

EFEMP1 inhibits migration of hepatocellular carcinoma by regulating MMP2 and MMP9 via ERK1/2 activity

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Abstract. The role of epidermal growth factor-containing fibulin-like extracellular matrix protein 1 (EFEMP1) inhibiting migration in hepatocellular carcinoma (HCC) remains unknown. Expression of EFEMP1 in HCC cell lines were quantified by western blotting and real-time PCR. The role of EFEMP1 in HCC cell migration was explored *in vitro* via siRNA and adding purified EFEMP1 protein. The associated molecule expression was detected by western blotting after downregulation of EFEMP1 and also tested by immunohistochemistry. Eight pairs of HCC non-HCC liver samples and 215 HCC samples were subjected to immunohistochemistry. EFEMP1 was highly expressed in 7,721 and HepG2 HCC cell lines while HuH7 HCC cell line expressed the lowest level of EFEMP1 compared with the others. Downregulating EFEMP1 by siRNA markedly increased the migration ability of HCC cells while adding purified EFEMP1 protein inhibited HCC cell migration. Downregulation of EFEMP1 increased the expression of ERK1/2, MMP2 and MMP9. Furthermore, U0126 (a highly selective and potent inhibitor of pERK1/2) could abrogate the migration ability enhanced by siRNA. Accordingly, MMP2 and MMP9 were inversely expressed with EFEMP1 expression by immunohistochemistry. EFEMP1 downregulated in HCC tissues, and lower EFEMP1 expression was significantly associated with HCC patients with ascites (P=0.050), vascular invasion (P=0.044), poorer differentiation (P=0.002) and higher clinical stage (P=0.003).

Introduction

Hepatocellular carcinoma (HCC) is the fifth most prevalent malignancy diagnosed worldwide. However, the etiology and progression events intrinsic to the malignant properties of HCC remain obscure.

Fibulin-3, also called epidermal growth factor-containing fibulin-like extracellular matrix protein 1 (EFEMP1), is a member of the fibulin family of extracellular glycoproteins distributed in various human tissues (1). They modulate cell growth, morphology, adhesion and motility (1). The fibulin-3 encodes a 493-amino acid protein with a molecular mass of 54 kD, and its gene, located at chromosome 2p16, contains 11 exons (1). Whether EFEMP1 promotes or inhibits cancer development remains obscure in some tumors (2-4). EFEMP1 was decreased due to methylation in HCC cases (5), and it was regarded as an oncogene and promotes HCC development (6). However, the underlying mechanism that EFEMP1 promotes HCC development has not yet been explored.

Here we found that down-regulation of EFEMP1 significantly promoted HCC cell migration as determined *in vitro*. Moreover, downregulation of EFEMP1 enhanced the expression of MMP2 and MMP9. Importantly, downregulation of EFEMP1 attenuated the expression of MMP2 and MMP9 at least partially via ERK1/2 activity. In clinical samples, MMP2 and MMP9 expression were greatly higher in low EFEMP1 expression samples. EFEMP1 was downregulated in HCC tissues, and lower EFEMP1 expression was significantly associated with patients with ascites (P=0.050), vascular invasion (P=0.044), poorer differentiation (P=0.002) and higher clinical stage (P=0.003).

Materials and methods

Cell lines. Five cell lines including SMMC-7721, Bel-7402, H2P, Huh7 and HepG2 were obtained from our laboratory cell bank (Guangzhou, China). All cells were grown in Dulbecco's modified Eagle's minimal essential medium (DMEM; Gibco, Carlsbad, CA, USA). All media were supplemented with 8% fetal bovine serum (FBS; Hyclone, Tauranga, New Zealand), 100 µg/µl streptomycin and 100 µg/µl penicillin in a 37°C incubator containing 5% CO₂.

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siRNA. Cells were seeded in 6-well plates at a density of 1×10^5 cells/well. Cationic lipid complex was prepared by incubating 50 nM siRNA with 5 μ l of Lipofectamine[®] RNAiMAX Transfection reagent (Invitrogen, Carlsbad, CA, USA) in 500 μ l of Opti-MEM[®] I Reduced Serum Medium (Invitrogen) for at least 20 min and added to the cell medium. After 6-h incubation, the medium was replaced with fresh medium. The cells were harvested at 24–72 h after transfection for analysis. The EFEMP1 siRNA-1 (#1), 5'-GCAAUGCACUGACGGAU-AUdTdT-3' and 3'-dTdTCGUUACGUGACUGCCUAUA-5' and the EFEMP1 siRNA-2 (#2), 5'-GAGUUCUACCUACGACAAAdTdT-3' and 3'-dTdTCUCAAGAUGGAUGCUGUUU-5' were synthesized by Ribobio (Guangzhou, China).

HCC cell line RNA extraction and qRT-PCR. Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instruction. One microgram of RNA from each sample was used for cDNA synthesis and quantitative real-time PCR analysis (both from Roche, Basel, Switzerland).

Western blotting. Standard western blotting was conducted for the protein expression analyses. The protein contents of cleared lysates were determined using a BCA Protein Quantitative Analysis kit (CoWin Biotech Co., Ltd., China). The membranes were incubated with primary antibodies overnight at 4°C and then with the appropriate secondary antibody. The following primary antibodies were used: EFEMP1 antibody (AP9095a), MMP-2 antibody (AM1844a), MMP-9 (AP6214a) (all from Abgent, San Diego, CA, USA), phospho-NF- κ B p65 antibody (3033; Cell Signaling Technology), anti-p44/42 MAPK (ERK1/2, 1:1,000), anti-phospho-p44/42 MAPK (ERK1/2, 1:1,000), and anti-GAPDH (1:2,000; Cell Signaling Technology, Danvers, MA, USA).

Migration assays. Migration was measured using Transwell cell culture chambers according to the manufacturer's manual. We used 24-well BioCoat cell culture inserts (BD) with a polyethylene terephthalate membrane (8- μ m porosity). Cells (0.5×10^5) were placed into the top chamber of each insert and incubated at 37°C for 24–48 h, according to the migration ability of different cell lines.

Clinical samples and characteristics. Paraffin-embedded HCC specimens (n=215) were analyzed. All cases had been clinically and histopathologically diagnosed at The First Affiliated Hospital of Sun Yat-Sen University (Guangzhou, China) from January 2014 to January 2015. The histology of the disease was determined according to the criteria of the World Health Organization. Tumor stage was defined according to the tumor-node-metastasis (TNM) classification of the American Joint Committee on International Union against Cancer. For research purposes, prior patient (or guardian) consent and approval of the Institutional Research Ethics Committee were obtained. These 215 cases had full detailed clinical data, including age, gender, ascites, cirrhosis, macroscopy, vascular invasion, differentiation, tumor size, AFP, CA125 and clinical stage. This study was approved by the Research Ethics Committee of The First Affiliated Hospital at Sun Yat-sen University.

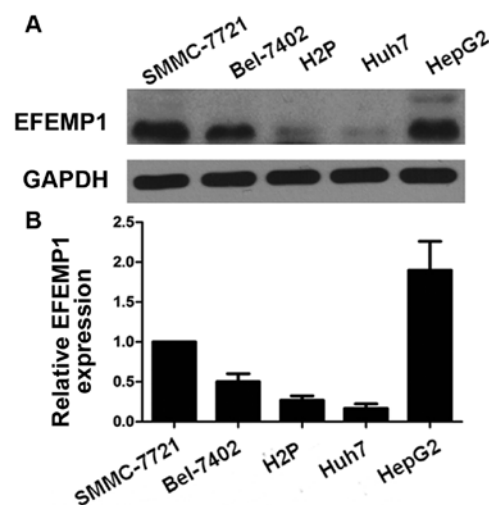


Figure 1. Expression of EFEMP1 in HCC cell lines. (A) Protein levels of EFEMP1 in HCC cell lines were detected by western blot analysis. (B) The mRNA levels of EFEMP1 in HCC cell lines were determined using qRT-PCR.

Immunohistochemistry. Four-micrometer-thick sections were deparaffinized, rehydrated in serially graded ethanol, heated in citric buffer (pH 6.0) once for 20 min in a microwave oven for antigen retrieval, and blocked with 3% hydrogen peroxide for 15 min. The samples were then labeled with EFEMP1 antibody (AP9095a, 1:100), MMP-2 antibody (AM1844a, 1:50), MMP-9 (AP6214a, 1:50) (all from Abgent) at 4°C overnight. The next day, after washing with phosphate-buffered saline (PBS), the sections were incubated with EnVision-HRP secondary antibody (Dako, Carpinteria, CA, USA) for 30 min at 37°C in a water bath, washed with PBS, stained with 0.5% diaminobenzidine and counterstained with Mayer's hematoxylin, then air dried, and mounted with resin.

Evaluation of immunohistochemistry. The immunohistochemical staining in HCC and non HCC liver samples were subjected to microscopy and image analysis (Nanozoomer, Hamamatsu, Japan). Briefly, after IHC staining, if a cell or tissue was stained from light yellow to brown, it would be recorded as positive immunostaining. The areas from cancer were selected for analysis. The intensity of the staining signal was measured and documented using the Image-Pro Plus 6.0 image analysis software (Media Cybernetics, Inc., Silver Spring, MD USA). The mean densitometry of the digital image (x400) is designated as representative IHC staining intensity. The signal density of tissue areas from three randomly selected visions were counted blindly and subjected for statistical analysis.

Statistical analysis. Data between two groups were evaluated using a two-tailed Student's t-test. Data among four groups were evaluated using One-way Anova. For all comparisons, a p-value <0.05 was considered statistically significant unless otherwise indicated.

Results

EFEMP1 knock-down increases HCC cell migration. We first determined the expression level of EFEMP1 in HCC cell lines using qRT-PCR and western blotting. SMMC-7721, HepG2

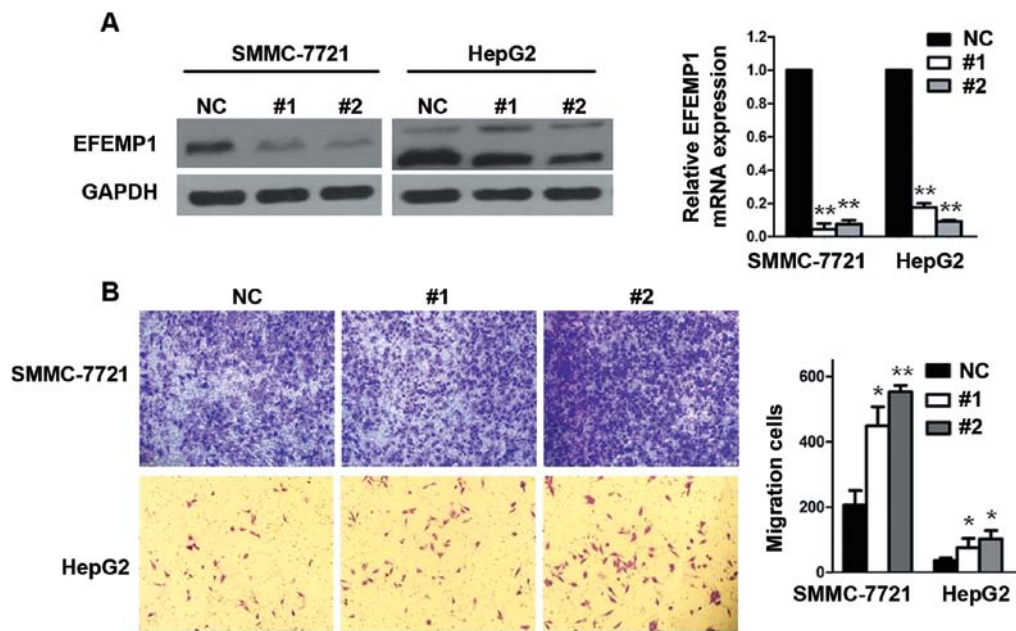


Figure 2. EFEMP1 siRNA promoted migration ability in SMMC-7721 and HepG2 cells. (A) EFEMP1 siRNA was noticeably downregulated in siRNA1 (#1) and siRNA2 (#2) compared with scramble siRNA (NC) and were detected by western blot analysis (left panel) and RT-PCR (right panel). (B) EFEMP1 siRNA significantly increased cell migration ability in HCC cells. * $P < 0.05$; ** $P < 0.01$.

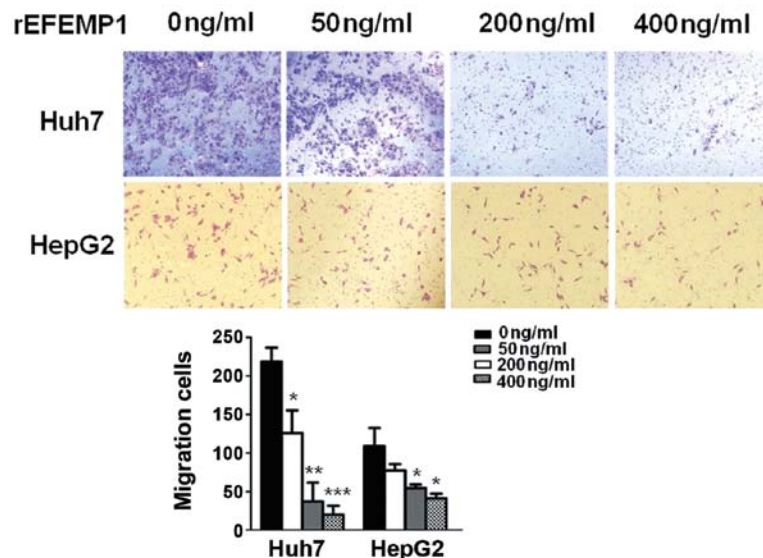


Figure 3. Purified EFEMP1 protein attenuated migration ability in Huh7 and HepG2 cells. In Huh7 cells, noticeable changes were found at all levels of purified EFEMP1 protein concentration compared with 0 ng/ml group. In HepG2 cells, significant decrease was found in 200 and 400 ng/ml compared with 0 ng/ml group. * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$.

and Bel-7402 expressed noticeable EFEMP1 level while Huh7 expressed the lowest EFEMP1 level (Fig. 1).

To confirm the effect of EFEMP1 on HCC cell migration, siRNA techniques were used to inhibit the endogenous expression of EFEMP1 (Fig. 2A). We chose relatively higher EFEMP1 expression HCC cell lines (SMMC-7721 and HepG2). Results showed that EFEMP1 inhibition significantly increased serum-induced transwell migration in these cells (Fig. 2B).

Exogenous EFEMP1 attenuated HCC cells migration. EFEMP1 is an extracellular matrix protein. HCC cells were

treated with purified EFEMP1 protein to induce exogenous overexpression of EFEMP1 in tumor microenvironment. The migratory ability of HCC cells after treatment with EFEMP1 protein (50, 200 and 400 ng/ml) were strongly reduced compared to that of the negative controls in Huh7 cells (Fig. 3). However, in HepG2 cells, significant changes were found only in very high concentration (200 and 400 ng/ml) of EFEMP1 protein (Fig. 3).

Downregulation of EFEMP1 increased the expression of pERK1/2, MMP2 and MMP9. To investigate the molecular mechanism of EFEMP1-mediated HCC cell migration, we

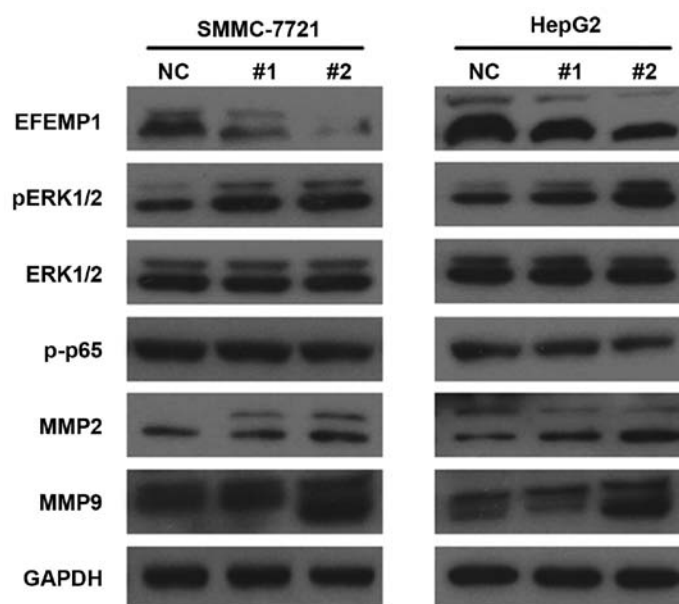


Figure 4. Downregulation of EFEMP1 promotes ERK1/2 phosphorylation, MMP2 and MMP9 expression in SMMC-7721 and HepG2 cells and MMP2 and MMP9 mRNA level in PBMC. EFEMP1 siRNA significantly increased pERK1/2 without changing total ERK1/2; and it also increased MMP2 and MMP9 expression. P-P65 (belongs to NF- κ B signaling pathway) expression was not affected.

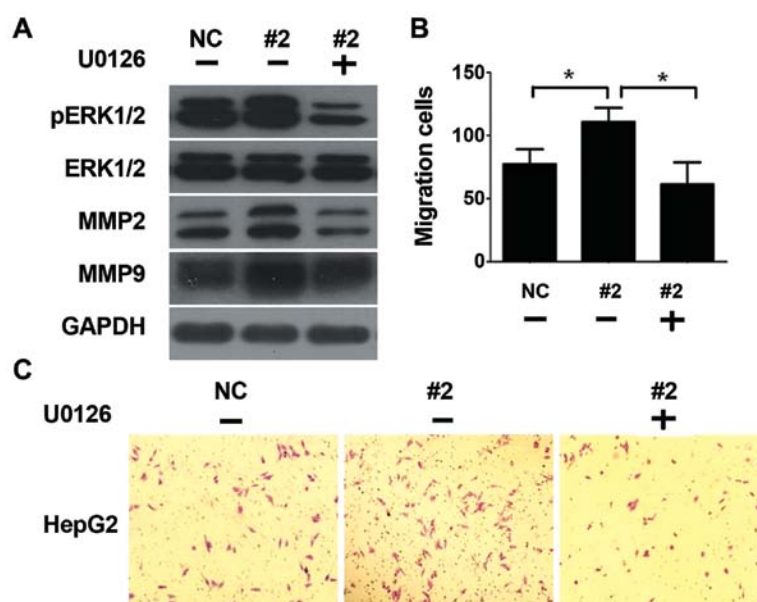


Figure 5. Potent inhibitor of pERK1/2 (U0126) abrogates the migration ability that EFEMP1 promoted by siRNA. (A) U0126 abrogated the expression of pERK1/2, MMP2 and MMP9 that #2 had increased. (B and C) U0126 abrogated the migration ability that #2 had increased. * $P < 0.05$.

found that MMP2 and MMP9 levels increased after down-regulating EFEMP1 (Fig. 4). In addition, pERK1/2 increased without changing ERK1/2 expression (Fig. 4).

We further used U0126, a highly selective and potent inhibitor of pERK1/2, to investigate the modulating role of EFEMP1. As was shown in Fig. 5A, the expression levels of pERK1/2 were significantly reduced after using U0126, accompanied by the decreasing of MMP2 and MMP9 levels. U0126 abrogate the migration ability enhanced by siRNA (Fig. 5B and C). These results suggest that down-regulation of EFEMP1 enhanced the expression of MMP2 and MMP9 at least partially via ERK1/2 activity.

EFEMP1 expression in HCC tissues is inversely associated with MMP2 and MMP9 levels. We next detected EFEMP1 expression in clinical samples by immunohistochemistry and found that EFEMP1 mainly located in the cytoplasm with minor nuclei distribution. In eight pairs of HCC tumor tissues and non-tumor liver samples (data not shown), EFEMP1 expression was much lower in HCC tissues than in non-tumor liver tissues.

We then detect EFEMP1, MMP2 and MMP9 in both high EFEMP1 expression and low EFEMP1 expression HCC samples. We found that low EFEMP1 expression samples expressed high MMP2 and MMP9 levels (Fig. 6A and B),

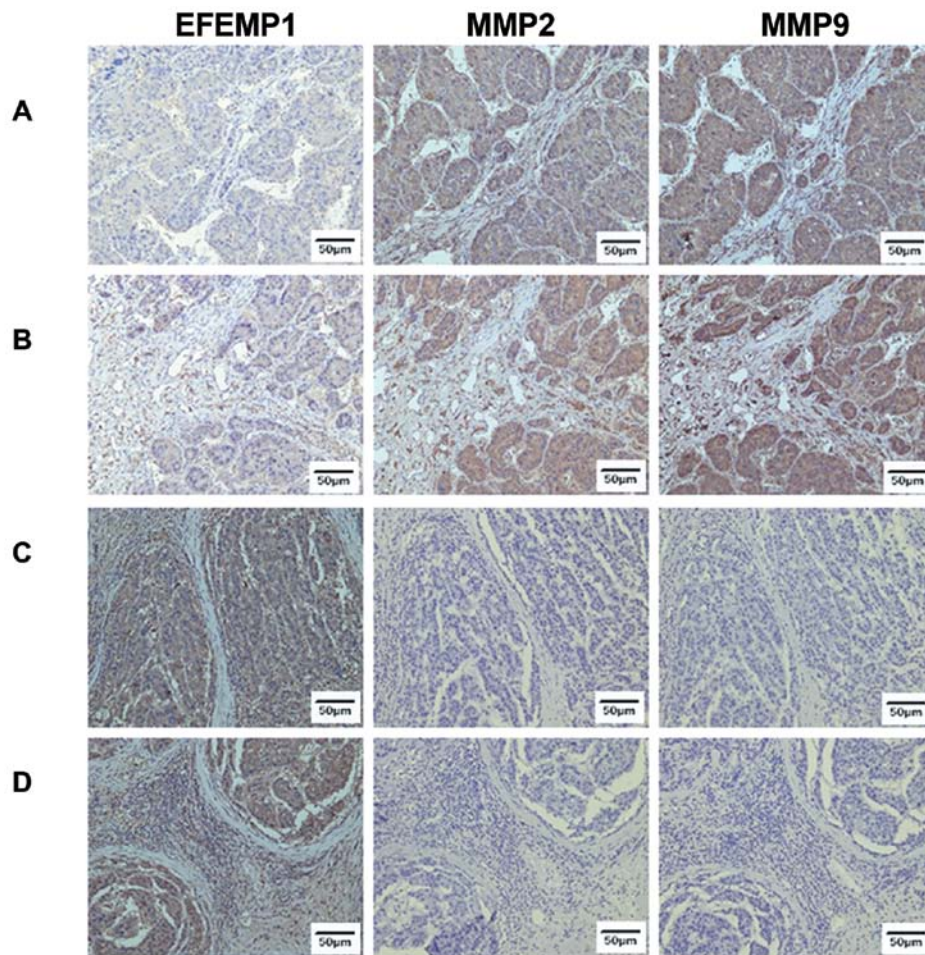


Figure 6. EFEMP1 expression in HCC tissues inversely associated with MMP2 and MMP9 levels. (A and B) Low EFEMP1 expression samples expressed high MMP2 and MMP9 levels. (C and D) High EFEMP1 expression samples expressed low MMP2 and MMP9 levels.

while high EFEMP1 expression samples expressed low MMP2 and MMP9 levels (Fig. 6C and D).

Association between EFEMP1 expression and clinicopathological features. We also investigated the relationship between EFEMP1 expression and clinical pathological features of HCC patients. A total of 215 patients had EFEMP1 expression of 0.232 ± 0.162 . The statistical analysis demonstrated that lower EFEMP1 expression was significantly associated with patients who had ascites ($P=0.050$), vascular invasion ($P=0.044$), poorer differentiation ($P=0.002$) and higher clinical stage ($P=0.003$) (Table I). However, patients who had higher AFP level showed no significant difference compared to patients who had normal AFP level in EFEMP1 expression.

Discussion

EFEMP1 as an antitumor glycoprotein in HCC has been confirmed in some studies (5,6). EFEMP1 was decreased in HCC patients and was associated with unfavorable prognosis, and it acts as an independent prognostic biomarker in HCC. Downregulation of EFEMP1 increased cell viability and promoted cell invasion in HCC cells (5). We found that knock-down of EFEMP1 promoted HCC cell migration. Adding purified EFEMP1 protein inhibited HCC cell migration. This

indicated that EFEMP1 inversely correlated HCC cell migration. Only very high concentration (200 and 400 ng/ml) of EFEMP1 protein could inhibit HepG2 migration. This might due to HepG2 expressing high EFEMP1, and therefore this cell line was not sensitive to low EFEMP1 protein concentration stimulation.

EFEMP1 (also called fibulin-3) is a member of the fibulin family of extracellular glycoproteins which are distributed in various human tissues (1). Luo *et al* (6) demonstrated that EFEMP1 was present both in the cytoplasm and the nucleus. However, we found that this protein mainly distributed in cytoplasm. This might due to the antibodies used from different companies. In agreement with our data, EFEMP1 protein expression was decreased in HCC patients. Lower EFEMP1 correlated with higher stage and poor differentiation. In addition, we found that HCC patients with ascites and vascular invasion had lower EFEMP1 expression. These results further confirmed that EFEMP1 played a pivotal role in HCC development. AFP assessment is comprehensively used in clinical test for HCC patients. Our data found that AFP was not associated with EFEMP1 expression in HCC patients. In addition, recent studies revealed that AFP assessment lacks adequate sensitivity and specificity for effective surveillance and diagnosis (7). Collectively, EFEMP1 might be of clinical significance in predicting the prognosis of HCC patients.

Table I. Correlation between EFEMP1 expression and clinico-pathologic variables.

Variable	Cases	EFEMP1 expression	P-value
Age (years)			0.541
≤40	46	0.242±0.161	
>40	169	0.230±0.163	
Gender			0.150
Male	191	0.226±0.161	
Female	24	0.282±0.165	
Ascites			0.050
Yes	40	0.187±0.151	
No	175	0.243±0.163	
Cirrhosis			0.931
No-mild	165	0.232±0.165	
Moderate-severe	50	0.234±0.152	
Tumor multiplicity			0.358
Single	157	0.239±0.167	
Multiple	58	0.216±0.147	
Macroscopy			0.128
Nodular	174	0.247±0.167	
Nodular merged	6	0.164±0.123	
Massive	5	0.161±0.075	
Giant	30	0.170±0.129	
Vascular invasion			0.044
Yes	27	0.174±0.121	
No	188	0.241±0.166	
Tumor size (cm)			0.159
≤5	104	0.248±0.162	
>5	111	0.217±0.161	
AFP (ng/ml)			0.069
≤20	96	0.255±0.161	
>20	119	0.214±0.161	
CA125 (U/ml)			0.829
≤35	161	0.234±0.162	
>35	54	0.228±0.163	
Stage			0.003
I	52	0.374±0.154	
II	50	0.248±0.137	
III	107	0.161±0.123	
IV	6	0.146±0.220	
Differentiation			0.002
Well-moderate	133	0.260±0.159	
Poor-undifferentiated	82	0.188±0.158	

Effects of EFEMP1 on tumor progression have been reported in two aspects. One aspect is its pro-tumor role. By increasing the expression of VEGF, overexpression of EFEMP1 in HeLa cells promotes angiogenesis, proliferation

and invasion (8). In pancreatic adenocarcinomas, EFEMP1 binds EGFR (competitive to EGF) leading to autophosphorylation of EGFR at Tyr-992 and Tyr-1068 and the subsequent phosphorylation of AKT and ERK and, then, accelerates pancreatic adenocarcinoma growth (9). By promoting Notch-1 cleavage and upregulating the active Notch-1 intracellular domain (NICD), EFEMP1 promoted glioma growth and reduce apoptosis (4). Another aspect is its antitumor role. Overexpression of EFEMP1 inhibited malignant glioma proliferation by suppressing EGFR-AKT signaling (3). Hwang *et al* reported that EFEMP1 inhibited nasopharyngeal carcinoma cell migration and invasion by decreasing the phosphorylation of AKT at Ser-473 (10). In addition, EFEMP1 sensitized pancreatic cancer cells to a PI3K/mTOR inhibitor by interacting with p27^{Kip1} (11). However, the mechanism of how EFEMP1 affects HCC cell migration remained obscure. In the present study, we found that EFEMP1 could negatively regulate the expression of pERK1/2 without changing total ERK1/2, which might explain the negative regulation role of EFEMP1 in HCC.

The antitumor and pro-tumor molecular mechanism of EFEMP1 may occur via different ways. One of the mechanisms may occur via an association with MMPs. Our previous study found that EFEMP1 can increase the expression and activity of MMP-2 and MMP-9 in malignant gliomas (12). However, another connection between EFEMP1 and MMPs is their opposite expression levels. EFEMP1 abrogated angiogenic activities and sprouting in MB114 cells by decreasing the expression of MMP-2 and MMP-3 (13). EFEMP1 is associated with decreased MMP-2 and MMP-7 levels in lung cancer (14) and MMP-2 and MMP-9 levels in endometrial carcinoma (15), which are somewhat similar to our data. We found that down-regulation of EFEMP1 increased the expression of MMP2 and MMP9, and MMP2 and MMP9 level in clinical samples showed the same tendency: the lower the EFEMP1 the higher the MMP2 and MMP9 expression was. Furthermore, such regulating role of EFEMP1 was modulated through ERK1/2 activity.

In summary, our findings provide an understanding that EFEMP1 negatively modulate the migration ability in HCC. Reduction of EFEMP1 promotes the migration ability though increasing MMP2 and MMP9 expression via ERK1/2 activity.

Acknowledgements

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