ICAM-1–Related Noncoding RNA in Cancer Stem Cells Maintains ICAM-1 Expression in Hepatocellular Carcinoma

Weixing Guo1, Shupeng Liu2, Yuqiang Cheng1, Lei Lu2, Jie Shi1, Guixia Xu2, Nan Li, Kai Cheng2, Mengchao Wu1, Shuqun Cheng1, and Shanrong Liu2

Abstract

Purpose: Portal vein tumor thrombus (PVTT) is a major complication of hepatocellular carcinoma (HCC) and is associated with poor survival. Long noncoding RNAs (IncRNA) contribute to HCC metastasis, but whether and how IncRNAs affect PVTT development remains unclear. In the present study, a novel highly expressed IncRNA (ICAM-1–related, ICR) was identified in ICAM-1+ cancer stem cells (CSC) in HCC. This IncRNA regulated CSC properties and contributed to PVTT development.

Experimental Design: We used microarray and bioinformatics analyses to identify differentially expressed IncRNAs. Real-time PCR and Western blotting were used to assess gene expression in cell lines and tumors. Sphere formation assays were performed to investigate stem cell properties of tumor cells in vitro. Retrospective and prospective studies were used to investigate the relationship between ICR expression and clinical outcomes.

Results: Compared with the corresponding primary tumors, PVTT expressed different IncRNAs and mRNAs, including the upregulated IncRNA ICR and ICAM-1. ICR regulated ICAM-1 expression by increasing the stability of its mRNA through RNA duplex formation, which modulated the CSC properties of ICAM-1+ HCC cells. ICR transcription in ICAM-1+ HCC cells was regulated by Nanog, and inhibition of ICR in situ significantly reduced ICAM-1 expression and ICAM-1+ HCC cells in tumors in vivo. Moreover, elevated ICR and ICAM-1 expression in tumors was correlated with PVTT development and poor clinical outcomes.

Conclusions: Our study demonstrates that ICR specifically regulates CSC properties of ICAM-1+ HCC cells and that ICR contributes to PVTT development. Therefore, ICR may be a promising target for HCC therapy.

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the second leading cause of cancer-related death worldwide, although substantial progress has been made regarding its diagnosis and treatment (1). Approximately 90% of cancer-associated mortality is due to tumor metastasis and relapse (2). In HCC, the poor prognosis is also related to metastasis (3). Portal vein tumor thrombus (PVTT), a special type of HCC metastases, is a major complication of HCC that is associated with poor survival (4). MRI and ultrasonography have demonstrated that approximately 50% to 80% of HCC cases are accompanied by portal or hepatic vein invasion (5). Although growing evidence indicates that noncoding RNA molecules are involved in HCC tumor metastasis and PVTT development (6–8), the mechanism underlying PVTT formation remains largely unknown.

Long noncoding RNAs (IncRNA) are a type of RNA composed of 200 to thousands of nucleotides that do not produce proteins (9). Emerging data have indicated that IncRNAs play important regulatory roles in diverse biologic cellular processes, including tumorigenesis and cancer progression (10, 11). Moreover, in HCC, IncRNAs, such as Highly Upregulated in Liver Cancer (HULC), High Expression in HCC (HEIH), and Microvascular Invasion in HCC (MVIH), were reported to be upregulated and to promote tumor microvascular invasion and metastasis (12–14). In addition, IncRNAs have also been reported to promote proliferation and stem cell–like properties of HCC cells (15). Cancer stem cells (CSC) are also inextricably involved in tumor metastasis (2, 16). In HCC, varied CSC subpopulations, such as EpCAM+ cells and CD24− cells, have been identified and found to correlate with tumor incidence and metastasis (17, 18). Although IncRNAs and CSCs have been found to be involved in tumor metastasis, whether and how they affect PVTT development remains unclear.

In the present study, we employed microarray analyses to investigate IncRNAs and mRNAs that are specifically highly expressed in PVTT tissues and the established PVTT cell line (CQST-2; ref. 19). Among the differentially expressed molecules, further analyses revealed that an IncRNA had approximately 800 bp complementary to the ICAM-1 mRNA sequence, thus termed as ICAM-1–related (ICR) IncRNA. ICR formed an RNA duplex with ICAM-1 to maintain ICAM-1 expression, thereby regulating...
the stem cell properties of ICAM-1⁺ HCC cells, as ICAM-1 was previously observed to be a CSC marker in HCC (20). In addition, retrospective and prospective analyses revealed that ICR expression was closely associated with PVTT development and HCC patients’ prognosis.

Materials and Methods

Patients and design
A total of 617 HCC patients who underwent partial hepatectomy at Eastern Hepatobiliary Surgery Hospital from 2001 to 2011 were included according to the guidelines as previously described (21). A total of 245 HCC patients with PVTT from 2001 to 2004 (Supplementary Table S1) were used for the training cohort. Another 372 patients from 2010 to 2011 were recruited in a prospective study and used as the validation cohort (Supplementary Table S2). The study was approved by the Ethics Committee of Eastern Hepatobiliary Surgery Hospital of Second Military Medical University. Written informed consent was obtained from all patients according to the regulations of the committee.

Cell lines
The human PVTT cell line CSQT-2 was established in our laboratory (19). Huh7 and Hep3B were obtained from the Japanese Collection of Research Bioresources and the American Tissue Culture Collection, respectively. The cell lines were maintained in high-glucose DMEM (Gibco BRL) supplemented with 10% FBS (Gibco BRL), 100 mg/mL penicillin G, and 50 μg/mL streptomycin (Gibco BRL) at 37°C in a humidified atmosphere containing 5% CO₂. At the beginning of this study, all the 3 cell lines were authenticated through testing the expression of α-fetoprotein and cytokeratin 19 by flow cytometry as what we did before in our previous study (20).

Statistical analyses, primers (Supplementary Table S3), antibodies (Supplementary Table S4), microarray data (GSE43630) analysis, and other materials and methods were listed in Supplementary Materials and Methods in detail.

Results
ICR was upregulated in PVTT tissues and CSQT-2 cells
To investigate IncRNAs that contribute to PVTT development, cancerous tissues were collected from 3 HCC patients with PVTT and then assessed using IncRNA microarrays to screen for IncRNAs that were specifically expressed in PVTT. Many IncRNAs were identified as differentially expressed between PVTT and the
corresponding primary tumors (Fig. 1A, left), indicating that the significantly altered expression of these lncRNAs may be involved in the pathogenesis of PVTT. In addition, many differentially expressed mRNAs were also identified in the microarray analysis between PVTT and the corresponding primary tumors (Fig. 1A, right). To verify the altered molecules, CSQT-2 cells and Hep3B cells were also assessed via microarray analyses. Similarly to the results obtained using tissues, many lncRNAs and mRNAs were found to be differentially expressed between the two cell lines (Supplementary Fig. S1). Among the differentially expressed RNAs, 32 lncRNAs (Supplementary Table S5) and 26 mRNAs (Supplementary Table S6) were found in both tissues and cell lines. These mRNAs may be candidate molecules involved in PVTT carcinogenesis. For further identification, the bio-software Blat was used to perform bioinformatic analyses to identify lncRNA–mRNA pairs among these altered RNAs, as lncRNAs can regulate gene expression based on sequence complements (22). Among the 11 lncRNA–mRNA pairs, lnc24236–ICAM-1 mRNA had an 812-bp continuous complementary sequence, which was the longest complementary sequence (Supplementary Table S7 and Supplementary Fig. S2). This observation indicates that lnc24236 may be involved in ICAM-1 regulation. Because ICAM-1 was highly expressed in HCC stem cells and plays pivotal roles in HCC carcinogenesis and metastasis, lnc24236 was thus selected for further analysis and termed ICR in the present study.

To ensure that ICR was a nontranslated transcript, we cloned the full-length transcript of ICR via 5′ rapid amplification of the cDNA ends (5′ RACE), even though a poly(A) tail was detected at the 3′-end of the ICR sequence via a DNAMAN analysis (Fig. 1B and C). We then calculated the coding probability (CP) of ICR using the Coding Potential Assessment Tool (23). The CP of ICR was 0.046, which was statistically associated with noncoding RNA (CP < 0.364; Supplementary Fig. S3). To verify ICR expression in the tumor tissues and cell lines, real-time PCR was performed using PVTT tissues (PVTT) and the corresponding primary HCC tumor tissues (Tumor) and cell lines (CSQT-2 and Hep3B), which were used in the microarray analysis. The expressions of ICR in PVTT and CSQT-2 were found higher than that in the corresponding control (Fig. 1D), which was consistent with the result from the microarray analysis. In addition, ICAM-1 expression was evaluated in these tissues and cell lines. Elevated expressions of ICAM-1 in PVTT and CSQT-2 were also observed (Supplementary Fig. S1B), indicating a potential relationship between ICAM-1 and ICR.

ICR/ICAM-1 expressions were involved in the prognosis of HCC patients with PVTT in the training cohort

We further examined whether ICR/ICAM-1 expression levels correlated with the outcome of PVTT patients after hepatectomy. The expression patterns of ICR and ICAM-1 in human HCC tumor tissues were firstly assessed using ISH. A small population of cells in the tumor tissues stained double positive for ICR (red) and ICAM-1 (green), although some ICAM-1+ cells stained negative for ICR (arrows; Fig. 2A; Supplementary Fig. S4A). The same expression pattern was found in PVTT tissue via staining, further confirming their expression correlation (Supplementary Fig. S4B). Then, real-time PCR was used to investigate the expression level of ICR and ICAM-1 in tumor tissues and paired PVTT tissues from a training cohort (cohort 1), which included 245 HCC patients with PVTT. The expression levels of both ICR and ICAM-1 were higher in PVTT tissues (PVTT) than those in the corresponding tumor tissues (Tumor; Fig. 2B). Moreover, ICR expression in PVTT was linearly correlated with ICAM-1 expression (Fig. 2C; r = 0.625; P < 0.001). To examined the relationship...
between ICR/ICAM-1 expression and the clinical outcomes of these HCC patients, the patients were classified into a high-ICR group (n = 123) and a low-ICR group (n = 122) and a high-ICAM-1 group (n = 125) and a low-ICAM-1 group (n = 120) according to the expression of ICR and ICAM-1, respectively, in their PVTT tissues (the median values were used as the cutoff points). The Kaplan–Meier analysis showed that the patients with higher ICR or higher ICAM-1 in the PVTT tissues had reduced overall survival (OS) than the corresponding low-expression groups (Fig. 2D). These results suggested the involvement of ICR and ICAM-1 in PVTT development and indicated that ICR and ICAM-1 could serve as prognostic predictors for HCC patients with PVTT.

ICR regulated ICAM-1 expression in HCC cells

We first showed that ICR expression was correlated with ICAM-1. Next, we investigated whether ICR regulated ICAM-1 expression. Three siRNAs targeting ICR at noncomplementary sequences were generated and transfected into CSQT-2 cells. All three siRNAs reduced ICR expression significantly, among which siRNA1 reduced ICR expression to the most extent (Supplementary Fig. S5A). When siRNA1 was transfected into CSQT-2, Hep3B, and Huh7 cells, ICAM-1 expression was markedly reduced at the mRNA and protein levels (Fig. 3A; Supplementary Fig. S5B, left). Transfection of siRNA3 also displayed the reduction of ICAM-1 mRNA and protein in the three cell lines, further verifying the downregulation of ICAM-1 by ICR reduction (Supplementary Fig. S5C). Moreover, the overexpression of ICR due to transfection of the cell lines with pDNA3.1-ICR (ICR) plasmids (Supplementary Fig. S5D) led to a significant upregulation of ICAM-1 mRNA and protein in the three cell lines, further verifying the downregulation of ICAM-1 by ICR reduction (Supplementary Fig. S5C). In this study, we hypothesized that ICR may upregulate ICAM-1 expression by binding to DNA or protein (9). Therefore, we hypothesized that ICR may upregulate ICAM-1 expression by binding to DNA or protein (9). Therefore, we hypothesized that ICR may upregulate ICAM-1 expression by binding to DNA or protein (9). Therefore, we hypothesized that ICR may upregulate ICAM-1 expression by binding to DNA or protein (9). Therefore, we hypothesized that ICR may upregulate ICAM-1 expression by binding to DNA or protein.
partially binding to ICAM-1 mRNA and increasing its stability. To investigate the possibility of RNA duplex formation, an RNase protection assay (RPA) was performed on RNA isolated from CSQT-2 cells. Subsequent real-time PCR analysis revealed that the overlapping portions of ICR and ICAM-1 were protected from degradation, indicating that ICR and ICAM-1 indeed form an RNA duplex (Fig. 3B, bottom plots and Supplementary Fig. S6). We further assessed the stability of ICAM-1 mRNA via real-time PCR after ICR downregulation or upregulation in vitro. The results demonstrated that the stability of ICAM-1 mRNA was decreased by the downregulation of ICR using siRNA1 and increased by the upregulation of ICR using a vector expressing a complementary sequence (Fig. 3C). The corresponding changes in ICAM-1 protein were also observed by the following Western blotting analysis (Fig. 3D). Because ICAM-1 expression was correlated with cell metastasis, we also investigated the metastatic capability of cells with modulated ICR expression by transwell migration assay. As shown in Fig. 3E and Supplementary Fig. S7, migration was significantly decreased when ICR was downregulated by siRNA1 transfection (siRNA1) and increased when ICR was upregulated by overexpression vector transfection (ICR), suggesting that ICR increased the migratory capability of HCC cells by upregulating ICAM-1 expression.

Collectively, our data demonstrated that ICR regulated ICAM-1 expression by increasing the stability of its mRNA through RNA duplex formation and then promoted HCC cells metastasis. ICR modulated the CSC properties of ICAM-1+ HCC cells Because ICAM-1+ HCC cells were previously identified as HCC CSCs (20), we next investigated whether ICR was involved in maintaining the CSC properties of ICAM-1+ cells due to its regulation of ICAM-1 expression in HCC cells. We first verified that ICR was only expressed in the ICAM-1+ cell subpopulation of HCCs. ICAM-1+ and ICAM-1- HCC cells were sorted in HCC cells (Huh7 and Hep3B) and primary tumor tissues (n = 6), and ICR expression was assessed by real-time PCR. Significantly elevated ICR expression was observed in ICAM-1+ cells from both cell lines and tumors compared with the corresponding ICAM-1- cells (Fig. 4A). To determine whether ICR is involved in maintaining the CSC properties of ICAM-1+ cells, lentiviruses containing either the shRNA expression vector targeting ICR (LV-ICR-shRNA1) or the mock sequence expression vector (LV-ICR-mock) were constructed and used to infect Huh7 or Hep3B cells. Four
days later, compared with LV-ICR mock infection, LV-ICR-shRNA1 infection reduced the number of ICAM-1^+ cells, as assessed by flow cytometry (Fig. 4B; Supplementary Fig. S8A). Subsequently, a sphere formation assay was performed to investigate the CSC properties of ICAM-1^+ cells in vitro. The number of hepatospheres was reduced in cultures infected with LV-ICR-shRNA1 compared with cultures infected with LV-ICR-mock (Fig. 4C; Supplementary Fig. S8B), indicating that ICR was involved in the self-renewal of ICAM-1^+ cells in vitro. The reduction of hepatospheres induced by ICR downregulation was further verified by another shRNA expression vector targeting ICR (LV-ICR-shRNA3) transfection (Supplementary Fig. S8C). We then assessed the possible role of ICR in vivo. Huh7 cells were injected subcutaneously into nude mice to establish a mouse model. First, ICAM-1 expression in Huh7 tumors was assessed 7 days after tumor cell implantation by fluorescence microscopy, and immunofluorescent staining found a minor population of CSC tumor cells expressing ICAM-1 (Fig. 4D, left and Supplementary Fig. S9). Then, LV-ICR-shRNA1 and LV-ICR-mock were intratumorally administered to these nude mice as described in Materials and Methods. As shown in Supplementary Fig. S8D, the administration of LV-ICR-shRNA1 successfully downregulated ICR expression compared with the LV-ICR-mock injection. ICAM-1 mRNA and protein expressions were also significantly reduced in vivo (Fig. 4D, right). Flow cytometry analysis revealed that the number of ICAM-1^+ cells was markedly reduced in the tumors from mice treated with LV-ICR-shRNA1 (ICR-shRNA1) compared with the mice treated with LV-ICR-mock (ICR-mock; Supplementary Fig. S8E). In parallel, treatment with LV-ICR-shRNA1 (ICR-shRNA1) slowed the tumor growth (Supplementary Fig. S8F). Collectively, these results demonstrate that ICR was involved in modulating ICAM-1^+ CSCs by regulating ICAM-1 expression in vitro and in vivo.

ICR was regulated by Nanog in ICAM-1^+ tumor cells

Having determined that ICR was involved in maintaining the CSC properties of HCC CSCs by regulating ICAM-1, we sought to elucidate the mechanism controlling ICR expression in CSCs. Bioinformatic analysis was performed to identify transcription factor binding sites in the ICR promoter. Because of the crucial role of Nanog in both ICAM-1^+ CSC maintenance and ICAM-1 expression (20) and the existence of two Nanog binding sites in the DNA sequence (~5 kb) upstream of ICR (Fig. 5A, left), we proposed that Nanog may also regulate ICR expression in CSCs. Chromatin immunoprecipitation experiments were then performed with CSCs enriched from tumor cell lines and primary cultures of HCC tissues to determine whether Nanog bound to these sites. As shown in Fig. 5A (right) and Supplementary Fig. S10, Nanog bound to site 1 in the ICR promoter, indicating that...
Nanog may regulate ICR transcription by binding to the ICR promoter.

To further confirm that ICR expression was regulated by Nanog, the pIRES2-EGFP-nanog plasmid was transfected into Huh7 and Hep3B cells. After validating the Nanog overexpression induced by pIRES2-EGFP-nanog transfection (Supplementary Fig. S11A), real-time PCR revealed that ICR expression was upregulated approximately 7- or 4-fold in cells transfected with pIRES2-EGFP-nanog (Nanog) compared with cells transfected with an empty pIRES2-EGFP vector (EGFP; Fig. 5B). Moreover, we also examined whether ICR expression can be reduced by downregulating Nanog expression in tumor cells. Because there is a minor population of Nanog+ cells in cell lines (<10%), we sorted the Nanog+ cells from Huh7 and Hep3B cells using the plasmid pH-nanog-promoter-EGFP (pH-NP-EGFP), which expresses GFP under the control of the human nanog promoter (–446 to +50 bp). After validating elevated Nanog expression (Supplementary Fig. S11B), GFP+ cells were transfected with the pLKO-nanog-shRNA1 plasmid. At 24 hours after transfection, ICR expression was reduced and accompanied by the downregulation of Nanog expression (N-shRNA1; Fig. 5C; Supplementary Fig. S11C). This result was further verified by transfection of another plasmid expressing shRNA targeting Nanog (pLKO-nanog-shRNA2; Supplementary Fig. S11C and S11D). Collectively, these results indicated that ICR transcription was directly regulated by Nanog in CSCs and that an ICR-mediated pathway was involved in CSC maintenance (Fig. 5D).

Relationship between ICR/ICAM-1 expression and the clinicopathologic features of HCC patients in validation cohort

To further verify the clinical significance of ICR and ICAM-1 in HCC, a prospective study including 372 HCC patients with or without PVTT from cohort 2 was performed as a validation cohort. According to the auxiliary (CT, MRI, and B-USG) and pathologic examinations as well as observations during surgery, the 372 HCC patients were divided into two groups: HCC without PVTT (n = 284) and HCC with PVTT (n = 88). Then, expressions of ICR and ICAM-1 were examined in the tumor tissues (Tumor) and paired peritumoral (PT) tissues using real-time PCR. As shown in Fig. 6A, the expression levels of ICR and ICAM-1 were higher in tumor tissues than in PT tissues in both groups. And the expression of ICR and ICAM-1 in tumor tissues from HCC patients with PVTT was strikingly higher than those from HCC patients without PVTT (Fig. 6A). We then analyzed the relationship between ICR/ICAM-1 expression and the clinicopathologic features of 372 HCC patients in the validation cohort. A, real-time PCR analysis of ICR (top) and ICAM-1 (bottom) expression in tumor tissues (Tumor) and corresponding peritumoral tissues (PT) from HCC patients with or without PVTT. The error bars represent the SD of data obtained in at least three independent experiments. **P < 0.05; ***P < 0.001. B, Kaplan-Meier’s analysis of correlations between overall survival (top) or DFS (bottom) of 372 HCC patients and ICR/ICAM-1 expression level. C, ROC analysis of the potential prognostic indicators determined by multivariate analysis. P < 0.05 for all. A, the area under the curve; FPF, false-positive fraction; TPF, true-positive fraction.
expression and the clinicopathologic features of HCC patients in cohort 2. We found that higher expression levels of ICR and ICAM-1 were associated with larger tumor size ($P = 0.0004$ and $P = 0.0019$, respectively), higher rate of intrahepatic metastasis ($P = 0.0003$ and $P = 0.0263$, respectively), and the more advanced tumor-node-metastasis (TNM) stage ($P = 0.001$ and $P = 0.0009$, respectively; Supplementary Table S8). In addition, high ICR expression was also related to high AFP levels ($P = 0.0224$) and intercurrent PVTT ($P = 0.001$; Supplementary Table S8). These findings provided further evidence for the relationship between ICR/ICAM-1 expression and PVTT, which was revealed by the studies conducted in cohort 1, and further clarified the close correlation between ICR/ICAM-1 and the clinicopathologic features of HCC patients.

Furthermore, we investigated the association between ICR/ICAM-1 and HCC patients’ prognosis. Univariate analysis revealed that the expression of ICR/ICAM-1 together with the presence of PVTT, tumor size, tumor encapsulation, intrahepatic metastasis, and TNM stage were significantly associated with both disease-free survive (DFS) and OS of HCC patients (Supplementary Table S9). These factors were further assessed by multivariate analysis using the Cox proportional hazards model. And the results showed that ICR was an independent risk factor of poor outcome of HCC patients (Supplementary Table S9).

Given the close link between ICR-ICAM-1 expression and HCC prognosis, we further analyzed their prognostic significance in HCC. Patients ($n = 372$) in cohort 2 were classified into four groups according to the combined expression of ICR/ICAM-1 in the corresponding tissues: group I ($n = 123$), high ICR and high ICAM-1; group II ($n = 63$), high ICR and low ICAM-1; group III ($n = 61$), low ICR and high ICAM-1; and group IV ($n = 125$), low ICR and low ICAM-1. As shown in Fig. 6B, the OS and DFS rates among the four groups were significantly different. The 3-year OS and DFS rates were 77.6% and 42.9% for group IV, respectively, and only 36.7% and 4.9% for group I, respectively. The potential prognostic indicators suggested by multivariate analysis were evaluated by ROC analysis. All the factors can serve as predictors of death and recurrence (Fig. 6C, $P < 0.05$ for all). For patient death, the areas under the curve (AUC) values for ICR and ICAM-1 were 0.701 (95% confidence interval [CI], 0.648–0.754; $P < 0.001$) and 0.697 (95% CI, 0.643–0.750; $P < 0.001$), respectively. For recurrence, the AUC values were 0.765 (95% CI, 0.712–0.817; $P < 0.001$) and 0.773 (95% CI, 0.719–0.828; $P < 0.001$), respectively (Fig. 6C). ROC analysis revealed that the expression levels of ICR and ICAM-1 are promising prognostic indicators for death and recurrence in HCC and exhibited stronger predictive potential than AFP levels and tumor size, which were also included in the assessment.

Discussion

LncRNAs are reported to have important epigenetic regulatory roles in diverse biologic cellular processes, including tumorigenesis and cancer metastasis. In this study, we found differently expressed lncRNAs in PVTT with primary HCC tissues. Among the differently expressed lncRNAs, ICR was highly expressed in PVTT. Further analyses found that ICR regulated ICAM-1 expression by increasing the stability of ICAM-1 mRNA through RNA duplex formation. In addition, ICR modulated the CSC properties of ICAM-1+ HCC cells in vitro and in vivo. And ICR expression in ICAM-1+ cells was regulated by the stemness-related protein Nanog. Moreover, we demonstrated that ICR expression was correlated with clinical PVTT incidence, aggressive tumor behavior, and poor clinical outcomes of HCC patients.

PVTT, as a special type of HCC metastasis, was previously reported to have different miRNAs expression patterns compared with primary HCC tissues (7). In the present study, we demonstrated that the expression of lncRNAs in PVTT differed from the corresponding tumors. This result explains why PVTT exhibits unique molecular characteristics and why PVTT displays different phenotypes, such as high mobility, with primary HCC tumors. In particular, we found that lncRNA ICR, highly expressed in PVTT, upregulated ICAM-1 expression, promoted HCC cells metastasis, and modulated the CSC properties of ICAM-1+ HCC cells. These findings may explain why PVTT displays high mobility and correlates with poor prognosis, since both ICAM-1 and CSCs were correlated with tumor metastasis. Obviously, there are many other lncRNAs differently expressed between PVTT and tumors, indicating that ICR is not the only lncRNA influencing the PVTT characteristics. In addition, one study reported that RNA polymerase II subunit 5 (RPB5)–mediating protein contributed to PVTT formation by maintaining tumorigenesis and metastases capacity via promoting IL6 production (6). Similarly to other biologic processes, PVTT formation also involves complex molecular networks, including miRNAs, lncRNAs, and proteins.

The correlation of ICR expression level with PVTT development and patients’ prognosis demonstrated that ICR may be a novel biomarker of PVTT development and a prognostic predictor for HCC patients. Major vascular invasion is one of the important factors contributing to the poor prognosis of HCC patients, and therapies for these patients still remain controversial. Resection may be the only therapeutic option that offers a possible cure for HCC patients with PVTT, though there is a high rate of recurrence after surgery (24). Among HCC patients who underwent hepatectomy, the PVTT status is correlated with the prognosis (24). Thus, factors associated with PVTT may aid in the determination of the appropriate surgical strategy. Our findings that ICR expression was correlated with PVTT development (Fig. 6) indicated the possible role of ICR in assessing the necessity of surgery, especially for liver transplantation. Patients with low ICR expression may be suitable for further surgical therapy. Of note, although ICR and ICAM-1 are located in overlapping positions in chromosome 19, they are transcribed from different strands and only about 800 bp overlapping segment located at the 5’end of ICR and at 3’end of ICAM-1. So ICR may be a single gene and not antisense lncRNA.

Our study is the first to demonstrate the heterogeneous expression of an lncRNA in tumor cells. Various lncRNAs have been correlated with diverse diseases, including cancer (25). Dysregulation of lncRNAs induces carcinogenesis and increases cancer invasiveness and metastasis (26–28). The effects of altered lncRNA expression on tumor cell biology have been primarily investigated in whole tumor cells and tissues (27). However, tumor cells are heterogeneous in tumor tissues, and advancements in experimental technologies have revealed varying heterogeneity in tumor cells, including cellular morphology, tumor histology, and genetic abnormalities (29). Very little is known regarding heterogeneous lncRNA expression. In our study, we observed distinct ICR expression levels in ICAM-1+ and ICAM-1− tumor cells in Huh7 and Hep3B cells and clinical HCC tissues, further confirming that the different
subpopulations may have distinct gene expression profiles and cell features in tumor tissues. Furthermore, ICR expression was correlated with aggressive tumor behavior and poor clinical outcomes, indicating the clinical significance of heterogeneous ICR expression in HCC tissues. These findings suggest that further attention should be paid to heterogeneous lncRNA expression in tumor development.

In summary, we demonstrated that the lncRNA ICR, which is regulated by Nanog, played a pivotal role in CSC maintenance and PVTT development by elevating ICAM-1 expression. The findings presented here indicate the crucial roles of IncRNAs in CSC maintenance and PVTT development and provide insight into IncRNA function. In addition, we demonstrated that ICR expression level was associated with HCC metastasis and HCC patient’s prognosis, suggesting that ICR may be a new prognostic indicator for HCC and may provide a specific target for the treatment of metastatic HCC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: W. Guo, S. Liu, M. Wu, S. Cheng, S. Liu
Development of methodology: W. Guo, Y. Cheng, J. Shi, N. Li, S. Cheng, S. Liu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): W. Guo, S. Liu, Y. Cheng, L. Lu, G. Xu, N. Li, S. Cheng
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): W. Guo, S. Liu, Y. Cheng, J. Shi, N. Li, K. Cheng, S. Cheng
Writing, review, and/or revision of the manuscript: W. Guo, S. Liu, Y. Cheng, S. Cheng, S. Liu
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Wu, S. Cheng
Study supervision: S. Cheng, S. Liu

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