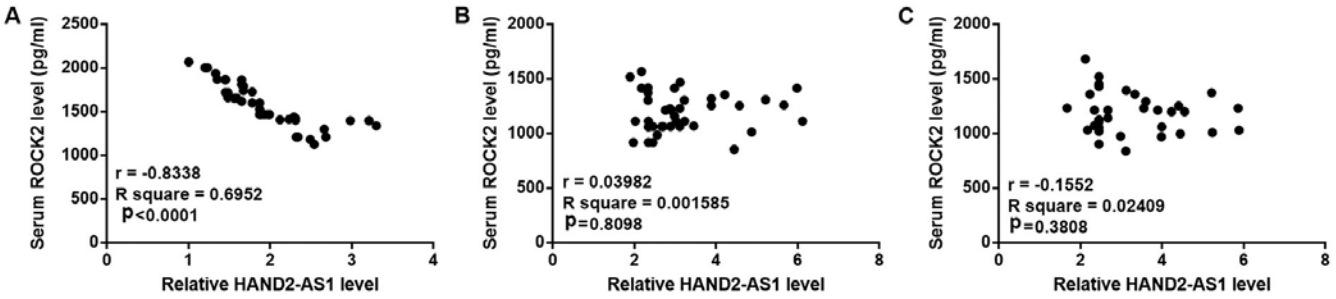

Figure 3. Serum levels of HAND2-AS1 and ROCK2 are negatively correlated in HCC patients. Serum levels of HAND2-AS1 and ROCK2 were negatively correlated in (A) HCC patients, but not in (B) HB patients and (C) healthy controls. HCC, hepatocellular carcinoma; HB, hepatitis B; ROCK2, Rho-associated protein kinase 2.

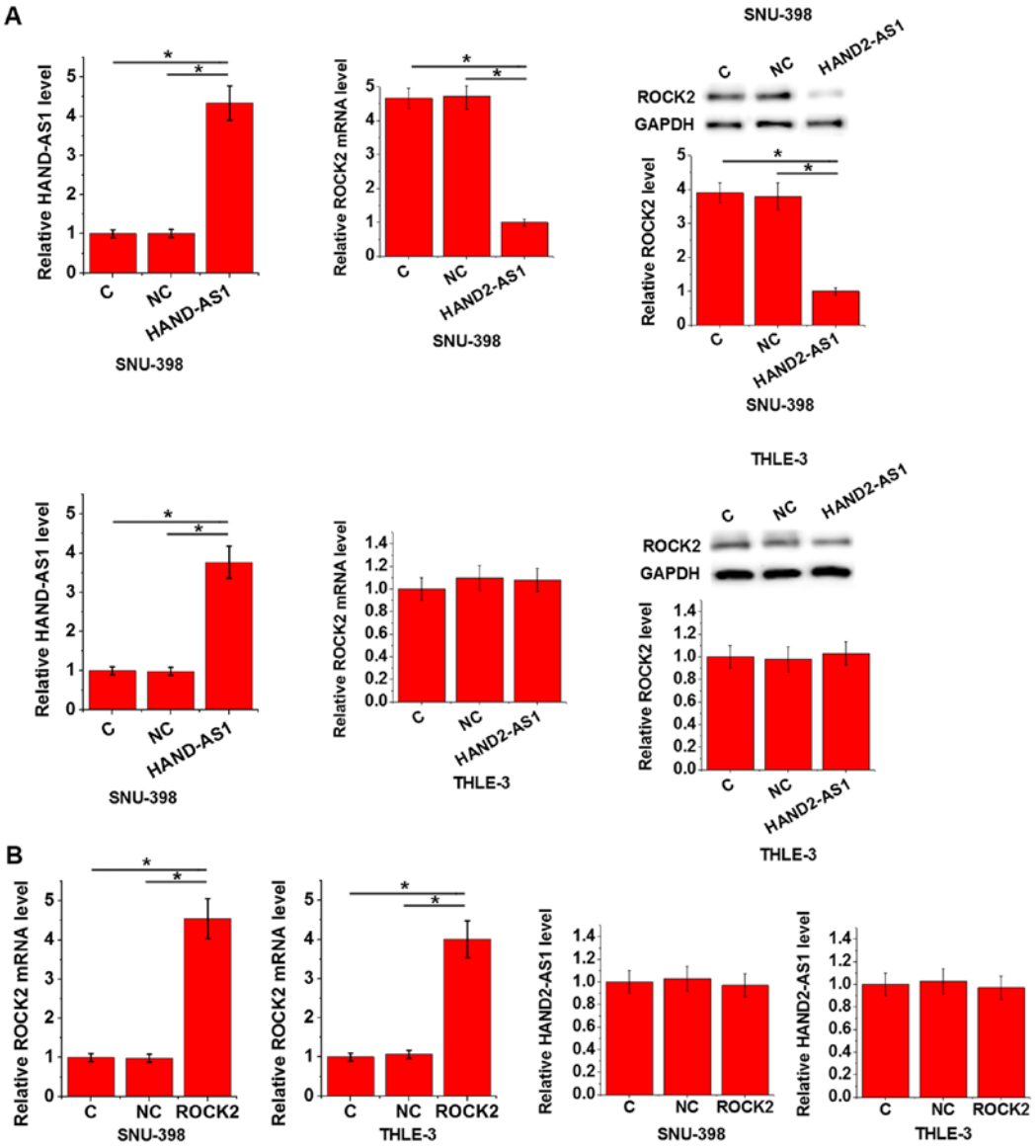

Figure 4. HAND2-AS1 overexpression leads to inhibited ROCK2 expression in HCC cells but not in normal liver epithelial cells. (A) Downregulation of ROCK2 expression was observed in HCC cells but not in normal liver epithelial cells after HAND2-AS1 overexpression, (B) while ROCK2 overexpression did not significantly affect HAND2-AS1 expression *P<0.05. HCC, hepatocellular carcinoma; ROCK2, Rho-associated protein kinase 2; NC, negative control; C, control.

unclear. The present study to the best of our knowledge first reported the involvement of HAND2-AS1 in HCC. The actions of HAND2-AS1 in HCC were also proved to be achieved probably through the downregulation of ROCK2.

Tumor metastasis globally affects gene expression (17). To simplify the story, the present study only included HCC patients at stages IA-IIIA (early stages before lymph node metastasis). Treatment outcomes of patients with metastatic HCC are

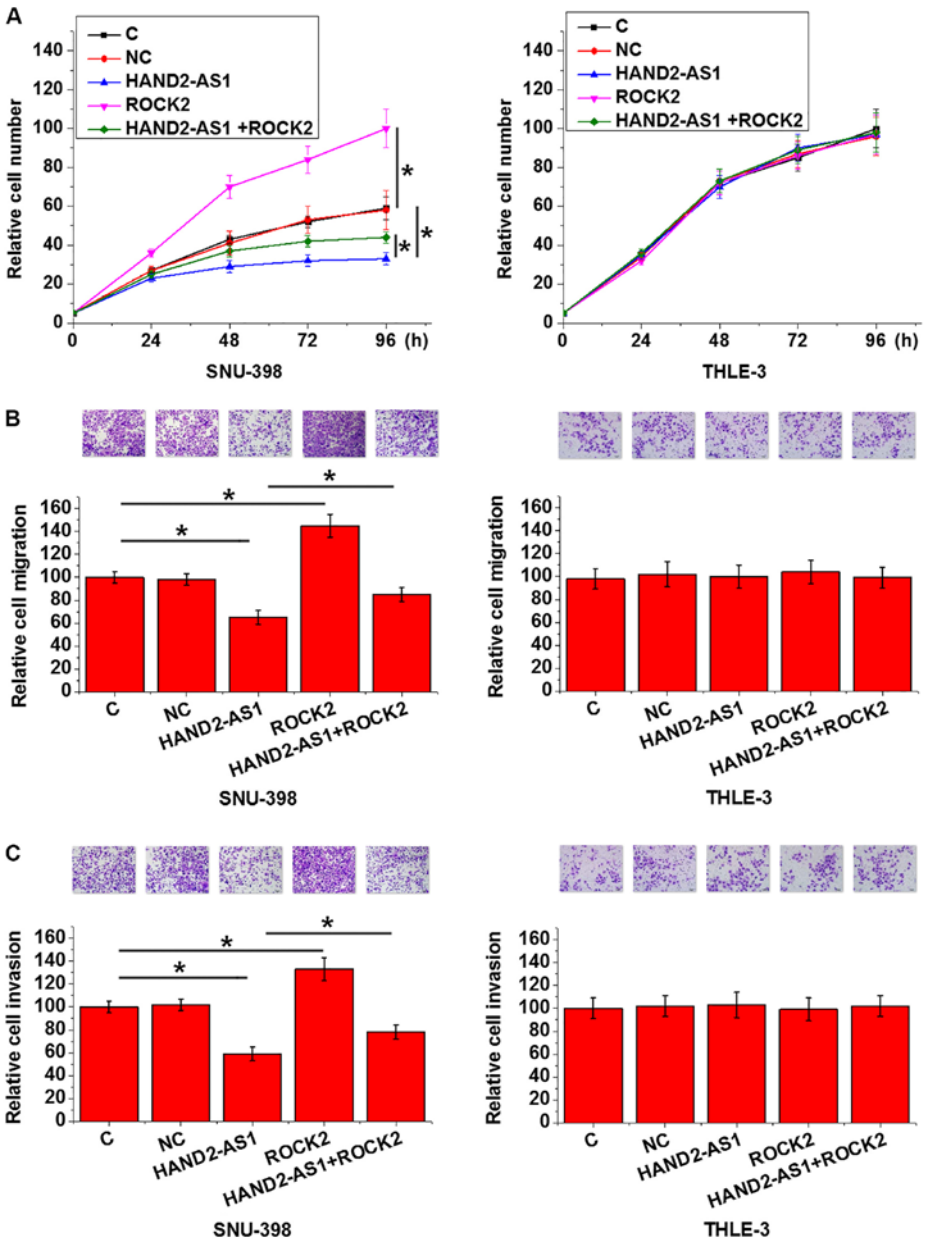

Figure 5. HAND2-AS1 and ROCK2 play opposite roles in the proliferation, migration and invasion of HCC cells. HAND2-AS1 overexpression significantly inhibited, while ROCK2 overexpression significantly promoted the (A) proliferation, (B) migration and (C) invasion of SNU-398 human HCC cell line but not cells of THLE-3 human normal liver epithelial cell line. Magnification, x40. *P<0.05. HCC, hepatocellular carcinoma; ROCK2, Rho-associated protein kinase 2; NC, negative control; C, control.

extremely poor (6). Therefore, early diagnosis and treatment are still critical for the survival of patients with HCC. Only HCC patients were enrolled at early stages to investigate the application potentials of HAND2-AS1 in the early detection of HCC. HAND2-AS1 is a tumor suppressor gene with downregulated expression pattern in colorectal cancer, endometrioid endometrial carcinoma and osteosarcoma (12-15). The present study revealed that serum levels of HAND2-AS1 were also reduced in HCC patients compared with HB patients and healthy controls. In effect, downregulation of HAND2-AS1 effectively distinguished HCC patients from HB patients and healthy controls. Therefore, HAND2-AS1 may have application potential in the early diagnosis of HCC. HBV or HCV infection is the major cause of HCC (4). No significant differences in serum levels of HAND2-AS1 and healthy controls were observed in the present study. Therefore, the downregulated HAND2-AS1 in HCC patients is unlikely to be caused by HBV infection. This study didn't include hepatitis C patients since most HCC patients (32/44) included in this study were infected by HBV and only 2 cases were infected by HCV.

As an oncogenic kinase protein, ROCK2 usually showed an upregulated expression pattern in the development of human cancers (18). Overexpression of ROCK2 promotes HCC through multiple pathways (19). Being consistent with a previous study (18), the present study found that the serum levels of ROCK2 were increased in HCC patients compared with those in HB patients and healthy controls, further confirming the oncogenic role of ROCK2 in HCC.

It has been well established that ROCK2 achieves its biological functions through the interactions with different functional molecules including lncRNAs (20), while crosstalk

between ROCK2 and lncRNAs in HCC still hasn't been reported. The present study proved that HAND2-AS1 is likely to be an upstream inhibitor of ROCK2 in the regulation of HCC cell proliferation, migration and invasion. The present study also speculated that the interaction between HAND2-AS1 and ROCK2 is indirect due to following observations: i) No significant correlation between serum levels of HAND2-AS1 and ROCK2 was observed in healthy controls; ii) HAND2-AS1 overexpression showed no significant effects on ROCK2 expression in normal liver cells.

It is also worth noting that HAND2-AS1 and ROCK2 showed no significant effects on the proliferation, migration and invasion of normal liver cells. This is possibly a result of certain signaling pathways activated in HCC may be mediating the actions of HAND2-AS1 and ROCK2 in HCC. Normal liver cells lack the activation of those pathways. Therefore HAND2-AS1 overexpression may serve as a promising target for the treatment of HCC by downregulating ROCK2. The present study was limited by the small sample size. Larger sample size studies may be needed in the future to further confirm the conclusions. Only one HCC cell line was used in this study. The authors' future studies will include more HCC cell lines to further confirm the present study's conclusions.

In conclusion, HAND2-AS1 levels in serum were decreased in HCC. Overexpression of HAND2-AS1 may inhibit the behaviors of HCC cells by downregulating ROCK2.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

LJ, YH and XL designed experiments. LJ, YH and GS performed experiments. JN, ZX, HL and YC collected and analyzed data. XL drafted the manuscript. All authors approved this manuscript.

Ethics approval and consent to participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The present study received approval from The Ethics Committee of Zhongshan Hospital, Shanghai before patient admission. Informed consent was obtained from all individual participants included in the study.

Patient consent for publication

Informed consent was obtained from all individual participants included in the study.

Competing interests

The authors declare that they have no competing interests.

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