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ISOC1 is a novel potential tumor suppressor in hepatocellular carcinoma

Jiao XIANG[1,#], Xiao-Qiang GAO[2,#], Xiang-Ling CHEN[1], Yin-Ying LU[1,3,*]

[1]Department of Liver Disease, Fifth Medical Center of Chinese PLA General Hospital, Beijing, China; [2]Department of Hepatobiliary Surgery, Affiliated Hospital of Guizhou Medical University, Guiyang, Guizhou, China; [3]Center for Synthetic and Systems Biology (CSSB), Tsinghua University, Beijing, China

**Correspondence: luyinying1973@163.com*
[#]Contributed equally to this work.

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Isochorismatase domain-containing 1 (ISOC1) plays a carcinogenic role in various tumors. However, its expression and role in hepatocellular carcinoma (HCC) have not been elucidated. This is the first study to investigate the involvement of ISOC1 in HCC growth and migration. ISOC1 expression was analyzed using public databases and clinical samples, and clinical specimens were analyzed by real-time quantitative polymerase chain reaction, western blotting, and immunohistochemistry. ISOC1 was also overexpressed in two HCC cell lines (Huh7 and HepG2) to explore how ISOC1 affects HCC cells. Finally, a nude mouse xenograft tumor model was used to investigate the role of ISOC1 in HCC cell tumorigenicity. ISOC1 was downregulated in HCC tissues compared to that in matched paracancerous tissues, and low ISOC1 expression was associated with a poor prognosis. The proliferation and single-cell colony-forming ability of the ISOC1-overexpressing cell lines Huh7 and HepG2 were significantly inhibited. Moreover, ISOC1 overexpression suppressed the migration and invasion abilities of HCC cells *in vitro*, and ISOC1 upregulation hindered tumor growth in the xenograft tumor model *in vivo*. Therefore, ISOC1 is a potential HCC suppressor protein.

Key words: hepatocellular carcinoma, ISOC1, migration, invasion, proliferation

In 2018, liver cancer was the sixth most common cancer [1] and the fourth leading cause of cancer death worldwide [2]. HCC is the most common type of primary liver cancer, accounting for 75–85% of cases, and has a high incidence rate in China [3]. In recent years, a series of immunotherapy [4–9] and targeted therapy [10–13] breakthroughs have been made. However, the overall HCC survival rate remains very low. Therefore, it is crucial to identify new molecular features and elucidate the underlying mechanisms for tumor formation.

Isochorismatase domain-containing 1 (ISOC1) is a gene belonging to the equiaxed enzyme hydrolases family, coding for a protein with putative isochorismatase activity, producing 2,3-dihydroxy-2,3-dihydroxybenzoate and pyruvate via hydrolysis [14], contains 298 amino acids, has a chromosomal position of 5q23.3, and is potentially located intracellularly in peroxisomes. The role of ISOC1 has been investigated in several cancers. For example, in breast cancer, ISOC1 is positively regulated by estrogen and negatively regulated by microRNA-130a during neutrophil development [15–17]. In uterine cancer, ISOC1 expression (including ISOC1 expression in leiomyoma) is significantly higher than in normal myometrium [18], and in pancreatic cancer, ISOC1 is highly expressed in cancerous tissue compared to normal tissue and plays an important role in pancreatic cancer cells growth and apoptosis [19]. Finally, in colon cancer, ISOC1 expression is significantly higher in cancerous tissue than in normal tissue, and it regulates the biological function of tumor cells through the AKT/GSK-3β signaling pathway [20]. However, the role of ISOC1 in HCC remains unknown.

This study compared ISOC1 expression in HCC tissues and matched adjacent non-tumor tissues. Further, ISOC1-overexpressed cell lines were used to explore the function of ISOC1 in HCC by *in vitro* and *in vivo* experiments.

Patients and methods

Patients and tissue samples. We retrospectively recruited 105 patients (age range, 30–70 years; mean age, 50.8±7.6 years) who underwent hepatectomy between 2017 and 2020 at the Department of Hepatic Surgery, Fifth Medical Center of the Chinese PLA General Hospital. The HCC specimens were diagnosed and confirmed by three pathologists. None

of the patients underwent any anticancer treatment before the surgery. This research was ratified by the Ethics Review Committee of the Fifth Medical Center of the Chinese PLA General Hospital (Number: 2019002D). The patients who participated in the study signed an informed consent form.

Cell culture. Two types of human HCC cell lines, Huh7 and HepG2 (Institute of Cell Research of the Chinese Academy of Sciences, Shanghai, China), were purchased and cultured in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum (FBS, Gibco, USA) in a humidified incubator at 37 °C with 5% CO_2. When the cell confluency reached 90%, 0.25% trypsin (Gibco, Cat. No. 15400054) was used for digestion and passaging.

Public data analysis. The HCC database (HCCDB) contains 15 public HCC gene expression datasets [21], including data from the Gene Expression Omnibus (GEO), the Liver Hepatocellular Carcinoma Project of The Cancer Genome Atlas (TCGA-LIHC), and the Liver Cancer – RIKEN, JP Project from International Cancer Genome Consortium (ICGC LIRI-JP). HCCDB shows the visual effect for some computer analysis results, such as differential expression analyses, survival analyses, and protein co-expression analyses. HCCDB data were evaluated for the ISOC1 gene expression patterns in HCC and adjacent tissues.

Quantitative real-time polymerase chain reaction (qPCR). Total RNA was isolated from the tumor tissues and corresponding non-tumor tissues (2 cm away from the tumor edge), and total complementary DNA (cDNA) was transcribed from the total RNA according to the manufacturer's protocol (GeneAll®Exgene™ Viral DNA/RNA, Cat. No.128-150). cDNA was used as the template for qPCR (Thermo Fisher Scientific, USA) with qPCR SuperMix (TransGen Biotech Co., Ltd., Beijing, China). The total reaction volume was 10 μl, and the reaction steps were 50 °C for 2 h, followed by 95 °C for 30 s, 95 °C for 5 s, 60 °C for 30 s, and 72 °C for 30 s for 40 cycles. The primer pair, 5'-CGACATGCACCGCAAATTCG-3' and 5'-TGAGCTGGATCTGCAACGG-3', was used to amplify the target gene (ISOC1), and the pair, 5'-TGATCTCACACAGCGACACCCA-3' and 5'-CACCCTGTTGCTGTAGCCAAA-3', was used to amplify the endogenous control gene (glyceraldehyde 3-phosphate dehydrogenase, GAPDH). Each sample was analyzed in triplicate, and all samples were analyzed thrice by qPCR. The differences in ISOC1 and GAPDH messenger RNA (mRNA) expression were compared using the $2^{-\Delta\Delta Ct}$ method [22].

Western blot analysis. Tissues and cells were washed gently three times with PBS, and 500 μl of radioimmunoprecipitation assay lysis buffer (Proteintech, Cat. No. B100020) was added to lyse the cells and extract the total protein. Equivalent amounts of protein sample were separated using 10% polyacrylamide gels and then transferred to a polyvinylidene fluoride membrane (Millipore, Cat. No. SF1J152I01). After blocking with 5% skim milk, the membranes were incubated with the primary rabbit anti-human ISOC1 antibody (Abcam, Cat. No. ab118245; 1:5000 dilution) for 12 h at 4 °C, then rinsed with TBS (phosphate-buffered saline with 0.1% Tween 20) four times for 5 min each. Goat anti-rabbit secondary antibody (Abcam, Cat. No. ab99697; 1:5000 dilution) was added, and the membrane was incubated at room temperature for 1 h, then washed four times with TBS. β-Actin was used as a control. After coating with a Pierce™ ECL Western Blotting Substrate kit (Thermo, Cat. No. 32106), the membrane was scanned using a gel imaging system (Thermo, Waltham, USA). The images were saved and exported, and ImageJ software 1.52v (National Institutes of Health, USA) was used to analyze the gray value of the images.

Immunohistochemistry. Five-micrometer sections of paraffin-embedded samples were rehydrated and placed in hydrogen peroxide, then incubated with normal goat serum. The primary rabbit anti-human ISOC1 antibody (Abcam, Cat. No. ab118245; 1:100 dilution) was added and incubated overnight at 4 °C. Thereafter, the horseradish peroxidase-conjugated secondary antibody (Zhongshan Golden Bridge Biotechnology, China) was added, and the slides were incubated at room temperature for 30 min. After staining in 3,3'-diaminobenzidine and counterstaining with hematoxylin for 5 min, the sections were sealed with resinene. Five non-repetitive fields were randomly selected for each slice using a microscope (Olympus, Japan) at 400× magnification. Image Pro Plus 7.0 software (United States) was used to analyze the results of immunohistochemistry with the mean ratios of integrated optical density (IOD) to the positive area (IOD/pixel).

Cell transfection. Human ISOC1 cDNA was amplified with the primer pair 5'-AGGTCGACTCTAGAGGATCCCGCCACCATGGCGGCTGCGGAGCCGGCGGTCC-3' and 5'-TCCTTGTAGTCCATACCTACTTTGGAAAGCAGACCCGAC-3' and sub-cloned into the plasmid GV492 to produce an ISOC1-overexpressing plasmid. This plasmid and the negative control (NC) plasmid were packaged into lentiviruses (Shanghai GeneChem Co., Ltd.). Huh7-NC and HepG2-NC cells were transfected with NC lentiviruses, while Huh7-ISOC1 and HepG2-ISOC1 cells were transfected with ISOC1-overexpressing lentiviruses. The lentiviruses were diluted for infection with a serum-containing medium, which was replaced after 12 h. Stable cells were selected using puromycin (1 μg/ml, Sigma).

Cell Counting Kit-8 (CCK-8) assay. Log-phase HCC cells were digested with trypsin, which was terminated with a serum-containing medium after the cells detached. All liquid was transferred to a centrifuge tube and centrifuged for 4 min at 1000× g. The cells were resuspended in 1 ml of the serum-containing medium after discarding the supernatant. The cells were cultured in 96-well plates (100 μl/4000 cells/well) at 37 °C and 5% CO_2 for 0 h, 24 h, 48 h, and 72 h, then the CCK-8 kit solution (Dojindo, Kumamoto Prefecture, Japan; 10 μl/well) was added. After incubation for 3 h, the optical density value of the cells was determined by a Scientific Microplate Reader (Thermo, USA).

Colony-formation assay. Log-phase HCC cells were collected using the same protocol as that used for the CCK-8 assay and then seeded in a six-well plate at a density of 1.2×10^3 cells/well. Fourteen days later, the cells were fixed using 4% paraformaldehyde and stained with Giemsa stain. Colonies were counted manually. The experiment was repeated three times.

Wound healing and transwell invasion assay. HCC cells (8.0×10^5/well) were seeded in a six-well plate. When the cells had ≥80% confluency, they were scratched with a pipet tip (10 μm). A serum-free medium was used to culture the cells to avoid the influence of cell proliferation. Images of the wound were taken at 0 h and 24 h after scratching using a light microscope (Olympus, Japan).

Transwell assay was used to investigate cell invasion. The cells (4.0×10^4/well) were seeded on the top of a Matrigel-coated membrane. Serum-free medium (0.5 ml) was added to the upper chamber, and 1 ml of media containing 10% FBS was added to the lower chamber. After incubating for 24 h, non-aggressive cells were wiped with cotton swabs, and invasive cells were stained with crystal violet. The invasive cell numbers per field of view in three random fields were plotted.

Animal experiment. Male BALB/c nude mice (Beijing Vital River Laboratory Animal Technology Co., Ltd) were acquired and raised by staff at the Animal Experimental Center of the Fifth Medical Center of the PLA General Hospital. All nude mice were 5 weeks old and weighed upon receipt. The mice were randomly divided into the NC and ISOC1 groups, and 5×10^6 Huh7-NC or Huh7-ISOC1 cells were injected subcutaneously. Tumor sizes were measured every 5 days. The mice were sacrificed by cervical dislocation 25 days after the injection, and the subcutaneous tumor tissues were quickly removed. The tumor size was estimated with the formula: $(\text{length}\times\text{width}^2)/2$. The animal experiment protocol was authorized by the Medical Experimental Animal Care Commission of the Fifth Medical Center of the Chinese PLA General Hospital.

Statistical analysis. Statistical analyses and plotting were performed with GraphPad Prism 8.0 (San Diego, CA, USA). Data of continuous variables were presented as the mean ± standard deviation. A t-test was used to compare the differences between the NC and ISOC1 groups, and Kaplan-Meier survival analysis and a log-rank statistical test were used to analyze the correlation between the ISOC1 expression level and HCC prognosis. Statistical significance was set at $p<0.05$.

Results

ISOC1 may be a novel HCC suppressor gene based on public data analysis. The HCC database standardizes numerous public HCC databases, allowing for easy access to several important sub-datasets. The GEO database showed that more than 50 human organs express the ISOC1 gene, and the average ISOC1 gene expression in the liver was 5.46, higher than most other tissues and organs (Figure 1A). Meanwhile, TCGA database showed that more than 30 types of cancerous tissues or adjacent tissues express ISOC1 to different degrees, and the average ISOC1 gene expression in HCC adjacent tissues and HCC cancer tissues was 11.25% and 10.27%, respectively. ISOC1 was downregulated in HCC tissues (Figure 1B) compared to that in the adjacent tissues. Three sub-datasets (HCCDB6, HCCDB15, and HCCDB18) were used to characterize the relationship between prognosis and ISOC1 gene expression in HCC (Figure 1C). The HCCDB15 dataset was derived from the TCGA-LIHC database, and 356 cancer tissue and 49 adjacent tissue samples were analyzed. The results showed that patients with higher ISOC1 gene expression had a longer overall survival time, and those with lower expression had a shorter overall survival time ($p=0.005$). The HCCDB6 dataset was derived from Series GSE14520, from the Affymetrix human genome U133A 2.0 array, which includes 225 liver cancer tissue and 220 adjacent cancer tissue samples. The HCCDB18 dataset was obtained from the ICGC LIRI-JP database. However, ISOC1 gene expression in HCC tissues was not correlated with prognosis in these datasets ($p=0.528$ and $p=0.0675$, respectively).

Downregulation of ISOC1 expression correlates with poor HCC prognosis. In total, 105 tumor and matched adjacent tissue pairs were collected for mRNA extraction and qPCR. The relative ISOC1 mRNA expression in HCC tissue (0.586±0.053) was significantly downregulated compared to the relative expression in the adjacent tissue (1.094±0.061; $p<0.001$; Figure 2A). Eighteen HCC and matched adjacent tissue pairs were randomly selected for protein extraction and western blotting. ISOC1 protein levels in the cancerous tissue and adjacent tissue were 0.53±0.26 and 1.11±0.46, respectively ($p<0.001$; Figure 2B). Similar results were observed by immunohistochemistry. The mean ratios of integrated optical density (IOD) to positive area are 0.68±0.13 and 0.31±0.12 respectively in adjacent and tumor tissues, ($p<0.05$; Figure 2C). Moreover, high ISOC1 mRNA expression in the tumor tissues positively correlated with poor HCC prognosis ($p<0.001$; Figure 2D).

Upregulation of ISOC1 expression inhibits proliferation and colony formation of HCC cells. Western blotting confirmed that the ISOC1 protein was stably overexpressed in Huh7-ISOC1 and HepG2-ISOC1 cells ($p<0.05$; Figure 3A) and the CCK-8 assay indicated that Huh7-ISOC1 and HepG2-ISOC1 cell proliferation was inhibited compared with the untreated cells (Figure 3B). The colony formation assay demonstrated that the Huh7-ISOC1 and HepG2-ISOC1 cells had less single-cell clone-forming ability than the negative control cell lines (Figure 3C).

ISOC1 overexpression suppresses HCC cell migration and invasion. The wound-healing test demonstrated that ISOC1 overexpression inhibited the migration ability of Huh7 and HepG2 cells (Figure 4A). Furthermore, the Transwell assay showed that the upregulation of ISOC1

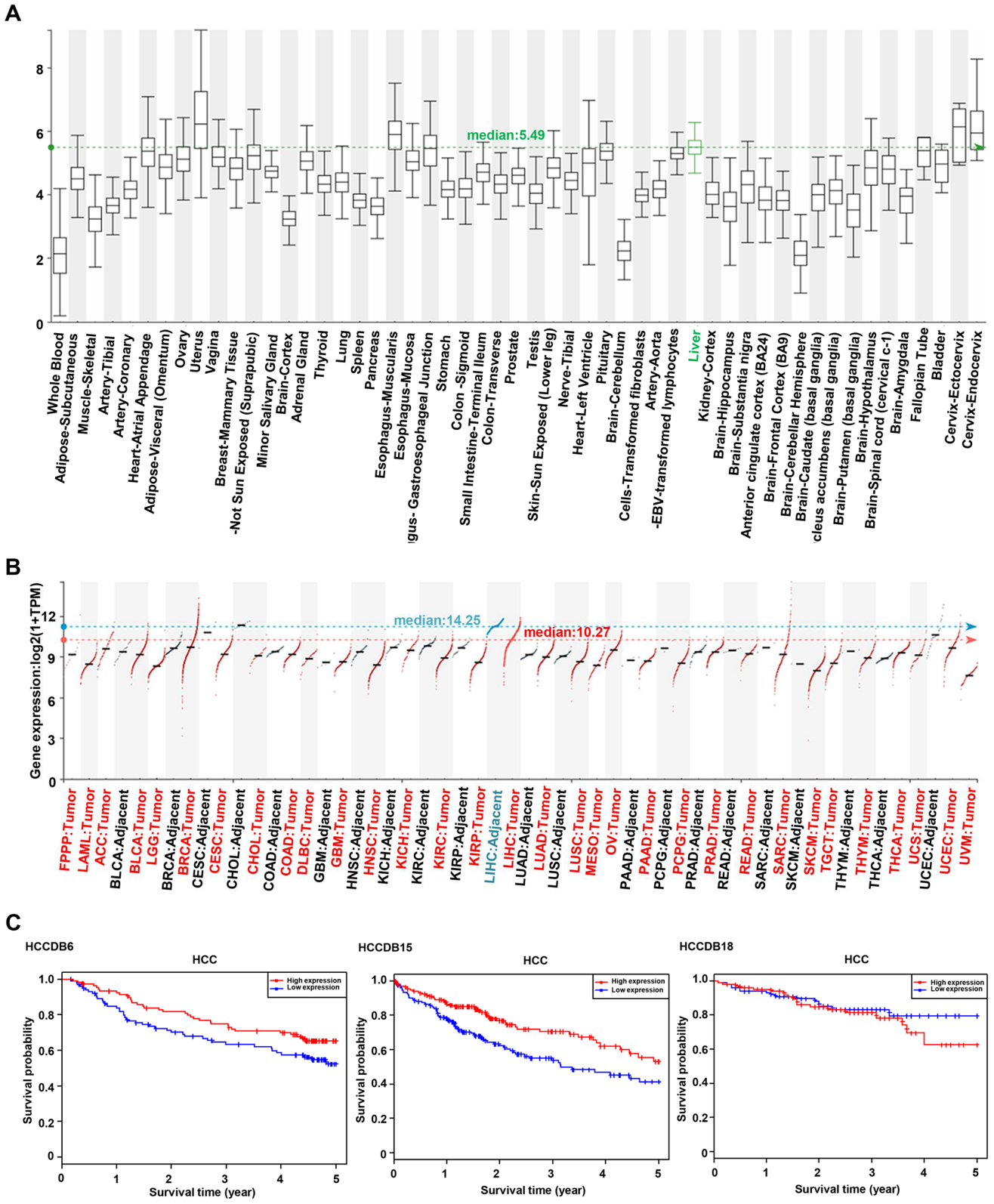

Figure 1. ISOC1 protein features checked using the HCC database. A) ISOC1 expression level in normal organs. B) ISOC1 expression level in tumor tissues and adjacent non-tumor tissues of different cancer types. C) Kaplan-Meier survival curves of HCC patients with high or low ISOC1 expression based on data present in the three HCCDB datasets. Abbreviations: ISOC1-Isochorismatase domain-containing 1; HCC-hepatocellular carcinoma; HCCDB-the HCC database

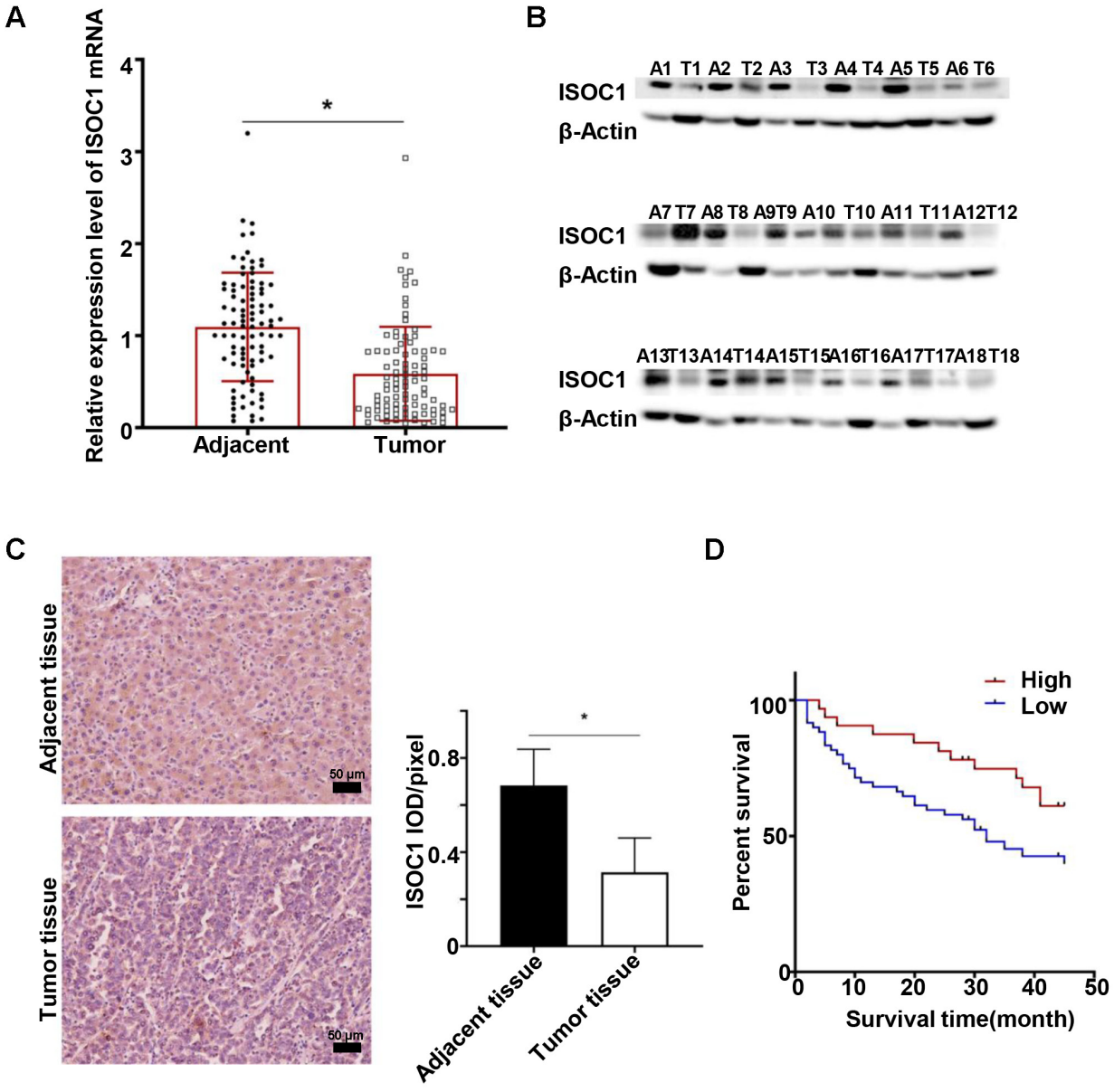

Figure 2. Expression and role of ISOC1 in HCC analyzed by clinical sample testing. A) The mRNA and B) protein ISOC1 expression levels between tumor tissues and adjacent non-tumor tissues. C) Immunohistochemistry for the ISOC1 protein expression. D) Kaplan-Meier survival curves of HCC patients with high or low ISOC1 expression (as determined by qRT-PCR) after curative resection. Abbreviations: ISOC1-Isochorismatase domain-containing 1; HCC-hepatocellular carcinoma

expression suppressed the invasive ability of HCC cells (Figure 4B), consistent with the wound-healing assay results.

ISOC1 overexpression inhibits xenograft tumor growth. The effects of ISOC1 on HCC cell tumorigenicity were investigated *in vivo* by implanting Huh7-ISOC1 and Huh7-NC cells into nude mice. Tumor growth was significantly inhibited in the Huh7-ISOC1 treated mice (Figure 5A), and the mean tumor volume of the Huh7-ISOC1 mice was significantly lower than the Huh-NC mice. ISOC1 protein expression in the tumor tissues was determined by western blotting and immunohistochemistry ($p<0.001$, Figures 5B, 5C).

Discussion

As a non-communicable disease, HCC is difficult to diagnose, particularly in the early stages. HCC is characterized by aggressive tumors that have a high proliferation rate and invasive ability [23], and the global incidence and

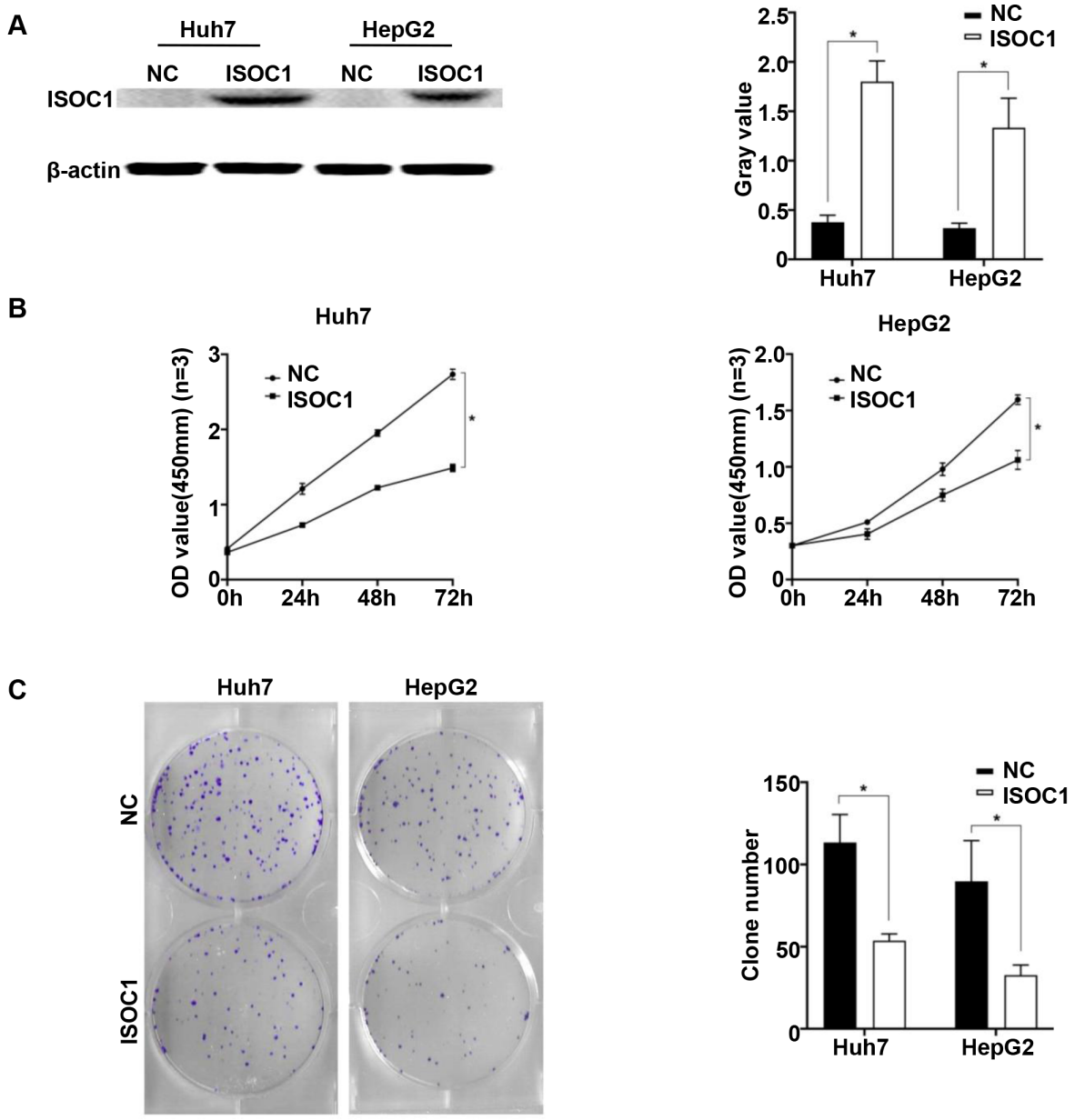

Figure 3. Overexpression of ISOC1 inhibits the proliferation and colony formation of HCC cell lines. NC indicates Huh7 and HepG2 cells transfected with a negative control lentivirus. ISOC1 indicates Huh7 and HepG2 cells transfected with an ISOC1-overexpressed lentivirus. A) ISOC1 protein expression in four cell types. B) Measuring HCC cell proliferation by the CCK-8 method. C) Testing the monoclonal formatting ability of four cell types. Abbreviations: ISOC1-Isochorismatase domain-containing 1; HCC-hepatocellular carcinoma; CKK-8-cell counting kit-8

mortality rates are increasing yearly [2]. Currently, even after multidisciplinary and comprehensive treatment, the HCC prognosis is still poor, with a recurrence rate of >50% [24]. Therefore, researchers are constantly working to identify new molecular features of HCC tumors and clarify the underlying mechanisms of tumor formation.

This study characterized the expression and function of ISOC1 in HCC. Based on clinical samples and public databases, we found that ISOC1 had a higher mRNA expression level in tumor tissues than that in adjacent paracancerous tissues, and the ISOC1 mRNA level positively correlated with HCC patient survival. We also found that HCC cell lines overexpressing ISOC1 had less proliferation, colony-forming, migration, and invasion abilities. ISOC1 overexpression also inhibited xenograft tumor growth in the mouse model. These results are inconsistent with those previously reported for ISOC1 in pancreatic and colon cancers [19, 20], where ISOC1 was presented as a tumor

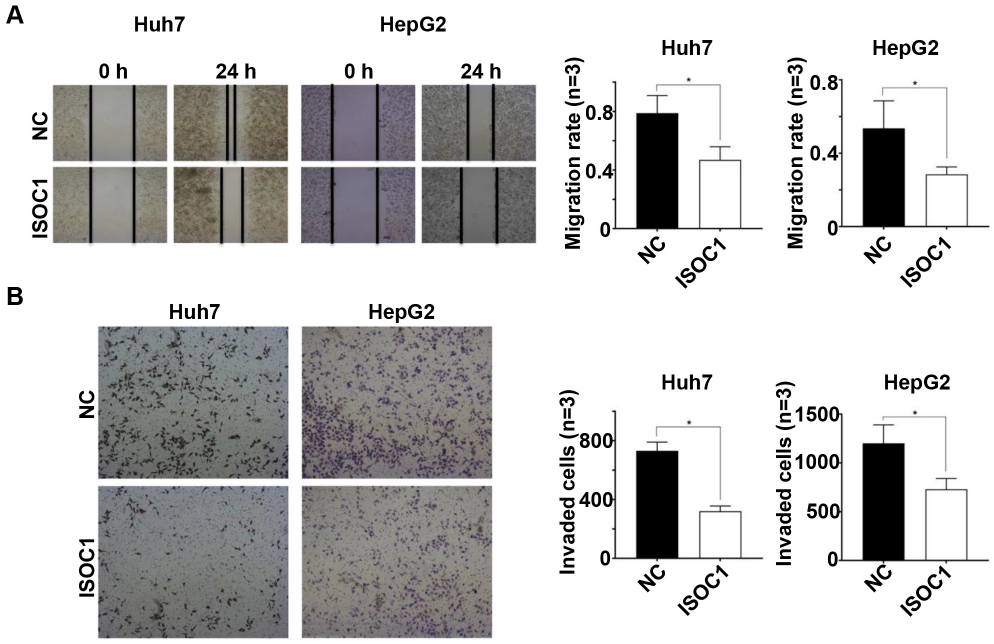

Figure 4. Relationship of ISOC1 with HCC cell migration and invasion. A) Wound healing assays for assessing HCC cell migration. Wound closure was determined 24 h after the scratch. (B) Quantifying cell invasion in a Matrigel-coated chamber by Transwell invasion assay. ISOC1: Isochorismatase domain-containing 1; HCC: hepatocellular carcinoma

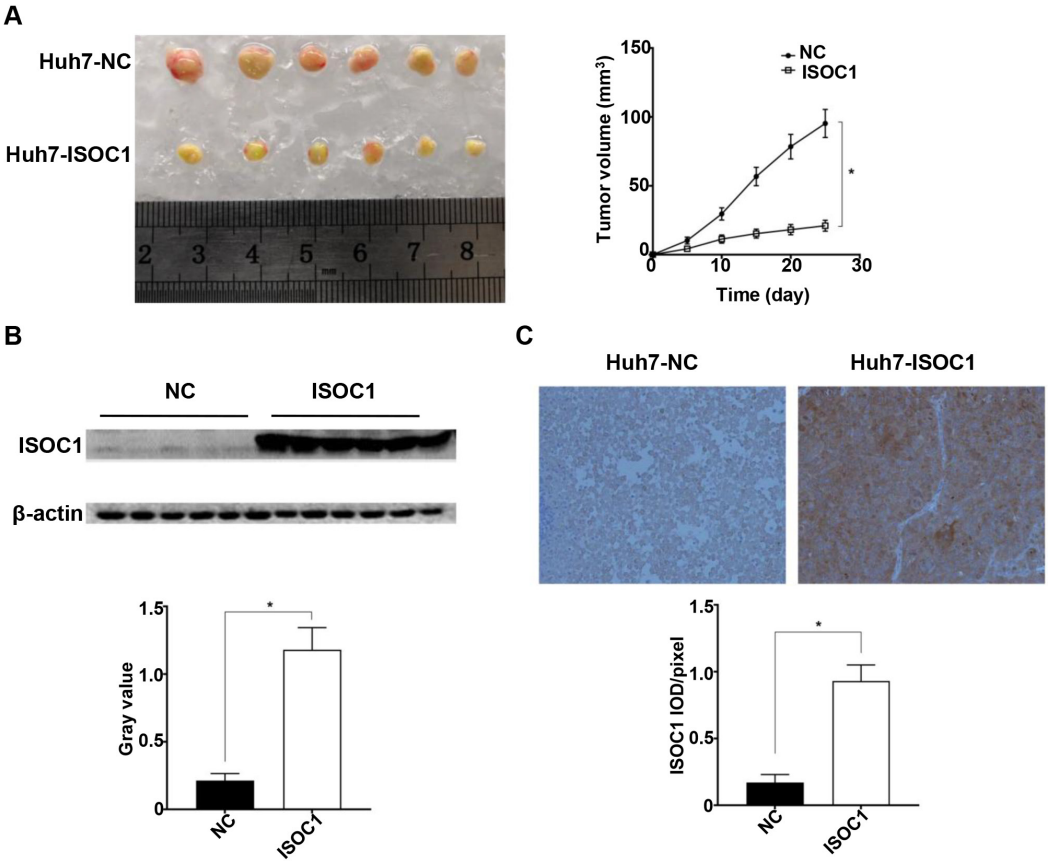

Figure 5. Effect of ISOC1 overexpression on a xenograft mouse model. Nude mice were subcutaneously injected with Huh7-ISOC1 or Huh7-NC cells and sacrificed on day 25. A) Photographs of the tumors. The tumor sizes were measured every 5 days. ISOC1 expression in mice tumor tissues was measured by B) western blot and C) immunohistochemistry. Abbreviations: ISOC1-Isochorismatase domain-containing 1

promoter. However, we found that ISOC1 acts as a tumor suppressor in HCC, suggesting that ISOC1 has different roles among cancer types.

Gao et al. reported that knockdown of ISOC1 induced apoptosis through the AKT/GSK-3β pathway in colon cancer cells [20], and Cheng et al. speculated that like ISOC2 (an ISOC1 homolog), ISOC1 might negatively regulate the tumor suppressor p16INK4a in pancreatic cancer [19]. Thus, the ISOC1 mechanism in HCC could incorporate these two pathways. Cancer is associated with aberrant metabolic processes, and peroxisomes are involved in the metabolism of bile acids, reactive oxygen species, very-long chains, and branched-chain fatty acids [25]. Therefore, HCC suppression by ISOC1 (a peroxisome-related protein) might be associated with peroxisome-regulated metabolic functions, which requires further investigation.

This is the first study to investigate the involvement of ISOC1 in HCC growth and migration. Our results suggest that ISOC1 is a potential therapeutic target for HCC treatment. However, the specific regulatory mechanism remains unclear and requires further investigation.

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