Loss of PIG3 increases HIF-1α level by promoting protein synthesis via mTOR pathway in renal cell carcinoma cells

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ABSTRACT

PIG3 is a target of the tumor suppressor p53 and is thought to be involved in p53-mediated cell apoptosis. Although PIG3 is similar to oxidoreductases involved in generating ROS, whether PIG3 would regulate HIF-1α was never characterized directly. Here we demonstrated that knockdown of PIG3 by transfecting with specific siRNA could increase the expression of HIF-1α in several human cancer cell lines, including CAKI, FTC-133 and A549. It indicates that PIG3 may be involved in the regulation of HIF-1α. Furthermore, we revealed that PIG3-siliencing increased HIF-1α protein level through promoting its protein biosynthesis via mTOR pathway. In addition, the effect of PIG3 on the production of HIF-1α was further related to VEGF secretion and cell migration. PIG3-downregulation increased the secretion of VEGF and promoted the migration of renal cancer cells obviously. Taken together, these data suggest that PIG3 was involved in HIF-1α regulation, and reveal a novel signaling pathway of PIG3/HIF-1α in the regulation of cell migration in renal cell carcinoma.

INTRODUCTION

Hypoxia-inducible factor-1α (HIF-1α) is a major transcription factor responsible for the induction of hypoxia-response elements (HREs)-containing genes that facilitate adaptation and survival of cells and the whole organism under hypoxia [1]. To date, there are more than 100 hypoxia-inducible genes identified with varying functions, including those involved in erythropoiesis/iron metabolism, angiogenesis, glucose, metabolism, cell proliferation/survival and apoptosis [2, 3]. Overexpression of HIF-1α was found in various human cancers, HIF-1α downstream genes have been identified that are widely involved in the malignant features of tumors, including angiogenesis, invasion, metastasis, and drug resistance [4-6].

HIF-1α is regulated in both oxygen-dependent and oxygen-independent manner. The ubiquitination-mediated degradation is the most important regulator of HIF-1α levels. In normoxia, hydroxylation of two proline residues and acetylation of a lysine residue in its oxygen-dependent degradation domain (ODDD) promote the association of HIF-1α with the von Hippel-Lindau (pVHL) ubiquitin E3 ligase complex, leading to HIF-1α degradation via ubiquitin-proteasome pathway [7-9]. Besides pVHL, p53, HSP90, cJun, etcetera, have also been found to relate to HIF-1a ubiquitination and stability [10]. In addition, the mitogen-activated protein kinase (MAPK) pathway and PI3K/AKT/mTOR pathway seem to play important role in HIF-1α expression [11-13]. Here, we found that knockdown of PIG3 mediated by siRNA transfection increased HIF-1α protein level.

PIG3 (p53 inducible gene 3), also called TP53I3 (tumor protein p53-inducible protein 3), is one of the P53
protein target originally indentified by Polyak et al [14]. To date, PIG3 has been found to participate in apoptosis, the generation of ROS, DNA damage response and mediating cancer cell death [15-17]. It is well known that PIG3 is a target of p53, but the down-stream signaling pathway of PIG3 is poorly understood.

In the present study, we showed that PIG3 may be involved in HIF-1α regulation. And PIG3 knockdown mediated by RNAi could up-regulate VEGF secretion via HIF-1α to promote renal cancer cell migration, which contributes to improve our knowledge of PIG3 function and HIF-1α regulation.

RESULTS

PIG3 regulates cellular HIF-1α protein level

We first investigated whether PIG3 regulated HIF-1α expression in human renal cell carcinoma cell. Knockdown the PIG3 expression by transfecting pig3-specific siRNA in CAKI-1 cells, then the PIG3-silencing cells were exposed to CoCl2-induced hypoxia-mimic system for 6 hours to detect the HIF-1α protein. As shown in Figure 1A, the protein levels of cellular HIF-1α were obviously higher than those in the hypoxic negative control cells. Similar results were found in other cell lines, such as follicular thyroid cancer FTC-133 cells and non-small-cell lung cancer A-549 cells.

Moreover, the similar profiles were showed by transfecting two different siRNAs targeting PIG3 into CAKI cell (Figure 1B) and the same story was found in hypoxic condition under 1% O2 (Figure 1C).

Furthermore, re-expression of PIG3 in siRNA experiment was done to rescue the phenotype of HIF-1α increase, avoiding off target-effects. We conducted PIG3 complementation experiments by transfecting a plasmid carrying a point mutant PIG3 sequence (Figure 1D) into the PIG3-silenced CAKI cells. This plasmid expresses same protein, but its transcripts could not be disrupted by PIG3-siRNA due to their unpairing (Figure 1D). PIG3 complementation effectively blocked the increase of HIF-1α protein levels induced by PIG3 loss (Figure 1D), indicating that PIG3 plays a causal role in the control of cellular HIF-1α protein levels.

Knockdown of PIG3 Up-regulates HIF-1α level through promoting protein biosynthesis

HIF-1α is regulated by various pathways at different levels [18]. Firstly, we detected the levels of HIF-1α mRNA in the PIG3-knockdown CAKI cells to examine whether PIG3 affects the transcription of HIF-1α. However, PIG3 silencing did not change the level of HIF-1α mRNA, indicating that PIG3 does not affect the transcription of the HIF-1α gene (Figure 2A).

Secondly, we investigated the alteration kinetics of the HIF-1α protein levels in CAKI cells transfected with negative control or PIG3 siRNA responding to hypoxia. As shown in Figure 2B, in PIG3-knockdown CAKI cells, the levels of HIF-1α protein went up earlier than those in the control cells on exposure to hypoxia. HIF-1α protein levels increased significantly at the 2-hour time point in PIG3-silencing cells. In contrast, in the control cells, at the same 2-hour time point, the levels of HIF-1α protein was much lower (Figure 2B). The result suggests that the loss of PIG3 is likely to promote the production of the HIF-1α protein.

To further examine whether the induction of HIF-1α protein in PIG3-knockdown cells is associated with its degradation, we exposed the cells to the protein biosynthesis inhibitor cycloheximide (CHX). The HIF-1α protein levels changed in the similar pattern in both cell (Figure 2C). The data indicate that PIG3 loss did not inhibit the protein degradation of HIF-1α.

PIG3-silencing promotes the production of HIF-1α via PI3K/mTOR pathway

It well known that PI3K/Akt/mTOR signaling pathway mediates HIF-1α translation in various cancer cells [11, 12, 19, 20]. We examined whether compounds targeting PI3K/mTOR pathway would attenuate the production of HIF-1α protein induced by PIG3-knockdown. As shown in Figure 3A, treatment of rapamycin at 10 nM for 24 h or wortmannin 100 nM for 8 h significantly inhibited PIG3-silencing-stimulated HIF-1α going up in CAKI cells.

To further determine the role of mTOR in PIG3-loss-induced HIF-1α expression, we examined up-regulation of HIF-1α protein level induced by PIG3 loss after down-regulation of Raptor by transiently transfection of siRNA. As shown in the Figure 3B, down-regulation of Raptor significantly inhibited PIG3-loss-induced increase of HIF-1α protein level. These results indicated that down-regulation of PIG3 may induce HIF-1α translation in hypoxia via mTOR pathway.

PIG3-silencing promotes the VEGF secretion and migration of renal cancer cells

Vascular endothelial cell growth factor (VEGF) is one of the major target genes HIF-1α [6, 21]. To test whether PIG3 knockdown impairs HIF-1α biological function, we detected the VEGF secretion in the PIG3-silenced CAKI and 769-P cells. As shown in Figure 4A, Silencing PIG3 significantly increased the protein secretion of VEGF under hypoxic conditions. VEGF is the most potent endothelial-specific mitogen and is known to directly participate in angiogenesis and metastasis.
Figure 1: Knockdown of PIG3 up-regulates HIF-1α. A. PIG3 down-regulation mediated by siRNA-transfection significantly increased HIF-1α protein level in CAKI, FTC-133 and A549 cells. Cells were transfected with PIG3-siRNA or negative control siRNA for 48 h before CoCl₂-induced hypoxia treatment. B., treatments with both PIG3 siRNA-2 and PIG3 siRNA-3 led to HIF-1α increase in CAKI cells under CoCl₂-induced hypoxia-mimic system for 6 hours. C., HIF-1α in PIG3 siRNA-1-transfected CAKI cells also increased under hypoxia (1% O₂) for 6 h. D. Complementation with PIG3 blocked HIF-1α increase induced by PIG3-siRNA. CAKI cells were transfected with the mutant PIG3 plasmid 24 h after PIG3-siRNA treatment. Protein levels were analyzed by Immunoblotting, GAPDH was employed a loading control. All the experiments above were conducted thrice.
Figure 2: Knockdown of PIG3 up-regulates prevents HIF-1α by promoting protein biosynthesis. A., Real-Time PCR analysis showed that PIG3-silencing in CAKI cells did not increase HIF-1α mRNA level. Data shown are fold-change relative to the HIF-1α levels in cells transfected with negative control siRNA (with normalization relative to GAPDH levels). B., Silencing PIG3 in CAKI cells affected the kinetics of the HIF-1α protein when the cells were exposed to hypoxic conditions. C., PIG3 silencing did not affect the degradation of HIF-1α protein under hypoxia. Forty-eight hours after transfection with PIG3-siRNA or negative control siRNA, CAKI cells were pretreated under hypoxia for 4 h followed by treatment with 100 μg/mL cycloheximide (CHX) to block protein synthesis for the indicated times. The mean values from two experiments are connected by the lines. Protein levels were analyzed by Immunoblotting, GAPDH was employed a loading control. All the experiments above were conducted at least twice.
To validate the role of PIG3 in metastasis-related events, we detected the migration of PIG3-silenced CAKI and 769-P cells. Knocking down PIG3 obviously increased the migration activities of CAKI and 769-P cells. However, HIF-1α-knockdown obviously blocked the increase of cell migration induced by PIG3 loss (Figure 4C), indicating that PIG3-loss increases cell migration in HIF-1α dependent way to some extent.

DISCUSSION

In the present study, we showed that down-regulation of PIG3 by specific siRNA transfection induced the expression of HIF-1α in CAKI cells. Similar results were replicated in another human cancer FTC-133 and A549 cell lines. PIG3 appeared to involve in regulating HIF-1α. Moreover, we found that knockdown of PIG3 would increase HIF-1α protein level via promoting its protein biosynthesis mediated by mTOR pathway. Furthermore, PIG3-silencing increased the secretion of VEGF and promoted the migration of renal cancer cells.

The relationship between p53 and HIF-1α has been the subject of several studies, which significantly affect cancer progression and compromise treatment outcomes [24]. Kaluzova et al. reported that activated p53 mediates an accelerated degradation of HIF-1α protein, without affecting significantly HIF-1α transcription [26]. While Munekazu et al. focused on P53-induced microRNA-107 inhibiting HIF-1α transcription and tumor angiogenesis [27]. It is also reported that p300 is related to the crosstalk between HIF-1 and p53 on the level of trans-activation [28]. It seemed that P53 regulates HIF-1α at various levels via different pathways [26-34]. As a down-stream target of p53, the PIG3 is mostly used as a long lived proapoptotic marker [14], and has also been shown to participate in the DNA damage response recently [15]. Although PIG3 activation leads to the ROS generation [16], there is no direct evidences show that PIG3 would regulate HIF-1α. Here we present the evidence to demonstrate that PIG3 functions as a new regulator of HIF-1α. Furthermore, we detailed that loss of PIG3 led to accumulation of HIF-1α by promoting the HIF-1α protein biosynthesis via PI3K/mTOR pathway.

VEGF is a main target of HIF-1α and has

Figure 3: PIG3-silencing promotes the production of HIF-1α via PI3K/mTOR pathway. A. CAKI cells were transfected with PIG3-siRNA for 24 h, then treatment with rapamycin 10 nM for 24 h or wortmannin 100 nM for 8 h before hypoxia treatment. B. CAKI cells were transfected with PIG3-siRNA for 24 h, then treatment with Raptor siRNA for 24 h before hypoxia treatment. All the experiments above were conducted thrice.
various effects, including inducing angiogenesis and promoting cell migration [21, 35]. In this regard we have demonstrated that knockdown of PIG3 promoted VEGF secretion and migration activity of renal cell carcinoma CAKI and 769-P cells.

In conclusion, our data provide more insights into of PIG3’s role in HIF-1α regulation, and the regulation network of the cellular HIF-1α. This will help us to

Figure 4: PIG3-silencing promotes the secretion of VEGF and the migration of CAKI and 769-P cells. A, Knocking down PIG3 and/or HIF-1α by transfecting with siRNA. B, ELISA assays showed that down-regulation of PIG3 led to an increase in the secretion of VEGF via HIF-1α in CAKI and 769-P cells exposed to hypoxia for 12 h. C, PIG3-silencing promoted CAKI and 769-P cells migration by targeting HIF-1α. All the experiments above were conducted thrice. Columns indicate the mean of three experiments; Bars, S.D.
understand the tumor progression as well as develop new therapeutic approaches.

**MATERIALS AND METHODS**

**Cell culture and reagents**

CAKI, FTC133 and A549 cells were obtained from ATCC and maintained in appropriate medium as suggested by ATCC. Cells were incubated in a humidified atmosphere of 95% air plus 5% CO\(_2\) at 37 °C. CoCl\(_2\) was obtained from Zhiyuan Chemical Reagent Co., Ltd. (Tianjin, China). MG-132 and CHX were obtained from MCE (Shanghai, China). Rapamycin was obtained from LC Laboratories (Woburn, MA, USA). Wortmannin was purchased from Selleckchem.cn (Shanghai, China).

**Transfection of siRNA**

Synthetic siRNA were purchased from Shanghai GenePharma Co., Ltd. The siRNA was transfected into cells using siRNA-Mate (Shanghai GenePharma Co., Ltd, Shanghai, China) according to the instructions of the manufacturer, with sequences as follows: pig3 : sense 5'-AAUAUGUUCAGGCUGAGACUAdTdT-3; 5'-UAGUCUCCAGCCUAGAAUUdTdT-3; hif-1α: 5'-TACGTTGTGAGTGGTATTATT; 5'-CUGAUGACCAGCAACUUGATT.

**Immunoblotting**

Immunoblotting was conducted with standard procedures [36], using antibodies against PIG3 (Origene, Rockville, MD), HIF-1α and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA), p-AKT, AKT, p-S6K1, S6K1 (Cell Signaling Technology, Beverly, MA).

**Real-time quantitative PCR**

Total RNA was extracted with Trizol according to the manufacturer’s instructions and was transcribed using Prime ScriptTM RT reagent Kit (TaKaRa, Dalian, China). The cDNA template was amplified by real-time PCR using SYBR-PremixExTaqTM Kit (TaKaRa, Dalian, China). The primer sequences were as follows: 5'- TCAATCCAAAGAGCCCCAAGTGC -3'(forward), 5'- TTTCGCTTTCTCTGAGCATTCT -3'(reverse) for hif-1α, 5'-GCACCGTCAAGGCGTGAGA-3'(forward), 5'-GGCTTTCGCCAATGTTGAGA-3' (reverse) for GAPDH. Thermal cycling was programmed as follows: 95°C for 30 sec followed by 40 cycles of 95°C for 5 sec, 60°C for 20 sec and 72°C for 15 sec, and then 72°C for 10 min. Gene expression was assessed by delta Ct method and mRNA levels of HIF-1α were normalized to those of GAPDH internal standard.

**Transwell assay**

Cell migration was evaluated using an 8-mm pore size Transwell system (Costar, Cambridge, MA, USA). Briefly, Cells were resuspended in serum-free RPMI-1640 at a density of 2×10^5 cells/mL. The top chamber of transwell was loaded with 100 μL of cell suspension and the bottom chamber was loaded with 0.6 mL of RPMI-1640 containing 10 % FBS. The total migrated cells to the lower chamber were fixed, stained with 0.1% crystal violet, and photographed after treatment. Crystal violet stained cells were dissolved with 10% acetic acid and OD value was measured at 595 nm.

**CONFLICTS OF INTEREST**

The authors declare that they have no conflict of interest.

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