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Profilin 1, negatively regulated by microRNA-19a-3p, serves as a tumor suppressor in human hepatocellular carcinoma

Zheyuan Wang[a], Zhiheng Shi[b], Lu Zhang[a], Huihan Zhang[a], Yawu Zhang[a,*]

[a] Department of General Surgery II, Lanzhou University Second Hospital, Lanzhou 730030, Gansu Province, China
[b] Beijing RDFZ Chaoyang Branch School, Beijing 100028, China

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ABSTRACT

Profilin 1 (PFN1) is a critical actin-regulatory protein; however, its functional role in hepatocellular carcinoma (HCC) progression remains to be further elucidated. In the present study, we observed that the expression levels of PFN1 were significantly decreased in HCC tissues and cell lines. Low PFN1 expression was significantly correlated with aggressive clinicopathological characteristics and poor prognosis of HCC patients. Further in vitro experiments demonstrated that overexpression of PFN1 remarkably inhibited the proliferation, migration, invasion and EMT of HCC cells. Moreover, we also found that PFN1 was a direct target gene of miR-19a-3p, and in HCC tissues, and there was a significantly inverse correlation between PFN1 mRNA and miR-19a-3p expression. Collectively, our results showed that PFN1 functions as a tumor suppressor in HCC, and might serve as a diagnostic and therapeutic target for HCC patients.

1. Introduction

Hepatocellular carcinoma (HCC), accounting for about 90% of all primary liver cancer cases, remains one of the most common and lethal malignancies worldwide [1,2]. Despite the recent advancements in the diagnostic and therapeutic methods, the long-term prognosis of HCC patients with advanced HCC remains dismal largely due to the high incidence of metastasis and recurrence after operation [3]. Hepatocarcinogenesis is a complex process involving accumulation of various genetic changes [4]. In this regard, it is of critical importance to understand the underlying molecular mechanisms of HCC progression.

Profilins (PFNs) constitute a group of evolutionarily conserved small actin-binding proteins that serve regulatory roles in many cellular activities, including proliferation and motility [5]. PFN1, the founding member of the family, has been linked to several types of human carcinomas. For example, in pancreatic cancer, PFN1 is downregulated in clinical tumor tissues, and overexpression of PFN1 impaired the malignant phenotypes of tumor cells [6]. In addition, PFN1 overexpression increased the sensitivity of breast cancer cells to camptothecin-induced apoptosis [7]. However, the functional role of PFN1 in HCC remains limitedly reported. Therefore, in this study, we sought to investigate the expression profile and biological function of PFN1 in HCC.

2. Materials and methods

2.1. Patients and tissue samples

96 paired HCC specimens and adjacent non-tumor tissues were collected from patients who underwent curative resection at Lanzhou University Second Hospital (Lanzhou, China). The clinicopathological characteristics of these patients were listed in Table 1. None of the patients received chemotherapy or radiotherapy before surgery. After collection, the fresh clinical tissue samples were snap-frozen in liquid nitrogen and stored at −80 °C until use. This study was approved by the Ethics Committees of Lanzhou University Second Hospital, and written informed consent was obtained from all patients or their relatives.

2.2. Cell culture and transfection

Four HCC cell lines, HepG2, SK-hep1, HCCLM3, Huh7, and one normal fetal hepatocyte cell line, LO2, were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 100 U/ml penicillin and 100 mg/ml streptomycin in a humidified atmosphere at 37 °C with 5% CO_2.

The PFN1 overexpression plasmid was established by inserting the full-length human PFN1 cDNA lacking the 3′UTR into the pcDNA3.1

* Corresponding author at: Department of General Surgery II, Lanzhou University Second Hospital, No. 82 Cuiyingmen, Chengguan District, Lanzhou 730030, Gansu Province, China.
E-mail address: zhangyawu123@163.com (Y. Zhang).

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Table 1
Association between PFN1 mRNA expression and clinicopathological characteristics of HCC patients.

Features	Total number (n = 96)	PFN1 mRNA expression		*P* value
		Low (n = 55)	High (n = 41)	
Age (years)				0.487
> 55	57	31	26	
≤55	39	24	15	
Gender				0.584
Male	65	36	29	
Female	31	19	12	
HBsAg status				0.379
Positive	61	37	24	
Negative	35	18	17	
AFP level (ng/ml)				0.122
> 20	60	38	22	
≤20	36	17	19	
Liver cirrhosis				0.299
With	55	34	21	
Without	41	21	20	
Tumor size (cm)				0.125
> 5	39	26	13	
≤5	57	29	28	
TNM stage				0.034
I + II	61	30	31	
III + IV	35	25	10	
Venous invasion				0.010
Present	30	23	7	
Absent	66	32	34	

vector (Invitrogen). The empty vector was used as the control. MiR-19a-3p mimics and miR-19a-3p inhibitor as well as their corresponding negative control were purchased from GenePharma (Shanghai, China). Cells were seeded into 6-well plates and transfected with the aforementioned plasmids and oligonucleotides using Lipofectamine 2000 (Invitrogen). After 48 h, the cells were collected for the following experiments.

2.3. RNA extraction and RT-qPCR analysis

Total RNA was extracted from tumor samples or cultured cells using Trizol Reagent (Invitrogen). cDNA was reverse-transcribed from 1 μg total RNA using the Reverse Transcription System Kit (TaKaRa; Dalian, China), and qPCR analysis was then performed on a 7500 HT Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using SYBR® Premix Ex Taq™ II (TaKaRa). Relative gene expression was analyzed using $2^{-\Delta\Delta Ct}$ method [8], and GAPDH or U6 was used as an endogenous control. Each experiment was performed in triplicate. The sequences of the primers were as follows:

miR-19a-3p, RT: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAGTT-3', forward primer: 5'-TGTGCAAATGTATGCAA-3' and reverse primer: 5'-GTGCAGGGTCCGAGGT-3';

U6, RT: 5'-AACGCTTCACGAATTTGCGT-3', forward primer: 5'-CTCGCTTCGGCAGCACA-3' and reverse primer: 5'-AACGCTTCACGAATTTGCGT-3';

PFN1, forward primer: 5'-TGGAGCGGATCCAGCGAAGG-3' and reverse primer: 5'-GGACACCAACCTCAGCTGGC-3';

GAPDH, forward primer: 5'-CGAGATCCCTCCAAAATCAA-3' and reverse primer: 5'-TTCACACCCATGACGAACAT-3'.

2.4. Western blot analysis

Total protein was extracted from tumor samples or cultured cells using radio immunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). The protein concentration was determined using the Enhanced BCA Protein Assay kit (Beyotime). Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis, and then transferred to nitrocellulose filter membranes (Hybond, Escondido, CA, USA). After blocking via 5% skimmed milk, the membranes were incubated with specific primary antibodies overnight at 4 °C, followed by incubation with HRP-conjugated secondary antibody for 2 h at room temperature. Immunoreactive bands were visualized using an electrogenerated chemiluminescence kit (Pierce Biotechnology, Rockford, IL, USA), and GAPDH served as a control to confirm equal loading. Each experiment was performed in triplicate.

2.5. MTT assay

Cell proliferation was detected using MTT (3-(4,5-dimethyl-thiazol-2-y1) 2,5-diphenyl tetrazolium bromide) assay. After transfection, cells were seeded in 96-well plates at a density of 5×10^3 cells/well. At indicated time points, 20 μl MTT (5 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) solution was added to the wells. After incubation for additional 4 h, the medium was removed, and formazan crystals were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich). The optical density (OD) was evaluated by measuring the absorbance at 490 nm on a microplate reader (MultiskanEX, Lab systems, Helsinki, Finland). Each experiment was performed in triplicate.

2.6. Colony formation assay

After transfection, cells were seeded in six-well plates at a density of 500 cells/well. The medium was replaced every 3 days, and the cells were cultured for 10 days. Then colonies were fixed by 4% paraformaldehyde, stained with 0.1% crystal violet and counted. Each experiment was performed in triplicate.

2.7. Wound healing assay

After transfection, cells were plated in 24-well plates and grown to confluence. Scratch wounds were then made in the cell monolayer using a sterile 100-μl pipette tip, and the debris was removed by washing the cells with serum-free medium. Cells were allowed to migrate for 24 h, and images were then captured. Each experiment was performed in triplicate.

2.8. Transwell assay

A 200 μl non-serum cell suspension containing 2×10^5 cells was seeded into the upper chambers of transwell plates (8-μm pore size; BD Biosciences, San Jose, CA, USA), whereas the lower chambers were filled with 600 μl medium containing 10% FBS. After 24 h of incubation, cells on the upper surface of the membrane were scraped off with a cotton swab, while cells that went through the membrane were fixed by 4% paraformaldehyde and stained with 0.1% crystal violet. The images were captured under a microscope. Each experiment was performed in triplicate.

2.9. Dual-luciferase reporter assay

The 3′-UTR of PFN1, containing putative miR-19a-3p-binding sites, was amplified by PCR and inserted into the psiCHECK-2 luciferase reporter vector (Promega, Madison, WI, USA). Cells were seeded into 24-well plates and cotransfected with 200 ng of the recombinant plasmids, 100 ng of miR-19a-3p mimics or mimics control, and the pRL-TK plasmid (Promega) using Lipofectamine 2000. 48 h after transfection, the luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega). Renilla luciferase activity was used as control. Each experiment was performed in triplicate.

2.10. Statistical analysis

All statistical analyses were performed using Graphpad Prism

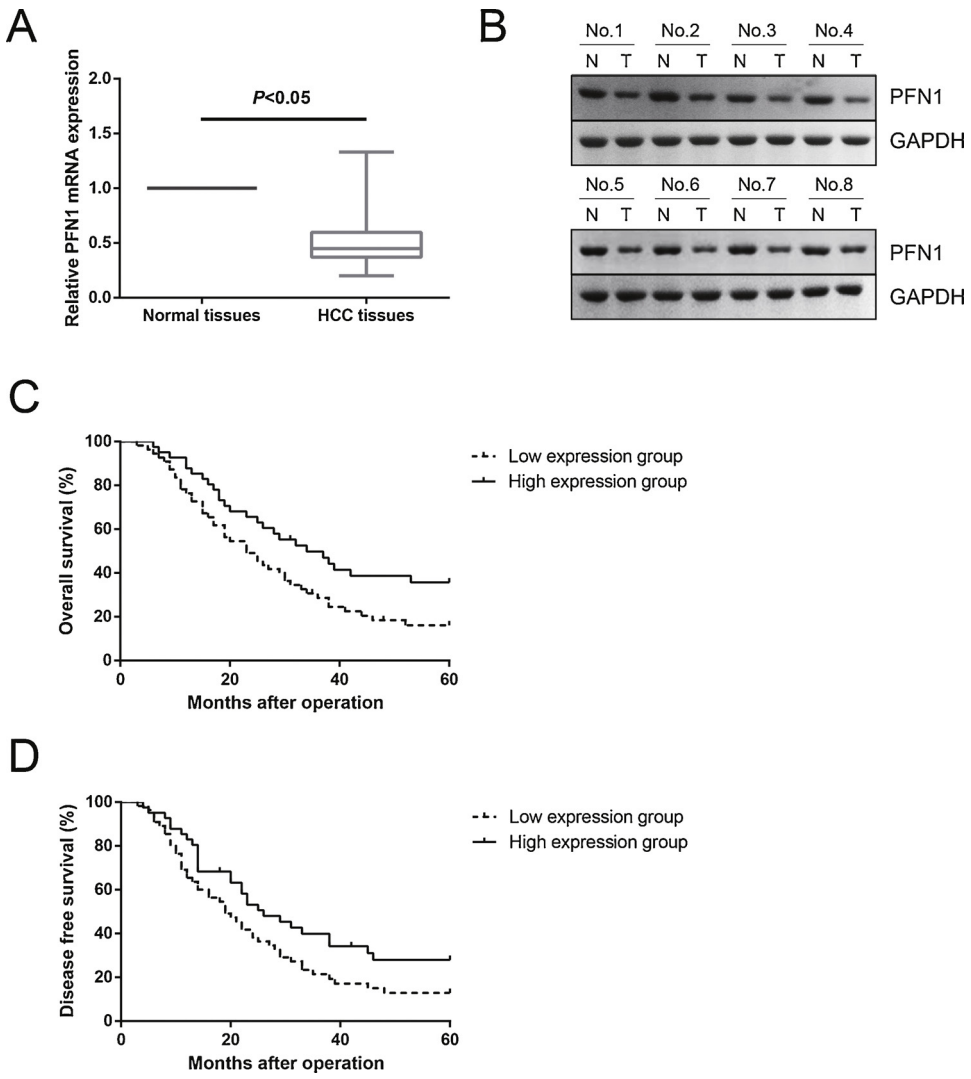

Fig. 1. PFN1 is downregulated in HCC. (A) RT-qPCR analysis of PFN1 mRNA expression in 96 pairs of HCC and adjacent non-tumor tissues. (B) Western blot analysis of PFN1 protein expression in eight pairs of HCC and adjacent non-tumor tissues. (C–D) Association of PFN1 mRNA expression with overall survival and disease free survival of HCC patients.

(version 6.01) software (GraphPad Software, Inc., La Jolla, CA, USA) and SPSS version 18.0 software (SPSS Inc., Chicago, IL, USA). Experimental results were expressed as the mean ± standard deviation (SD), in which the differences were analyzed by Student's *t*-test or ANOVA test. The survival curves were plotted using the Kaplan-Meier method, and the differences in survival probabilities were estimated using the log-rank test. Pearson's correlation analysis was performed to identify the correlation between PFN1 mRNA and miR-19a-3p expression in HCC tissues. A value of $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. PFN1 is downregulated in HCC

First, we noticed that the mRNA expression levels of PFN1 were significantly decreased in HCC tissues than in adjacent non-tumor tissues (Fig. 1A). The protein expression levels of PFN1 were also reduced in eight randomly selected HCC specimens (Fig. 1B). Next, we classified the 96 HCC patients into two groups according to PFN1 mRNA expression: low expression group (n = 55) and high expression group (n = 41). We found that low expression of PFN1 mRNA was significantly correlated with TNM stage ($P = 0.034$) and venous invasion ($P = 0.010$) of HCC patients (Table 1). In addition, patients with HCC tumors expressing high PFN1 levels had significantly longer overall survival ($P = 0.026$; Fig. 1C) and disease free survival ($P = 0.045$; Fig. 1D) than those expressing low PFN1 levels.

3.2. PFN1 inhibits HCC cell proliferation

Then we detected the expression levels of PFN1 in normal fetal hepatocyte cell line LO2 and four HCC cell lines (HepG2, SK-hep1, HCCLM3, Huh7), and the results showed that the mRNA expression levels of PFN1 were significantly decreased in HCC cells (Fig. 2A). To further investigate the functional role of PFN1 in HCC progression, we overexpressed PFN1 by transfection of pcDNA3.1-PFN1 into two HCC cell lines: HCCLM3 and HepG2 (Fig. 2B). By performing MTT assay, we observed that PFN1 overexpression remarkably inhibited the proliferation of HCCLM3 and HepG2 cells (Fig. 2C). Similarly, as shown in Fig. 2D, the number of colonies formed by HCCLM3 and HepG2 cells were significantly decreased by PFN1 overexpression.

3.3. PFN1 inhibits HCC cell migration and invasion

We then investigated whether PFN1 affected HCC cell migratory and invasive abilities. In wound healing assay, we found that the cells

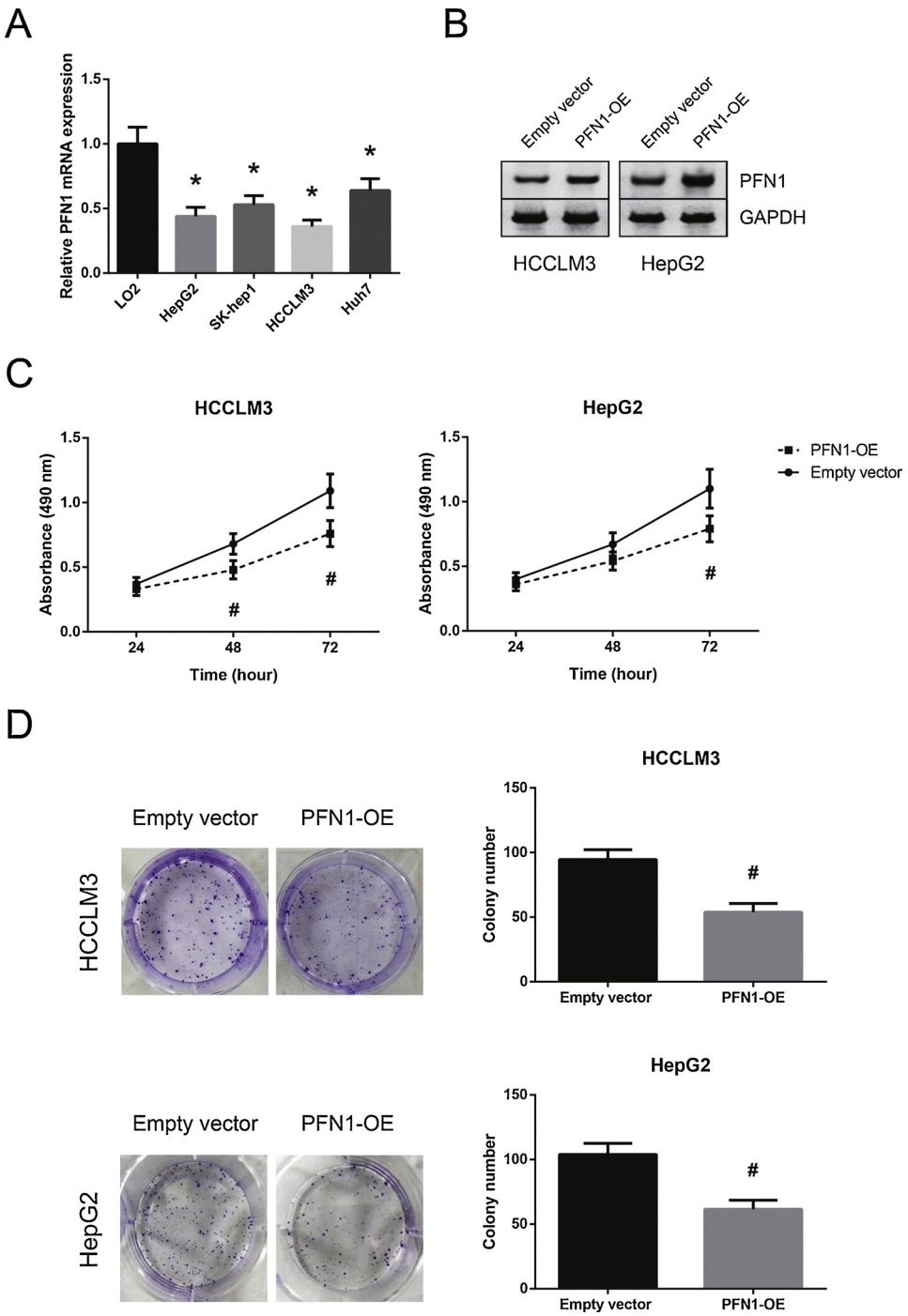

Fig. 2. PFN1 inhibits HCC cell proliferation. (A) RT-qPCR analysis of PFN1 mRNA expression in a panel of HCC cell lines and normal LO2 cells. (B) Western blot analysis of PFN1 protein expression in HCCLM3 and HepG2 cells after transfection. (C) MTT assay was performed to detect the proliferation of HCCLM3 and HepG2 cells after transfection. (D) Colony formation assay was performed to detect the colony formation abilities of HCCLM3 and HepG2 cells after transfection. $^{*}P < 0.05$ vs. LO2 cells, $^{\#}P < 0.05$ vs. empty vector-transfected cells.

transfected with pcDNA3.1-PFN1 migrated more slowly than those transfected with empty vector (Fig. 3A). In addition, as indicated by transwell assay, PFN1 overexpression notably reduced the number of migrated or invaded HCC cells (Fig. 3B).

3.4. PFN1 inhibits EMT in HCC cells

We then explored whether PFN1 regulated EMT in HCC cells. The expression levels of epithelial and mesenchymal protein markers in HCC cells were detected by western blot analysis, and the results demonstrated that PFN1 overexpression significantly increased the expression of E-cadherin and decreased the expression of N-cadherin and Vimentin in HCCLM3 and HepG2 cells (Fig. 4).

3.5. PFN1 is a direct target of miR-19a-3p in HCC

Through TargetScan database (http://www.targetscan.org/vert_71/) [9], we identified the potential miR-19a-3p binding sites in the PFN1 3′-UTR (Fig. 5A). The binding sites are highly conserved among species. Moreover, the results of dual-luciferase reporter assay showed that co-

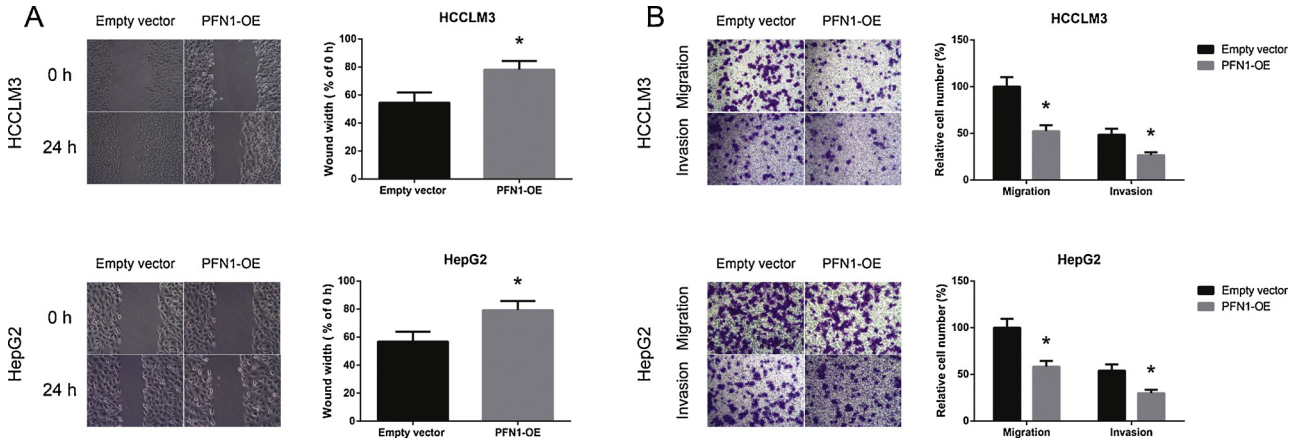

Fig. 3. PFN1 inhibits HCC cell migration and invasion. (A) Wound healing assay was performed to detect the migratory ability of HCCLM3 and HepG2 cells after transfection. (B) Transwell assay was performed to detect the migratory and invasive abilities of HCCLM3 and HepG2 cells after transfection. *$P < 0.05$ vs. empty vector-transfected cells.

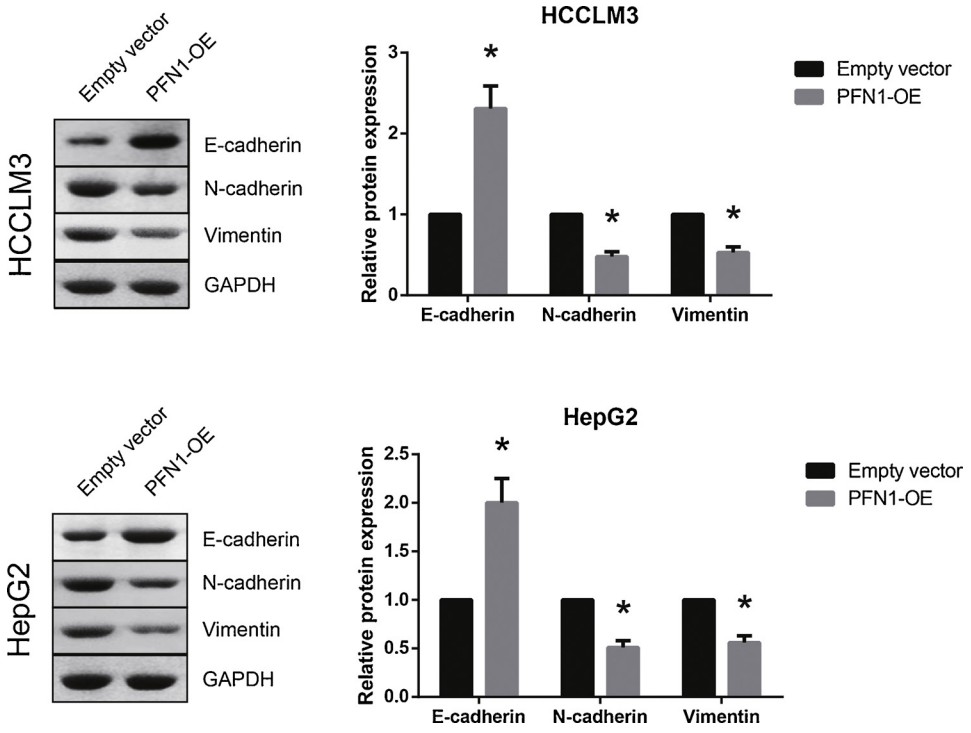

Fig. 4. PFN1 inhibits EMT in HCC cells. Western blot analysis of EMT-related protein expression levels in HCCLM3 and HepG2 cells after transfection. *$P < 0.05$ vs. empty vector-transfected cells.

transfection with miR-19a-3p mimics remarkably reduced the luciferase activity of reporter contained the WT but not the MUT PFN1 3′-UTR in HepG2 cells (Fig. 5B). We further found that miR-19a-3p overexpression decreased, whereas miR-19a-3p knockdown increased the expression levels of PFN1 mRNA and protein in HepG2 cells (Fig. 5C-D).

3.6. PFN1 and miR-19a-3p expression is inversely correlated in HCC

We further confirmed that the expression levels of miR-19a-3p were significantly increased in HCC cells (Fig. 6A). The expression levels of miR-19a-3p were also remarkably increased in HCC tissues than in adjacent non-tumor tissues (Fig. 6B). Moreover, as indicated by Pearson's correlation analysis, there was a significantly inverse correlation between PFN1 mRNA and miR-19a-3p expression in HCC tissues ($r = -0.21$, $P = 0.04$; Fig. 6C).

4. Discussion

Cancer cells exhibit abnormal actin remodeling profiles. PFN1 is a ubiquitously expressed actin-associated protein, and its functional role in cancer biology has recently emerged. A previous study reported that Guttiferone K suppresses the motility and metastasis of HCC cells by restoring aberrantly reduced PFN1 [10]. Our study also showed that PFN1 was downregulated in human HCC tissues, and its low expression was closely associated with unfavorable clinicopathological characteristics and prognosis of HCC patients. These results suggested that PFN1 may be a promising diagnostic and prognostic indicator of HCC patients.

Given the clinical significance of PFN1 in HCC, to elucidate the biological function of PFN1 in HCC, we further performed in vitro functional experiments and the results showed that overexpression of PFN1 significantly inhibited HCC cell proliferation, migration and

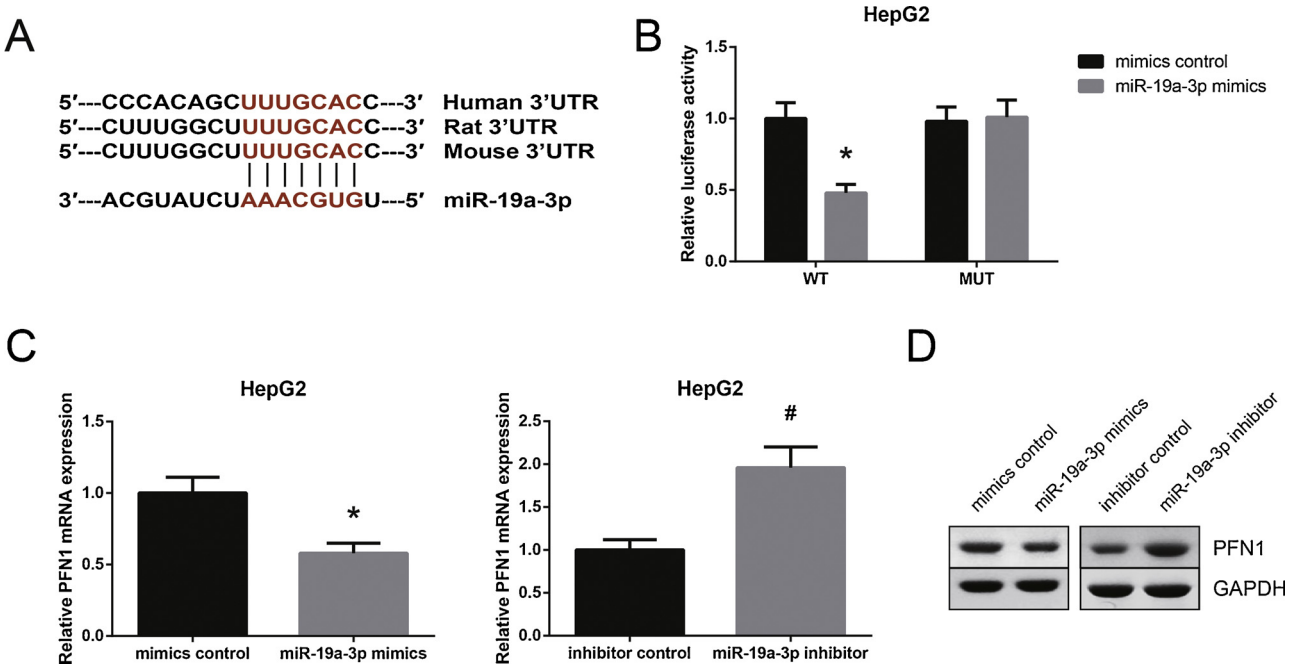

Fig. 5. PFN1 is a direct target of miR-19a-3p in HCC. (A) The predicted binding sites of miR-19a-3p on the PFN1 3′-UTR. (B) Dual-luciferase reporter assay was performed to validate the binding relationship between miR-19a-3p and PFN1 3′-UTR. (C) RT-qPCR analysis of PFN1 mRNA expression in HepG2 cells after transfection. (D) Western blot analysis of PFN1 protein expression in HepG2 cells after transfection. $^{*}P < 0.05$ vs. miR-19a-3p mimics-transfected cells, $^{\#}P < 0.05$ vs. miR-19a-3p inhibitor-transfected cells.

invasion. Local and systemic metastasis is one of the major causes leading to the unfavorable prognosis of HCC patients, and epithelial-mesenchymal transition (EMT) plays a crucial role in early steps of HCC invasion and metastasis [11]. EMT is a complex and multi-step program characterized by the loss of epithelial markers, including E-cadherin, and the acquisition of mesenchymal markers, including N-cadherin and Vimentin [12]. In this study, we also found that overexpression of PFN1 suppressed EMT of HCC cells. All of the above observations indicated that PFN1 might serve as a tumor suppressor in HCC.

MicroRNAs (miRNAs, miRs), a class of small non-coding RNAs, often regulate downstream gene expression by binding to the 3'-UTRs of target mRNAs [13]. In triple-negative breast cancer, miR-182 exerts its regulatory functions through its targeting of PFN1 [14]. We therefore speculated that the tumor suppressive role of PFN1 in HCC might be also regulated by miRNAs. A recent study reported that miR-19a-3p was upregulated in HCC specimens, and ectopic expression of miR-19a-3p

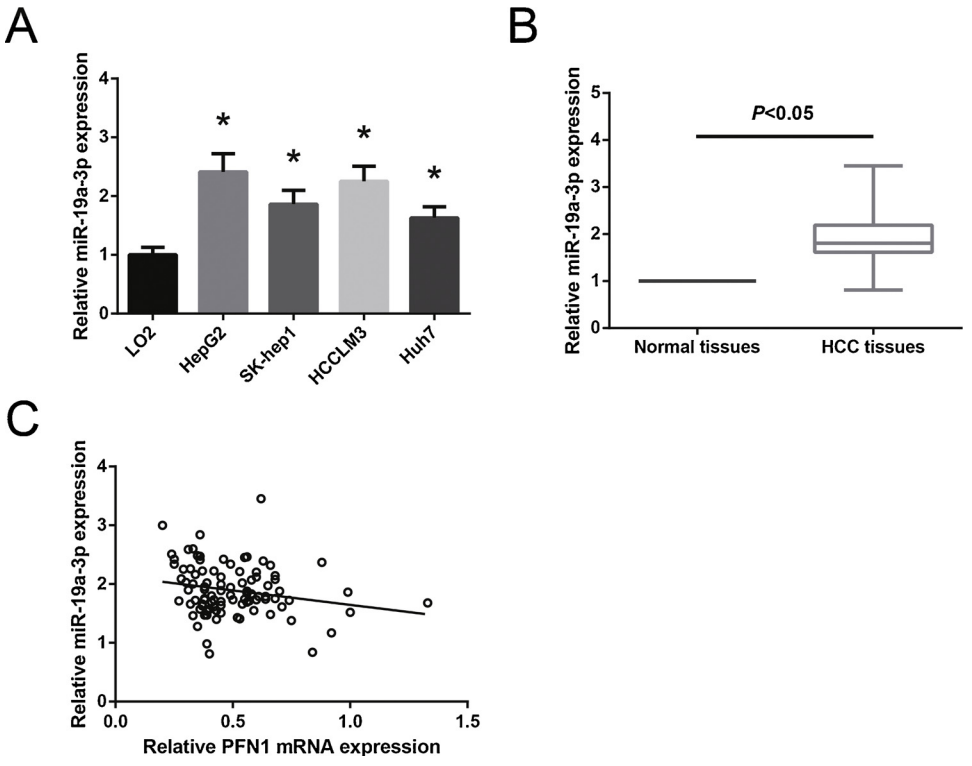

Fig. 6. PFN1 and miR-19a-3p expression is inversely correlated in HCC. (A) RT-qPCR analysis of miR-19a-3p expression in a panel of HCC cell lines and normal LO2 cells. $^{*}P < 0.05$ vs. LO2 cells. (B) RT-qPCR analysis of miR-19a-3p expression in 96 pairs of HCC and adjacent non-tumor tissues. (C) Correlation analysis of PFN1 mRNA and miR-19a-3p expression in HCC tissues.

contributes to HCC metastasis [15]. Here, through bioinformatic prediction and experimental validation, we considered that PFN1 might be a direct downstream target of miR-19a-3p in HCC, and an inverse correlation between the expression of miR-19a-3p and PFN1 mRNA was also identified in our clinical HCC samples.

In conclusion, this study might be the first direct report to investigate a potential regulatory role of PFN1 in HCC. We found that overexpression of PFN1 suppressed the malignant phenotypes of HCC cells, and its expression in HCC is partly regulated by miR-19a-3p. Our data suggested that PFN1 might be a potential diagnostic biomarker and therapeutic target for HCC patients.

Declaration of conflict of interest

None.

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