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Original Article

CYP2J2 promotes the development of hepatocellular carcinoma by increasing the EETs production to improve HIF-1α stability

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Abstract: Objective: This study aimed to explore the function and mechanism of Cytochrome P450 2J2 (CYP2J2) epoxygenase and epoxyeicosatrienoic acids (EETs) in the malignant development of hepatocellular carcinoma (HCC). Method: The expressional levels of EETs and CYP2J2 in HCC tissues and cell lines were quantified by ELISA, western blot and RT-qPCR, respectively. The effects of EET and CYP2J2 on HCC development were analyzed by CCK-8 assays, flow cytometry analysis, colony formation and transwell assays. The effect of CYP2J2-EET metabolism on stability of HIF-1α was detected by western blot experiments. HIF-1α inhibitor, YC-1, was used to probe the relationship between HIF-1α and metastasis of HCC cells. Finally, xenograft experiments were established to investigate the function of CYP2J2-EETs metabolism in HCC tumorigenesis *in vivo*. Result: CYP2J2, 11, 12-EET and 14, 15-EET were up-regulated in HCC tissues and Huh-7, HepG2 cell lines. Addition of exogenous 14, 15-EET accelerated proliferation and metastasis of HCC cells. Knockdown of CYP2J2 inhibited growth and metastasis of HCC cells and malignant xenograft, which was obviously reversed by addition of 14, 15-EET. Moreover, in Huh-7 and HepG2 cells, CYP2J2-EET metabolism elevated the expression of HIF-1α and its downstream factors including VEGFA, PDK1, GLUT1 and DDIT4 through suppressing the expression of PHD. Treatment of YC-1 remarkably suppressed the HCC cells proliferation and restored the effect of 14, 15-EET on tumor size *in vivo*. Conclusion: The up-regulated levels of CYP2J2 and 14, 15-EET in HCC cells improved the stability of HIF-1α thourgh inhibiting PHD expression, which further promoted the malignant development of HCC.

Keywords: Hepatocellular carcinoma, CYP2J2, 14, 15-EET, HIF-1α, PHD

Introduction

Hepatocellular carcinoma (HCC), the most frequent type of primary liver cancer, has become the leading cause of cancer-related death worldwide, especially in countries or regions with high infection rates of hepatitis B virus (HBV) and hepatitis C virus (HCV) [1]. In spite of the improvement in clinical diagnosis and therapeutic strategies for HCC treatment, the 5-year survival rate of patients with advanced HCC remains poor [2, 3]. Therefore, investigations of the malignant mechanism of HCC and effective therapeutic targets are urgently required.

Human Cytochromes P450 (CYPs) 2 epoxygenases consisting of CYP2C (CYP2C8, CYP2C9, CYP2C19), CYP2J (CYP2J2) and CYP2S1 are mostly expressed in tissues such as the heart and liver, as well as in epithelial cells, cardiomyocytes, and endothelial cells [4, 5]. In the CYP450 epioxidase pathway, CYP2 family members are responsible for the transformation of arachidonic acid (AA) to epicosatetraenoic acid (EET) that can be rapidly hydrolyzed by soluble epioxide hydrolase (sEH) to less biologically active dihydroxy-eicosatrienoic acids (DHETs) [6]. There are four isomers of EETs (5, 6-EET, 8, 9-EET, 11, 12-EET, 14, 15-EET) generated by CYP2 family members, of which 11, 12-EET and

14, 15-EET are the predominant isomers of EETs in blood vessels and various types of cells [7, 8]. EETs are lipid molecule involved in a series of physiological processes by acting as autocrine and paracrine mediators [9, 10]. EETs are supposed to activate intracellular pathways to exert a variety of biological effects, such as cell migration, proliferation, and inflammation through interacting with PPARα, ion channel or G-protein coupled receptor GPR40 [11-13].

Previous studies implied oncogenic role of CYP2J2 and EETs in the development of both solid tumors and hematological malignancies [14, 15]. Ectopic expression of CYP2J2 significantly affects the proliferation, invasion of tumor cells while inhibition of CYP2J2 reduces the malignancy of tumor cells [15]. Despite the acceleration on metastasis and proliferation of tumor cells, elevated level of EETs also contributes to the induction of epithelial-mesenchymal transition (EMT), and is involved in the chemotherapy-resistance in breast cancer [16]. In liver cancer, CYP2J2 expression is up-regulated, and studies in HCC cell lines preliminarily demonstrate that CYP2J2 promotes HepG2 cells proliferation [17, 18]. However, the mechanism by which CYP2J2 and metabolites EETs affect the malignant progression of HCC has not been fully elucidated.

In hypoxic microenvironment of solid tumor, due to the inactivity of HIF-1α-degrading enzymes prolyl-4-hydroxylase domain proteins (PHDs), HIF-1α is accumulated and further promotes tumor growth and migration by enhancing angiogenesis and regulating cell metabolism [19, 20]. In the present study, we found that the up-regulated expression of CYP2J2 and decreased level of sEH in HCC increased the amount of EETs. Besides, the level of PHD was reduced and the stability of HIF-1α was elevated in HCC. The accumulated HIF-1α activated the transcription of HIF-1α downstream factors including VEGF, which in turn accelerated the development of HCC.

Materials and methods

Cell culture and clinical samples

Huh-7, HepG2 and normal liver epithelial cell line LO2 were purchased from the American Type Culture Collection (ATCC, VA, USA), and were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, CA, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, UT, USA), 1% penicillin-streptomycin (Invitrogen, CA, USA). All the cells were maintained at 37°C in humidified 5% CO_2 atmosphere. For hypoxic cell culture, cells were maintained in hypoxic work station (Invivo$_2$ 400, Ruskinn, UK) with hypoxic atmosphere containing 1% O_2, 5% CO_2, and 94% N_2.

Peripheral blood was collected from patients with HCC (n=20) and healthy control (n=20) who were enrolled from the Affiliated Wuxi No.2 People's Hospital of Nanjing Medical University. All participants signed the official informed consent forms. The project was approved by the Ethical and Scientific Committee of the Affiliated Wuxi No.2 People's Hospital of Nanjing Medical University.

Total RNA isolation and real time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, CA, USA) according to the user's guidance. Reverse transcription was conducted by AMV Reverse Transcriptase kit (Thermo Fisher Scientific, MA, USA). RT-qPCR was performed using SYBR Green Realtime PCR Master Mix (TOYOBO, Osaka, JPN), and PCR procedure was shown as follow: pre-denaturation at 95°C for 5 min; 95°C for 30 s, 60°C for 30 s, extension at 72°C for 30 s, 30 cycles, followed by a final extension at 72°C for 10 min. Intracellular level of GAPDH was used as internal control, and fold changes of indicated genes were calculated by $2^{-\Delta\Delta CT}$ method.

Establishment of stable cell lines

Lentivirus package system containing shRNA sequences targeting CYP2J2 (sh-CYP2J2) and scramble shRNA sequence (sh-NC) were purchased from Shanghai GenePharma Co., Ltd (Shanghai, CHN). The sequences for shRNAs targetting CYP2J2 were as follow: sh-CYP2J2-1: AUAUUUCUUCACAAACAGCUG; sh-CYP2J2-2: UAAGAACUGCAGAUAUGUCAC. Transient transfection was conducted using Lipofectamine 2000 reagent (Invitrogen, CA, USA) following commercial manual. The lentivirus used for cell transducing was packaged in HEK293T cells according to commonly protocol.

Cell counting Kit 8 (CCK-8) assay

Huh-7 and HepG2 cells were seeded at density of 6×10^4 cells/well on 48 well plates, and were incubated with 100 nm of 14, 15-EET (Cayman chemical, MI, USA) for 48 h. Cells were harvested at 24 h, 48 h, 72 h and 96 h post 14, 15-EET treatment to assess the cell proliferation. Both experimental and control cells were incubated with 100 μl DMEM medium containing 10 μl CCK-8 reagent (Beyotime, Shanghai, CHN) for 2 h at 37°C. The optical density (OD) value at 450 nm was recorded by microplate reader (Bio-Rad, VT, USA).

Enzyme-linked immunosorbent assay (ELISA)

Peripheral blood and experimental cell culture medium were centrifuged at 1,000 g for 10 min at 4°C, and the supernatant was collected. ELISA assays of EETs were performed by commercial kit following manufacturer's instructions (Shanghai Huzhen Industrial Co., Ltd, Shanghai, CHN).

Colony formation assay

Huh-7 and HepG2 cells were seeded into 6 well plates at density of 1000 cells per well. Experimental cells treated with 100 nm 14, 15-EET and control cells were normally cultured in DMEM medium containing 10% FBS for 7 days. Then, all the cell colonies were fixed with 4% paraformaldehyde for 15 min and stained with 0.1% crystal violet (Sigma-Aldrich, MO, USA) for another 10 min. After twice phosphate-buffered saline (PBS) washing, the colonies were scanned for photograph record (Nikon, Tokyo, Japan) and counting.

In vitro migration assay

The migration assays for Huh-7 and HepG2 cells were carried out in 6-well transwell chambers (BD Biosciences, CA, USA) with 8 μm-pore sized polycarbonate membrane. A number of 1×10^6 cells were seeded into the upper chamber and incubated with 100 nm 14, 15-EET (control group was cultured without EET). Volume of 600 μl medium containing 10% FBS was added to the bottom chamber. After 2 h, cells in the bottom chamber were fixed with 4% paraformaldehyde and then stained with 0.1% crystal violet at room temperature for 40 min. After PBS washing, the cells were photographed (Nikon, Tokyo, Japan) and counted for analysis.

Cell cycle and cell apoptosis assays

Apoptosis of Huh-7, HepG2 cells were detected using Annexin V-FITC/propidium iodide (AV/PI) Apoptosis Detection Kit (Abcam, Cambridge, UK) according to the manufacturer's instructions. Cell cycle was determined using Cell Cycle Analysis Kit (Beyotime, Shanghai, CHN) following user's guidance. Briefly, cells were fixed in 5 ml ice-cold 75% ethanol at 4°C overnight. After removing the supernatant and twice washing, the cells were gently re-suspended in 500 μl staining buffer supplemented with 10 μl RNase A and 25 μl propidium iodide (PI), followed by incubation in dark at 37°C for 30 min. Apoptosis and cell cycle were examined and analyzed by flow cytometry using FACS Calibur (BD Biosciences, CA, USA).

Western blot

Total protein isolated from experimental and control cells was quantified using BCA protein assay kit (Bio-Rad, CA, USA). Protein extraction was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and was electro-transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, MA, USA) for target bands detection. After blocking by 5% non-fat milk for 1 h at room temperature, the PVDF membrane was incubated with corresponding primary antibodies at 4°C overnight. Then, the membrane was washed 3 times by 1×TBST buffer, and was incubated with HRP-labeled secondary antibodies at 37°C for 2 h. Target protein were visually detected and photographed by the ECL Plus Western blotting detection system (Bio Rad, CA, USA). Quantification of protein expression was analyzed by ImageJ software of version 1.41 (National Institutes of Health, MD, USA).

Xenograft experiments

Four to six weeks old male BALB/c nude mice were randomly divided into eight groups (6 mice per group) based on different treatment: Huh-7-sh-NC, Huh-7-sh-CYP2J2, Huh-7-sh-CYP-2J2+14, 15-EET, Huh-7-sh-CYP2J2+14, 15-EET+YC-1 (Sigma-Aldrich, MO, USA), HepG2-sh-NC, HepG2-sh-CYP2J2, HepG2-sh-CYP2J2+14, 15-EET, HepG2-sh-CYP2J2+14, 15-EET+YC-1 (Sigma-Aldrich, MO, USA). All the mice were maintained under specific pathogen-free facility. Huh-7-sh-NC, Huh-7-sh-CYP2J2, HepG2-sh-NC and HepG2-sh-CYP2J2 cells (2×10^6) were

inoculated subcutaneously into mice. Tumors volume were measured every 7 days and tumor growth curves were plotted. At 42 days post implantation, mice were euthanized by cervical dislocation. The xenograft tumors were removed and weighed. All the experiments protocols were in accordance with the ethical standards of the Affiliated Wuxi No.2 People's Hospital of Nanjing Medical University.

Statistical analysis

All the data were presented as the mean ± standard deviation (SD). Student's *t*-test and one-way analysis of variance (ANOVA) was performed to analyze difference among groups, as appropriate. The gray value of protein bands was calculated by ImageJ software (National Institutes of Health, MD, USA). All experiments were repeated three times and the statistical analyses were performed using software package GraphPad Prism 8.0 (GraphPad Software, CA, USA). $P<0.05$ was considered as statistically significant.

Results

The up-regulation of CYP2J2 and down-regulation of sEH led to the increased production of EETs in HCC

To investigate the amount of EETs in HCC, we first quantified the level of 5, 6-EET, 8, 9-EET, 11, 12-EET, 14, 15-EET in serum from patients with HCC ($n=20$) and healthy individuals ($n=20$) using ELISA assays. Compared with healthy group, only the expression of 11, 12-EET and 14, 15-EET in serum from HCC patients was significantly increased ($P<0.01$), while the level of 5, 6-EET, 8, 9-EET did not obviously change ($P>0.05$, **Figure 1A**). To validate the up-regulation of EETs, we further analyzed EETs level in LO2 cell lines and HCC cell lines including Huh-7 and HepG2. Similarly, the amount of 11, 12-EET and 14, 15-EET was greatly up-regulated in Huh-7, HepG2 when compared with LO2 ($P<0.01$). And, there was no difference in the expressional level of 5, 6-EET, 8, 9-EET between LO2 and HCC cell lines (**Figure 1B**). CYP2J2, along with CYP2C8, CYP2C9, are the main producers of EETs, and sEH is responsible for mediating the transformation of EETs to DHETS. To explore the association between increased production of EETs and the regulators of EETs in HCC, we detected endougenous protein level of CYP2J2, CYP2C8, CYP2C9, CYP2S1 and sEH in both HCC tissues and cell lines. Western blot assays revealed that CYP2J2 was significantly up-regulated and sEH expression was down-regulated in HCC tissues when compared with adjacent normal liver tissue (**Figure 1C**). In Huh-7 and HepG2 cell lines, the abundance of CYP2J2 was also increased and the level of sEH was markedly decreased ($P<0.01$) (**Figure 1D** and **1E**).

14, 15-EET promoted the proliferation and migration of HCC cells

To define the role of EET in tumor cell growth, Huh-7 and HepG2 cells treated with 14, 15-EET were harvested at indicated time points for CCK-8 analysis. Compared with cells without 14, 15-EET treatment (Blank), addition of 100 nm of 14, 15-EET significantly promoted HCC cells proliferation (**Figure 2A**). Apoptosis assays revealed that EET treatment effectively reduced apoptosis rates of Huh-7 and HepG2 cells (**Figure 2B**). The similar results were observed in colony formation experiments. As shown in **Figure 2C**, exogenous addition of 14, 15-EETs greatly improved the colony formation of Huh-7 and HepG2 cells. Compared with blank group, 14, 15-EET treatment dramatically accelerated the mobility of Huh-7 and HepG2 cells (**Figure 2D**).

CYP2J2 affected the proliferation and migration of HCC cells through regulating EETs production

To elucidate the function of CYP2J2 in HCC cells, Huh-7 and HepG2 cells with deficient CYP2J2 expression (sh-CYP2J2) were established (**Figure 3A**). HCC cells transduced with sh-CYP2J2-1 was selected for further study because of the higher transducing efficiency. Knockdown of CYP2J2 significantly reduced the production of 11, 12-EET, 14, 15-EETS but not 5, 6-EET and 8, 9-EET (**Figure 3B**). The CCK-8 assays revealed that knockdown of CYP2J2 restricted the proliferation of Huh-7 and HepG2 cells, while exogenous addition of 100 nm 14, 15-EET restored the proliferation of HCC cells transduced with sh-CYP2J2 (**Figure 3C**). Inhibition of CYP2J2 expression markedly promoted apoptosis of Huh-7 and HepG2 cells, while addition of 14, 15-EET decreased the apoptosis rates of Huh-7 and HepG2 cells transduced with sh-CYP2J2 (**Figure 3D**).

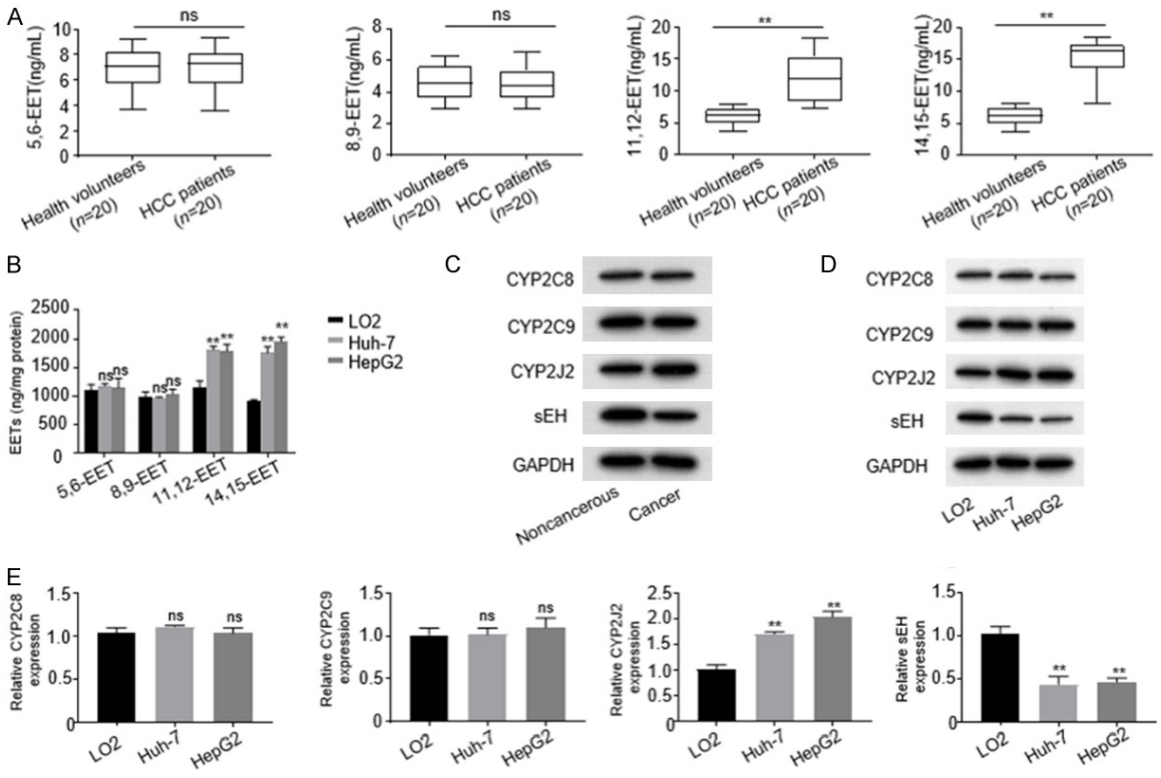


Figure 1. Elevated CYP2J2 expression and epoxyeicosatrienoic acids (EETs) metabolism in HCC tissue and cell lines. (A) ELISA assays were conducted to quantify the amount of 5, 6-EET, 8, 9-EET, 11, 12-EET and 14, 15-EET in serum from HCC patients (*n*=20) and healthy volunteers (*n=20*) (A). ***P*<0.01, ns, *P*>0.05, compared to healthy volunteers; (B) The levels of 5, 6-EET, 8, 9-EET, 11, 12-EET and 14, 15-EET in HCC cell lines (Huh-7, HepG2) and normal hepatic cells LO2 were detected by ELISA analysis. **, *P*<0.01, ns, *P*>0.05, compared to LO2 cells; (C) The protein levels of CYP2C8, CYP2C9, CYP2J2 and sEH were analyzed by western blot assays in HCC and adjacent tissue; (D) The protein levels of CYP2C8, CYP2C9, CYP2J2 and sEH were analyzed by western blot assays in Huh-7, HepG2 and LO2 cells; (E) The mRNA abundance of CYP2C8, CYP2C9, CYP2J2 and sEH were quantified by RT-qPCR in Huh-7, HepG2 and LO2 cells. ***P*<0.01; ns, *P*>0.05, compared to LO2 cells.

Addition of 14, 15-EET in HCC cells transduced with sh-CYP2J2 obviously rescued the inhibitory effect of down-regulated CYP2J2 on the colony formation and migration of Huh-7 and HepG2 cells (**Figure 3E** and **3F**).

CYP2J2 and EET played critical role in maintaining the stability of HIF-1α

In order to deeply probe the relationship between 14, 15-EET and the stability of HIF-1α, we detected the expression of HIF-1α and its negative regulator PHD in HCC cells. Compared with LO2 cells, HIF-1α and its downstream factors including VEGFA, PDK1, GLUT1 and DDIT4 were highly expressed in Huh-7 and HepG2 cells, but the expression of PHD was exactly the opposite (**Figure 4A** and **4B**). Besides, knockdown of CYP2J2 reduced HIF-1α and downstream molecules expression and improved the abundance of PHD in Huh-7 and HepG2 cells when compared with LO2 cells. However, ectopic addition of 14, 15-EET into Huh-7 and HepG2 cells transduced with sh-CYP2J2 greatly increased the expression of HIF-1α, VEGFA, PDK1, GLUT1 and DDIT4 and suppressed the PHD level (**Figure 4C** and **4D**). These results indicated that CYP-mediated EET production could reduce PHD expression and improve the stability of HIF-1α.

Inhibition of HIF-1α reduced the proliferation and migration of HCC cells

To study the role of HIF-1α in HCC cells, we repressed the expressional level of HIF-1α downstream factors by HIF-1α inhibitor, YC-1 [3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole] [21] (**Figure 5A** and **5B**). The CCK-8, colony formation and transwell experiments demonstrat-

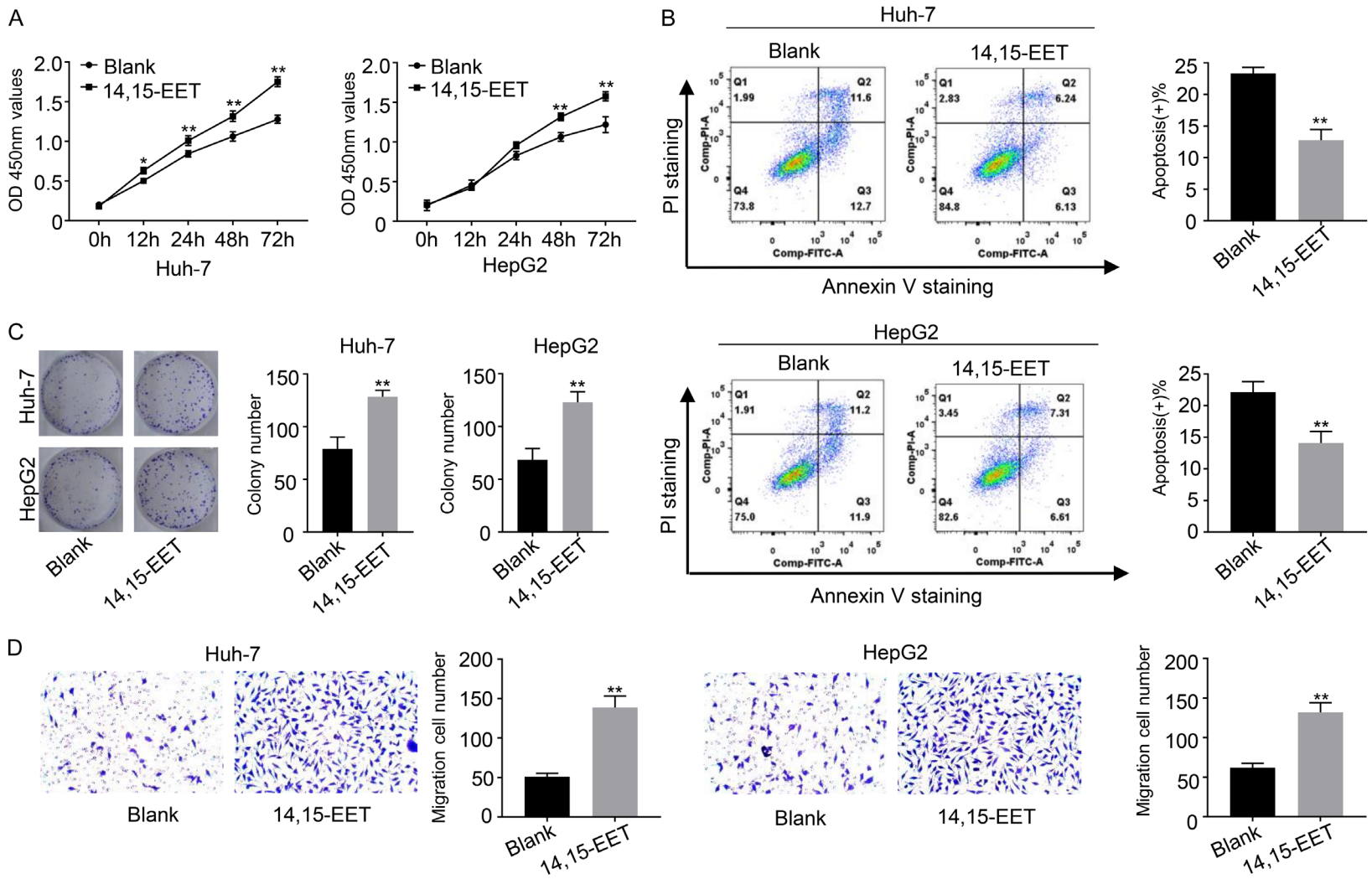
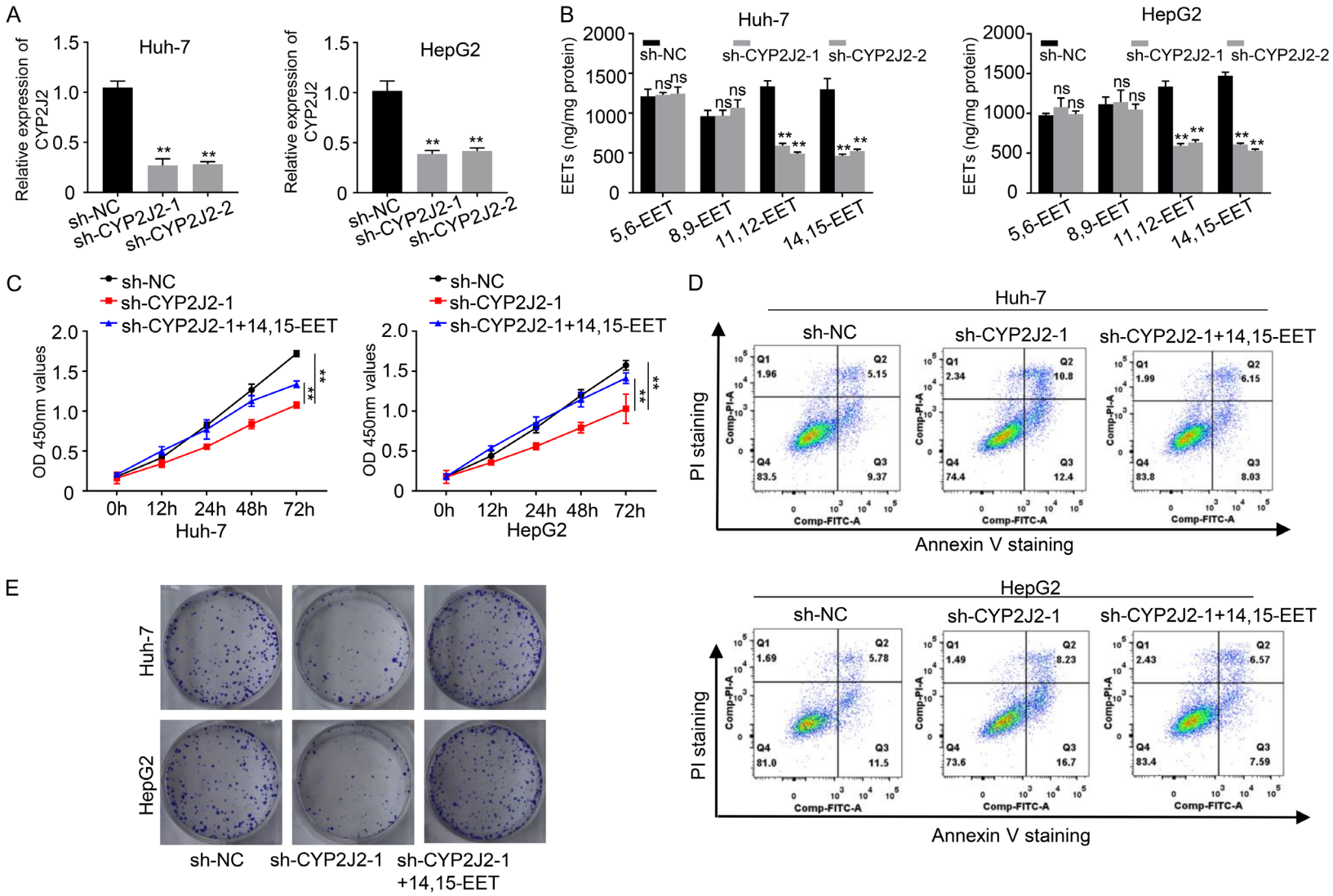


Figure 2. Exogenous addition of EET promoted proliferation, invasion and migration of HCC cells. A. CCK-8 analysis was performed to detect the cell growth of Huh-7 and HepG2 treated with or without 14, 15-EET (100 nM); B. The apoptosis rate of Huh-7 and HepG2 cells incubated with or without 14, 15-EET (100 nM) was detected by flow cytometry; C and D. The colony formation and transwell assays in Huh-7 and HepG2 cells treated with or without 14, 15-EET (100 nM). The quantification results were presented in the right panel. *P<0.05, **P<0.01, compared to Blank group.


A
Huh-7
HepG2
Relative expression of CYP2J2
sh-NC
sh-CYP2J2-1
sh-CYP2J2-2
B
Huh-7
HepG2
EETs (ng/mg protein)
5,6-EET
8,9-EET
11,12-EET
14,15-EET
C
sh-NC
sh-CYP2J2-1
sh-CYP2J2-1+14,15-EET
OD 450nm values
0h 12h 24h 48h 72h
Huh-7
HepG2
D
Huh-7
HepG2
PI staining
Annexin V staining
Comp-FITC-A
Comp-PI-A
E
Huh-7
HepG2
sh-NC
sh-CYP2J2-1
sh-CYP2J2-1 +14,15-EET

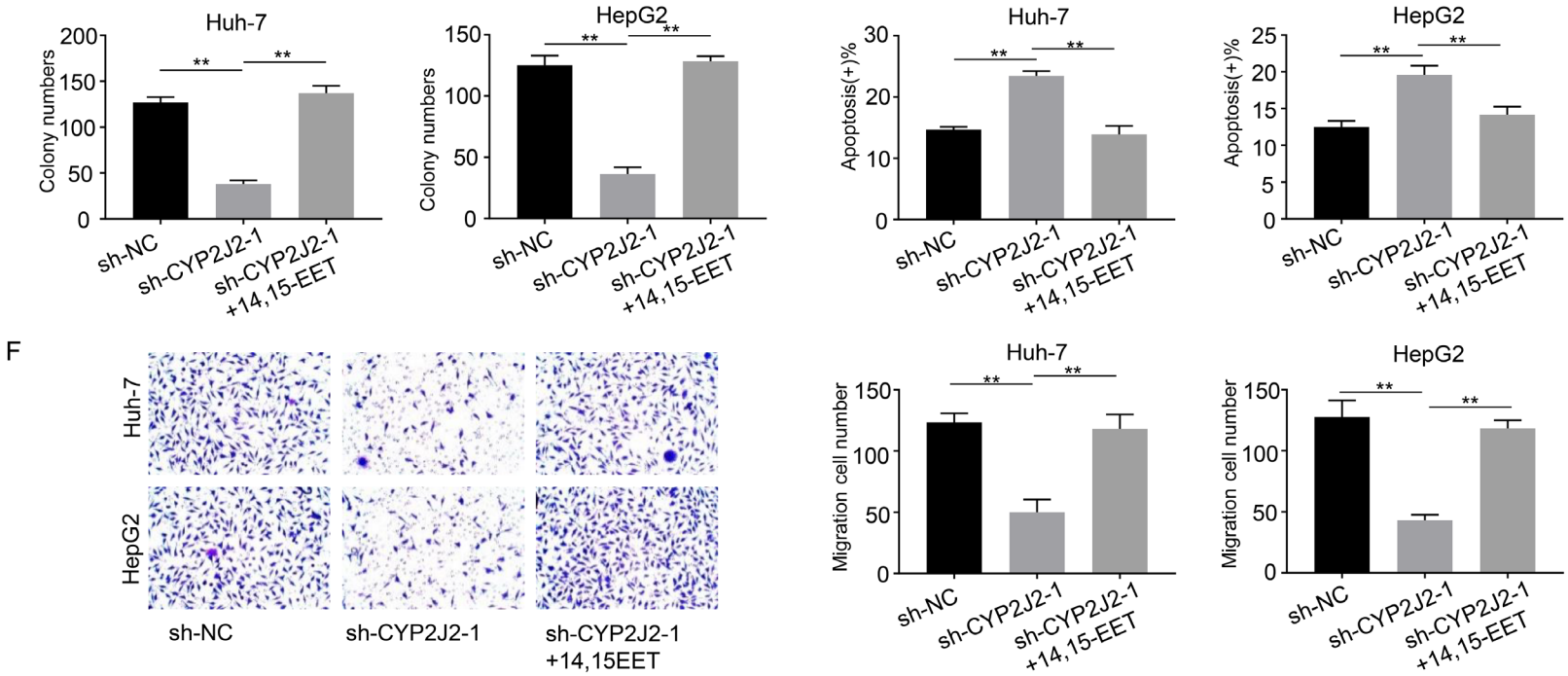


Figure 3. CYP2J2 modulated proliferation, invasion and migration of HCC cells through up-regulating the level of EET. A. The knockdown efficiency of HCC cells transduced with sh-CYP2J2 was verified by RT-qPCR. $**P<0.01$, ns, $P>0.05$ compared to sh-NC; B. ELISA assays to quantify the amount of EETs production in Huh-7-shCYP2J2 and HepG2-sh-CYP2J2 cell lines. $**P<0.01$, ns, $P>0.05$ compared to sh-NC; C. The CCK-8 experiments in Huh-7/HepG2-sh-NC, Huh-7/HepG2-sh-CYP2J2 treated with or without 14, 15-EET (100 nM); D. Apoptosis analysis in Huh-7/HepG2-sh-NC, Huh-7/HepG2-sh-CYP2J2 treated with or without 14, 15-EET (100 nM). E and F. The colony formation and transwell experiments in Huh-7/HepG2-sh-NC, Huh-7/HepG2-sh-CYP2J2 treated with or without 14, 15-EET (100 nM). $**P<0.01$.

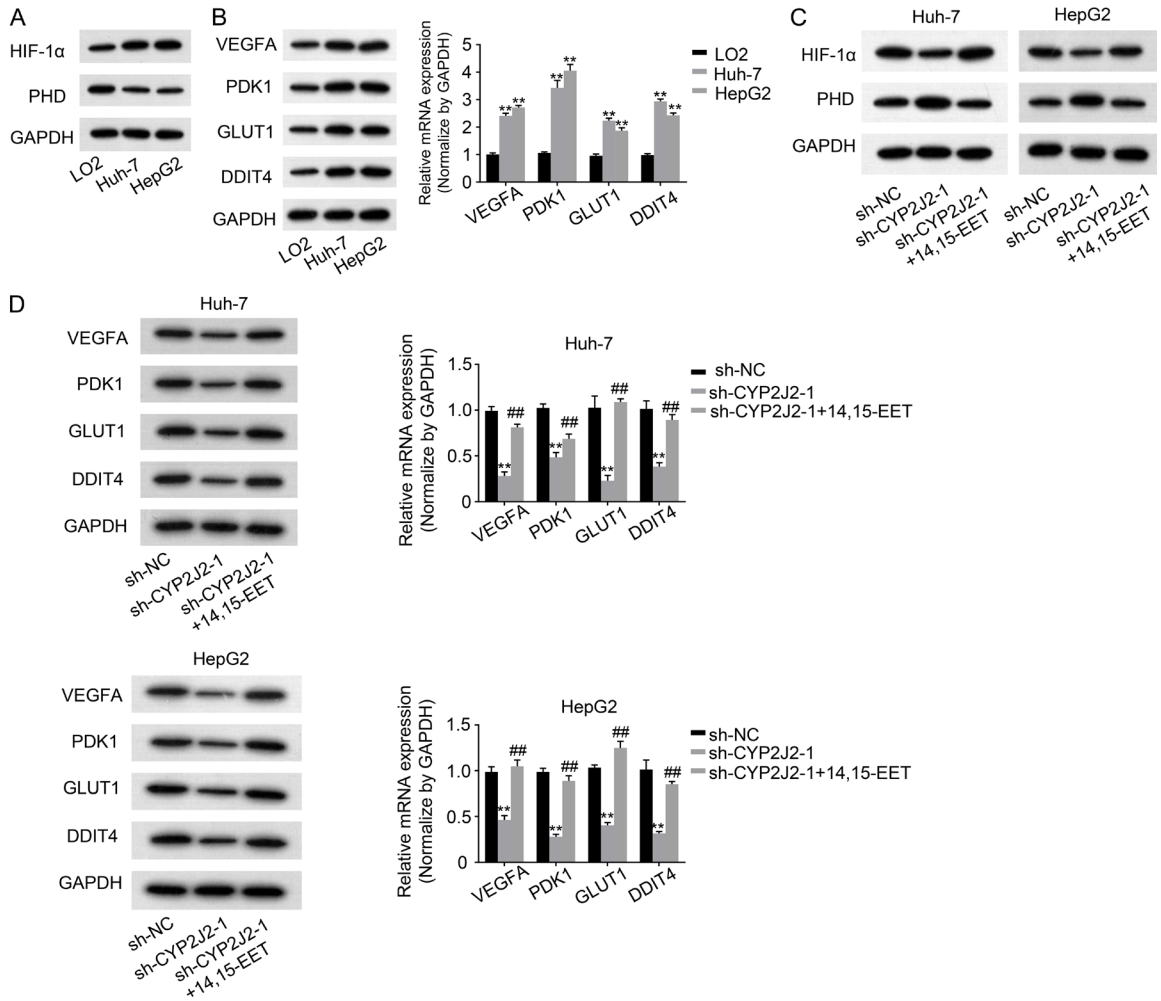
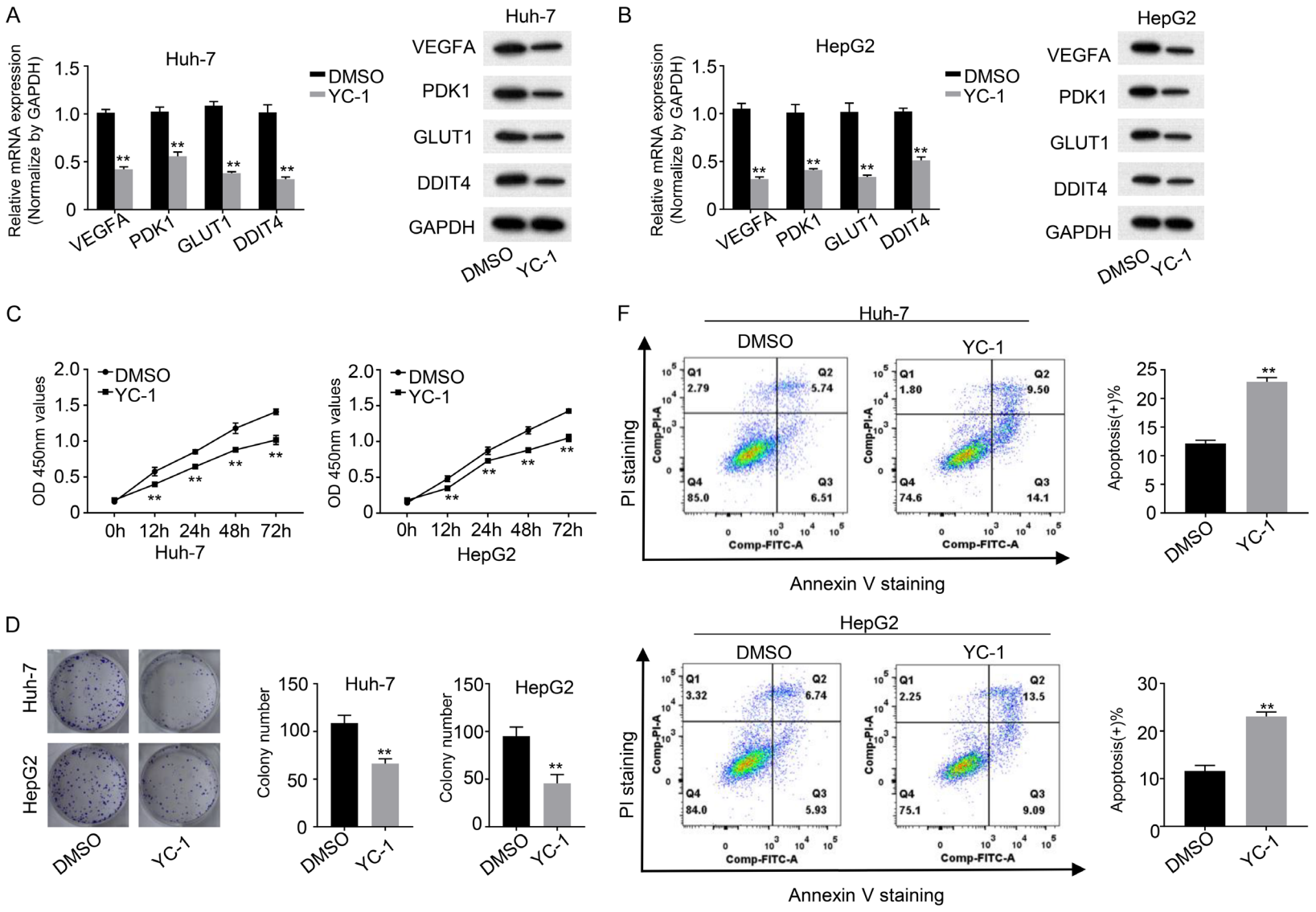


Figure 4. CYP2J2-EET metabolism maintained the stability of HIF-1α via reducing PHD level. A and B. Western blotting was performed in Huh-7, HepG2 and LO2 cells to detect the protein level of PHD, HIF-1α and its downstream factors including VEGFA, PDK1, GLUT1 and DDIT4. $^{**}P<0.01$ compared to LO2 cells; C and D. Protein expression of PHD, HIF-1α and its downstream factors were detected by western blot assays in Huh-7/HepG2-sh-NC, Huh-7/HepG2-sh-CYP2J2 treated with or without 14, 15-EET (100 nM). The grey value of protein bands were calculated by ImageJ software and were exhibited in the right panel. $^{**}P<0.01$ compared to sh-NC group, $^{\#\#}P<0.01$, compared to sh-CYP2J2-1 group.

ed that YC-1 treatment significantly reduced the proliferation and mobility of HCC cells when compared with DMSO group (**Figure 5C-E**). Inversely, HIF-1α treatment greatly improved the apoptosis rate of Huh-7 and HepG2 cells (**Figure 5F**).

Inhibition of HIF-1α alleviated HCC development promoted by 14, 15-EET

Aiming to investigate whether CYP2J2 and EETs affected tumorigenesis *in vivo*, Huh-7-sh-CYP2J2, Huh-7-sh-NC, HepG2-sh-CYP2J2 and HepG2-sh-NC cells were inoculated into nude mice by subcutaneous injection to perform tumor growth assays. As shown in **Figure 6A**, inhibition of CYP2J2 expression significantly attenuated tumors size *in vivo*. Besides, the weight of tumors was also less in Huh-7-sh-CYP2J2 and HepG2-shCYP2J2 cells injected mice when compared with control mice (**Figure 6B**). Consistent with cellular experiments, 14, 15-EET treatment obviously promoted tumor growth and increased the weight of tumor *in vivo* (**Figure 6A** and **6B**). Moreover, in sh-CYP2J2+14, 15-EET group, addition of YC-1 partially attenuated the pro-tumor effect of 14, 15-EET on tumor growth (**Figure 6A** and **6B**).


A
Huh-7
Relative mRNA expression (Normalize by GAPDH)
DMSO
YC-1
VEGFA
PDK1
GLUT1
DDIT4
GAPDH
B
HepG2
C
OD 450nm values
0h 12h 24h 48h 72h
Huh-7
HepG2
D
Colony number
F
Huh-7
HepG2
PI staining
Annexin V staining
Comp-FITC-A
Comp-PI-A
Q1 2.79
Q2 5.74
Q4 85.0
Q3 6.51
Q1 1.80
Q2 9.50
Q4 74.6
Q3 14.1
Q1 3.32
Q2 6.74
Q4 84.0
Q3 5.93
Q1 2.25
Q2 13.5
Q4 75.1
Q3 9.09
Apoptosis(+)%

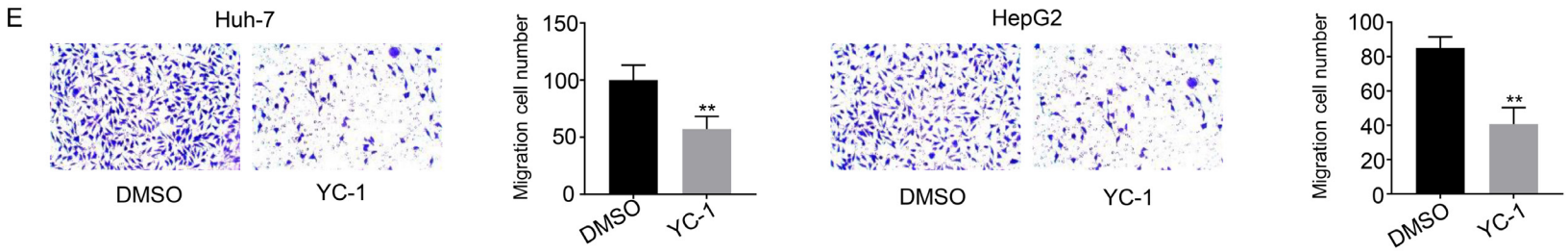


Figure 5. Inhibition of HIF-1α attenuated the proliferation and metastasis of HCC cells. A and B. RT-qPCR and western blot assays to quantify the endogenous level of HIF-1α downstream factors in Huh-7 and HepG2 cells; C-E. The CCK-8, colony formation and transwell experiments in Huh-7 and HepG2 cells treated with or without YC-1 (10 μM); F. Apoptosis analysis in Huh-7 and HepG2 cells treated with YC-1 (10 μM) or DMSO. $^{**}P<0.01$ compared to DMSO group.

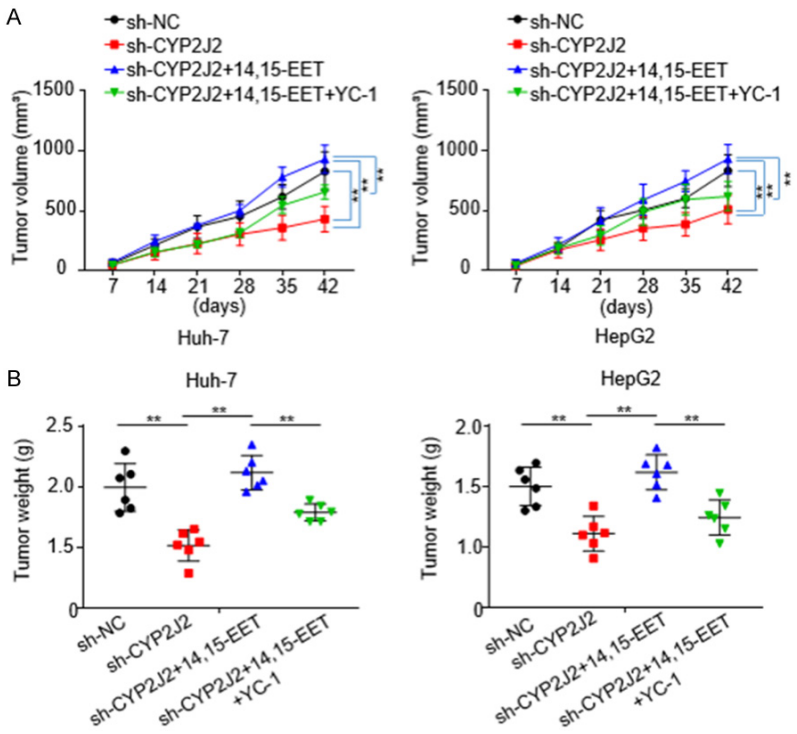


Figure 6. Inhibition of HIF-1α alleviated HCC development promoted by 14, 15-EET treatment. A and B. Tumor volume curve and tumor weight of mice subcutaneously injected with HCC cells with combination of 14, 15-EET (30 μg/kg/2 d) and YC-1 (10 mg/kg/2 d) or 14, 15-EET (30 μg/kg/2 d) alone. $^{**}P<0.01$.

Discussion

In order to explore more effective strategies for clinical treatment of HCC, better understanding of molecular mechanism underlying the tumorigenesis of HCC is crucial. CYP2J2 is one member of CYP450 family that is responsible for metabolism of arachidonic acids to EETs. Therefore, overexpression of CYP2J2 or inhibition of sEH can maintain high level of EETs. In the current study, ELISA experiments showed that the amounts of 11, 12-EET, 14, 15-EET but not 5, 6-EET and 8, 9-EET were elevated in HCC tissue and cell lines in comparison with healthy sample and normal cells, which was result from the increased level of CYP2J2 and less amount of sEH in HCC tissue and cell lines (**Figure 1**).

Except for the function of CYP2J2-EETs pathway in inflammation, angiogenesis, cardiovascular diseases and metabolic related diseases, accumulating researches suggest the increased abundance of CYP2J2 and EETs possibly contributes to the malignant development of tumor. In both HCC tissues and cell lines, the level of CYP2J2 was obviously elevated. The other two member of CYP 450 family, CYP2C8 and CYP2C9 have been implied correlated with the prognosis of cancer [22], indicating the potential role of CYP2J2 in HCC prognosis prediction. In next step, with more collection of clinical data, the prognosis value of CYP2J2 will be investigated. *In vitro* experiments revealed that both 14, 15-EET and CYP2J2 could promoted the cell proliferation, invasion and mobility of HCC cells. Moreover, the effect of CYP2J2 and 14, 15-EET on HCC tumor growth and metastasis was further confirmed by tumorigenesis studies established in xenograft mice model (**Figures 2**, **3** and **6**).

CYP2J2-EET pathway has been extensively investigated in metabolic disease and angiogenesis, and is associated with several critical signaling pathway such as PPARs [23], TGF-β/smad [24]. As for carcinoma, CYP2J2 and 14, 15-EET may contribute to the development and drug-resistance of cancer through PI3K/AKT pathway [18]. Recent report in breast cancer implied that 14, 15-EET could induce EMT phenotype [16]. In general, the malignant process of HCC is accompanied by angiogenesis and EMT. Hypoxia is one characteristics of solid tumor microenvironment and main element that induce the occurrence of angiogenesis and EMT phenotype [25, 26]. The heterodimeric transcription factor hypoxia inducible factor is a hypoxia responsive factor comprising a hypoxia-induced subunit HIF-1*α* and a constitutive subunit HIF-1*β*. Under normoxia, HIF-1α is hydroxylate on two proline residues by prolyl hydroxylases (PHDs) and then degraded by von Hippel-Lindau (pVHL)-mediated ubiquitin-proteasome pathway. In hypoxic conditions, PHDs is inhibited, so that HIF-1α is stabilized, and translocates into the nucleus and dimerizes with HIF-1β to activate the transcription of downstream genes containing the hypoxia response element (HRE). The accumu-

lation of HIF-1α has been proved to be closely associated with tumor-associated angiogenesis, proliferation and metastasis of tumor cells [27]. Increased abundance of HIF-1α caused by inhibition of PHD promotes the protective function of EET on cardiomyocytes [28]. According to these finding, we speculated that CYP2J2-EET pathway might participate in HCC development through stabilizing HIF-1α. In the current study, the protein level detection proved that HIF-1α together with its downstream factors including VEGFA, PDK1, GLUT1 and DDIT4 were up-regulated in HCC cell lines while the expression of PHD was significantly reduced. Conversely, knockdown of CYP2J2 enhanced the abundance of PHD, resulting in obvious degradation of HIF-1α in HCC cells. However, the effect of knockdown of CYP2J2 on HIF-1α was rescued by addition of 14, 15-EET, indicating that elevated level of CYP2J2 or 14, 15-EET could promote the stability of HIF-1α via repressing PHD expression in HCC cells (**Figure 4**). In HCC cells, we found treatment of HIF-1α inhibitor (YC-1) could attenuate the proliferation and metastasis of HCC cells. Similar results were also observed in xenograft mice, addition of YC-1 reduced the HCC cell growth and metastasis promoted by 14, 15-EET addition (**Figures 5** and **6**). Despite the oxygen-dependent manner, HIF-1α is also modulated by oxygen-independent manner. We have clarified the relation between CYP2J2-EET pathway enhanced level of HIF-1α through inhibiting PHD, but the mechanism underlying the effect of CYP2J2-EET on PHD expression still needed to be elucidated. Additionally, HIF-1α is critical for hypoxia-mediated sorafenib resistance in HCC [29]. The effect of CYP2J2-EET pathway on chemotherapy resistance also deserved to be deeply verified.

In summary, we defined an oncogenic role of CYP2J2-EET pathway in the development of HCC. The up-regulated CYP2J2 and down-regulated sEH in HCC tissue and cells resulted in the accumulating of certain type of EETs (11, 12-EET, 14, 15-EET) in HCC. The elevated CYP2J2-EET increased the abundance of HIF-1α by inhibiting PHD expression, which in further significantly promoted the malignant development of HCC. Collectively, these results suggested that CYP2J2 was one potential prognosis marker for HCC patients, and CYP2J2-EET pathway might be novel therapeutic target for HCC treatment.

Conclusion

The present research indicates that increased expression of CYP2J2 in HCC cells up-regulates the expression of 14, 15-EET, which inhibits the expression of PHD and improves the stability of HIF-1α. The accumulated HIF-1α further promotes the malignant development of HCC. Hence, CYP2J2-EET metabolism pathway may serve as therapeutic target for HCC clinical treatment.

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Disclosure of conflict of interest

None.

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