LIFR functions as a metastasis suppressor in hepatocellular carcinoma by negatively regulating phosphoinositide 3-kinase/AKT pathway

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Abstract

Hepatocellular carcinoma (HCC) is one of the leading causes for cancer related mortality worldwide. Poor prognosis of HCC patients is mainly due to frequent metastasis and recurrence. Deregulation of metastasis suppressors in malignant cells plays critical roles during cancer metastasis. Thus, novel metastasis suppressors are urgently needed to be uncovered to shed new light on molecular mechanisms driving HCC metastasis. In the present study, decreased expression of leukemia inhibitory factor receptor (LIFR) was demonstrated in HCC, and its expression levels were even lower in HCC with metastasis. Downregulated LIFR expression predicted poor prognosis in HCC patients. LIFR was an independent and significant risk factor for their recurrence and survival. Silencing LIFR resulted in forced metastasis of HCC cells, whereas ectopic overexpression of LIFR attenuated migration and invasion of HCC cells in vitro and in vivo. Moreover, LIFR knockdown could activate phosphoinositide 3-kinase/V-akt Murine Thymoma Viral Oncogene Homolog (PI3K/AKT) signaling through enhancing phosphorylation of Janus kinase 1 (JAK1), which successively promoted matrix metalloproteinase 13 (MMP13) expression and HCC metastasis. Combination of LIFR and p-AKT or MMP13 was a more powerful predictor of poor prognosis for HCC patients. Together, these findings conclude that LIFR functions as a novel metastasis suppressor in HCC and may serve as a prognostic biomarker for HCC patients.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer with the worldwide incidence of more than 700 000 new cases annually, and is currently the third leading cause of cancer related mortality worldwide (1,2). Frequent intrahepatic or pulmonary metastasis makes predominant contributions to high recurrence and mortality rate of HCC (3). However, molecular mechanisms underlying HCC metastasis remain largely unknown. Identifying novel molecules of HCC progression and elucidating molecular mechanism responsible for post-surgical recurrence and metastasis will contribute to improving overall survival (OS) of HCC patients. Leukemia inhibitory factor receptor (LIFR) was originally isolated by expression screening of a complementary DNA library.
using radiiodinated leukemia inhibitory factor (LIF) as a probe (4). LIFR is highly homologous with the membrane-spanning 130 kD glycoprotein (gp130), an interleukin-6 signal transducer (LILST) and belongs to gp130 receptor family (6). It functions through heterodimerization with gp130 to transduce signals induced by IL-6-related cytokines, including LIF, cardiomyosin-1 (CT-1), oncostatin M (OM) and ciliary neurotrophic factor (CNTF) (6). The biological roles of those cytokines are highly diverse, ranging from maintenance of stem cell pluripotency, hepatoprotective activities, glucose uptake, to modulation of cell proliferation and differentiation.

Recently, roles of LIFR in some cancers have been disclosed. LIFR is down-regulated in human breast cancer, and it functions as a breast cancer metastasis suppressor. It promotes localization of Scribble to the cell membrane, which in turn activates Hippo signaling, leading to the phosphorylation and functional inactivation of the transcriptional activator Yes-associated protein 1 (YAP1), and thus suppresses tumor metastasis. In addition, downregulation of LIFR also significantly correlates with poor clinical outcomes of breast cancer patients (7,8). It has been reported that LIFR was clearly reduced in tumor tissues because of the frequent hypermethylation of its promoter in HCC (9,10). We previously found that LIFR was a potential immunomarker in distinction of well-differentiated HCC from dysplastic nodules and downregulation of LIFR could enhance the colony formation of HCC cells (11). However, roles of LIFR in development and progression of HCC remain to be revealed.

In this study, we investigated clinicopathologic significance of LIFR expression and addressed underlying molecular mechanism of LIFR-modulated metastasis in HCC. Our data provide the first evidence that LIFR is a valuable independent factor for predicting the prognosis of HCC, and downregulation of LIFR indicates poor prognosis for HCC patients. LIFR functions as a novel HCC metastasis suppressor by negatively regulating phosphoinoside 3-kinase/V-akt Murine Thymoma Viral Oncogene Homolog (P3K-AKT-MMP13) cascade.

Materials and methods

Patients and specimens

Specimens for construction of tissue microarrays derived from HCC patients who underwent curative resection at the Eastern Hepatobiliary Surgery Hospital (EHHB) (Second Military Medical University, Shanghai, China). Criteria for inclusion and exclusion of patients used in the current study have been reported previously (12). None of the patients received any preoperative anticancer treatment. Patients in prognostic group (n = 222) were followed until October 2010, and follow-up range was 0.3–141 months. Computed tomography and/or magnetic resonance imaging, and an elevated serum AFP level (>20ng/mL as positive) were used to verify tumor recurrence in suspected cases. OS was defined as interval between surgery and death or between surgery and the last observation point. Time to recurrence (TTR) was defined as interval between date of surgery and date of any diagnosed relapse. About 165 of 222 patients had hepatitis B virus background. Tumor stage was defined according to the Barcelona Clinic Liver Cancer Staging System (13). Tumor differentiation was graded according to the Edmondson–Steiner grading system. Micrometastases, which were only observed under microscope, were defined as tumors adjacent to the border of main tumor (14).

Immunohistochemistry and scoring

Immunohistochemistry (IHC), signal evaluation and integrated optical density (IOD) analysis were performed as described previously (15). Photographs of three representative fields were captured under high-power magnification (>200); identical settings were used for each photograph. IOD was counted and measured using Image-Pro Plus v6.0 software (Media Cybernetics, Bethesda, MD), and mean IOD was calculated from three photographs per specimen. Semiquantitative scores were also used to analyze immunostaining of each HCC case in tissue microarray. Intensity of staining was categorized into −, +, ++ or ++++, denoting negative, weak, moderate or strong staining, respectively. Extent of immunostaining was categorized into 0 (0–5%), 1 (6–25%), 2 (26–50%) or 3 (>51%) on the basis of the percentage of positive cells. Three random microscope fields per tissue were captured. The sum of intensity and extent of staining was used as final score of expression level, and determined by the formula: final score = intensity score × percentage score. The final score of ≤3 was defined as low expression, and > 3 as high expression.

Tissue microarray was stained for expression analysis of LIFR (sc-659, 1:50 dilution), p-AKT (Cell Signaling 4060, 1:50 dilution) and matrix metalloproteinase 13 (MMP13) (sc-30073, 1:50 dilution). Immunostaining scores were independently evaluated by two pathologists who were blinded to clinical outcome, as described previously (12).

Cell culture

Human normal liver cell line L02 was obtained from Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Human HCC cells (HepG2, Hep3B, SK-Hep1 and PLC) were purchased from the American Type Culture Collection. Human HCC cells (SMCC7721, MHCC97H, MHCC97L and HCCLM3) were provided by the Liver Cancer Institute of Zhongshan Hospital of Fudan University (Shanghai, China). MHCC97H, MHCC97L and HCCLM3 cells are metastatic cell lines with same genetic background but different lung metastatic potentials (16). Huh7 cells were purchased from Riken Cell Bank (Tsukuba, Japan). Cells were cultured in Dulbeccos modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 μg/mL streptomycin at 37°C in a 5% CO₂ incubator. Cells were treated with DAPT (Beta Amyloid inhibitor), Cyclopamine (Hh pathway-related protein SMO inhibitor), SB431542 (TGFR1 receptor inhibitor), AG490 (JAK2 inhibitor), U0126 (MEK inhibitor), SP600125 (JNK1/2/3 inhibitor), SB203580 (p38 inhibitor), Wortmannin/LY294002 (PI3K inhibitor) or CLE2198 (MPP13 activity inhibitor) at dose of 10, 10, 10, 1, 5, 10 μM, 100 μM/50 μM or 10 μg/mL, respectively, according to manufacturer’s instructions.

Lentivirus production and stable cell lines

Viral packaging was performed by cotransfection of pWPXL, pWPXL-LIFR, shLIFR#1 or shLIFR#2 with packaging plasmid psPAX2 and envelope plasmid pMD2.G (Addgene) using Lipofectamine 2000 (Invitrogen) in HEK 293T cells. Viruses were harvested at 48h after transfection, and viral titers were determined. Target cells, including Huh7, HepG2, MHCC97H and HCCLM3, were infected with 1×10⁵ recombinant lentivirus-transducing units in the presence of 6 μg/ml polybrene (Sigma).

In vitro cell behavior assays

For migration and invasion assays, transwell filter chamerps (BD Biosciences) and transwells coated with Matrigel (BD Biosciences) were utilized according to manufacturer’s instructions. Cells were seeded on the upper chamber in serum-free DMEM media, and DMEM with 10% fetal bovine serum was contained in the lower chamber as a chemoattractant. For migration assay, 5×10⁴ cells were seeded on the upper chamber, and 1×10⁵ cells were plated for invasion assay. After incubation at 37°C
for 24 or 48 h, non-migrated or non-invaded cells remaining in the top chamber of the transwell were carefully removed, and the cells adhering to the lower side were fixed and stained in a dye solution containing 0.1% crystal violet and 20% methanol. Migratory or invasive cells were imaged and counted through an IX71 inverted microscope (Olympus). Six random microscopic fields were counted per well for each group, and these experiments were repeated at least three times independently. For cell proliferation assay, cells (1×10³) were seeded in 100 μl complete culture media in 96-well microplates for various time periods. Cell Counting Kit-8 assay (Dojindo Laboratories, Kumamoto, Japan) was performed to measure cell viability according to manufacturer’s instructions.

**In vivo metastasis and tumorigenesis assays**

For orthotopic liver tumor model, 2×10⁶ cells were suspended in 44 μl of a mixture of DMEM/Matrigel (BD Biosciences) (volume 1:1), and were orthotopically inoculated into the left hepatic lobe of each 6-week-old male nude mouse with a microsyringe through an 8-mm transverse incision in the upper abdomen under anesthesia. Mice inoculated with HCC cells were killed 4 weeks (HuH7 cells) or 6 weeks (HCCLM3 cells) after inoculation. Consecutive sections of individual lung and liver tissues were subjected to hematoxylin and eosin (H&E) staining. For pulmonary metastatic tumors, 10μm-thick sections of paraffin embedded tissues were stained with H&E. For liver metastatic tumors, 5μm-thick sections of paraffin embedded tissues were examined microscopically by H&E staining for the presence of metastatic nodules.

**RNA isolation and real-time PCR**

Total RNA was extracted using TRIzol reagent (Invitrogen) and reversely transcribed using PrimerScript™ RT Reagent Kit (TaKaRa Biotechnology). Real-time polymerase chain reaction (real-time PCR) was subsequently performed according to manufacturer’s instructions (TaKaRa Biotechnology). Expression levels were normalized against β-actin, and relative expression levels were displayed using 2^−ΔΔCt method. Primer sequences used are listed in Supplementary Table 1, available at Carcinogenesis Online.

**Western blot analysis**

Briefly, cell lysates were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Non-specific binding sites were blocked with 5% milk in phosphate buffered saline tween-20 for 2h at room temperature. Blots were incubated with primary antibody overnight at 4°C and subsequently probed with horseradish peroxidase-conjugated secondary antibodies. Chemiluminescence detection was performed using SuperSignal West Femto Maximum Sensitivity Substrate Kit (Pierce). Membranes were exposed and recorded with Molecular Imager ChemiDoc XRS+ System (Bio-Rad, CA), and density of bands was quantified with ImageLab software (Bio-Rad). GAPDH on same membrane was used as a loading control. Antibody information is listed in Supplementary Table 2, available at Carcinogenesis Online.

**Statistic analysis**

Differences among variables were assessed by χ² analysis or two-tailed Student t test. Kaplan–Meier analysis was used to assess survival. Log-rank tests were used to compare survival of patients between subgroups. Multivariate analyses were performed by multivariate Cox proportional hazard regression model. Data were presented as mean ± SEM. Differences were considered to be statistically significant for P < 0.05.

**Results**

**LIFR is frequently downregulated in human HCC**

To clarify the role of LIFR in HCC, we evaluated expression of LIFR in human HCCs. Oncomine data-mining analysis was firstly performed. LIFR downregulation was obviously found in 11 of 15 cancer types, such as colorectal cancer, gastric cancer, liver cancer, etc (Supplementary Figure 1A, available at Carcinogenesis Online). In liver cancer datasets, both Wurmbach liver dataset (17) and Chen liver dataset (18) showed decreased levels of LIFR expression in HCC (Supplementary Figure 1B and C, available at Carcinogenesis Online) and in hepatocellular adenoma (Supplementary Figure 1D, available at Carcinogenesis Online) compared to normal liver tissues. Correspondingly, as shown in Figure 1A, levels of LIFR mRNA were significantly downregulated in 90.0% (36/40, ratio of 2^−ΔΔCt: N/T ≥ 2.0) of HCC samples, which was also confirmed by western blot analysis (Figure 1B). We next performed IHC analysis of LIFR expression using tissue microarrays consisting of 50 paired HCC tissue samples. Immunohistochemical results showed the staining density of LIFR in peritumor group (N) was markedly stronger than that in HCC group (T) and LIFR was frequently decreased in 90.0% (45/50, ratio of IOD: N/T ≥ 2.0) of the HCC patients (Supplementary Figure 2A, available at Carcinogenesis Online). To further validate the results, we analyzed LIFR expression in an independent cohort of 77 HCC patients by IHC. Similarly, LIFR expression was significantly down-regulated in 90.9% (70/77) of these cases (Supplementary Figure 2B, available at Carcinogenesis Online).

**Downregulated expression of LIFR predicts poor prognosis for HCC patients**

To investigate the clinical significance of LIFR downregulation in HCC, we observed expression of LIFR in normal liver cell line and various HCC cell lines with varying metastatic capabilities. The protein levels of LIFR obviously decreased in relatively highly metastatic HCC cells (MHCC97L, MHCC97H and HCCLM3) (Figure 1C). Next, we performed tissue microarray analysis of HCC specimens to explore the association of LIFR with features of tumor metastasis. LIFR expression was significantly reduced in HCC tissues with metastasis (n = 45) contrast to those without metastasis (n = 47) (Figure 1D), suggesting the role of LIFR in tumor invasion and metastasis.

Additionally, to determine whether LIFR expression is associated with disease recurrence and poor survival in HCC, tissue microarray analysis of HCC tissues from 222 patients who underwent liver resection was carried out. All 222 HCC cases were divided into two groups based on the results from IHC: a group with high expression of LIFR (moderate and strong immunostaining of LIFR, n = 72) and a group with low expression of LIFR (negative and weak immunostaining of LIFR, n = 150) (Figure 1E). We observed that downregulation of LIFR in HCC tissues was significantly correlated with several aggressive clinico-pathological characteristics (Supplementary Table 3, available at Carcinogenesis Online), including a high serum α-fetoprotein level (>20ng/ml; P = 0.001), a higher tumor-node metastasis (TNM) stage (P = 0.003), poor differentiation (P = 0.027) and microvascular invasion (P = 0.026). More importantly, as shown in Figure 1F, patients with low expression of LIFR exhibited worse overall survival (OS; median OS times were 28 and 72 months, respectively; P = 0.015) and shorter time to recurrence (TTR; median
TTR times were 19 and 47 months, respectively; \( P = 0.006 \) than those with high expression of LIFR.

We further explored prognostic value of LIFR in specific subgroups of HCC patients. The OS rate of patients with up-regulated LIFR was obviously increased compared to patients with down-regulated LIFR in HBsAg-positive group (\( P = 0.026 \) (Supplementary Figure 3A, available at Carcinogenesis Online) and in alpha fetal protein (AFP) ≤ 20 ng/ml group (\( P = 0.031 \)) (Supplementary Figure 3D, available at Carcinogenesis Online). The tumor recurrence rate of patients with up-regulated LIFR was significantly decreased compared to patients with down-regulated LIFR in HBsAg-negative group (\( P < 0.001 \)) (Supplementary Figure 3B, available at Carcinogenesis Online), in barcelona clinic liver cancer stage 0+A (\( P = 0.023 \)) (Supplementary Figure 3C, available at Carcinogenesis Online), in AFP ≤20 ng/ml group (\( P = 0.032 \)) (Supplementary Figure 3D, available at Carcinogenesis Online), and in TNM I stage (\( P < 0.001 \)) (Supplementary Figure 3E, available at Carcinogenesis Online). Our results also indicated 5-year OS rate in group with LIFR overexpression was significantly higher than that in group with low expression (\( P = 0.004 \)), and LIFR exhibited prognostic significance in early recurrence group (≤2 year after surgery) (\( P = 0.007 \)) (Supplementary Figure 3F, available at Carcinogenesis Online).

Univariate analysis showed that HBsAg (\( P = 0.021 \)), TNM (\( P = 0.003 \)), tumor number (\( P < 0.001 \)), tumor differentiation (\( P = 0.045 \)), microvascular invasion (\( P = 0.001 \), barcelona clinic liver cancer...
LIFR negatively regulates metastasis of HCC cells

In view of significant correlation between expression level of LIFR and clinical invasive characteristics in HCC patients, it suggests that LIFR plays a negative role in tumor metastasis. To explore roles of LIFR in HCC, shRNA technique was utilized to generate stable HCC cell lines with knockdown LIFR. Huh7 or HepG2 cells having low metastatic potential were infected with lentivirus-mediated LIFR shRNAs (shLIFR#1, shLIFR#2), and stable transfectants were established (Figure 2A and B). The transwell migration assay and matrigel invasion chamber assay revealed that silencing endogenous LIFR significantly enhanced cell migration and invasion in both Huh7 and HepG2 cells (Figure 2A and B), but did not affect cell proliferation (Supplementary Figure 4A, available at Carcinogenesis Online). We further examined effects of LIFR on growth and pulmonary metastasis of HCC cells in vivo metastatic assays. Huh7 cells stably infected with shLIFR#1 were used for orthotopic liver tumor model studies to evaluate HCC growth and pulmonary metastasis. Mouse lungs were stained with H&E and number of lung metastatic foci was calculated. Results showed that LIFR knockdown resulted in more and larger lung micrometastases in Huh7 cells (Figure 2C), whereas tumor size was not altered (Supplementary Figure 4B, available at Carcinogenesis Online), which revealed that LIFR did not affect hepatoma cell growth in vivo. In addition, we injected HepG2-shLIFR#1 cells into lateral tail vein of nude mice and evaluated metastatic growth in the lungs. Six weeks later, micrometastatic lesions were microscopically detected and histologically examined. Results showed that mice bearing tumors with LIFR knockdown had significantly more numbers of lung metastatic nodules than mice bearing control tumors (Figure 2D; Supplementary Figure 4C and D, available at Carcinogenesis Online). Similar results were also obtained using two different siRNAs targeting different parts of the LIFR mRNA. Endogenous LIFR in Huh7 and HepG2 cells was inhibited by LIFR siRNAs (Supplementary Figure 5A, available at Carcinogenesis Online), and metastatic potential of HCC cells was markedly increased without affecting cell growth (Supplementary Figure 5B–D, available at Carcinogenesis Online). In contrast, after overexpression of LIFR in HCC cells, transwell migration and matrigel invasion assays disclosed that migratory and invasive abilities of HCC cells were attenuated (Figure 3A and B), and number of lung metastatic foci was further decreased in vivo (Figure 3C). However, the cell growth was not altered in vivo whereas overexpression of LIFR occurs. Taken together, these results indicate that LIFR is capable of manipulating the aggressive and highly metastatic phenotype of HCC cells both in vitro and in vivo.

LIFR suppresses metastasis of HCC cells by inhibiting MMP13

To understand underlying molecular mechanism of LIFR-mediated inhibition for HCC metastasis, we explored downstream targets using tumor metastasis PCR array analysis. Results showed that mRNA levels of 14 genes, including CST7, CXCR4, DENR, EPHB2, ETV4, FXYD5, IGFI, IL1B, ITGAV, KISS1, MMP13, MTA1, MYCIL1 and TNFSF10, were altered by depletion of LIFR in HepG2 or Huh7 cells (fold change > 2.0). Of these differentially expressed genes, MMP13 was the most distinctive one for its fold change >2.0 in both cell lines, and upregulation of MMP13 was confirmed (Figure 4A and Supplementary Table 5, available at Carcinogenesis Online). Additionally, ETV4, MTA1 and TNFSF10 were chose to be validated by real-time PCR (Supplementary Figure 6, available at Carcinogenesis Online). To verify MMP13 as a functional downstream target of LIFR, we detected levels of MMP13 protein in HCC cells which had stably silenced LIFR and found that LIFR depletion notably increased MMP13 expression (Figure 4B). Then after stably silencing LIFR in HepG2 or Huh7 cells, we transfected MMP13 siRNAs to knockdown MMP13 or used MMP13 inhibitor (CL82198) to inhibit its activity. Results indicated that silencing MMP13 or suppressing MMP13 activity could significantly inhibit LIFR knockdown-induced migration and invasion in both cell lines (Figure 4C–E). In vivo, a negative correlation between expression levels of LIFR and MMP13 was also observed in xenograft tumor samples (Figure 4F). In addition, HCCLM3 and MHCC97H cells, which express low levels of LIFR and high levels of MMP13 contrast to Huh7 and HepG2 cells, were transfected with MMP13 siRNAs or treated with CL82198 (Supplementary Figure 7A and B, available at Carcinogenesis Online). Metastatic potential of HCC cells was attenuated through inhibition of MMP13 (Supplementary Figure 7C–F, available at Carcinogenesis Online). Collectively, these findings suggest that MMP13 can promote cell migration and invasion, and act as an important mediator in HCC metastasis induced by downregulation of LIFR.

LIFR inhibits migration and invasion of HCC cells by blocking PI3K-AKT-MMP13 signaling

To elucidate molecular mechanisms for LIFR as a metastatic suppressor in HCC, signaling pathways regulated by LIFR were analyzed by detecting phosphorylated forms of AKT (p-AKT), phosphatase-like forms of YAP1 (p-YAP1), and STAT3 (p-STAT3) in HCC cells treated with CL82198 (a PI3K/AKT inhibitor) or treated with interferon-α (IFN-α). In the cell lines that were stably transfected with LIFR siRNAs or treated with CL82198, the phosphorylation activities of PI3K/AKT and STAT3 were significantly increased, whereas YAP1 was significantly reduced. The results indicated that LIFR negatively regulates metastasis of HCC cells by blocking PI3K-AKT-MMP13 signaling.
Additionally, our data also showed that downregulation of AKT1 or inactivation of p-AKT impaired both MMP13 expression and metastatic potentials in HCCLM3 and MHCC97H cells (Supplementary Figure 9C–E, available at Carcinogenesis Online).

Janus kinase 1 (JAK1)-associated PI3K signaling involves many important biological processes, such as enhancing cytokine expression in human airway epithelial cells (19). Janus kinases are constitutively associated with gp130 (20, 21). We therefore detected status of p-JAK1, pPI3K, p-AKT and MMP13 expression in HCC cells after altering LIFR expression. Results indicated that knockdown of LIFR enhanced levels of p-JAK1, pPI3K, p-AKT and MMP13 expression, while overexpression of LIFR conversely inhibited the levels of p-JAK1, pPI3K, p-AKT and MMP13 (Figure 5F). The PI3K/AKT signaling pathway plays an important role in EMT (22). To determine whether decreased LIFR could promote EMT process, we analyzed EMT markers in HepG2 cells after silencing LIFR. EMT markers exhibited no changes between LIFR-depleted cells and control cells (Supplementary Figure 10, available at Carcinogenesis Online), suggesting that migration and invasion facilitated by LIFR knockdown was not a phenotypic requirement of EMT. Thus, these studies indicate that LIFR negatively regulates cell metastasis at least partly through JAK1/PI3K/AKT/MMP13 pathway.

Combination of LIFR and p-AKT or MMP13 exhibits improved prognostic accuracy for HCC

Our above results in this study found that expression level of LIFR was a valuable independent factor for predicting the prognosis of HCC, and MMP13 as well as p-AKT were functional.
downstream molecules of LIFR. We therefore analyzed whether the combination of LIFR and p-AKT or MMP13 was a more powerful tool for prognosis prediction. As shown in Figure 6A, tissue microarray analysis of 204 HCC specimens revealed a negative correlation of LIFR expression with p-AKT level. Based on the results from immunohistochemistry, all 204 specimens were divided into three groups: high expression of LIFR and low levels of p-AKT (LIFR\textsuperscript{HIGH} and p-AKT\textsuperscript{LOW}, \(n = 30\)), both high or low levels of LIFR and p-AKT (LIFR\textsuperscript{HIGH} and p-AKT\textsuperscript{HIGH} or LIFR\textsuperscript{LOW} and p-AKT\textsuperscript{LOW}, \(n = 105\)), and low expression of LIFR and high levels of p-AKT (LIFR\textsuperscript{LOW} and p-AKT\textsuperscript{HIGH}, \(n = 69\)). In comparison with other two groups, HCC patients with low expression of LIFR and high level of p-AKT exhibited poorest OS (median OS time was 19 months; \(P = 0.003\)) and shortest TTR (median TTR time was 8 months; \(P = 0.004\)) (Figure 6B). Similarly, a negative correlation between LIFR and MMP13 was also observed by immunostaining (Figure 6C). About 204 specimens were also divided into three groups: high LIFR expression and low MMP13 expression (LIFR\textsuperscript{HIGH} and MMP13\textsuperscript{LOW}, \(n = 34\)), both high or low levels of LIFR and MMP13 (LIFR\textsuperscript{HIGH} and MMP13\textsuperscript{HIGH}, or LIFR\textsuperscript{LOW} and MMP13\textsuperscript{LOW}, \(n = 90\)), and low LIFR expression and high MMP13 expression (LIFR\textsuperscript{LOW} and MMP13\textsuperscript{HIGH}, \(n = 80\)). In contrast to other two groups, HCC patients with low LIFR expression and high MMP13 expression displayed poorest OS (median OS time 18 months, \(P < 0.001\)) and shortest TTR (median TTR time was 9 months; \(P = 0.006\)) (Figure 6D). These data suggested that the combination of LIFR and p-AKT or MMP13 was a more powerful predictor of poor prognosis for HCC patients.

**Discussion**

Cancer metastasis plays important roles in malignant progression, in which inactivation or downregulation of metastasis suppressors is crucial (23). In this study, we found that expression of LIFR was commonly downregulated in HCC (Figure 1A and B), and even lower in HCC tissues from patients who developed metastases (Figure 1D). The downregulation of LIFR was correlated with aggressive clinicopathological characteristics (Supplementary Table 3, available at Carcinogenesis Online). HCC patients with low levels of LIFR showed poorer prognosis than patients with high levels of LIFR (Figure 1F). Prognostic significance of LIFR also existed in AFP-negative patients and in patients occurring early recurrence (Supplementary Figure 3D and F, available at Carcinogenesis Online). These data suggest that LIFR is a good prognostic marker for HCC patients, even in specific subgroups, for whom prognosis is difficult to predict by conventional clinical indexes. Then, to investigate function of LIFR, a series of in vitro and in vivo studies were performed. Results
demonstrated that knockdown of LIFR markedly facilitated cell migration and invasion whereas overexpression of LIFR significantly inhibited metastatic potential of HCC cells without affecting cell proliferation (Figures 2 and 3; Supplementary Figure 4, available at Carcinogenesis Online), supporting that LIFR was a potential metastasis suppressor in HCC.

To disclose executor regulated by LIFR in tumor metastasis, tumor metastasis PCR array analysis was used, and MMP13 was identified as a target (Figure 4A). Levels of MMP13 expression were found to be upregulated under the condition of LIFR knockdown, while overexpression of LIFR resulted in downregulation of MMP13 (Figure 4B; Supplementary Figure 9B, available at Carcinogenesis Online). Functionally, inactivation or depletion of MMP13 in HCC cells could significantly impair metastatic capabilities that had been enhanced by downregulation of LIFR (Figure 4D and E). Furthermore, combined evaluation of LIFR and MMP13 was a more powerful prognostic marker in patients with HCC (Figure 6D). MMP13, known as collagenase-3, is a proteolytic enzyme with high proteolytic activity toward fibrillar types I, II and III collagens (24). Studies of MMP13 in various cancers indicate that upregulation of MMP13 contributes to metastasis of cancer cells. Increased expression of MMP13 can

Figure 4. MMP13 is a functional downstream target of LIFR. (A) Tumor Metastasis RT2 Profiler PCR Array was utilized to profile HepG2 or Huh7 cells transfected with siRNA targeting LIFR (siLIFR#3) or negative control siRNA (siNC). Fourteen 2.0-fold or more differentially expressed genes were identified. fold change: siLIFR#3/siNC ratio; Red: log₂(fold change) > 1, Green: log₂(fold change) < −1, Black: −1 < log₂(fold change) < 1. Upregulated mRNA levels of MMP13 were verified by qRT-PCR in siLIFR#3 transiently transfected HCC cells. (B) Expression of indicated molecules in HepG2 or Huh7 cells infected with Scr, shLIFR#1 or shLIFR#2 lentivirus, were detected by western blot. (C, D) After transfecting MMP13 siRNA (siMMP13#1, siMMP13#2, siMMP13#3) or negative control siRNA (siNC), immunoblots for MMP13 expression and transwell assays were performed in HepG2-shLIFR#1 cells or Huh7-shLIFR#1 cells. (E) After treating with CL82198 (10 µg/ml), in vitro migration and invasion properties of HepG2-shLIFR#1 cells and Huh7-shLIFR#1 cells were assessed by transwell migration and invasion assays. Bars represent the means ± SEM of three independent experiments. (F) LIFR and MMP13 were detected by IHC in xenograft tumor tissues from the indicated cells (magnification, ×200). *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.
Figure 5. Reduced expression of LIFR promotes HCC metastasis via PI3K-AKT-MMP13 signaling. (A) Western blot analysis of MMP13 for Huh7-shLIFR#1 cells or HepG2-shLIFR#1 cells treated with various chemical inhibitors. (B) Levels of p-AKT(Ser473), AKT, and MMP13 were detected in LIFR knockdown Huh7 and HepG2 cells by immunoblots. (C) Huh7-shLIFR#1 or HepG2-shLIFR#1 cells were transfected with AKT1 siRNA. Expression levels of AKT1 and MMP13 were detected, and migration and invasion were measured using transwell assays. (D) Huh7-shLIFR#1 or HepG2-shLIFR#1 cells were treated with 10 µM LY294002. Expression levels of AKT, p-AKT(Ser473) and MMP13 were detected, and metastatic properties were measured. The data are presented as the mean ± SEM (from six random 200× magnification fields). (E) LIFR and p-AKT were detected by IHC in xenograft tumor tissues from indicated cells (magnification, ×200). (F) Western blot analysis for Jak1 (p-Jak1 Tyr1022/1023), PI3K (pPI3K Tyr473), AKT (p-AKT Thr308), and MMP13 in indicated cell lysates. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.
mediate Oct-3/4 enhanced degradation of surrounding extracellular matrix in glioblastomas (25). Tumor-derived MMP13 is correlated with aggressive tumor phenotypes, and inversely correlated with the overall survival of breast cancer patients (26). However, roles of MMP13 in HCC remain unclear. Our above results indicate that MMP13 can promote metastasis of HCC cells, and it is a functional mediator of LIFR downregulation which promotes migration and invasion of HCC cells.

To determine pathways regulated by LIFR, we analyzed levels of p-AKT, p-YAP, pSTAT3, p-p38 and pERK in HCC cells depleting LIFR. The results excluded involvement of other cascades except PI3K-AKT pathway (Supplementary Figure 8, available at Carcinogenesis Online). Particularly, p-YAP level exhibited no difference between LIFR knockdown cells and control cells, indicating that molecular mechanism underlying the role of LIFR in HCC is different from breast cancer in which LIFR suppresses tumor metastasis by activation of Hippo-YAP pathway (7). This discrepancy may be due to the complexity of cancers, including heterogeneities, specific mechanical and biological properties, and distinct surrounding microenvironment in different...
cancers (3,27). Maybe cell heterogeneities like epithelial and mesenchymal phenotypes also affected LIFR’s roles.

We further identified pathways in regulation of MMP13 expression by treating HCC cells with various small chemical inhibitors against key factors of different signal pathways, including Cycloamine (Hh pathway-related protein SMO inhibitor), DAPT (Beta Amyloid inhibitor), AG490 (JAK2 inhibitor), SB431542 (TGFβ1 receptor inhibitor), U0126 (MEK inhibitor), SP600126 (JNK1/2/3 inhibitor), SB203580 (p38 inhibitor) or Wortmannin/LY294002 (PI3K inhibitor). We found that only inhibitors of PI3K/AKT signaling obviously inhibited MMP13 expression (Figure 5A; Supplementary Figure 9A, available at Carcinogenesis Online). Meanwhile, we verified that decreased LIFR could increase levels of p-AKT and MMP13 expression, whereas overexpressed LIFR decreased levels of p-AKT and MMP13 expression (Figure 5B; Supplementary Figure 9B, available at Carcinogenesis Online). In addition, silencing AKT1 or inactivation of phosphorylated AKT molecules in HCC cells markedly suppressed MMP13 expression and correspondingly inhibited migration and invasion which had been facilitated by depletion of LIFR. Taken together, these results show that LIFR can inhibit HCC metastasis by suppressing PI3K-AKT-MMP13 signaling. It is consistent with previous report in which MMP13 is increased by FAK/PI3K/AKT/mTOR pathway to mediate migration of human chondrosarcoma cells induced by Endothelin-1 (28).

It is well known that gp130 functions as a common signal transducer for IL-6, LIF and OM (29). It can convert LIFR into a high-affinity binding site for the ligands by heterodimerization (30). Jak-Tyk kinases constitutively associate with gp130 and LIFR, and their activation occurs as a result of ligand-induced dimerization of LIFR and gp130 (20). Meanwhile, signaling via receptors that contain gp130 indicates that JAK1 is an obligatory kinase, and JAK2 and TYK2 serve as additional components (31). Cytoplasmic JAKs are crucial components of diverse signaling pathways that govern cellular survival, proliferation, differentiation and apoptosis. JAK kinase is required for optimal activation of the PI3K-AKT pathway following the interaction of cytokine receptors with their ligands (32). In present study, we found levels of MMP13 protein were not altered in HCC cells treated with AG490, an inhibitor of JAK2 (Figure 5A; Supplementary Figure 9A, available at Carcinogenesis Online). However, levels of pJAK1, pPI3K, p-AKT and MMP13 were increased by LIFR knockdown, while decreased by LIFR overexpression (Figure 5F), supporting that JAK1/PI3K/AKT/MMP13 signaling involved in the LIFR-suppressed metastasis of HCC.

In conclusion, our findings unravel a novel mechanism in HCC that LIFR binds to the co-receptor (gp130) and inactivates the Jak1-Pi3K-Akt phosphorylation cascade, which successively inhibits expression of MMP13 and leads to retard metastasis of HCC cells (Figure 6E). LIFR alone, or combination of LIFR with MMP13 or p-AKT, is a potential marker for predicting prognosis of HCC.

Supplementary material
Supplementary Materials and methods, Tables 1–7 and Figures 1–10 can be found at http://carcin.oxfordjournals.org/

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