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PHF8 Plays an Oncogene Function in Hepatocellular Carcinoma Formation

Hong Ye,* Qing Yang,† Shujie Qi,* and Hairong Li‡

*Department of Gastroenterology, Jiaozhou People's Hospital, Jiaozhou, Shandong Province, P.R. China

†Department of Pathology, Jiaozhou People's Hospital, Jiaozhou, Shandong Province, P.R. China

‡Department of Traditional Chinese Medicine, Jiaozhou People's Hospital, Jiaozhou, Shandong Province, P.R. China

Hepatocellular carcinoma (HCC) has high morbidity and mortality rates, and the number of new cases and deaths from liver cancer are increasing. However, the details of the regulation in HCC remain largely unknown. Plant homeodomain finger protein 8 (PHF8) is a JmjC domain-containing protein. Recently, PHF8 was reported to participate in several types of cancer. However, the biological function and clinical significance of PHF8 in HCC remain unknown. In this study, we investigate the role of PHF8 in HCC growth and metastasis. We used bioinformatics analysis and identified the differentially expressed PHF8 in primary HCC and metastasis HCC. Immunohistochemistry analysis demonstrated that PHF8 was expressed higher in human HCC tissues than in corresponding adjacent noncancerous tissues. Silencing PHF8 in HCC cells significantly decreased the cells' ability of proliferation, migration, invasion, and sphere formation. On the contrary, overexpression of PHF8 promoted these properties. In addition, the analysis *in vivo* showed that PHF8 overexpression promoted tumor formation and metastasis in nude mice. In the end, the RNA-sequence assay showed that CUL4A is upregulated by the PHF8. Taken together, these results demonstrated that PHF8 was a novel oncogene in HCC, which may contribute to therapeutic approaches aimed at targeting components of the PHF8 and provide new insights into the mechanisms governing the developmental programs in HCC.

Key words: Hepatocellular carcinoma (HCC); PHF8; Proliferation; Metastasis

INTRODUCTION

Hepatocellular carcinoma (HCC) has become the most common leading cause of cancer-associated mortality globally¹, which has high morbidity and mortality rates, and the number of new cases and deaths from liver cancer are increasing². However, the details of the regulation in HCC remain largely unknown.

Histone modification pattern change is involved in the development and metastasis of cancer. Histone demethylases have been linked to various cancers. For instance, LSD1 inhibits breast cancer's metastasis, but promotes proliferation, migration, and invasion of non-small cell lung cancer (NSCLC)³. KDM5B promotes bladder and lung tumor progression but serves as a tumor suppressor in melanoma⁴. Histone demethylases play important roles during HCC initiation and metastasis as well⁵. Previously, investigators also considered targeting histone demethylases as a new weapon in the fight against cancer⁶. Thus, illustrating the role of histone demethylases in HCC is critical for the treatment of this disease. The KDM7

family of histone demethylases, which consists of three members, KDM7A, KDM7B [plant homeodomain finger protein 8 (PHF8)], and KDM7C, is characterized by the presence of a C-terminal JmjC domain and a PHD finger domain in the N-terminal portion⁷. The members of this family have been associated with tumor formation processes⁷. PHF8 catalyzes the removal of H3K9me2/me1 and monomethylated histone H4 lysine 20 (H4K20me1); its PHD finger binds H3K4me3 and contributes to the demethylase activity of the protein at appropriate target sites⁸. Recently, PHF8 was reported to participate in several types of cancer, including prostate cancer, leukemia, and esophageal squamous cell carcinoma⁹. Previous data identified that PHF8 was overexpressed in prostate cancer with an impact on cell proliferation, migration, and invasion. However, the biological function and clinical significance of PHF8 in HCC remain to be investigated.

In the present study, we found that the PHF8 was upregulated in HCC and correlates with poor prognosis

Address correspondence to Hong Ye, Department of Gastroenterology, Jiaozhou People's Hospital, Guangzhoubei Road #88, Jiaozhou, Shandong Province 266300, P.R. China. E-mail: yehong_001@126.com

of HCC. PHF8 enhanced metastasis and progression of HCC cells *in vitro* and *in vivo*. Thus, we identified PHF8 as an oncogenic protein in HCC and suggested that PHF8 might be a potential therapeutic target for preventing HCC progression.

MATERIALS AND METHODS

Cell Lines

The HCC cell lines were purchased from ATCC. The HCC cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 37°C in an atmosphere containing 5% CO₂.

Western Blot

Cells were lysed with cell lysis buffer (Beyotime, Zhejiang, P.R. China). Total proteins were applied to SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred to PVDF membranes, followed by blocking in the buffer containing 5% fat-free dry milk. The membranes were then probed with indicated antibodies overnight, and then washed and incubated with conjugated secondary antibodies for 1.5 h and finally visualized using Chemiluminescent ECL reagent (Vigorous Biotechnology, Beijing, P.R. China).

Quantitative Real-Time PCR (qPCR)

Total RNA samples were extracted from lung cancer tissues or cells with TRIzol reagent (TaKaRa, Dalian, P.R. China). cDNA was synthesized from 1 µg of total RNA with One Step RT-PCR Kit (TaKaRa). qPCR was performed with the SYBR Green (TaKaRa) detection method on an ABI-7500 RT-PCR system (Applied Biosystems, Foster City, CA, USA).

Immunohistochemical Analysis

Immunohistochemistry analysis was performed on samples of liver adenocarcinoma tissues and corresponding adjacent noncancerous tissues. The samples were fixed with 10% formalin, embedded in paraffin, and sliced into 5-µm sections. The sections were then stained. Staining was observed in five randomly selected high-power fields. The staining intensity was based on the average percentage of positive cells. The scoring results were analyzed by two investigators.

Colony Formation Assay

As for colony formation assays, the cells were seeded (100/35-mm dish) in triplicate and cultured for 14 days. Cell colonies were fixed, stained with 0.25% crystal violet in 50% ethanol for 20–30 min, air dried, and counted. A region containing >50 cells was considered one colony. The number of colonies was recorded.

Transwell Assay

The invasion ability of HCC cells was performed using 24-well Transwells (8-mm pore size; Corning Life Sciences, Corning, NY, USA) coated with 1 mg/ml of Matrigel (BD Sciences, San Jose, CA, USA). The cells were seeded in the upper chamber of the wells in 100 µl of FBS-free medium, and 500 µl of 20% FBS medium was added to the lower chambers. Following incubation for 24 h, the cells remaining on the upper membrane were removed with cotton wool. Cells that had invaded through the membrane were stained with methanol and 0.1% crystal violet, and photographed with a phase-contrast inverted microscope (Olympus, Tokyo, Japan). The cells from at least five random microscopic fields (×40) were counted.

Soft Colony Formation Assay

Cells were suspended in 1.5 ml of complete medium supplemented with 0.45% low-melting point agarose. The cells were placed in 35-mm tissue culture plates containing 1.5 ml of complete medium and agarose (0.75%) on the bottom layer. The plates were incubated at 37°C with 5% CO₂ for 2 weeks. Cell colonies were stained with 0.005% crystal violet and analyzed using a microscope.

Tumor Metastasis Model

Nude mice were maintained in a specific pathogen-free facility. Cells were injected into the tail veins of mice. After 2 months, the mice were sacrificed by anesthesia with chloral hydrate. The lung tissues were dissected out for hematoxylin and eosin (H&E) staining.

TCGA RNASeqV2 Expression Correlation and Outcome Analysis

The Kaplan–Meier overall survival plot and the log-rank *p* value for The Cancer Genome Atlas (TCGA) HCC were generated using R with HCC samples (TCGA Network, 2015) that had RNASeqV2 data and clinical information with at least 6 months of follow-up information or a death event. The samples were split according to whether the expression measured by RNASeqV2 fell into the lower one-third quantile or the upper two-thirds quantile of expression.

TCGA Genetic Alteration Analysis

Analysis of genetic alterations in HCC TCGA data was performed on cBioPortal (<http://www.cbioportal.org>). A Fisher's exact test was performed on the confusion matrix of co-occurrence of alterations in PHF8 and CUL4A. cBioPortal (<http://www.cbioportal.org>) containing samples of HCC and its GISTIC 2.0 changes in copy number, all exon group sequencing mutation data, and RNASeqV2 RSEM expression data were used in this study. The *p* values were calculated using a pairwise *t*-test

in R, performing pairwise comparisons between group levels with a Holm correction for multiple testing.

Statistical Analysis

SPSS 20.0 software (IBM, Armonk, NY, USA) was used for statistical analysis. The significance of differences between groups was estimated by Student's *t*-test and chi-square test as appropriate. The survival curves were calculated by the Kaplan–Meier method with the log-rank test applied for comparison. Survival data were evaluated using univariate and multivariate Cox proportional hazards model. Variables with a value of $p < 0.05$ in univariate analysis were used in subsequent

multivariate analysis on the basis of Cox regression analyses.

RESULTS

High Expression of PHF8 Predicts Poor Survival

We used datasets from TCGA databases; genes that are dysregulated in primary liver cancer and metastasis liver cancer are outlined (Fig. 1A). Moreover, bioinformatics analysis identified 10 differentially expressed genes including *PHF8* (Fig. 1B). These data analyses indicate that *PHF8* may play an important role in human liver cancer metastasis. To further explore whether PHF8

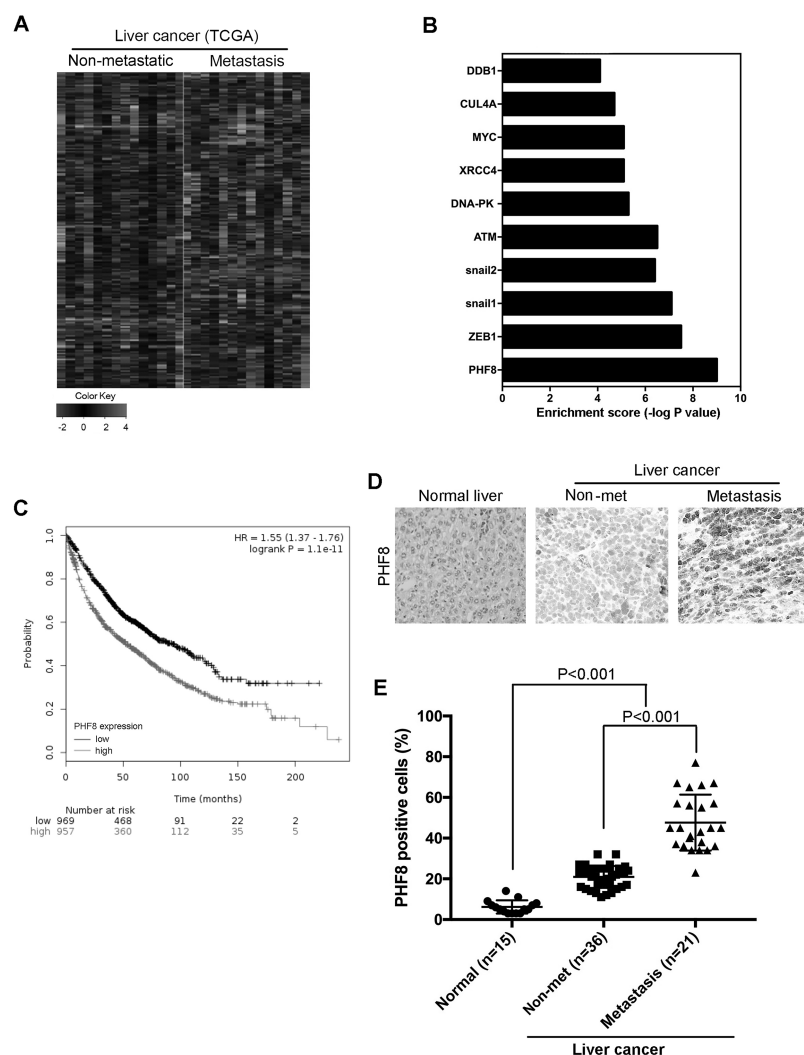


Figure 1. High PHF8 expression level predicted poor survival. (A) Heat map of differentially expressed genes in primary liver cancer and metastasis liver cancer in The Cancer Genome Atlas (TCGA) dataset. (B) Bioinformatics analysis identified 10 differentially expressed genes in primary liver cancer and metastasis liver cancer. (C) Kaplan–Meier curve comparing time to survival between hepatocellular carcinomas (HCCs) with the plant homeodomain finger protein 8 (PHF8). (D) Patients with higher expression of PHF8. PHF8 protein levels were analyzed by immunohistochemistry (IHC) staining. (E) The graph showed quantitative analysis. All experiments were repeated three times. Error bars indicated standard deviation.

participates in human liver cancer, we analyzed the correlation between PHF8 mRNA level and survival using the Kaplan–Meier method. Significantly, high PHF8 level predicted poor survival (Fig. 1C). The finding indicates that PHF8 is involved in human HCC. Then we examined the expression of PHF8 in paired human HCC specimens by immunohistochemistry (IHC). The expression level of PHF8 protein in HCC tissues was significantly higher than those in peritumor tissues (Fig. 1D and E). More importantly, we found that PHF8 overexpression was significantly correlated with distant metastasis (Fig. 1E).

Stable Construction of Cell Lines

To study the potential oncogenic mechanism of PHF8 in hepatoma cells, we surveyed the level of endogenous PHF8 expression among various hepatoma cell lines. As shown in Figure 2A and B, PHF8 was highly expressed in invasive cancer cells (HCCLM3, SNU-182, and MHCC97H), compared with noninvasive liver cell

line (HepG2, SK-HEP-1). To further assess the potential function of PHF8 in hepatocellular carcinoma, we next employed a loss-of-function strategy in PHF8 high-expressing human liver cancer cell lines SUN-182 using lentivirus-mediated short hairpin RNAs (shRNAs). qPCR and Western blot analysis showed that the knockdown of PHF8 by shRNAs (shPHF8 #1, #2) significantly decreased both the mRNA and protein expression of PHF8 (Fig. 2C and D). On the contrary, stably overexpressed PHF8 significantly elevated PHF8 mRNA and protein levels compared with the control (Fig. 2E and F). When infected with retrovirus carrying shPHF8, PHF8 protein level was significantly decreased in SUN-182 HCC cell lines.

PHF8 Promotes the Malignant Tumor Characteristics of HCC Cells

Colony formation assay showed that knockdown of PHF8 led to a slight decrease in colony number (Fig. 3A), whereas overexpression of PHF8 showed a

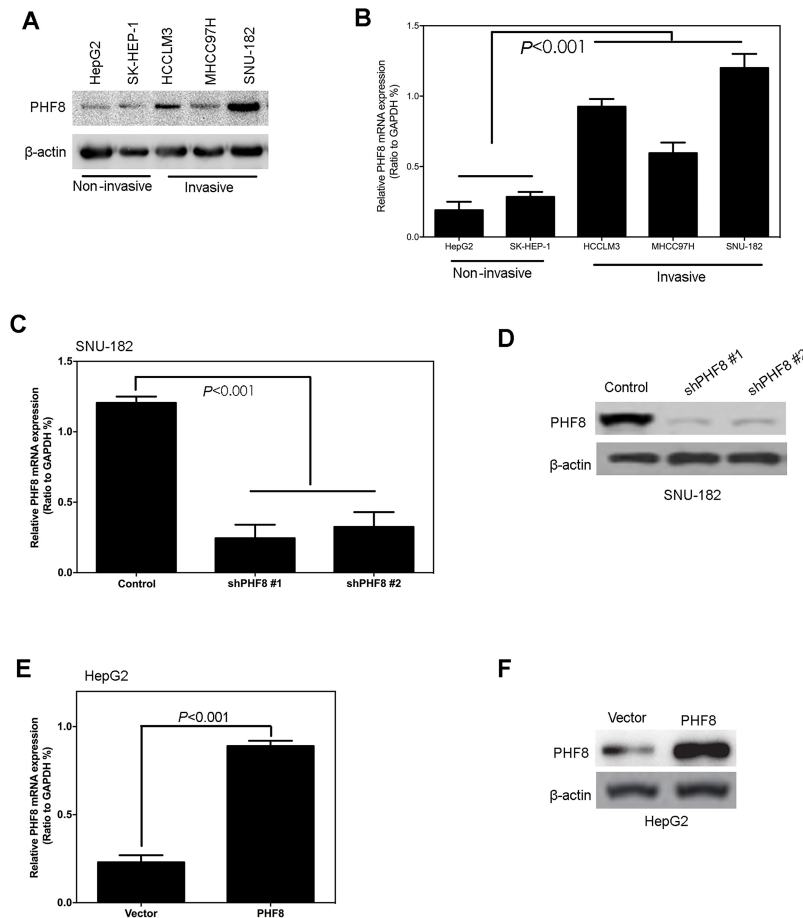


Figure 2. Stable construction of cell lines. PHF8 expression was analyzed by Western blotting (A) and quantitative qPCR (B) in liver cancer cell lines. PHF8 expression was analyzed by qPCR (C) and Western blotting (D) in SUN-182 cells. PHF8 expression was analyzed by qPCR (E) and Western blotting (F) in HepG2. All experiments were repeated three times. Error bars indicated standard deviation.

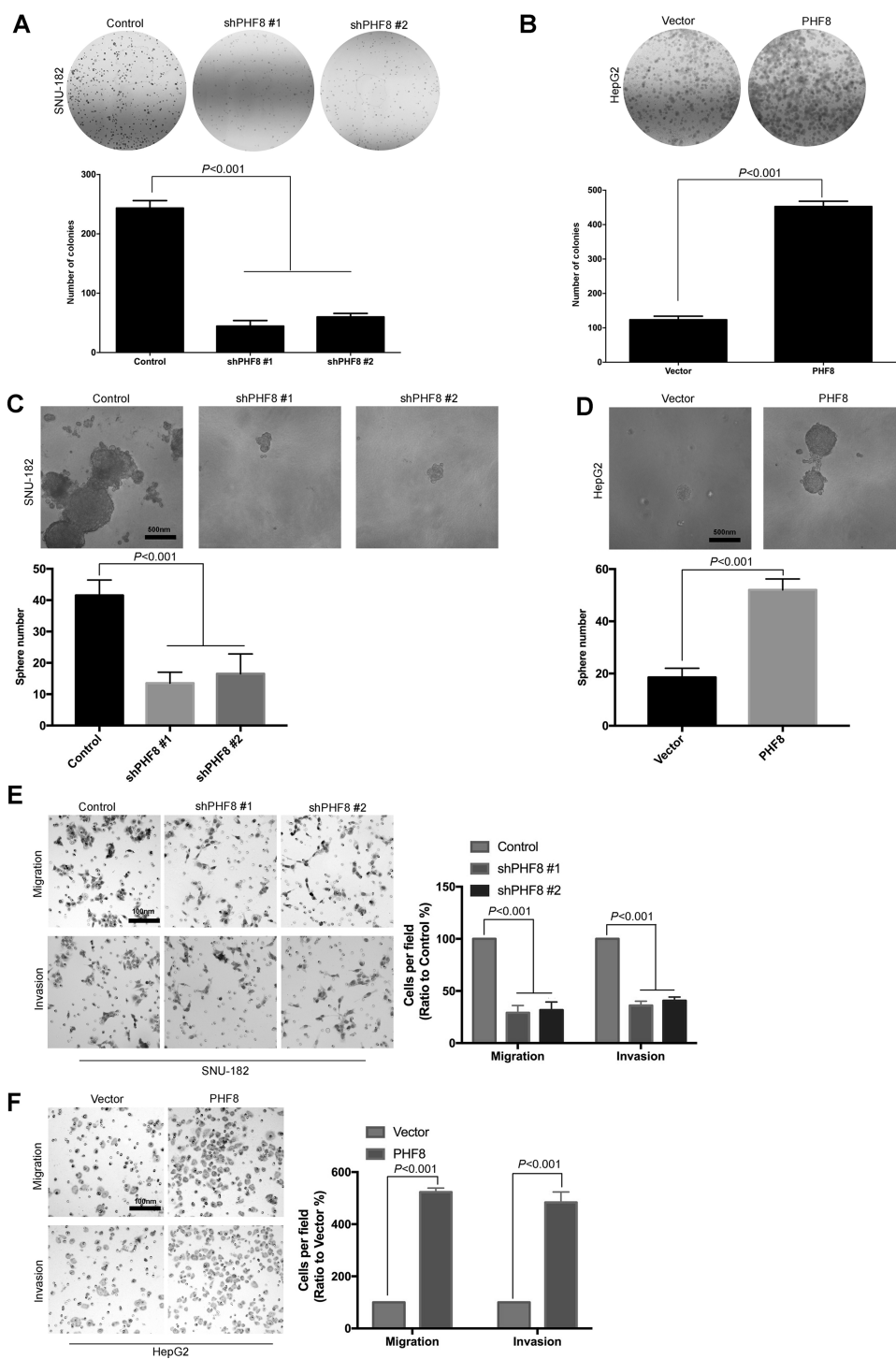


Figure 3. PHF8 promoted HCC cell proliferation, migration, and invasion in vitro. Colony formation assays in SNU-182 cells with PHF8 knockdown (A) and in HepG2 cells with PHF8 overexpression (B) were performed. The graph showed quantitative analysis. PHF8 overexpressed HepG2 cells (C) formed much larger and a greater number of tumor spheres than SNU-182 cells with PHF8 knockdown (D). PHF8 inhibition decreased cells' ability of migration and invasion in SNU-182 cells (E). PHF8 overexpression increased cells' ability of migration and invasion in HepG2 cells (F). All experiments were repeated three or four times. Error bars indicated standard deviation.

significant increase in the number of colonies of HepG2 cells (Fig. 3B). The result suggested that PHF8 could promote proliferative capacity of HCC cells. Recent research showed that PHF8 was associated with cancer stem cell-enriched populations of several epithelial tumor cells. We next investigated whether PHF8 was associated with cancer stem cell-like traits of human liver cancer cell lines. We observed that PHF8-overexpressed cells have a higher capacity for self-renewal. Compared with control cells, significantly fewer and smaller hepatospheres were observed in PHF8 knockdown cells (Fig. 3C). PHF8-overexpressed cells formed much larger and a greater number of tumor spheres compared with the control (Fig. 3D). The results suggested that PHF8 could promote the cancer stem-like capacity of HCC cells. Previous research has found that PHF8 could promote epithelial-to-mesenchymal transition in breast cancer cells¹⁰. We

confirmed that PHF8 also can promote HCC cell migration and invasion. Silencing PHF8 significantly decreased the migration and invasion of HCC cells (Fig. 3E), whereas overexpression of PHF8 led to increase in migration and invasion compared with the control (Fig. 3F).

PHF8 Promoted HCC Tumor Formation In Vivo

To extend our in vitro observations, we investigated whether PHF8 could regulate tumorigenic capacity of HCC cells in vivo. HepG2-PHF8 cells and their control cells were subcutaneously injected into nude mice. Tumor size was measured up to 28 days after injection. As expected, the tumors from HepG2-PHF8 cells grew more rapidly at the implantation site than the control cells (Fig. 4A–C). These results suggested that PHF8 might be an important regulator of proliferation in HCC cells in vivo.

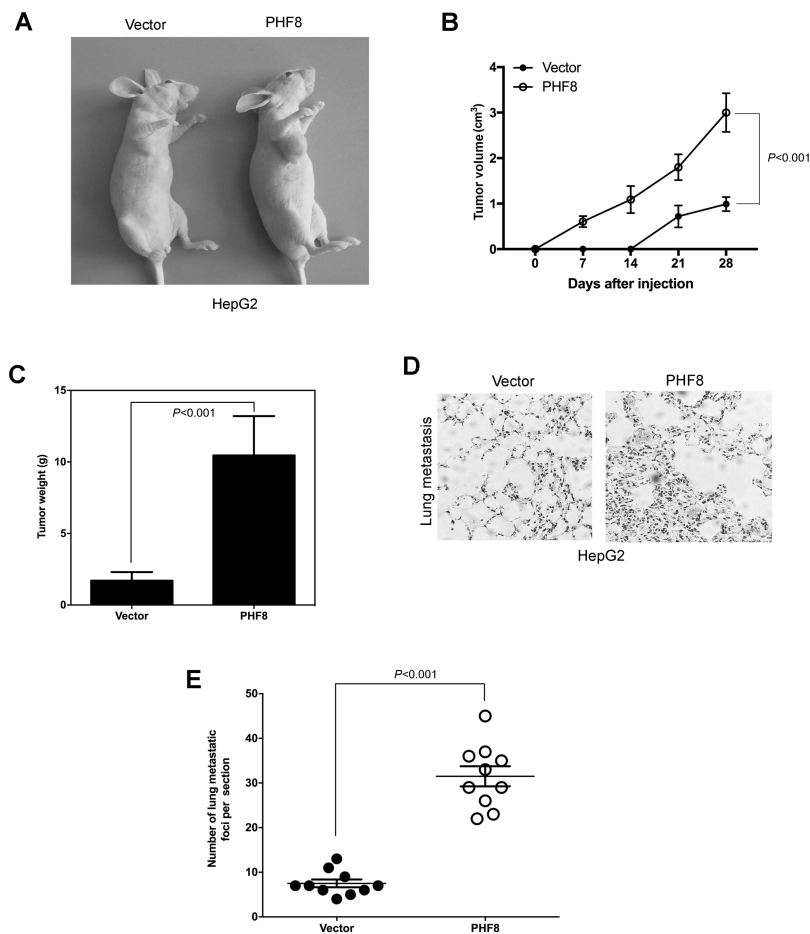


Figure 4. PHF8 overexpression promoted HCC cell tumor formation and metastasis in vivo. (A) Representative images of tumor from PHF8 overexpression and its control HepG2 cells. (B) Growth curve of tumors formed by PHF8 overexpression and its control HepG2 cells. (C) The weight of tumors formed by PHF8 overexpression and its control HepG2 cells. (D) Representative images of lung tissues measured by hematoxylin and eosin (H&E) stain with injection of PHF8 overexpression and its control HepG2 cells into the tail vein. (E) The numbers of metastatic foci per section in the lung of individual mouse with injection of PHF8 overexpression and its control HepG2 cells into the tail vein. All experiments were repeated three or four times. Error bars indicated standard deviation.

Overexpression of PHF8 Promoted HCC Cell Metastasis In Vivo

Due to PHF8's contribution to migration and invasion in human liver cancer cells in vitro, we wondered whether PHF8 could participate in the metastasis in vivo. Cells were injected in tail veins of nude mice. Two months later, the mice were sacrificed by anesthesia with chloral hydrate. The lung was dissected

out for H&E staining. As shown in Figure 4D and E, PHF8-overexpressed cells enhanced distant metastasis abilities compared with the control.

PHF8 Induced the Expression of CUL4A

To better understand the mechanisms of how PHF8 is engaged in proliferation, migration, and invasion functionality, we performed gene expression profiling on HepG2-PHF8 and its control cells. RNA-sequence

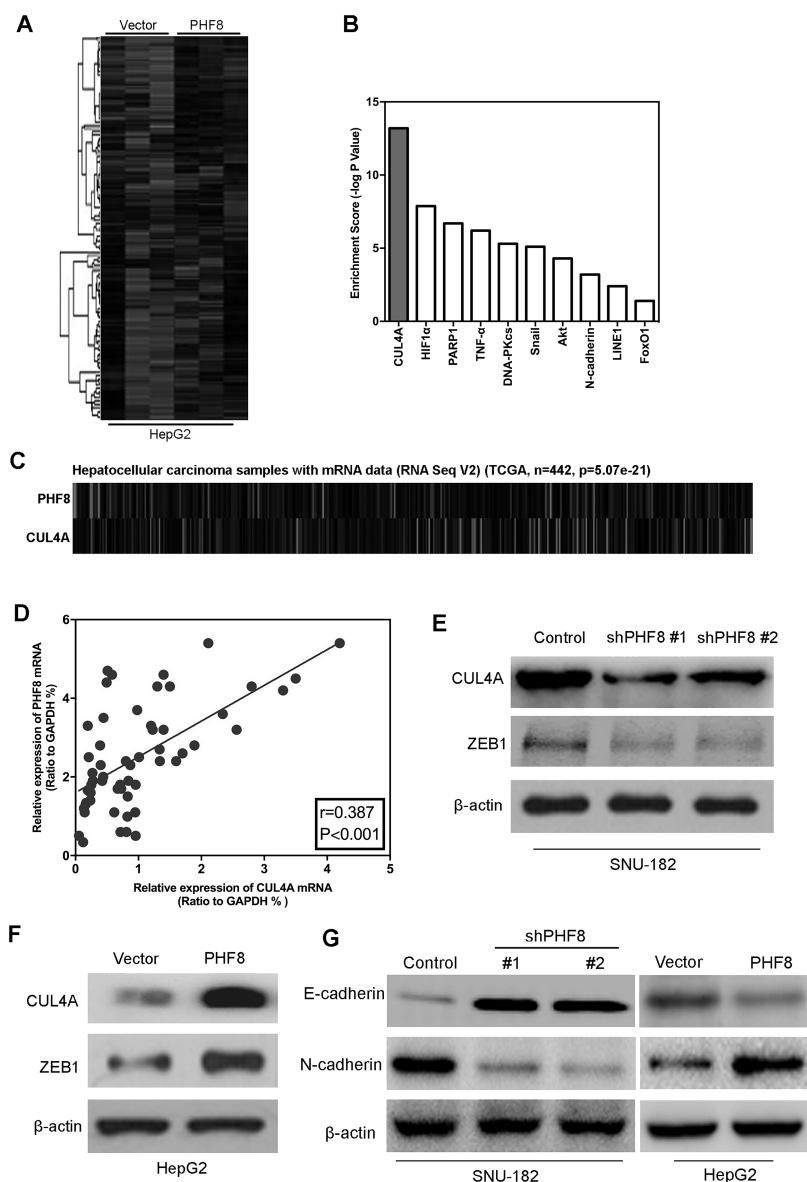


Figure 5. PHF8 induced the expression of CUL4A. (A) Gene expression profiling on PHF8 overexpression and its control HepG2 cells was assayed by RNA-sequence analyses. (B) Gene set enrichment analysis showed the top 10 regulated by PHF8 overexpression in HepG2 cells. (C) The data from TCGA showed that the expression of PHF8 was positively correlated with CUL4A expression in HCC. (D) CUL4A mRNA expression was positively correlated with PHF8 mRNA in HCC tissues. (E) The expression of CUL4A and its target gene ZEB1 was assayed by Western blot in PHF8 knockdown and its control SNU-182 cells. (F) The expression of CUL4A and its target gene ZEB1 was assayed by Western blot in PHF8 overexpression and its control HepG2 cells. (G) The expression of E-cadherin and N-cadherin was assayed by Western blot in indicated cells.

analysis identified a list of significantly differentially expressed genes after PHF8's overexpression, including the downregulation of CUL4A (Fig. 5A and B). The data from TCGA showed that the expression of PHF8 was positively correlated with CUL4A expression (Fig. 5C). The same correlation was also observed in our HCC tissues by measuring the mRNA expressions of PHF8 and CUL4A using qPCR (Fig. 5D). Together with previous reports linking CUL4A with tumorigenesis and metastasis in carcinomas^{11–13}, we examined whether CUL4A could be a downstream target of PHF8. Expression of CUL4A in the cells with altered PHF8 expression was evaluated by Western blot. Silencing PHF8 in SNU-182 cells dramatically decreased CUL4A expression (Fig. 5E). Moreover, overexpression of PHF8 in HepG2 cells greatly increased CUL4A expression (Fig. 5F). It has been demonstrated that CUL4A targets many proteins for degradation, such as ZEB1¹⁴. Thus, we measured the expression level of ZEB1 in PHF8-altered cells. The results showed that silencing PHF8 significantly inhibited the expression of ZEB1 (Fig. 5E), while ectopic expression of PHF8 drastically upregulated its expression (Fig. 5F). CUL4A has been reported to regulate EMT¹⁴, so we examined whether PHF8 can alter EMT marker expression in liver cancer cells. As shown in Figure 5G, the epithelial cell marker E-cadherin dramatically decreased after PHF8 overexpression in HepG2 cells, and the mesenchymal cell marker N-cadherin was significantly upregulated. On the contrary, silencing PHF8 in SNU-182 cells has reversed the results. All of these results indicated that CUL4A was the downstream target of PHF8.

DISCUSSION

Emerging evidence has suggested that overexpression of PHF8 was associated with several different types of cancers, including prostate cancer¹⁵, esophageal squamous cell carcinoma¹⁶, lung cancer⁹, and breast cancer¹⁷. However, the role of PHF8 in HCC is still unclear.

In this study, we performed a study to investigate the role of PHF8 in HCC and its potential prognostic value. We found that the expression of PHF8 was high in HCC cell lines and tissues compared with the control, which was associated with poor patient prognosis further. We also found that decreased expression of PHF8 in HCC cells inhibited the cells' ability of migration and invasion significantly compared with the control. Furthermore, we also found that the expression of CUL4A was affected with PHF8 synchronously, indicating that PHF8 might modulate HCC's progression through CUL4A and its target genes, such as ZEB1. These results suggest that PHF8 is an important oncogene that is involved in initiation, growth, and metastasis of hepatocellular carcinoma.

The initiation and progression of tumor have been recognized as a complex process, which is relevant to both genetic and epigenetic alterations¹⁸. Furthermore, global changes in histone modification patterns are functionally associated with cancer development and recurrence^{19–21}. Epigenetic mechanisms have been proven to be important in cancer development²². Histone methylation, a common form of epigenetic regulation controlled by both methyltransferases and demethylases, has been reported to play fundamental roles in many cellular processes²².

As a histone demethylase, PHF8 could activate its target genes through demethylation of the transcriptionally repressive marks, H3K9me2/1 and H4K20me1²³. PHF8 could bind to H3K4me3 via its PHD finger, which is typically enriched at the transcription start sites (TSSs) and may play a role in their recruitment to target promoters²⁴. Histone demethylase PHF8 acts on mH4K20me1, monomethylated and dimethylated H3 lysine 9 (H3K9me1/2), and dimethylated H3 lysine 27 (H3K27me2), serving as a transcription coactivator^{25–27}. Bioinformatics analysis has shown that PHF8 binding sites overlap with the consensus sequences of several transcription factors significantly: E2F1, ETS-1 SP1, FOXO1, TCF, and MYC-MAX²⁸. These data indicate that PHF8 may exert distinct functions in different types of cancer¹⁰.

In the present study, we show that PHF8 also participates in the regulation of liver cancer as a histone demethylase. Thus, PHF8 might be a potential therapeutic target for preventing HCC progression.

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