RETRACTED ARTICLE

RETRACTED ARTICLE: PHD2 acts as an oncogene through activation of Ras/Raf/MEK/ERK and JAK1/STAT3 pathways in human hepatocellular carcinoma cells

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ABSTRACT

Background: Prolyl hydroxylase domain proteins (PHD2) is an oxygen sensor that is able to induce hypoxia-inducible factor-α (HIF-α) degradation under normoxic condition. The present paper designed to reveal the function of PHD2 in hepatocellular carcinoma (HCC) cells proliferation, migration and invasion.

Methods: qRT-PCR and Western blot were carried out to see the expression of PHD2 in HCC tissues and cell lines. PHD2 expression in Huh7 and HepG3B cells was overexpressed or suppressed by transfection and then the changes of cell proliferation, migration and invasion were detected by CCK-8 assay, transwell assay and Western blot.

Results: PHD2 was highly expressed in HCC tissues and cell lines (Huh7, Hep3B, SK-HEP-1, HCCLM3 and MHCC97) as relative to para-cancerous non-tumour tissues and a normal hepatocyte line MIHA. PHD2 overexpression promoted Huh7 and Hep3B cells viability, migration and invasion. Meanwhile, CyclinD1, c-Myc, MMP-2, MMP-9 and Vimentin were up-regulated, while p53 was down-regulated by PHD2 overexpression. PHD2 silence led to a contrary impact. Further, PHD2 overexpression up-regulated Ras and Raf expression and induced phosphorylation of MEK, ERK, JAK1 and STAT3.

Conclusion: PHD2 exhibited pro-tumour functions in HCC cells. PHD2 promoted HCC possibly through Ras/Raf/MEK/ERK and JAK1/STAT3 pathways.

HIGHLIGHTS

1. PHD2 is highly expressed in HCC tissue and cell lines;
2. PHD2 promotes the proliferation of Huh7 and HepG3B cells;
3. PHD2 enhances Huh7 and HepG3B cells migration and invasion;
4. PHD2 activates Ras/Raf/MEK/ERK and JAK1/STAT3 signalling.

Introduction

Prolyl hydroxylase domain proteins (PHDs) are oxygen sensor proteins located intracellular that directly sense oxygen partial pressure and they exert activities depending on the intracellular oxygen partial pressure. Under normoxic conditions, PHDs hydroxylate two proline residues (pro402 and pro564) of hypoxia-inducible factor-α (HIF-α) at the oxygen-dependent degradation domain and lead to HIF-α degradation in a von-Hippel-Lindau tumour suppressor protein (pVHL)-dependent way [1]. However, under hypoxic conditions, the PHD-dependent hydroxylation is limited. HIF-α is accumulated and then involves in the regulation of hypoxia adaption [1]. In mammalian cells, three subtypes of PHDs are identified, including PHD1, PHD2 and PHD3, among which PHD2 is the most abundant one and is the main factor in modulating the level of HIF-α [2]. So, PHD2 has been considered as a key enzyme in regulating hypoxic response.

During the development of solid tumours, hypoxic microenvironment is formed due to rapid growth of tumour cells which consumed a great deal of oxygen. In the clinic, the value of hypoxia as a tumour hallmark has been recognized and its level is correlated with tumour metastasis and poor survival in a broad spectrum of cancers [3]. Hepatocellular carcinoma (HCC) is one such solid tumour that hypoxic condition contributes a lot in the progression of tumours [4]. HCC is a common primary liver cancer with a high incidence of distal metastasis. Tumour excision combined with other treatments has been applied in the clinic to manage this cancer. However, the outcome is still unsatisfactory, since recurrence is observed in about 50–60% of HCC patients [5]. The main reason for such high recurrence rate after treatment is micro-metastasis and rapid growth of tumours [6].

Considering PHD2 and solid tumours are both correlated with hypoxic conditions, the link between PHD2 and human tumours has been explored. For instance, high expression of PHD2 could indicate poor prognosis of lung adenocarcinoma, indicating its application value as a tumour biomarker [7]. Besides, PHD2 was reported to be essential for regulating...
glioblastoma progression through a HIF-α-dependent way [8]. Our previous study illustrated that PHD2 expression was associated with HCC progression and poor prognosis of patients with this cancer [9]. However, the deep functions of PHD2 in the onset and progression of HCC were not comprehensively analyzed.

In the present paper, we designed to investigate the function of PHD2 in the proliferation, migration and invasion of two HCC cell lines (Huh7 and HepG3B). Then, this study attempted to find out the underlying mechanisms which involved in PHD2’s functions. This study may deepen our understanding of PHD2 and suggest PHD2 as a novel target for the management of HCC.

Materials and methods

Clinical specimens

Twenty five pairs of HCC tissue specimens and their para-carcinoma non-tumour tissues were harvested from patients with HCC during tumour resection in The First Affiliated Hospital of Zhengzhou University (Zhengzhou, China), from December 2017 to November 2018. Written informed consent was signed before the usage of their specimens. No other therapies were applied in these individuals before tumour resection. Tissue specimens were rinsed by PBS twice and then used in qRT-PCR analysis for testing expression of PDH2. The study was approved by the Ethics Committees of The First Affiliated Hospital of Zhengzhou University and executed under the standard.

Cell culture

Human HCC cell lines Huh7 and HCCLM3 were from China Centre for Type Culture Collection (Wuhan, China), Hep3B and SK-HEP-1 were from American Type Culture Collection (ATCC, Manassas, VA, USA), and MHCC97 was a gift from Liver Cancer Institute and Zhongshan Hospital (Shanghai, China). A human normal hepatocyte line MIHA was from Shanghai Suer ShengWu Technology (Shanghai, China). The study was approved by the Ethics Committees of The First Affiliated Hospital of Zhengzhou University and executed under the standard.

Cell viability

Post-transfection, Huh7 and HepG3B cells were gathered and replanted in 96-well plates with 5000 cells/well. Forty eight hours later, 20 μL CCK-8 (Daiichi Molecular Technologies, Kyoto, Japan) was added. The plates were hatched at 37 °C for 2 h, after which the optical density at 450 nm was monitored by a Microplate Reader (Bio-Rad, Hercules, CA, USA).

Transwell assay

Cell migration was tested by utilizing a 24-well transwell system (Costar-Corning, New York, NY, USA). Cell invasion was tested as cell migration assay except the inserts were pre-coated with matrigel (Millipore, Bedford, MA, USA). In short, transfected Huh7 and HepG3B cells were gathered into serum-free medium and placed into the upper side of the system with 1 × 10^5 cells/mL. The lower side was filled with complete culture medium. Twenty four hours later, cells in lower side were stained with crystal violet (Sangon Biotech) and counted.

Western blot

Proteins in Huh7 and HepG3B cells following transfection as well as in tissue specimens were extracted by RIPA butter (Beyotime). Immunoblotting was carried out by using antibodies against PHD2 (orb6804), CyclinD1 (orb77046), p53 (orb178524), c-Myc (orb89268), MMP-2 (orb11061), MMP-9 (orb97357), Vimentin (orb34040), Ras (orb379158), Raf (orb38563), t-MEK (orb27588), p-MEK (orb14875), t-ERK (orb95116), p-ERK (orb10060), t-JAK1 (orb214134), p-JAK1 (orb223036), t-STAT3 (orb89397), p-STAT3 (orb14778) and β-actin (orb10033, San Francisco, CA, USA). Positive bands were developed by BeyoECL Plus (Beyotime) and the grey levels were quantified by Image Lab™ software (Bio-Rad).

Statistics

Data presented as mean ± SD. Statistical analyses were done by SPSS 19.0 software (Chicago, IL, USA) with ANOVA or
Student t-test. Statistical differences were set at $p < .05$ and indicated as asterisks in figures.

**Results**

**Elevated PHD2 expression occurred during HCC**

qRT-PCR analysis was utilized for testing PHD2 expression in 25 pairs of HCC tissues and the para-cancerous non-tumour tissues. As seen in Figure 1(A), PHD2 levels in tumour tissues were significantly high as relative to the non-tumour tissues ($p < .001$). Three tumour tissues and three para-cancerous non-tumour tissues were randomly selected, and protein levels of PHD2 in these tissues were measured by Western blot. Consistently, the mRNA and protein levels of PHD2 were higher in HCC cell lines (Huh7, Hep3B, SK-HEP-1, HCCLM3 and MHCC97) and a human normal hepatocyte line MIHA were measured by qRT-PCR and Western blot. *$p < .05$, **$p < .01$, ***$p < .001$. 

Figure 1. Elevated PHD2 expression occurred during HCC. (A) qRT-PCR analysis was utilized for testing PHD2 expression in 25 pairs of HCC tissues and the para-cancerous non-tumour tissues. (B) Three tumour tissues (#1, 2, 3) and three para-cancerous non-tumour tissues (#1, 2, 3) were randomly selected. Protein levels of PHD2 in these tissues were measured by Western blot. (C) mRNA and (D) protein levels of PHD2 in HCC cell lines (Huh7, Hep3B, SK-HEP-1, HCCLM3 and MHCC97) and a human normal hepatocyte line MIHA were measured by qRT-PCR and Western blot. *$p < .05$, **$p < .01$, ***$p < .001$. 

Figure 2. PHD2 expression in Huh7 and Hep3B cells was altered by transfection. A PHD2 expressing plasmid (pEX-PHD2) and two specific shRNA against PHD2 (sh-PHD2#1 and sh-PHD2#2) were transfected into Huh7 and Hep3B cells. Protein levels of PHD2 in (A) Huh7 and (B) Hep3B cells were tested by Western blot. *$p < .01$, **$p < .001$.
level than other three cell types, the two cell lines were utilized in the following.

**PHD2 accelerated Huh7 and Hep3B cells proliferation**

A PHD2 expressing plasmid (pEX-PHD2) and two kinds of specific shRNA against PHD2 (sh-PHD2#1 and sh-PHD2#2) were transfected into Huh7 and Hep3B cells. Viability of (A) Huh7 and (B) Hep3B cells was tested by CCK-8 assay. Protein levels of CyclinD1, p53 and c-Myc in (C, E) Huh7 and (D, F) Hep3B cells were examined by Western blot. *p < .05, **p < .01, ***p < .001.

Figure 3. PHD2 accelerated Huh7 and Hep3B cells proliferation. A PHD2 expressing plasmid (pEX-PHD2) and two kinds of specific shRNA against PHD2 (sh-PHD2#1 and sh-PHD2#2) were transfected into Huh7 and Hep3B cells. Viability of (A) Huh7 and (B) Hep3B cells was tested by CCK-8 assay. Protein levels of CyclinD1, p53 and c-Myc in (C, E) Huh7 and (D, F) Hep3B cells were examined by Western blot. *p < .05, **p < .01, ***p < .001.

Next, cell viability and expression of proliferation-associated proteins were analyzed. Figure 3(A,B) showed that viability of Huh7 and Hep3B cells was promoted by pEX-PHD2 and declined by sh-PHD2#1 and sh-PHD2#2 (p < .01, Figure 2(A,B)).

Efficiency tested by Western blot revealed that PHD2 level was significantly overexpressed by pEX-PHD2 (p < .001) while declined by both sh-PHD2#1 and sh-PHD2#2 (p < .01, Figure 2(A,B)).
Meanwhile, protein levels of CyclinD1 and c-Myc were elevated by pEX-PHD2 \( (p < .001) \) and repressed by two kinds of sh-PHD2 \( (p < .05, \text{Figure 3(C–F)}) \). Contrary trends were observed in p53 expression, as pEX-PHD2 suppressed p53 expression \( (p < .05) \), while both sh-PHD2#1 and sh-PHD2#2 raised it \( (p < .001, \text{Figure 3(C–F)}) \).

**Figure 4.** PHD2 enhanced Huh7 and Hep3B cells migration and invasion. A PHD2 expressing plasmid (pEX-PHD2) and two glasses of specific shRNA against PHD2 (sh-PHD2#1 and sh-PHD2#2) were transfected into Huh7 and Hep3B cells. Migration of (A) Huh7 and (B) Hep3B cells was tested by Transwell assay. Invasion of (C) Huh7 and (D) Hep3B cells was tested by Transwell assay. \* \( p < .05 \), \* \* \( p < .01 \), \* \* \* \( p < .001 \).
PHD2 enhanced Huh7 and Hep3B cells migration and invasion

The role of PHD2 in HCC cells migration and invasion was also explored. Figure 4(A,B) displayed that migration of Huh7 and Hep3B cells was significantly promoted by pEX-PHD2 and repressed by sh-PHD2 (sh-PHD2#1 and sh-PHD2#2) \((p < .05 \text{ or } p < .01)\). Similar invasion of these two cell lines was enhanced by pEX-PHD2 and suppressed by two classes of sh-PHD2 \((p < .05 \text{ or } p < .01, \text{Figure 4}(C,D))\).

The expression of migration- and invasion-associated proteins was then examined to further confirm the role of PHD2. Figure 5(A–D) displayed that MMP-2, MMP-9, and Vimentin protein levels were raised by pEX-PHD2 \((p < .01 \text{ or } p < .001)\) and lowered by both sh-PHD2#1 and sh-PHD2#2 \((p < .05)\). Figure 5.

PHD2 activated ras/raf/MEK/ERK and JAK1/STAT3 signalling

The deep signalling pathways responsible for PHD2’s tumour promoting effects were then explored. Results in Figure 6(A–D) displayed that protein expression of Ras and Raf, as well as the phosphorylation of MEK and ERK were notably increased by pEX-PHD2 \((p < .001)\) and lowered by sh-PHD2#1 and sh-PHD2#2 \((p < .05 \text{ or } p < .01)\). Also, the phosphorylation of JAK1 and STAT3 was raised by pEX-PHD2 \((p < .01 \text{ or } p < .001)\) and lowered by both sh-PHD2#1 and sh-PHD2#2 \((p < .05, \text{Figure 6}(E–H))\).

Discussion

The significance of PHD2 expression during HCC has been revealed in our previous study, in which high level of PHD2 has been demonstrated to be associated with the clinical features and poor prognosis of patients with HCC [9]. The data in the present work was in line with previous findings. As compared to para-cancerous non-tumour tissues and a human normal hepatocyte line MIHA, PHD2 expression was much high in HCC tissues and HCC cell lines (Huh7, Hep3B, SK-HEP-1, HCCLM3, and MHCC97). Further data illustrated that high expression of PHD2 may participate in the progression of HCC. PHD2 overexpression accelerated the proliferation, migration, and invasion of Huh7 and Hep3B cells, while PHD2 silence suppressed them. Additionally, PHD2 exerted its function probably through Ras/Raf/MEK/ERK and JAK1/STAT3 pathways.

Hypoxic microenvironment is a common feature during the progression of HCC [4]. HCC cells can survive and rapidly proliferate even under hypoxic conditions. In this procedure, the balance between tumour-promoting proteins and anti-tumour proteins is disturbed. p53 is a well-known anti-tumour factor that has been considered as “molecular police” to monitor the integrity of genome during DNA replication [10]. Once
activated, p53 will bind to DNA and induce the accumulation of p21. p21 binds with the complex of Cyclin-CDK and ultimately induces cell cycle arrest. c-Myc is a proto-oncogene that is essential for the proliferation of most tumour cells [11]. High expression of c-Myc will mediate the stabilization of cell-cycle proteins, like CyclinB1 [12]. Herein, the viability of Huh7 and Hep3B cells was found to be raised by PHD2 overexpression, which accompanied by CyclinD1 and c-Myc up-regulation and p53 down-regulation. All these data indicated the pro-proliferating function of PHD2 in HCC cells.

Figure 6. PHD2 activated Ras/Raf/MEK/ERK and JAK1/STAT3 signalling. A PHD2 expressing plasmid (pEX-PHD2) and two types of specific shRNA against PHD2 (sh-PHD2#1 and sh-PHD2#2) were transfected into Huh7 and Hep3B cells. Protein levels of Ras, Raf, MEK, and ERK in (A, C) Huh7 and (B, D) Hep3B cells were examined by Western blot. Protein levels of JAK1 and STAT3 in (E, H) Huh7 and (F, G) Hep3B cells were examined by Western blot. *p<.05, **p<.01, ***p<.001.
The ability of invasion and metastasis are important factors that lead to poor prognosis of patients with HCC. Investigating the mechanisms of tumour invasion and metastasis has a promise in improving the outcome of HCC patients [13]. The present paper found that PHD2 overexpression was able to accelerate the migration and invasion of Huh7 and Hep3B cells. Besides, the motility of tumour cells is mediated by epithelial-mesenchymal transition (EMT) related factors, including MMPs and Vimentin. MMPs are responsible for degradation of extracellular matrix (ECM) that confers cell metastatic potential [14]. Vimentin is another hallmark of EMT, as mesenchymal cells are characterized by high level of Vimentin [15]. Herein, MMP-2, MMP-9 and Vimentin were all found to be up-regulated by PHD2 overexpression, which further confirmed the pro-migrating and pro-invasive functions of PHD2 in HCC cells.

Sporadic literatures studied PHD2 in human cancer and its contradictory role has been reported [16]. Some researchers reported PHD2 as a tumour suppressor as its expression was able to suppress the initiation of melanoma [17]. Likewise, the silence of PHD2 would overcome apoptosis of breast cancer cells, which helped tumour cells against glucose starvation [18]. However, some researchers suggested the pro-tumour functions of PHD2. As reported by Leite de Oliveira et al., genetic inactivation of PHD2 showed anti-tumour and anti-metastatic effects of which sensitized tumour cells to chemotherapy [19]. Later literature further confirmed this result that PHD2 promoted metastasis in a spontaneous tumour model [20]. It seems that PHD2 functions as pro- or anti-tumour factor depending on different cell types [16]. In the present paper, pro-tumour function of PHD2 has been observed in Huh7 and Hep3B cells, which added our understanding of PHD2 in human cancers.

Initially, PHD2 has been considered to exert its role in carcinogenesis via HIF-dependent signalling [21]. However, recent paper illustrated that PHD2 also worked through HIF-independent pathways, like AKT/mTOR [17], PP2A/B55α [18], EGFR [22], NF-αB [23], and TGF-β1 [24]. The present paper illustrated Ras/Raf/MEK/ERK and JAK1/STAT3 as two novel signalling pathways that can be activated by PHD2 in the tumour cells. Ras is a proto-oncogene and acts as an upstream activator of Raf. Raf binds with Raf and activates Raf indirectly, generally through tyrosine kinase. Once activated, Raf induces the activation of MEK/ERK signalling and impacts the proliferation, migration and invasion of various cancers, including HCC [25,26]. JAK1/STAT3 is another signalling that can quickly respond to various stresses, like cytokine stimulation [27]. Activation of JAK1/STAT3 signalling promotes the initiation and progression of cancers, through controlling cell proliferation, differentiation and metastasis [28–30]. This study further suggested the effect of PHD2 via HIF-independent pathways and two novel such pathways were revealed, i.e. Ras/Raf/MEK/ERK and JAK1/STAT3 pathways.

To conclude, the present paper illustrated the pro-tumour functions of PHD2 in HCC cells. Its expression was high in HCC tissues and cell lines as compared to non-tumour tissues and normal cell lines. PHD2 overexpression largely accelerated HCC cell proliferation, migration and invasion. PHD2 promoted HCC possibly through HIF-independent pathways, including Ras/Raf/MEK/ERK and JAK1/STAT3 pathways.

Author contributions
Junqiang Guo conceived and designed the experiments. Junqiang Guo and Zhi Lan performed the experiments and analyzed the data. Junqiang Guo and Zhi Lan wrote the manuscript.

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All authors are informed and agree to publish. The authors declare that they have no competing interests.

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Data availability
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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