

Lrig1 is a positive prognostic marker in hepatocellular carcinoma

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Background: The prevalence of hepatocellular carcinoma (HCC) is increasing worldwide. As a consequence, there is an urgent need for identifying molecular markers of HCC development and progression. Recently, several studies have suggested that the Lrig1 may have prognostic implications in various cancer types, but its clinical value in HCC is not well evaluated.

Materials and methods: In this study, the expression level of Lrig1 was examined in 133 HCC tissues and adjacent normal tissues by immunohistochemistry. Furthermore, potential associations between Lrig1 expression and the carcinoma clinical parameters were investigated, including recurrence and survival rate. We silenced the Lrig1 in the normal liver cell line (LO2) and liver cancer cell line (Hep-G2) in vitro by the small interference RNA and detected its influence on proliferation, migration, and invasion.

Results: The expression of Lrig1 was significantly downregulated in liver cancer tissues and cell lines, and its expression levels were related to tumor size, tumor–node–metastasis staging and tumor recurrence. Furthermore, analysis of 6-year survival of 133 HCC patients showed that those with stronger Lrig1 expression had significantly longer overall survival time than those with weaker Lrig1 expression. In addition, decreased expression of Lrig1 in vitro promoted the growth, migration, or invasion of normal liver cells and cancer cells.

Conclusion: Our findings demonstrate that Lrig1 could serve as a potential marker in the prognosis of patients with HCC. We also revealed that Lrig1 might be involved in the metastatic progression of liver cancer. However, its clinical value should be further investigated in the future.

Keywords: Lrig1, hepatocellular carcinoma, prognosis, immunohistochemistry, siRNA, invasion, migration

Introduction

Hepatocellular carcinoma (HCC), a major pathological type of primary liver cancer, is one of the serious threats to human health and the third leading cause of cancer-related deaths worldwide.^{1,2} The highest incidence rate of HCC has been reported in the developing world and is steadily increasing across the developed world.³ Currently, surgical operation is the most effective treatment of HCC. However, after hepatic resection and liver transplantation, the recurrence incidence and prognosis are still not optimistic. Totally 65%–80% of the cases recur after surgical therapy, and the crucial postoperative 5-year survival rate ranges from 30% to 40%.^{4,5} The late diagnosis for this rapidly progressing tumor still remains an obstacle in the improvement of prognosis for HCC patients. Therefore, identifying an effective biomarker for early diagnosis and improving treatment strategies are required.

The leucine-rich repeats and immunoglobulin-like domains (Lrig) family comprises three paralogous genes, termed *Lrig1*, *Lrig2*, and *Lrig3*, which are widely expressed in human

and mouse tissues. Moreover, *Lrig1* is a transmembrane protein that is involved in the regulation of cellular signal transduction, whose transcript is located at chromosome 3p14.3, a region frequently deleted in various types of human cancers.⁶ In the last decade, *Lrig1* has been hypothesized to function as a tumor suppressor in vivo and in vitro. Recently, experimental and clinical data have subsequently supported this hypothesis. Numerous previous reports have shown that *Lrig1* is downregulated in several types of tumors, such as cutaneous squamous cell carcinoma, renal cell carcinoma, glioblastoma, and breast cancer.^{7–10} Recently, researchers revealed that the mechanism of *Lrig1* may involve the inhibition of the PI3K/Akt and Ras/Raf/ERK signal transduction pathways.¹¹ However, the expression of *Lrig1* and its clinical significance in HCC remain unclear. In addition, Yokdang et al¹² found that *Lrig1* may represent a barrier to epithelial-to-mesenchymal transition (EMT). Depletion of endogenous *Lrig1* in human mammary epithelial cells expands the stem cell population, augments mammosphere formation, and accelerates EMT. *Lrig1* is frequently methylated in human colorectal cancer. Lower expression of its mRNA and protein may also contribute to tumor growth.¹³ These findings provide evidence indicating that *Lrig1* functions as a key mediator of metastatic progression. However, very little is known of *Lrig1* function in this regard.

In the present study, we aimed to clarify the relationship between the expression of *Lrig1* and prognosis of HCC patients and the epigenetic regulation and biological functions of *Lrig1* in liver cancer. Here, we investigated the expression of *Lrig1* in 133 patients with HCC by immunohistochemistry (IHC) and compared three liver cancer cell lines with the normal liver cell line by reverse transcription polymerase chain reaction (RT-PCR). Furthermore, we estimated the correlation of *Lrig1* expression with clinicopathological parameters and prognostic indicators of HCC patients. Finally, we downregulated *Lrig1* in normal and cancerous liver cell lines in vitro using interfering RNA (iRNA) technology and evaluated the cell proliferation, migration, and invasion after *Lrig1* deficiency. Herein, we expect that our research could provide some clues to understand the function of *Lrig1* in liver cancer.

Materials and methods

Patient and tissue specimens

A total of 133 pairs of paraffin-embedded primary HCC tissue specimens and adjacent non-cancerous liver tissue specimens were obtained from patients who had undergone surgery in Liaocheng People's Hospital from January 2006 to January 2010. The clinical diagnoses were made by a hepatoma specialist and were histologically confirmed by

a pathology report. None of the patients had undergone any preoperative treatment before surgery, such as transcatheter arterial chemoembolization or chemotherapy. The study was approved by the Human Ethics Committee of Liaocheng People's Hospital and Tongji Medical College. All patients provided written informed consent.

IHC and analysis

The paraffin-embedded specimens obtained from all HCC patients were cut into 4 μm sections. The sections were then dewaxed with xylene and rehydrated using a series of graded alcohols after being baked. Then, the endogenous peroxidase activity was blocked and the sections were incubated overnight with the *Lrig1* rabbit polyclonal antibody (working dilution 1:100, #ab 197985; Abcam, Cambridge, MA, USA). Subsequently, the sections were incubated with horseradish peroxidase (HRP)-conjugated second antibody. Assessment of the staining was made under a light microscope by two experienced pathologists who did not know the exact condition of the patient. Scoring depended on the staining intensity and proportion as previously described.¹⁴ For each tissue core, the intensity of staining was categorized as follows: 0, negative; 1, weak; 2, moderate, and 3, strong. Based on the proportion of staining, the degree was scored on a scale of 0 (<5%, absent), 1 (5%–25%, sporadic), 2 (25%–50%, focal), and 3 (>50%, diffuse). The final score of each staining was obtained by multiplying the two scores. The IHC score ranged from 0 to 9. IHC scores less than 4 points were determined as negative.

Cell culture and transfection

The LO2, SMMC-7721, Hep-G2, and Hep-3B cells were provided by the Department of Liver Surgery of Tongji Hospital. The cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin (complete medium) in a humidified atmosphere containing 5% CO₂ at 37°C. For transfection, the cells were plated in 24-well culture plates at a density of 1×10⁵ cells/well. Twenty-four hours after plating, the cells were transfected with three different *Lrig1* siRNA fragments and negative control siRNA (siNC), which were designed and synthesized by Ribbio (Guangzhou, People's Republic of China) for an additional 24 h: *Lrig1* siRNA-1 (5'-TCTGCAAATGGCAACCATT-3'), *Lrig1* siRNA-2 (5'-ACTCTCTGAGATTGACCCT-3'), and *Lrig1* siRNA-3 (5'-CGACATCCATTGCATTTAA-3'). The liver cells were transfected separately with siRNAs and siNC by Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) to knockdown the *Lrig1*.

Quantitative real-time PCR

The total RNA was isolated from the cells using TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. RT-PCR was performed using an SYBR Master Mix kit (Takara, Kusatsu, Japan) on the LightCycler 480 Real-Time PCR System (Roche, Basel, Switzerland). The sequences of primers for the *Lrig1* were as follows: forward: 5'-AGCTAACCATCTTATGAGTGCC-3' and reverse: 5'-CTCAGAAGCAGCAAATTCACA-3'. Each sample was measured in triplicate. A mean value was used to determine mRNA levels using the comparative Ct method, using the formula $2^{-\Delta\Delta C_t}$, and the mRNA levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH; forward: 5'-GACAAAATGGTGAAGGTCGGT-3' and reverse: 5'-GAGGTCAATGAAGGGTTCG-3').

Cell growth assay

Cell growth ability was exhibited by the cell counting kit-8 (CCK-8) from Dojindo Laboratories (Kumamoto, Japan). Cells were transplanted into a 96-well plate at a density of 8×10^2 cells/well and were continuously incubated for 24, 48, 72, 96, and 120 h at 37°C in 5% CO₂. After incubation, at designed different times, 10 μL of CCK-8 was added to each well, and following incubation for 1 h, the absorbance at 450 nm was measured to calculate the number of cells. The analysis of each well type was repeated six times independently.

Cell migration and invasion assays

Cell culture was performed in transwell chambers (8-μm pore size; Corning Incorporated, Corning, NY, USA). For the migration assay, 5×10^4 cells were plated into 200 μL RPMI-1640 medium in the upper chamber and were cultured for 36 h. For the invasion assay, the insert membranes were coated with diluted Matrigel (San Jose, BD Biosciences, San Jose, CA, USA), but were cultured under the same conditions. Finally, the membranes were removed and stained with hematoxylin, and images were captured using an inverted microscope (Olympus BX51; Olympus Corporation, Tokyo, Japan).

Western blotting

The protein levels were quantified by standard Western blotting procedures. Protein extracted from cells was separated on 8% sodium dodecyl sulfate polyacrylamide electrophoresis gels and transferred to nitrocellulose membranes. After being blocked with 5% non-fat milk in Tris-buffered saline for 3 h, the membranes were incubated with indicated primary antibodies (dilution 1:200; Bioss, Beijing, People's Republic of

China) at 4°C overnight, followed by incubation with HRP-conjugated secondary antibody for 3 h. GAPDH (Sungene, Tianjin, People's Republic of China) was used as a loading control for comparison between samples.

Statistical analysis

All statistical analyses were carried out using the SPSS statistical software package (version 16.0; SPSS Inc., Chicago, IL, USA). Survival curves were calculated by Kaplan–Meier analysis and compared using the log-rank test. Correlations between Lrig1 expression and the clinical variables were analyzed using the Pearson's chi-squared test. The Student's *t*-test was used for other comparisons. All *P*-values were two-sided, and *P*-values <0.05 were considered to be statistically significant.

Results

Lrig1 has low expression in human HCC tissue and cell lines

To elucidate the biological significance of Lrig1 in HCC, we examined the expression of Lrig1 in primary paired HCC tissue samples. While normal liver specimens showed positive protein expression, primary liver cancer tissues showed negative protein expression (Figure 1A and B). Low Lrig1 expression was found in 18 of the 133 (13.5%) primary HCC specimens, compared with 68/133 (51.1%) in adjacent non-tumorous (NT) tissues (*P*<0.05; Figure 1C). In addition, we also compared three HCC cell lines with the normal liver cell line by quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis. *Lrig1* expression at the mRNA level in three HCC cell lines evaluated (SMMC-7721, Hep-G2, and Hep-3B) was found to be lower (particularly in SMMC-7721 and Hep-3B cells) compared with the normal cell line LO2 (Figure 1D). Thus, these data suggested that Lrig1 lost was significantly higher in HCC tissues and cell lines than that in NT tissues and cell lines.

Correlation of Lrig1 with clinicopathological variables

To verify the biological function of Lrig1 in HCC, we correlated Lrig1 status in 133 HCC samples with widely recognized clinicopathological features. The low expression of Lrig1 in HCC was associated with tumor size (>5 cm; *P*<0.01), tumor–node–metastasis (TNM) stage (*P*<0.05) and tumor recurrence (*P*<0.01; Table 1). In contrast, Lrig1 expression displayed no association with gender, age, AFP, HBsAg, tumor number, tumor differentiation, and vascular invasion (*P*>0.05). These data suggest that Lrig1 might play a tumor suppressor role in HCC.

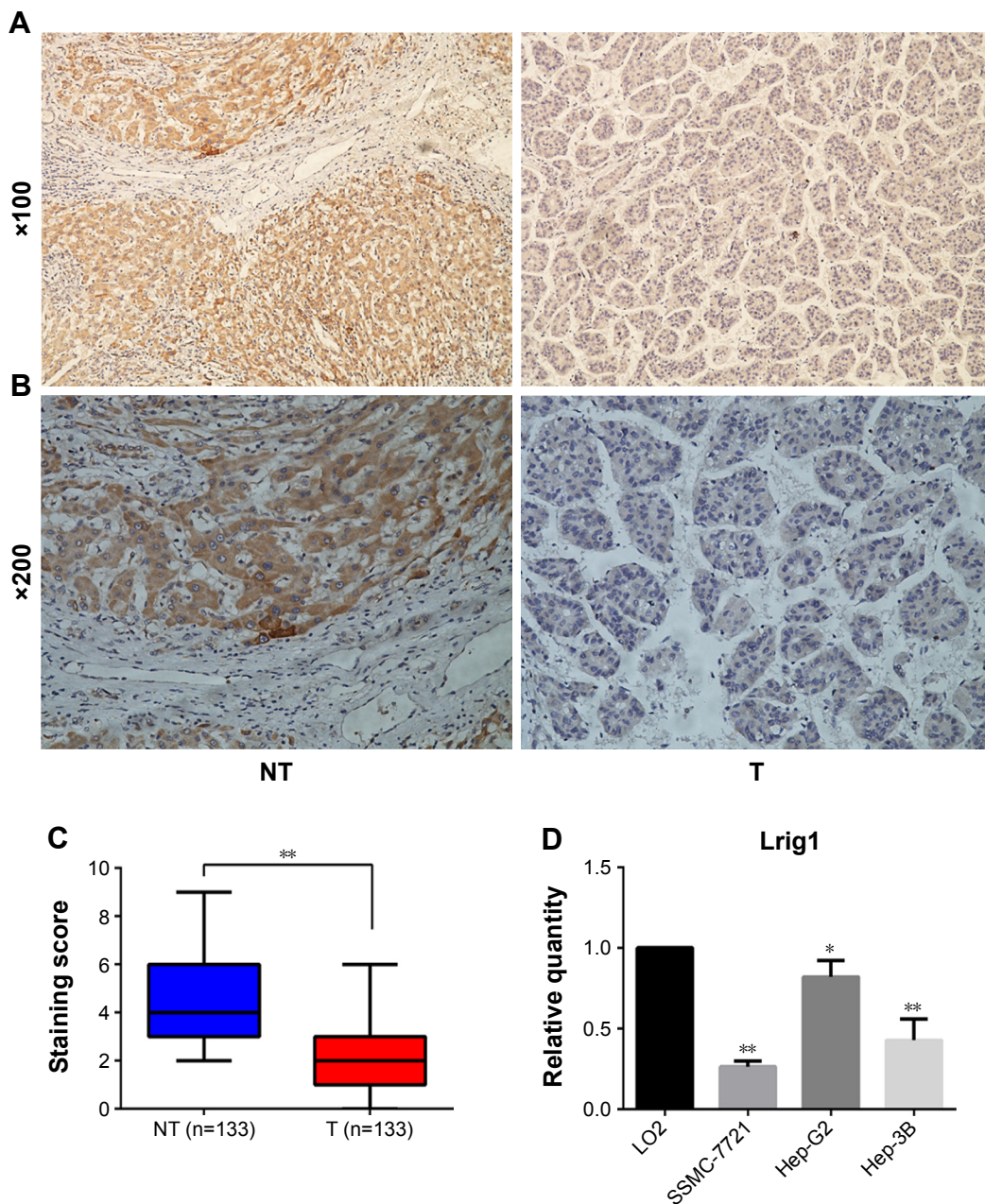


Figure 1 Lrig1 is significantly downregulated in HCC.

Notes: (A and B) IHC assays of Lrig1 expression in 133 paired HCC and adjacent NT tissues. The left panel represents high Lrig1 expression in adjacent NT tissues. The right panel represents low Lrig1 expression in HCC (magnification: A, $\times 100$; B, $\times 200$). (C) Lrig1 expression levels were compared with HCC and adjacent NT specimens. Statistical analysis was performed by paired samples t-test. (D) RT-qPCR showed that relative *Lrig1* mRNA expression was lower in SSMC-7721, HepG2 and Hep3B than in LO2 cells (* $P < 0.05$ and ** $P < 0.01$).

Abbreviations: HCC, hepatocellular carcinoma; IHC, immunohistochemistry; NT, non-tumorous; T, tumorous; RT-qPCR, quantitative reverse transcription polymerase chain reaction.

Lrig1 is significantly associated with positive prognosis in HCC patients

To examine whether Lrig1 expression is associated with patients' overall survival, we evaluated via IHC by comparing patients with negative protein expression and those with strong protein expression (n=133). Kaplan–Meier curves showed that HCC patients with high Lrig1 expression had

a significantly higher 6-year survival rate than those with negative Lrig1 expression ($P < 0.05$; Figure 2).

Inhibition of Lrig1 expression in HCC cell lines

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis showed relatively higher expression

Table 1 Correlation of Lrig1 expression with clinicopathological parameters

Clinical feature	No of patients (n=133)	Lrig1 expression		P-value
		Low	High	
Gender				0.104
Female	54	23	31	
Male	79	45	34	
Age (years)				0.183
≤52	60	35	25	
>52	73	33	40	
AFP (ng/mL)				0.999
≤400	89	45	44	
>400	44	23	21	
HBsAg				0.905
Negative	23	12	11	
Positive	110	56	54	
Tumor size (cm)				<0.010
≤5	83	32	51	
>5	50	36	14	
Tumor number				0.880
Single	115	58	57	
Multiple	18	10	8	
Tumor differentiation				0.239
I-II	103	56	47	
III-IV	30	12	18	
Vascular invasion				0.711
No	89	44	45	
Yes	44	24	20	
TNM stage				0.014
I	78	32	46	
II	25	18	7	
III	30	18	12	
Recurrence status				<0.010
No	76	30	46	
Early	29	22	7	
Late	28	16	12	

Abbreviation: TNM, tumor–node–metastasis.

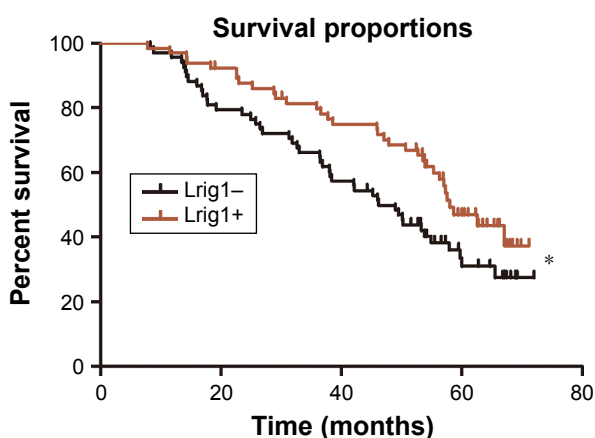


Figure 2 Lrig1 is significantly associated with positive prognosis in HCC patients. **Notes:** Based on Lrig1 immunostaining analysis of tumors, HCC patients were divided into low-Lrig1 expression and high-Lrig1 expression groups. Follow-up investigation of the 6-year survival study showed that the patients with high Lrig1 clearly presented a better outcome than the low-Lrig1 patients (* $P<0.05$). **Abbreviation:** HCC, hepatocellular carcinoma.

of *Lrig1* in Hep-G2 cells than the other two liver cancer cell lines tested (SMMC-7721 and Hep-3B; Figure 1D). Accordingly, we selected LO2 and Hep-G2 as the optimal cells to transfect with three different *Lrig1*-targeting siRNAs (*siLrig1-1*, *siLrig1-2*, and *siLrig1-3*) and siNC in order to investigate the biological function of Lrig1 in HCC cell lines. The knockdown efficiency of Lrig1 was evaluated by RT-qPCR and Western blot analysis. There is an ~70% decrease in *Lrig1* gene expression and Lrig1 expression in *siLrig1-2* and *siLrig1-3*-transfected cells compared with those treated with *siLrig1-1* and siNC (Figure 3). Therefore, *siLrig1-2* and *siLrig1-3* can be chosen in the following studies.

Lrig1 knockdown increases liver cell proliferation, migration, and invasion

Human normal liver cell line (LO2) and HCC cell line (Hep-G2) were treated with siNC and *Lrig1*-siRNA (*siLrig1-2*), respectively. As the results in Figure 4 show, the decreased expression of Lrig1 in the *Lrig1*-siRNA treatment cells significantly promoted the proliferation of the cells, compared with the siNC-treated cells and untreated cells ($P<0.05$). In migration assay, LO2 cells treated with *Lrig1*-siRNA showed a high level of penetration through the membranes compared with those treated with siNC and untreated cells ($P<0.05$). The role of Lrig1 in tumor invasion was apparent in the *Lrig1*-siRNA treatment Hep-G2 cells, compared with the negative control groups ($P<0.01$). Taken together, these data suggest that Lrig1 promotes the growth and metastasis of HCC cells in hepatocarcinogenesis. These results further support that Lrig1 is involved in promoting the progression of liver cancer (Figures 3 and S1).

Discussion

In recent years, there are multiple clinical factors considered to be involved in the stratification of HCC patients, including tumor size, serum AFP level, TNM stage, and so on.¹⁵ Although these factors can be used to predict prognosis, the prognosis of HCC patients remains dismal.^{16,17} At present, the diagnosis of HCC is mainly based on biopsy and radiology, but it is unable to discover this disease at the early stage because of the low sensitivity of serum AFP and ultrasonic and other examinations.¹⁸ Because of late diagnosis of HCC, only limited therapeutic options with marginal clinical benefits are available for the majority of HCC patients. To further understand the tumorigenesis and development of HCC, novel predictive biomarkers are needed.

Lrig1 is a negative regulator of growth factor signaling that has been confirmed to function as a tumor suppressor

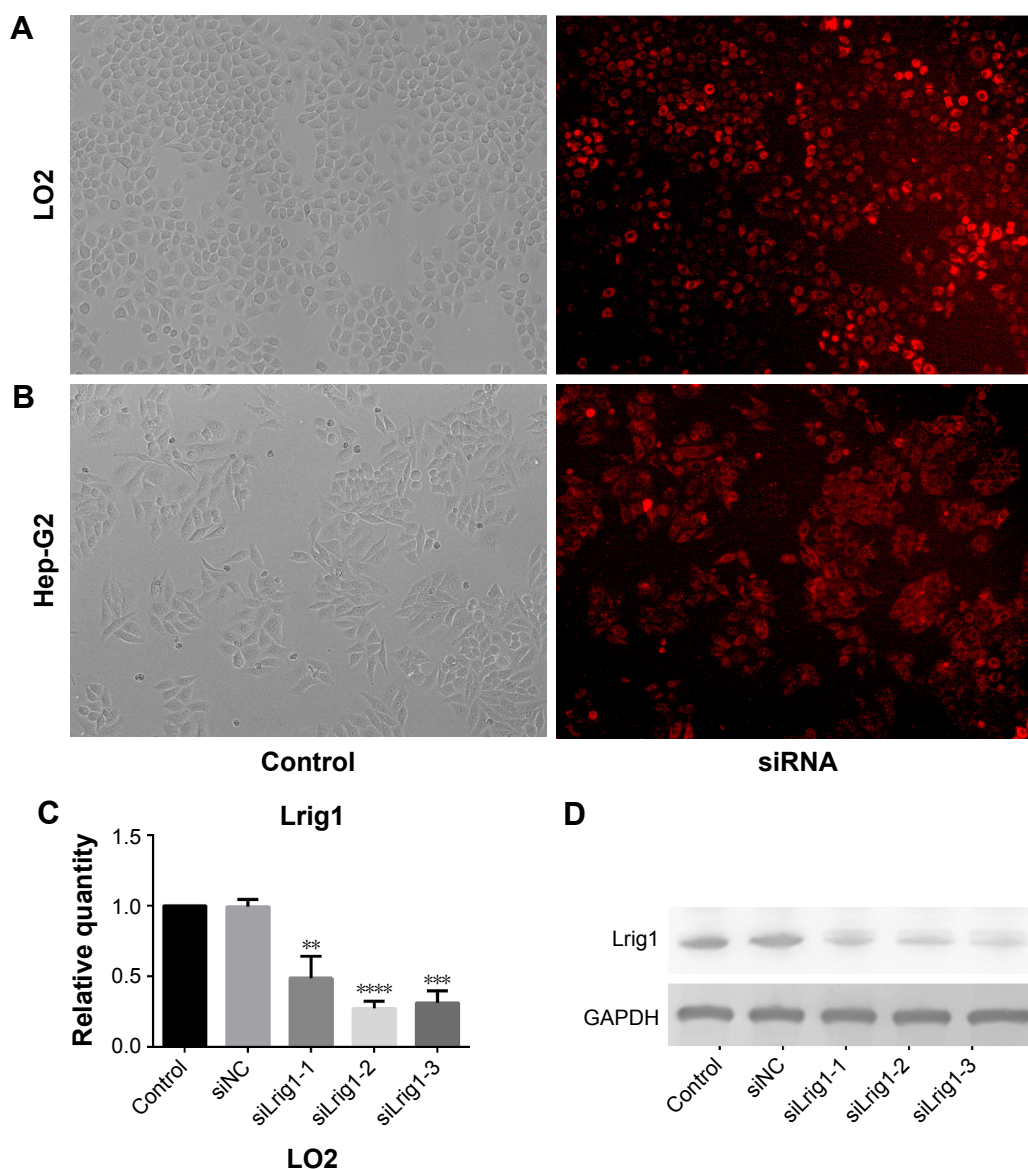


Figure 3 Lrig1 knockdown in LO2 and Hep-G2 cell lines.

Notes: (A and B) The representative image of LO2 and Hep-G2 cells after Lrig1 siRNA stable transfection by fluorescence microscopy (left: control; right: siLrig1-2) (magnification: $\times 200$). (C and D) Lrig1 expression was evaluated by real-time PCR and Western blot after 48 h of siRNA transfection. Lrig1 mRNA and protein levels in the siRNA-2 and siRNA-3 groups were significantly decreased to ~30% of the Lrig1 expression in untreated cells. Each bar represents mean values \pm SD from three independent experiments. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PCR, polymerase chain reaction; SD, standard deviation; siNC, negative control siRNA.

in various cancers. Over the years, numerous efforts have been made to look through the relationship between Lrig1 and the pathogenesis and prognosis of tumor patients. In fact, the findings of previous reports have shown that the high expression of Lrig1 is significantly associated with the better prognosis of cancer patients. In a meta-analysis of gene expression and patient outcome in ovarian serous carcinoma, *Lrig1* was identified as one single gene prognostic biomarker that predicted survival across all the data sets.¹⁹ In an immunohistochemical analysis of 347 non-small cell lung cancer cases, Kvarnbrink et al²⁰ found out that high expression of Lrig1 was an independent prognostic factor that correlated

with good survival, particularly among patients with lung adenocarcinoma. However, there are no reports to demonstrate that address the specific function of Lrig1 in the HCC patients. Lrig1 is found low expressed in human HCC tissue and cells in our study. We also analyzed the expression of Lrig1 and its correlation with the clinicopathologic factors and survival rate in 133 total patient-matched tissues. Among the clinicopathological parameters, low Lrig1 expression was significantly correlated with tumor size, TNM stage, and recurrence status. Poor TNM stage and early recurrence status were significantly associated with poor survival of cancer. In brief, negative Lrig1 expression was likely correlated with

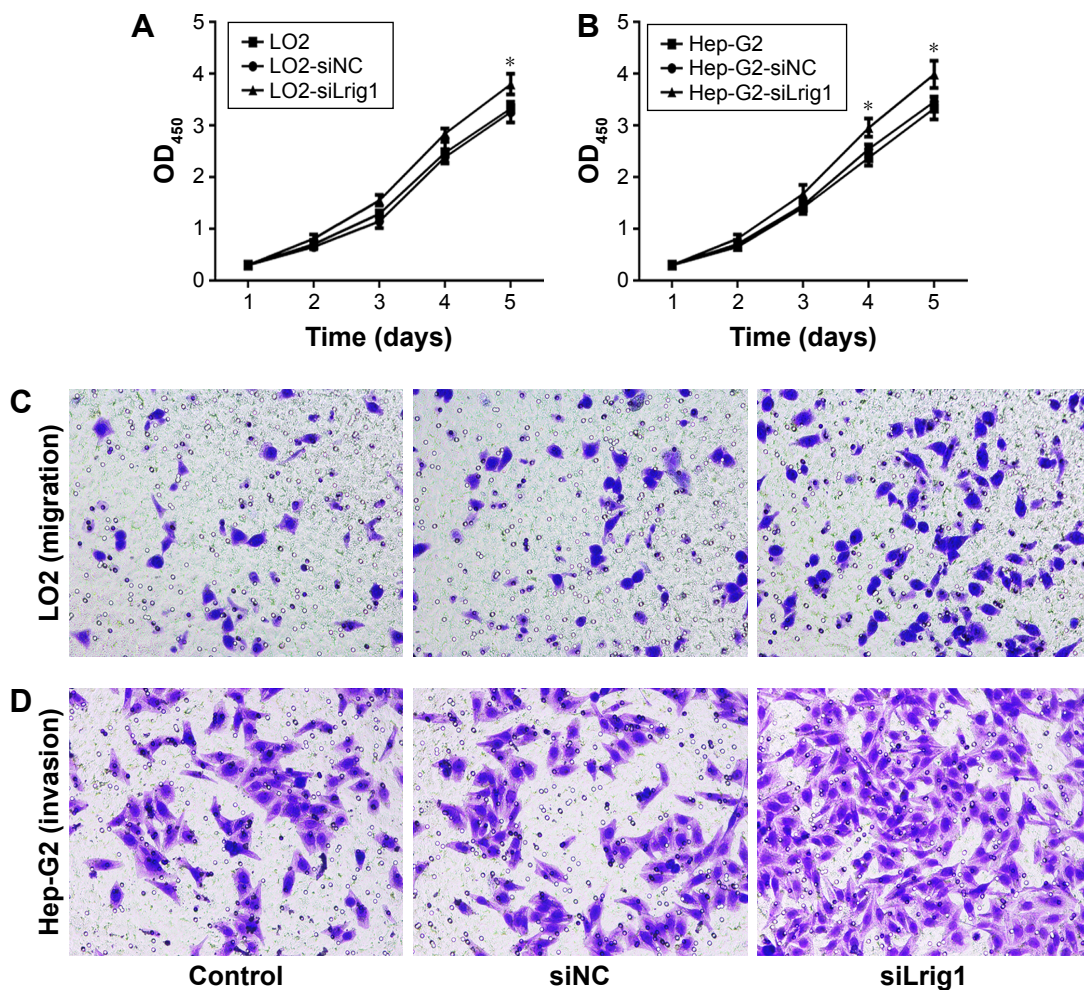


Figure 4 Lrig1 knockdown promotes proliferation, migration, and invasion in liver cancer cell line.

Notes: (A and B) CCK-8 assay shows that down expression of Lrig1 promotes cell growth in the LO2 and Hep-G2 cell lines when compared to untreated and negative-control transfectants ($*P < 0.05$). (C) Transwell assay demonstrated that LO2 cells in the Lrig1 siRNA group have increased migratory capacity compared to those in the negative control group. (D) Low expression of Lrig1 promotes increased Hep-G2 cell invasion compared to other study groups (representative images are shown; magnification $\times 200$).

Abbreviations: CCK-8, cell counting kit-8; OD, optical density; siNC, negative control siRNA.

poor prognosis of liver cancer. To the best of our knowledge, this is the first evidence showing that Lrig1 could be used as a potential positive prognostic marker for HCC patients.

Long-term survival of patients with HCC after curative resection is confounded by its high recurrence rate, which is mainly caused by invasion and metastasis.²¹ Therefore, identification of metastatic factors and understanding of the underlying molecular pathways involved in metastatic progression are critical. Recently, low expression of Lrig1 was revealed to be an independent risk factor for cancer invasion and metastasis.²²⁻²⁴ The research group of Xie et al²⁵ has found that downregulation of Lrig1 resulted in significantly enhanced capabilities of proliferation and invasion in the glioma cells via activation of epidermal growth factor receptor, protein kinase B (Akt), and c-Myc signaling molecules. It is still unknown whether the Lrig1 expression level

plays a role in the regulation of liver cancer cell biological behaviors, such as cell growth and invasion, through some specific signaling pathways. To address these questions, we investigated the biological function of Lrig1 in human liver cancer cells. Downregulation of Lrig1 can promote the growth, invasion and migration of liver cancer cells. These data provided evidence to believe that Lrig1 plays a vital role in cell growth and metastasis and is a promising target for anticancer therapy.

Conclusion

Prior to this study, expression of Lrig1 has never been reported in human liver cancer. Here, our findings of this study also show that Lrig1 may be a potential target in the invasion and metastasis of liver cancer. This suggests a novel approach to investigate the mechanism of the underlying

molecular pathways involved in metastatic progression. Furthermore, we demonstrated that low expression of Lrig1 can promote the tumorigenicity of liver cancer. However, its relevance to the prognosis of HCC is poorly understood, and further studies on these mechanisms are still needed in the future.

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary material

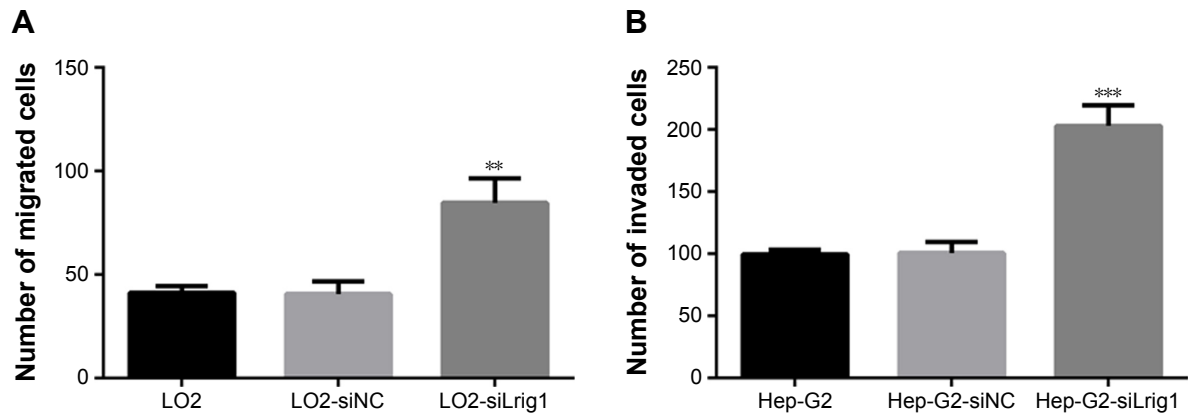


Figure S1 Transwell migration and invasion assay for the analysis of cell migratory and invasive capacity.

Notes: (A) The migratory abilities of the LO2-siLrig1 cells (approximately twofold) were significantly increased compared with those of the control cells. (B) The invasive abilities of the HepG2-siLrig1 cells (approximately twofold) were significantly increased compared with those of the control cells. All experiments were performed at least in triplicate, and the values are the mean values \pm SD (** $P < 0.01$ and *** $P < 0.001$).

Abbreviations: SD, standard deviation; siNC, negative control siRNA.

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