Novel Role for Epidermal Growth Factor-Like Domain 7 in Metastasis of Human Hepatocellular Carcinoma

Fan Wu,1 Lian-Yue Yang,1,2 Yun-Feng Li,1 Di-Peng Ou,2 Dong-Ping Chen,1 and Chun Fan1

Epidermal growth factor-like domain 7 (Egfl7) is a recently identified secreted protein that is believed to be primarily expressed in endothelial cells (ECs). Although its expression was reported elevated during tumorigenesis, whether and how Egfl7 contributes to human malignancies remains unknown. In the present study overexpression of Egfl7 was found predominantly in hepatocellular carcinoma (HCC) cells in HCC tissues and closely correlated with poor prognosis of HCC. The expression of Egfl7 in cancer cells was further verified with HCC cell lines including HepG2, MHCC97-L, and HCCLM3, and the Egfl7 expression levels positively correlated with metastatic potential of HCC cell lines was tested. To functionally characterize Egfl7 in HCC, we depleted its expression in HCCLM3 cells by using small interfering RNA. Interestingly, reduction of Egfl7 expression resulted in significant inhibition of migration but not growth of HCCLM3 cells. Biochemical analysis indicated that Egfl7 could facilitate the phosphorylation of focal adhesion kinase (FAK) and therefore promote the migration of HCCLM3 cells. In addition, this effect was almost completely blocked by inhibition of epidermal growth factor receptor (EGFR), indicating that the activation of FAK by Egfl7 is mediated through EGFR. Finally, we used a mouse model to demonstrate that down-regulation of Egfl7 was associated with suppression of intrahepatic and pulmonary metastases of HCC. Collectively, our study shows for the first time that overexpression of Egfl7 in HCC and Egfl7 promotes metastasis of HCC by enhancing cell motility through EGFR-dependent FAK phosphorylation.

Conclusion: Our study suggests Egfl7 as a novel prognostic marker for metastasis of HCC and a potential therapeutic target.

(HEPATOLOGY 2009;50:1839-1850.)

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third most common cause of death from cancer, resulting in more than 600,000 deaths each year.1,2 As surgical techniques have progressed, hepatic resection has evolved into a safe procedure with low operative mortality at large centers.3 However, the overall survival of patients with HCC remains unsatisfactory because of a high incidence of recurrence and metastasis after hepatic resection.4,5 Thus, the inhibition of recurrence and metastasis is of great importance in the treatment of HCC.

Although the recurrence and metastasis of HCC is a multifactorial, multistep, and complex process,6 available information suggests that this process is to a large extent attributable to the ability of cell migration.7-9 In recent decades, various molecules have been reported to play a

Abbreviations: AFP, alpha-fetoprotein; ANLT, adjacent nontumorous liver tissue; EC, endothelial cell; Egfl7, epidermal growth factor-like domain 7; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FAK, focal adhesion kinase; HCC, hepatocellular carcinoma; PDGF, platelet derived growth factor; PDGFR, platelet derived growth factor receptor; qRT-PCR, real-time quantitative reverse transcription-polymerase chain reaction; siRNA, short interfering RNA; TGF-α, transforming growth factor-α.

From the 1Liver Cancer Laboratory and 2Department of Surgery, Xiangya Hospital, Central South University, Changsha, Hunan, China.

Received March 17, 2009; accepted July 21, 2009.

Supported by grants from the National Key Technologies R and D Program of China (No. 2001BA703B04, No. 2004BA703B02), National Keystone Basic Research Program of China (No. 2004CB720303), National High Technology Research and Development Program of China (No. 2006AA02Z4B2), National Science Fund for Distinguished Young Scholars of China (No. 30328028), National Natural Science Foundation of China (No. 30571826), Clinical Subjects’ Key Project of Ministry of Health (2007-2009), and National Science & Technology Major Projects (2009ZX09103-681).

Address reprint requests to: Lian-Yue Yang, M.D., Ph.D, Liver Cancer Laboratory, Department of Surgery, Xiangya Hospital, Central South University, Changsha 410008, Hunan, China. E-mail: lianyueyang@hotmail.com; fax: +86-731-4327332.

Copyright © 2009 by the American Association for the Study of Liver Diseases.

Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.23197

Potential conflict of interest: Nothing to report.

Additional Supporting Information may be found in the online version of this article.
role in HCC cell migration such as OPN, KAI-1, and PAK1. We recently showed that RhoC is involved in migration of HCC cells. Although these findings represent significant progress in the field, mechanisms underlying HCC cell migration are not fully understood, raising a need for further studies.

Epidermal growth factor-like domain 7 (Egfl7) is a recently identified secreted protein that contains two EGF-like domains and is conserved across species. Egfl7 was initially believed to be exclusively expressed in endothelial cells (ECs) and essential in the process of vascular development during embryogenesis of zebrafish. Interestingly, Egfl7 expression is high during embryonic and neonatal development, down-regulated in almost all mature tissues, and increases again during vascular injury and tumorgenesis. Moreover, recent studies have demonstrated that Egfl7 can regulate collective migration of ECs and act as a chemoattractant for cell migration. Focal adhesion kinase (FAK) phosphorylation is an important event required for cell migration. In Egfl7-deficient mice, the phosphorylation of ECs was significantly reduced, implicating the potential role of Egfl7 in regulation of cell motility. However, evidence for the function of Egfl7 in human malignancies is still limited. Therefore, we carried out the present study to determine the expression of Egfl7 in human HCC tissues as well as cell lines to elucidate the function of Egfl7 in the metastasis of HCC by characterizing its role in cell migration using both in vitro and in vivo models.

Materials and Methods

Patients and Tissue Specimens. Specimens of HCC tissues were obtained from 112 HCC patients who underwent hepatic resection at the Department of Surgery, Xiangya Hospital of Central South University (CSU) from February 1998 to December 2005. These patients included 98 males and 14 females with a median age of 48 years (range: 16-73). Among these 112 cases of HCC, matched fresh specimens of HCC and adjacent nontumorous liver tissue (ANLT) from 31 cases were collected for real-time quantitative reverse-transcription polymerase chain reaction (RT-PCR) and western blot detection. Six samples of normal liver tissues obtained from patients with cavernous hemangioma who underwent hepatic resection were also included as a control. Prior informed consent was obtained and the study protocol was approved by the Ethics Committee of Xiangya Hospital.

qRT-PCR. Real-time PCR was performed as described. The primers of Egfl7 were as follows: forward, 5′-GCACCGTCAAGGGCTGAAC-3′; reverse, 5′-GGGTTAGGGGTCTCTATAG-3′. GAPDH expression was determined as a control using primers: forward, 5′-GCACCGTCAAGGGCTGAAC-3′; reverse, 5′-TGGTGAAGCGCCAGTGA-3′. The results were analyzed using the 2-ΔΔCt method and the formula was: ΔΔCt = (CtHCC-CtGAPDH) - (CtANLT-CtGAPDH).

Western Blot. Total protein was extracted and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto PVDF membrane. The blotted membranes were incubated antihuman Egfl7 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), antihuman FAK antibody (Santa Cruz), or antihuman phosphorylated FAK antibody (Santa Cruz) and then secondary antibody (KPL, Gaithersburg, MD) in order. Beta-actin protein was also determined by using the specific antibody (Sigma, St. Louis, MO) as a loading control. NIH 3T3 cells which do not normally express Egfl7 were used as a negative control for antihuman Egfl7 polyclonal antibody.

Histoimmunofluorescence. The slides were incubated with goat antihuman Egfl7 antibody (1:50 dilution, Santa Cruz) and the Cy3-labeled donkey antigoat immunoglobulin G (IgG; 1:1000 dilution, Beyotime Institute of Biotechnology, Jiangsu, China) for 1 hour at 37°C. The slides were then incubated with a mouse antihuman alpha-fetoprotein (AFP) antibody (1:50 dilution, Zhongshan Goldenbridge Biotechnology, Beijing, China) and FITC-labeled goat antimouse IgG (Beyotime Institute of Biotechnology) for 1 hour at 37°C. Fluorescence was analyzed with a Nikon 108 fluorescence microscope.

Immunohistochemistry. Formalin-fixed paraffin sections were stained for Egfl7 (Santa Cruz Biotechnology, 1:200) using the streptavidin-peroxidase system (Zhongshan Goldenbridge Biotechnology). Negative control slides were probed with normal goat serum under the same experimental conditions. The immunohistochemical staining was scored using a four-point scale according to the percentage of positive hepatocytes: 0, ≤10% positive; 1+, 11%-25% positive; 2+, 26%-50% positive; 3+, ≥51% positive. The protein expression of Egfl7 was thus defined as negative if scored 0, and 1+, 2+, and 3+ as positive. Egfl7 expression in HCC specimens was also divided into a low expression group (0 or 1+) and a high expression group (2+ or 3+).

Follow-Up and Prognostic Study. Follow-up data were obtained after hepatic resection for all 112 patients. The follow-up period was defined as the interval between the date of operation and that of the patient’s death or the last follow-up. Deaths from other causes were treated as censored cases. Recurrence and metastasis were diagnosed by clinical examination, serial AFP level mensuration, and...
ultrasonography or computed tomography (CT) scan. Nine conventional variables together with Egfl7 expression were tested in all 112 patients: age, gender, liver cirrhosis, serum AFP level, Edmondson-Steiner grade, capsular formation, size of the tumor, number of tumor nodes, and vein invasion.

**Cell Lines and Cell Culture.** HCCLM3 and MHCC97-L cell lines were purchased from the Liver Cancer Institute of Fudan University. HepG2, CCL13, and 293T cell lines were purchased from the American Type Culture Collection (Rockville, MD). These cell lines were cultured in low glucose Dulbecco’s modified Eagle media supplemented with 10% fetal bovine serum and incubated in 5% CO2 at 37°C.

**Construction of siRNA Plasmid Vector.** The short interfering RNA (siRNA) expressing vector pLKO.1.puro was purchased from Addgene (Cambridge, MA). Three putative candidate sequences were designed by using Oligoengine software and their specificity confirmed by nucleotide BLAST searches. The three putative candidate sequences and a scramble sequence were as follows (the 19-nucleotide sense or antisense strands are in bold letters, and stem loop sequences are in italics): sequence-1: sense 5’-GCCGATCATCATTATAAAGCTCGAG-CCGAGCCTTATAAATGATGTC-3’, antisense 5’-AAATCCAAAAGTACATCCATTATAAAGCTCGAG-CAGCTTATAATGGATGTAC-3’; sequence-2, sense 5’-CCGGCCGGCAGACG-ACTTCTCCCTGAGGGAGAAATGC-CCGCGGCTTTTTT-3’, antisense 5’-AAATCCAAAAGCAGGAGAAGTCGAGCCGGCGATGAC-3’; sequence-3, sense 5’-CGGAGCATTCCACAGGGCCATGTCG-3’, antisense 5’-AAATCCAAAACATCGCCCTGTGGATGACTCCTGAGATCCTACACAGGGCCATG-3’; control sequence, sense 5’-CCGGAGCTTTCACCTACAAACCTGCTCAGAGCTTG-3’, antisense: 5’-AAATCCAAAACAGGTTCCAGCTCTCCCACACCGGTCGCCGCTGCAG-3’.

**Transfection.** The lentiviral packaging cells, 293T cells, were transfected with recombinant lentiviral expression plasmid pLKO.1.puro and packaging plasmid pH8.2delataR dvpr and pCMV-VSV-G at 70% confluence with the use of FuGENE 6 transfection reagent (Invitrogen, Carlsbad, CA) to produce lentivirus. Media containing lentivirus were added to the cells supplied with polybrene (8 μg/mL) for 4 hours. After 12 hours the original medium was replaced with fresh medium and lentivirus was added again.

**Wound Healing and Transwell Assay.** The methods for wound healing and the Transwell assay have been described.13,28,29 These experiments were performed in triplicate.

**MTT Assay and Cytoimmunofluorescence.** The procedures for MTT assay and cytoimmunofluorescence have also been described.13,29

**HCC Metastatic Mouse Model.** A metastatic hepatocellular carcinoma model in mice was established according to the existing protocol.30 Briefly, cells (5 × 10⁶) of HCCLM3Egfl7RNAi⁺ or HCCLM3Egfl7RNAi⁻ were injected subcutaneously into the left upper flank regions of three experimental mice (3–4 weeks of age, male, BALB/c) and the subcutaneous tumor tissues were removed and implanted into the livers of two groups of nude mice with eight mice in each group. Mice with small tumor colonies seen with the naked eye around the local tumor were considered intrahepatic metastasis-positive. The lung tissue of each mouse was fixed, embedded, sectioned serially, stained with hematoxylin and eosin (H&E), and observed under a microscope. Mice whose metastatic hepatocellular carcinoma cells were found on any slide of lung sections were considered lung metastasis-positive. The expression of Egfl7 and phosphorylated FAK in local tumor tissues were determined by immunohistochemistry. In addition, the microvessel density (MVD) in local tumor was also calculated as described.31 The Animal Use Committee of the Xiangya Hospital approved all protocols for treating animals.

**Statistical Analysis.** Fisher’s exact test was used for statistical analysis of categorical data, whereas independent t tests were used for continuous data. Survival curves were constructed using the Kaplan-Meier method and evaluated using the log-rank test. In addition, the Cox proportional hazards regression model was established to identify factors that were independently associated with overall survival. Tests were considered significant at P < 0.05.

**Results**

**Egfl7 Was Overexpressed in Human HCC Tissues.** To examine Egfl7 expression in HCC, 31 cases of HCC tissues and the corresponding ANLTs was measured by qRT-PCR and the results showed that the average expression level of Egfl7 mRNA in HCC tissues was 3.68 times that in ANLTs (Fig. 1A). Consistent with the messenger RNA (mRNA) levels, the Egfl7 protein levels in HCC tissues were significantly higher than those in ANLTs (0.85 ± 0.22 versus 0.27 ± 0.07, P = 0.002; Fig. 1B). Although the Egfl7 expression in ANLTs was slightly higher than in normal liver tissue, this difference was not statistically significant (P > 0.05; Fig. 1A,B).

**HCC Cells Express Egfl7.** To validate the data gen-
erated from qRT-PCR and western blot, we performed immunofluorescence analysis of the Egfl7 expression. As presented in Fig. 1B, HCC cells in tumor tissues, which were identified by anti-AFP antibody labeling, were positively stained by a special anti-Egfl7 antibody. The merged images show that Egfl7 and AFP were colocalized, indicating that the Egfl7 protein in HCC tissues was mainly expressed in HCC. We further confirmed the Egfl7 expression in three HCC cell lines with different metastatic potential\(^3\): HepG2, MHCC97-L, and HCCLM3, with a liver cell line CCL13 as a control. Among the three cell lines analyzed, HCCLM3 cells have the highest Egfl7 expression \((P < 0.05)\), followed by MHCC97-L and HepG2 \((P < 0.05)\) (Fig. 1C), implicating a potential role for Egfl7 in HCC metastasis.

**Correlations of Egfl7 Expression with Clinicopathologic Characteristics and Prognosis of HCC.** Positive Egfl7 expression (Fig. 2A) was detected in 90.2\% of HCC tissues \((101/112)\). The Egfl7 expression levels were found to be significantly higher in HCCs with multiple nodules...
P/H11005 0.020), without capsules (P/H11005 0.037), and with vein invasion (P/H11005 0.031) (Table 1). According to the immunohistochemistry results, all 112 HCC patients were divided into two groups: the high expression group (n = 65) and low expression group (n = 47). HCC patients within the high expression group had either worse disease-free survival (median disease-free survival time, 303 days versus 544 days, P/H11005 0.037; Fig. 2B) or worse overall survival (median survival time, 395 days versus 616 days, P/H11005 0.008; Fig. 2C) than those within the low expression group. By multivariate Cox regression analysis, high Egfl7 expression (relative risk [RR], 1.56; P/H11005 0.027) was found to be an independent prognostic factor for overall survival (Table 2).

**Suppression of Egfl7 Expression by siRNA.** To understand how Egfl7 contribute to the progression of HCC, we employed siRNA to inhibit the expression of Egfl7 in HCC cells and characterized the cells for their tumorigenicity. Western blot was performed to assess the ability of three candidate sequences and a scramble sequence (the control sequence, sequence C) to down-regulate Egfl7 in HCCLM3 cells. As shown in Fig. 3A, sequence 1 inhibited the Egfl7 protein more than 80%. The other two sequences, candidates 2 and 3, only resulted in 50%-70% inhibition efficiency, whereas the scramble sequence did not cause detectable changes of Egfl7 expression. Then the HCCLM3 cells transfected with the pLKO.1puro plasmid containing sequence 1 and the scramble sequence were termed, for convenience, HCCLM3Egfl7RNAi+ and HCCLM3Egfl7RNAi−, respectively. The expression of Egfl7 protein was markedly decreased in HCCLM3Egfl7RNAi+ cells compared with HCCLM3Egfl7RNAi− cells, which showed a more than 80% inhibitory efficiency (Fig. 3B).

**Egfl7 Affects Invasion and Migration but Not Proliferation of HCCLM3 Cell.** Because the levels of Egfl7 expression correlated with the metastatic potentials (Fig. 1C), we asked whether Egfl7 depletion could impact the ability of cell migration. As shown in Fig. 3C, the wound healing assay showed that the closure of HCCLM3Egfl7RNAi+ was significantly slower than that of HCCLM3Egfl7RNAi− (29% versus 71%, P < 0.05; Fig. 3C), suggesting a role for Egfl7 in regulation of HCCLM3 cell migration. To confirm the results, we performed a Transwell assay. The data indi-

### Table 1. Correlation Between Egfl7 Expression and Clinicopathologic Variables of HCC

| Clinicopathologic Variables | n | 0   | 1+ | 2+ | 3+ | P Value |
|-----------------------------|---|-----|----|----|----|---------|
| Gender                      |   |     |    |    |    |         |
| Male                        | 98| 9   | 31 | 42 | 14 | 0.711   |
| Female                      | 14| 2   | 5  | 6  | 3  |         |
| Age (years)                 |   |     |    |    |    |         |
| ≤65                         | 92| 6   | 30 | 41 | 15 |         |
| >65                         | 20| 5   | 6  | 7  | 2  | 0.632   |
| Liver cirrhosis             |   |     |    |    |    |         |
| Presence                    | 85| 8   | 26 | 37 | 14 |         |
| Absence                     | 27| 3   | 10 | 11 | 3  | 0.422   |
| Tumor size (cm)             |   |     |    |    |    |         |
| ≤5                          | 34| 4   | 11 | 13 | 6  | 0.351   |
| >5                          | 78| 7   | 25 | 35 | 11 |         |
| Capsular formation          |   |     |    |    |    |         |
| Presence                    | 25| 6   | 9  | 8  | 2  |         |
| Absence                     | 87| 5   | 27 | 40 | 15 | 0.037   |
| Tumor nodule number         |   |     |    |    |    |         |
| Solitary                    | 33| 5   | 15 | 11 | 2  | 0.200   |
| Multiple                    | 79| 6   | 21 | 37 | 15 |         |
| Edmondson-Steiner grade     |   |     |    |    |    |         |
| I-II                        | 27| 3   | 9  | 12 | 3  |         |
| III-IV                      | 85| 8   | 27 | 34 | 14 | 0.416   |
| Venous invasion             |   |     |    |    |    |         |
| Presence                    | 64| 2   | 19 | 29 | 14 |         |
| Absence                     | 48| 9   | 17 | 16 | 3  | 0.031   |

Fig. 2. Immunohistochemistry of Egfl7 expression in HCC tissues and its prognostic implication. (A-D) In these representative images, Egfl7 expression is seen in more than 51% of cancer cells (scored as 3+, A), 26%-50% of cancer cells (scored as 2+, B), 11%-25% of cancer cells (scored as 1+, C), and the negative control (D) is also included to show the specificity of the antibody. Original magnification ×400. (E) Estimated disease-free survival according to the expression of Egfl7 in 112 cases of HCCs (the Kaplan-Meier method). Log-rank test shows that HCC patients in the high Egfl7 expression group have poorer disease free survival than those in the low Egfl7 expression group (P = 0.037). (F) Overall survival was analyzed in the same cohort of HCC patients and the results showed that HCC patients in the high Egfl7 expression group also have poorer overall survival than those in the low Egfl7 expression group (P = 0.008).
cate that the numbers of HCCLM3Egfl7RNAi−/H11001 cells that passed through the Matrigel was 47% of that of HCCLM3Egfl7RNAi−/H11002 cells (53 ± 7 versus 113 ± 25, P = 0.05; Fig. 3D). Considering the possibility that the rate of cell proliferation can impact the rate of cell migration, we performed an MTT assay to compare the proliferation rates of HCCLM3Egfl7RNAi−/H11001 and HCCLM3Egfl7RNAi−/H11002 cells. Interestingly, no significant difference in proliferation between these cells was observed (Fig. 3E). Together, our results suggest that while having little effect on proliferation, Egfl7 seems to play a role in HCC cell migration.

Egfl7 Facilitates FAK Phosphorylation and Promotes Motility of HCCLM3Egfl7RNAi− Cells. A recent study indicated that FAK phosphorylation was significantly reduced in ECs of Egfl7-deficient mouse, suggesting that Egfl7 may promote cell motility by facilitating FAK phosphorylation. We thus compared the phosphorylation status of FAK between HCCLM3Egfl7RNAi−/H11001 and HCCLM3Egfl7RNAi−/H11002 cells. Our results show that the phosphorylated FAK decreased significantly in HCCLM3Egfl7RNAi−/H11001 cells compared to HCCLM3Egfl7RNAi−/H11002 cells, whereas FAK levels were close in these cells (Fig. 4A). To determine if the observed difference in FAK phosphorylation was because of Egfl7, we treated HCCLM3Egfl7RNAi−/H11001 cells with 50 ng/mL recombinant Egfl7 protein or phosphate-buffered saline (PBS) (as control). We found the FAK phosphorylation level in HCCLM3Egfl7RNAi−/H11001 cells was significantly induced by recombinant Egfl7 protein (Fig. 4B). To examine the biological consequence to FAK phosphorylation, we examined the pattern and morphology of F-actin. As shown in Fig. 4C, recombinant Egfl7 protein stimulated the reorganization of actin leading to the formation of stress-fiber-like structures transversing in HCCLM3Egfl7RNAi−/H11001 cells. Additionally, the wound healing assay (74% versus 49%, P = 0.05) as well as the Transwell assay (108 ± 27 versus 48 ± 9, P = 0.05) showed that the recombinant Egfl7 protein enhanced the migration of HCCLM3Egfl7RNAi−/H11001 cells (Fig. 4D,E), consistent with the role of Egfl7 in controlling cell motility.

EGFR Inhibitor Blocks the Effect of Egfl7 on FAK Phosphorylation and Cell Motility. Egfl7 has two EGF-like domains in its protein structure and is a secreted protein just like EGF. We therefore asked whether Egfl7 might activate FAK through EGF receptor (EGFR). To test this hypothesis, we treated HCCLM3Egfl7RNAi−/H11001 cells with 15 μM EGFR inhibitor or PBS (as control) and then

Table 2. Multivariate Analysis by a Cox Proportional Hazards Regression Model

| Variables                      | n    | RR (95% CI)   | P Value | RR (95% CI)   | P Value |
|-------------------------------|------|---------------|---------|---------------|---------|
| Gender                        |      |               |         |               |         |
| Male                          | 98   | 1             |         | 1             |         |
| Female                        | 14   | 1.07 (0.64–1.83) | 0.399  | 1.13 (0.71–1.96) | 0.422  |
| Age (years)                   |      |               |         |               |         |
| ≤65                           | 92   | 1             |         | 1             |         |
| >65                           | 20   | 1.03 (0.55–1.79) | 0.560  | 1.09 (0.65–1.87) | 0.617  |
| Liver cirrhosis               |      |               |         |               |         |
| Absence                       | 27   | 1             |         | 1             |         |
| Presence                      | 85   | 0.93 (0.48–1.56) | 0.631  | 0.98 (0.53–1.62) | 0.691  |
| Serum AFP level (ng/mL)       |      |               |         |               |         |
| ≤20                           | 41   | 1             |         | 1             |         |
| >20                           | 71   | 1.56 (1.01–2.51) | 0.032  | 1.61 (1.03–2.59) | 0.043  |
| Tumor size (cm)               |      |               |         |               |         |
| ≤5                            | 34   | 1             |         | 1             |         |
| >5                            | 78   | 1.17 (0.71–1.88) | 0.315  | 1.73 (1.12–2.67) | 0.056  |
| Capsular formation            |      |               |         |               |         |
| Presence                      | 25   | 1             |         | 1             |         |
| Absence                       | 87   | 1.75 (1.15–2.83) | 0.039  | 1.81 (1.17–2.88) | 0.067  |
| Edmondson-Steiner grade       |      |               |         |               |         |
| III–IV                        | 85   | 1             |         | 1             |         |
| I–II                          | 27   | 1.21 (0.88–1.95) | 0.227  | 1.27 (0.91–2.12) | 0.336  |
| Tumor nodule number           |      |               |         |               |         |
| Solitary                      | 33   | 1             |         | 1             |         |
| Multiple (≥2)                 | 79   | 1.67 (1.07–2.53) | 0.027  | 1.63 (1.05–2.50) | 0.041  |
| Venous invasion               |      |               |         |               |         |
| Absence                       | 48   | 1             |         | 1             |         |
| Presence                      | 64   | 1.72 (1.13–2.63) | 0.011  | 1.55 (1.01–2.42) | 0.031  |
| Egfl7 expression              |      |               |         |               |         |
| Low                           | 47   | 1             |         | 1             |         |
| High                          | 65   | 1.75 (1.15–2.79) | 0.009  | 1.56 (1.01–2.44) | 0.027  |
stimulated these cells with 50 ng/mL recombinant Egfl7 protein. We found that the FAK phosphorylation level in HCCLM3 cells treated with 50 ng/mL recombinant Egfl7 protein was not increased in response to Egfl7 protein stimulation, whereas the FAK phosphorylation level was elevated in those cells treated with PBS (Fig. 5A). We also examined the cytoskeletal changes induced by 50 ng/mL recombinant Egfl7 protein. As shown in Fig. 5B, the reorganization of actin stimulated by recombinant Egfl7 protein was inhibited by EGFR inhibitor. Moreover, the wound healing assay (27% versus 73%, \( P < 0.05 \)) and the Transwell assay (49 ± 5 versus 107 ± 17, \( P < 0.05 \)) all showed that EGFR inhibitor blocked the stimulatory effect of Egfl7 on the cell motility of HCCLM3 Egfl7RNAi+ cells (Fig. 5C, D). We also investigated whether Egfl7 could enhance production of transforming growth factor-\( \alpha \) (TGF-\( \alpha \)), which is known to be produced by HCC and also works through EGFR.33 However, neither the suppression of Egfl7 in HCCLM3 cells nor the stimulation of recombinant Egfl7 protein to HCCLM3 Egfl7RNAi+ cells could change the expression of TGF-\( \alpha \) protein (Supporting Fig. 1).

In Vivo Inhibition of HCC Metastasis in the HCCLM3 Egfl7RNAi+ Group. To validate the observations
obtained from in vitro studies, we examined the in vivo relevance of the potential role for Egfl7 in HCC tumorigenesis by using a mouse metastasis model. We found that the average size of liver local tumors in the HCCLM3Egfl7RNAi/H11001 group was dramatically smaller than those in the HCCLM3Egfl7RNAi/H11002 group (Fig. 6A). And the expression of Egfl7 and phosphorylated FAK were all significantly decreased in the HCCLM3Egfl7RNAi/H11001 group than those in the HCCLM3Egfl7RNAi/H11002 group (Fig. 6B). However, expression of TGF-α was not significantly different in these two groups (Supporting Fig. 2). In light of the in vitro results implicating a role for Egfl7 in HCC cell migration, we examined the mice for liver and lung metastasis of the carcinoma cells. Two of eight mice in the HCCLM3Egfl7RNAi+ group showed intrahepatic metastasis (25%), which is significantly lower than that in the HCCLM3Egfl7RNAi− group (seven of eight, 88%; P < 0.01), as shown in Fig. 6C. Pulmonary metastasis was observed in the lung tissue sections of only one mouse in the HCCLM3Egfl7RNAi+ group (one of eight, 13%), much less than the ratio of pulmonary metastasis in the HCCLM3Egfl7RNAi− group (four of eight, 50%; P <
also as shown in Fig. 6C. In addition, we also found that the average MVD in tumors of HCCLM3Egfl7RNAi/H11001 group was significantly decreased compared to the HCCLM3Egfl7RNAi/H11002 group (Fig. 6D). Together, these data support an important role for Egfl7 in HCC metastasis.

Discussion

Although Parker et al. previously reported that Egfl7 expression increases during tumorigenesis, it is unclear whether and how Egfl7 contributes to the development of human cancer. We show that both Egfl7 mRNA and protein increased significantly in HCC tissues when compared with the corresponding ANLTs. Recently, Díaz et al. also reported that Egfl7 mRNA increased in human colon cancer tissues. These data indicate that Egfl7 may be involved in the development of human malignancies such as HCC and colon cancer. Although our results showed that Egfl7 mRNA and protein levels are slightly higher in ANLTs than those in normal liver tissues ($P > 0.05$), the ANLTs from our HCC patients are usually associated with liver cirrhosis, which is considered a precancerous lesion of HCC. That may cause a slight increase of Egfl7 expression in ANLT over normal liver tissue.

Although Egfl7 was initially believed to be specifically expressed by ECs, a nonendothelial expression of Egfl7 in primordial germ cells was documented recently. The expression of Egfl7 in other cell types has not been reported. Our immunofluorescence stains show a distribution of the Egfl7 protein in HCC cells as evidenced by its colocalization with AFP, a hallmark for HCC cells. Analysis of Egfl7 expression in HCC cell lines confirmed its expression in cancer cells. Our results provide the first evidence for Egfl7 expression in HCC cells.

Analysis of the association of Egfl7 expression and the clinicopathologic characteristics in 112 HCC patients re-
reveals that Egfl7 expression is significantly correlated with multiple tumor nodes, capsular formation, and vein invasion of HCC, which are widely accepted markers for metastasis and poor prognosis of HCC.36,37 The Kaplan-Meier analysis shows that the HCC patients with high Egfl7 expression in general had worse prognosis than those with low expression. A multivariate Cox regression analysis indicates that high Egfl7 expression is an independent risk factor for the prognosis of HCC patients, suggesting that Egfl7 may be a useful prognostic biomarker of HCC.

Of particular interest is the correlation between Egfl7 expression and the ability of HCC cells to metastasize in three HCC cell lines, HepG2, MHCC97-L, and HCCLM3.32 HepG2 cells exhibited a moderate metastatic potential, whereas HCCLM3 cells are highly invasive as demonstrated by extensive metastases by way of both subcutaneous and orthotopic inoculation.38 Egfl7 expression was markedly higher in HCCLM3 cells when compared with HepG2 cell lines and MHCC97-L, suggesting an association of Egfl7 overexpression with the metastasis potential of HCC.

To gain insight into a role for Egfl7 in HCC tumorigenicity, we employed siRNA to knockdown the Egfl7 expression in HCCLM3 cells. Our results show that depletion of Egfl7 expression resulted in marked inhibition of HCCLM3 cell migration with no significant effect on proliferation. Our data extend the critical role of Egfl7 in regulating cell migration of ECs and fibroblasts20,21 to human HCC carcinoma cells. A recent study by Schmidt et al.20 indicated that FAK phosphorylation, a critical event in processes of cell migration, adhesion, and growth39 of ECs was significantly reduced in Egfl7-deficient mice, indicating that Egfl7 may promote cell motil-

![Fig. 6. Knockdown of Egfl7 inhibits the growth and metastasis of HCCLM3 cells in vivo. (A) The size of liver tumors in the HCCLM3Egfl7RNAi− group was dramatically smaller than that of the HCCLM3Egfl7RNAi+ group. Black arrows: local tumors at the sites of implantation. (B) H&E staining was used in the HCCLM3Egfl7RNAi+ group (a) and the HCCLM3Egfl7RNAi− group (b) and immunohistochemistry examination for Egfl7 and phosphorylated FAK expression in tumor was also performed in the HCCLM3Egfl7RNAi− group (c,e) and the HCCLM3Egfl7RNAi− group (d,f). Original magnification ×400. (C) Metastasis of HCCLM3; (a) intrahepatic metastasis of HCCLM3Egfl7RNAi− group (yellow arrow, local tumor; green arrows, metastatic nodules); (b) pulmonary metastasis of HCCLM3Egfl7RNAi− group (blue arrow, metastatic HCCLM3 cells in the lung tissues of the mice, H&E stain, original magnification ×400). (D) MVD was calculated in tumors of the HCCLM3Egfl7RNAi− group (a) and HCCLM3Egfl7RNAi− group (b) and the results showed that the average MVD in tumors of the HCCLM3Egfl7RNAi− group was significantly lower than that in the HCCLM3Egfl7RNAi− group (24 ± 11 versus 49 ± 23, P < 0.05). *P < 0.05.](image)
ity through facilitating FAK phosphorylation. Our results also showed that the phosphorylated FAK level in HCCLM3Egfl7RNAi cells was down-regulated significantly but the total FAK level did not change. The reduced FAK phosphorylation in HCCLM3Egfl7RNAi cells was due to Egfl7 depletion because addition of recombinant Egfl7 protein restored the FAK phosphorylation. Associated with FAK phosphorylation are the corresponding changes in F-actin organization and cell migration induced by recombinant Egfl7 protein in HCCLM3Egfl7RNAi cells, consistent with the idea that Egfl7 regulates cell motility through FAK phosphorylation.

As a secreted protein, Egfl7 would likely induce the phosphorylation of FAK, which is located in the cytoplasm as a nonreceptor tyrosine kinase, by interacting with a cell surface receptor. Many growth factors such as EGF and PDGF can induce FAK phosphorylation through their membrane receptors (e.g., EGFR and PDGFR). Based on two EGF-like domains included in the structure of Egfl7, it was predicted that Egfl7 may induce FAK phosphorylation through EGF just like EGF. Indeed, treatment of HCCLM3Egfl7RNAi cells with EGFR inhibitor blocked the effects of recombinant Egfl7 protein on FAK phosphorylation, F-actin reorganization, and cell motility, supporting the hypothesis that FAK activation by Egfl7 was mediated by EGFR and the Egfl7/EGFR/FAK pathway may be critical in controlling HCC cell motility. To explore the other potential mechanisms involved in the function of Egfl7, we also determined whether Egfl7 could play a role through TGF-α, which is known to be produced by HCC and also works through their membrane receptors (e.g., EGFR and PDGFR). Based on two EGF-like domains included in the structure of Egfl7, it was predicted that Egfl7 may induce FAK phosphorylation through EGF just like EGF. Indeed, treatment of HCCLM3Egfl7RNAi cells with EGFR inhibitor blocked the effects of recombinant Egfl7 protein on FAK phosphorylation, F-actin reorganization, and cell motility, supporting the hypothesis that FAK activation by Egfl7 was mediated by EGFR and the Egfl7/EGFR/FAK pathway may be critical in controlling HCC cell motility. To explore the other potential mechanisms involved in the function of Egfl7, we also determined whether Egfl7 could play a role through TGF-α, which is known to be produced by HCC and also works through their membrane receptors (e.g., EGFR and PDGFR). Indeed, treatment of HCCLM3Egfl7RNAi cells with EGFR inhibitor blocked the effects of recombinant Egfl7 protein on FAK phosphorylation, F-actin reorganization, and cell motility, supporting the hypothesis that FAK activation by Egfl7 was mediated by EGFR and the Egfl7/EGFR/FAK pathway may be critical in controlling HCC cell motility. To explore the other potential mechanisms involved in the function of Egfl7, we also determined whether Egfl7 could play a role through TGF-α, which is known to be produced by HCC and also works through their membrane receptors (e.g., EGFR and PDGFR). Indeed, treatment of HCCLM3Egfl7RNAi cells with EGFR inhibitor blocked the effects of recombinant Egfl7 protein on FAK phosphorylation, F-actin reorganization, and cell motility, supporting the hypothesis that FAK activation by Egfl7 was mediated by EGFR and the Egfl7/EGFR/FAK pathway may be critical in controlling HCC cell motility. To explore the other potential mechanisms involved in the function of Egfl7, we also determined whether Egfl7 could play a role through TGF-α, which is known to be produced by HCC and also works through their membrane receptors (e.g., EGFR and PDGFR). Indeed, treatment of HCCLM3Egfl7RNAi cells with EGFR inhibitor blocked the effects of recombinant Egfl7 protein on FAK phosphorylation, F-actin reorganization, and cell motility, supporting the hypothesis that FAK activation by Egfl7 was mediated by EGFR and the Egfl7/EGFR/FAK pathway may be critical in controlling HCC cell motility. To explore the other potential mechanisms involved in the function of Egfl7, we also determined whether Egfl7 could play a role through TGF-α, which is known to be produced by HCC and also works through their membrane receptors (e.g., EGFR and PDGFR). Indeed, treatment of HCCLM3Egfl7RNAi cells with EGFR inhibitor blocked the effects of recombinant Egfl7 protein on FAK phosphorylation, F-actin reorganization, and cell motility, supporting the hypothesis that FAK activation by Egfl7 was mediated by EGFR and the Egfl7/EGFR/FAK pathway may be critical in controlling HCC cell motility.

The role for Egfl7 in regulation of cell migration was further validated by our in vivo study, in which we show that depletion of Egfl7 expression was associated with reduced FAK phosphorylation in local tumors. More important, the metastatic ratio of metastatic sites in the Egfl7-deficient group were all significantly decreased compared with the control group, supporting an important role for Egfl7 in regulation of metastasis of HCC. Our in vitro data have shown that Egfl7 influences the migration but not the growth of HCCLM3 cells. However, we found that the suppression of Egfl7 inhibited the growth of the xenotransplants of these cells in mouse models. In light of the important role of Egfl7 in angiogenesis, we propose that the suppression of Egfl7 might inhibit the angiogenesis of the tumors leading to the slower growth. Therefore, MVD in tumors of HCCLM3Egfl7RNAi and HCCLM3Egfl7RNAi was analyzed and the data exhibited a significantly lower average MVD in HCCLM3Egfl7RNAi tumors than that in HCCLM3Egfl7RNAi tumors, indicating the inhibition of angiogenesis induced by Egfl7 suppression, which supported our presumption.

In conclusion, our study has shown for the first time that Egfl7 is expressed in HCC cells and its overexpression significantly correlates with a poor prognosis of HCC. Furthermore, we have demonstrated the critical role of Egfl7 in metastasis of HCC by enhancing cell motility through EGFR-mediated FAK phosphorylation. Collectively, our data suggest Egfl7 as a novel prognostic marker and a potential therapeutic target for metastasis of HCC.

References
1. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin 2005;55:74-108.
2. Roberts LR. Sorafenib in liver cancer—just the beginning. N Engl J Med 2008;359:620-622.
3. Song TJ, Ip EW, Fong Y. Hepatocellular carcinoma: current surgical management. Gastroenterology 2004;127:5248-5260.
4. Marrero JA, Fontana RJ, Barrat A, Akiari F, Conjeevaram HS, Su GL, et al. Prognosis of hepatocellular carcinoma: comparison of 7 staging systems in an American cohort. Hepatology 2005;41:707-716.
5. Bruix J, Sherman M. Management of hepatocellular carcinoma. Hepatology 2005;42:1208-1236.
6. Farazi PA, DePinho RA. Hepatocellular carcinoma pathogenesis: from genes to environment. Nat Rev Cancer 2006;6:674-687.
7. Yamazaki H, Wyckoff J, Coneidels J. Cell migration in tumors. Curr Opin Cell Biol 2005;17:559-564.
8. Yamasaki D, Kurisu S, Takenawa T. Regulation of cancer cell motility through actin reorganization. Cancer Sci 2005;96:379-386.
9. Chambers AF, Groom AC, MacDonald IC. Dissemination and growth of cancer cells in metastatic sites. Nat Rev Cancer 2002;2:563-572.
10. Ye QH, Qin LX, Forgues M, He P, Kim JW, Peng AC, et al. Predicting hepatitis B virus-positive metastatic hepatocellular carcinomas using gene expression profiling and supervised machine learning. Nat Med 2003;9:416-423.
11. Yang JM, Peng ZH, Si SH, Liu WW, Luo YH, Ye ZY. KAI1 gene supresses invasion and metastasis of hepatocellular carcinoma MHCC97-H cells in vitro and in animal models. Liver Int 2008;28:132-139.
12. Ching YP, Leong YY, Lee MF, Xu HT, Jin DY, Ng IO. P21-activated protein kinase is overexpressed in hepatocellular carcinoma and enhances cancer metastasis involving c-Jun NH2-terminal kinase activation and paxillin phosphorylation. Cancer Res 2007;67:3601-3608.
13. Wang W, Wu F, Fang F, Tao YM, Yang LY. Inhibition of invasion and metastasis of hepatocellular carcinoma cells via targeting RhoC in vitro and in vivo. Clin Cancer Res 2008;14:6804-6812.
14. Wang W, Yang LY, Huang GW, Lu WQ, Yang ZL, Yang JQ, et al. Genomic analysis reveals RhoC as a potential marker in hepatocellular carcinoma with poor prognosis. Br J Cancer 2004;90:2349-2355.
15. Aravalli RN, Steer CJ, Cressman EN. Molecular mechanisms of hepatocellular carcinoma. Hepatology 2008;48:2047-2063.
16. Llovet JM, Bruix J. Molecular targeted therapies in hepatocellular carcinoma. HEPATOLOGY 2008;48:1312-1327.
17. Soncin F, Mattot V, Lionneton F, Spruyt N, Lepretre F, Begue A, et al. VE-statin, an endothelial repressor of smooth muscle cell migration. EMBO J 2003;22:5700-5011.
18. Fitch MJ, Campagnolo L, Kuhnert F, Stuhlmann H. Egfl7, a novel epidermal growth-factor-domain gene expressed in endothelial cells. Dev Dyn 2004;230:316-324.
19. Parker LH, Schmidt M, Jin SW, Gray AM, Beis D, Pham T, et al. The endothelial-cell-derived secreted factor Egfl7 regulates vascular tube formation. Nature 2004;428:754-758.
20. Schmidt M, Paes K, De Mazière A, Szymczek T, Yang S, Gray A, et al. EGFL7 regulates the collective migration of endothelial cells by restricting their spatial distribution. Development 2007;134:2913-2923.
21. Campagnolo L, Leaby A, Chitnis S, Koschnick S, Fitz MJ, Fallon JT, et al. EGFL7 is a chemottractant for endothelial cells and is up-regulated in angiogenesis and arterial injury. Am J Pathol 2005;167:275-284.
22. Cox BD, Natarajan M, Stettner MR, Gladson CL. New concepts regarding focal adhesion kinase promotion of cell migration and proliferation. J Cell Biochem 2006;99:35-52.
23. Gandemer V, Rio AG, de Tayrac M, Sibut V, Mottier S, Ly Sunnaram B, et al. Five distinct biological processes and 14 differentially expressed genes characterize TEL/AML1-positive leukemia. BMC Genomics 2007;8:385.
24. Diaz R, Silva J, Garcia JM, Lorenzo Y, Garcia V, Peña C, et al. Deregulated expression of miR-106a predicts survival in human colon cancer patients. Genes Chromosomes Cancer 2008;47:794-802.
25. Fang F, Luo LB, Tao YM, Wu F, Yang LY. Decreased expression of inhibitor of growth 4 correlated with poor prognosis of hepatocellular carcinoma. Cancer Epidemiol Biomarkers Prev 2009;18:409-416.
26. Wu F, Liu SY, Tao YM, Ou DP, Fang F, Yang LY. Decreased expression of methyl methansulfonate and UV sensitive gene clone 81 is related to poor prognosis of patients with hepatocellular carcinoma. Cancer 2008;112:2002-2010.
27. Yang LY, Tao YM, Ou DP, Wang W, Chang ZG, Wu F. Increased expression of Wiskott-Aldrich syndrome protein family verprolin-homologous protein 2 correlated with poor prognosis of hepatocellular carcinoma. Clin Cancer Res 2006;12:5673-5679.
28. Ou DP, Tao YM, Tang FQ, Yang LY. The hepatitis B virus X protein promotes hepatocellular carcinoma metastasis by upregulation of matrix metalloproteinases. Int J Cancer 2007;120:1208-1214.
29. Wang W, Wu F, Fang F, Tao YM, Yang LY. Rhoc is essential for angiogenesis induced by hepatocellular carcinoma cells via regulation of endothelial cell organization. Cancer Sci 2008;99:2012-2018.
30. Lu XD, Qin WX, Li JJ, Tan N, Pan DN, Zhang HT, et al. The growth and metastasis of human hepatocellular carcinoma xenografts are inhibited by small interfering RNA targeting to the subunit ATP6L of proton pump. Cancer Res 2005;65:6843-6849.
31. Yang LY, Lu WQ, Huang GW, Wang W. Correlation between CD105 expression and postoperative recurrence and metastasis of hepatocellular carcinoma. BMC Cancer 2006:6:110.
32. Li Y, Tian B, Yang J, Zhao L, Wu X, Ye SL, et al. Stepwise metastatic human hepatocellular carcinoma cell model system with multiple metastatic potentials established through consecutive in vivo selection and studies on metastatic characteristics. J Cancer Res Clin Oncol 2004;130:460-468.
33. Schiffer E, Housset C, Cacheux W, Wendum D, Desbois-Mouthon C, Rey C, et al. Gefitinib, an EGFR inhibitor, prevents hepatocellular carcinoma development in the rat liver with cirrhosis. HEPATOLOGY 2005;41:307-314.
34. Campagnolo L, Moscatelli I, Pellegrini M, Siracusa G, Stuhlmann H. Expression of EGFL7 in primordial germ cells and in adult ovaries and testes. Gene Expr Patterns 2008;8:389-396.
35. Yokoo H, Kondo T, Fujii K, Yamada T, Todo S, Hirohashi S. Proteomic signature corresponding to alpha fetoprotein expression in liver cancer cells. HEPATOLOGY 2004;40:609-617.
36. Velañquez RF, Rodríguez M, Navascués CA, Linares A, Pérez R, Sotorrios NG, et al. Prospective analysis of risk factors for hepatocellular carcinoma in patients with liver cirrhosis. HEPATOLOGY 2003;37:520-527.
37. Poon RT, Fan ST, Lo CM, Liu CL, Wong J. Difference in tumor invasiveness in cirrhotic patients with hepatocellular carcinoma fulfilling the Milan criteria treated by resection and transplantation: impact on long-term survival. Ann Surg 2007;245:51-58.
38. Zhang SZ, Pan FY, Xu JF, Yuan J, Guo SY, Dai G, et al. Knockdown of c-Met by adenovirus-delivered small interfering RNA inhibits hepatocellular carcinoma growth in vitro and in vivo. Mol Cancer Ther 2005;4:1577-1584.
39. Parsons JT. Focal adhesion kinase: the first ten years. J Cell Sci 2003;116:1409-1416.
40. Li S, Hua ZC. FAK expression regulation and therapeutic potential. Adv Cancer Res 2008;101:45-61.
41. Sieg DJ, Hauck CR, Ilac D, Klingbeil CK, Schaefer E, Damsky CH, et al. FAK integrates growth-factor and integrin signals to promote cell migration. Nat Cell Biol 2000;2:249-256.