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Original Article

14-3-3ζ up-regulates hypoxia-inducible factor-1α in hepatocellular carcinoma via activation of PI3K/Akt/NF-κB signal transduction pathway

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Abstract: 14-3-3ζ protein, a member of 14-3-3 family, plays important roles in multiple cellular processes. Our previous study showed that 14-3-3ζ could bind to regulate the expression of hypoxia-inducible factor-1α (HIF-1α), which is induced by hypoxia and a crucial factor for induction of tumor metastasis. Moreover, we also have confirmed the response of 14-3-3ζ to hypoxia in our unpublished data as well. Thus, in the present study, we attempted to reveal that whether the regulation effect of 14-3-3ζ on HIF-1α functioned in a similar pattern as hypoxia. Stable regulation of 14-3-3ζ in human HCC cell line SMMC-772 and HCC-LM3 was achieved. The regulation of 14-3-3ζ on HIF-1α mRNA transcription was evaluated by luciferase activity assay and quantitative real-time PCR (qPCR). The effect of 14-3-3ζ on the production of HIF-1α and pathways determining HIF-1α's response to hypoxia was assessed using western blotting assay. Our results showed that regulation of 14-3-3ζ expression influenced the activity of HIF-1α, phosphatidyl inositol 3-kinase (PI3K), Akt, extracellular signal-regulated kinase 1/2 (ERK1/2), and nuclear factor kappa B (NF-κB). Blocking of these pathways using indicated inhibitors revealed that 14-3-3ζ enhanced the production of HIF-1α via the activation of PI3K/Akt/NF-κB pathway, which was identical to hypoxia induced HIF-1α expression. For the first time, our study described the key role of 14-3-3ζ in the HIF-1α production in HCC cells. And the molecule exerted its function on HIF-1α both by directly binding to it and via PI3K/Akt/NF-κB signal transduction pathway.

Keywords: 14-3-3ζ, Akt, hypoxia, hypoxia-inducible factor-1α, nuclear factor kappa B, phosphatidyl inositol 3-kinase

Introduction

Hepatocellular carcinoma (HCC) is one of the most common human cancers, ranking the eight in frequency worldwide. Generally, development of HCC depends on the background of chronic inflammatory liver disease, which is caused by viral infection and exposure to biological or chemical carcinogens [1]. On a global scale, the major disease areas of HCC include Asia, Africa, and southern Europe. At the molecular level, numerous clinical and histopathologic evidence suggest that HCC is a heterogeneous disease, structural mutations of *p53*, *β-catenin*, *AXIN1*, and other known tumor suppressor genes all participating in the oncogenesis and development of HCC [2-4]. Therefore, although owing to the advancing of surgical and early diagnostic techniques, the prognosis of HCC patients has improved [5, 6], the exact mechanism of HCC in most patients still remains unexplored.

A key factor determining the neovascularization of HCC is hypoxia induced factor-1α (HIF-1α) [7]. HIF-1α is the unique, O_2-regulated subunit targeting to proteasome degradation by ubiquitination, and the activity of HIF-1 mainly depends on HIF-1α [8, 9]. A series of genes and proteins that involves in the survival of tumor cells under hypoxia conditions are regulated by HIF-1α [10-13]. Thus, it is reasonable to infer the potential role of HIF-1α in HCC prevention and treatment. In our previous studies, we have found that one member of 14-3-3 proteins family, 14-3-3ζ, has the ability to bind to HIF-1α and up-regulate its

expression in HCC (unpublished data). This regulation will then influence the activation of epithelial-mesenchymal transition (EMT) and expression of VEGF, matrix metalloprotease 9 (MMP-9), and MMP-2 (unpublished data). Expression of the above three molecules are all closely related to the growth, invasion, and metastasis of carcinoma [14-18]. In addition, 14-3-3ζ overexpression could increase tumor cell proliferation via the activation of Akt in PI3K/Akt pathway of HCC [19, 20]. Taken together, these findings provided a novel therapeutic target for improvement of HCC in the future. However, we also find that hypoxia, which would up-regulate the transcription of HIF-1α via PI3K/Akt/NF-κB, could induce the expression of 14-3-3ζ as well (unpublished data). Therefore, it was significant to explore whether 14-3-3ζ could regulate HIF-1α via the similar pattern as hypoxia except for binding to it.

In the present study, we sought to determine the possible regulation effect of 14-3-3ζ on HIF-1α via PI3K/Akt/NF-κB signal transduction pathway, which was identical to the pattern of hypoxia. Stable regulation of 14-3-3ζ was achieved in human HCC cell lines. The effect of 14-3-3ζ on the transcription and expression of HIF-1α, vascular endothelial growth factor (VEGF), phosphatidyl inositol 3-kinase (PI3K), Akt, extracellular signal-regulated kinase (ERK), and nuclear factor kappa B (NF-κB) were evaluated by quantitative real-time PCR (qPCR) and western blotting assay. We hoped that our findings on 14-3-3ζ could facilitate to underlie the mechanism of the progression of HCC and help with the improvement of the cancer patients' condition in clinic.

Materials and methods

Chemicals and cell cultures

The human HCC cell line SMMC-7721 (low metastatic potential) was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The human HCC cell line HCC-LM3 (high metastatic potential) in was provided by Prof. Weizhong Wu (Zhong Shan Hospital, Fu Dan University, Shanghai, China). Cells were cultured in DMEM/F-12 medium supplemented with 10% (v/v) fetal calf serum and 1% (v/v) antibiotics mixture in 95% air and 5% CO_2 at 37°C in a humidified incubator. All the cells were passaged every 2-3 days to maintain logarithmic growth and cells between passage 3 and 6 were used for further experiments. The PI3K inhibitor LY294002, NF-κB inhibitor PDTC, and ERK1/2 inhibitor PD98059 were purchased from Sigma-Aldrich, St Louis, MO, USA and dissolved in DMSO.

Overexpression of 14-3-3ζ gene in SMMC-772 cell line

Expression vector for 14-3-3ζ, pcDNA3 (ID: 9002) was obtained from Addgene, a non-profit plasmid repository (Cambridge, MA, American). The vector was constructed by William Sellers. For transfection, SMMC-772 cells was adjusted to 1×10^4/mL and incubated on slides in one well of 24-well plates for 24 h. Transfection was performed with Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol and subsequent selection was conducted using 400-μg/mL puromycin (Amresco, Solon, USA). SMMC-772 cells were grouped into three groups: 1) mock group, cells were incubated with transfection agent without expression vectors. 2) pcDNA3 group, cells were incubated with blank vector pcDNA3. 3) 14-3-3ζ group, cells were incubated with pcDNA3-14-3-3ζ plasmid. Each treatment was represented by six replicates. All the cells were cultured for 24 h before sampling for further experiments.

shRNA interference of 14-3-3ζ gene in HCC-LM3 cell line

For shRNA interference, the pLKO-1-sh14-3-3ζ plasmid was obtained by cloning the plasmid harboring 14-3-3ζ-small hairpin RNA into the PLKo vector (Invitrogen, St. Louis, MO, USA). HCC-LM3 cells were grouped into three treatments and transfection was conducted as described above: 1) mock group, cells were incubated with transfection agent without interference vectors. 2) shcon group, cells were incubated with blank vector pLKO-1. 3) sh14-3-3ζ group, cells were incubated with pLKO-1-sh14-3-3ζ plasmid. Each treatment was represented by six replicates. All the cells were cultured for 24 h before sampling for further experiments.

Quantitative real-time PCR (qPCR)

The whole RNA in different treatments was extracted using Trizol method according to the manufacture's instruction. And β-actin was

selected as the reference gene. The RNA was reversely transcribed to cDNA using RT-PCR kit (Fermentas), and the final qPCR reaction mixture of volume 20 μL contained 10 μL of SYS BR Primix Ex Taq 2, 0.5 μL of each primers (HIF-1α, forward: 5'-TCATCCAAGAAGCCCTAACG-3', reverse: 5'-TTGCTTTCTCTGAGCATTCTG-3'. VEGF, forward: 5'-CGAAACCATGAACTTTCTGC-3', reverse: 5'-CCTCAGTGGGCACACACTCC-3'. β-actin forward: 5'-TCATGTTTGAGACCTTCAA, reverse, 5'-GTCTTTGCGGATGTCCACG-3'), 1 μL of the cDNA template, and 8 μL of Rnase free H_2O. Thermal cycling parameters for the amplification were as follows: a denaturation step at 94°C for 30 min, followed by 40 cycles at 95°C for 5 s, 60°C for 30 s and 72°C for 15 s. Relative gene expression was evaluated with Data Assist Software version 3.0 (Applied Biosystems/Life Technologies). The relative expression levels of *KiSS-1* were determined according to the expression of $2^{-\Delta\Delta ct}$.

Western blotting assay

Protein products of 14-3-3ζ, HIF-1α, VEGF, PI3K, phosphorylated Akt (p-Akt), Akt, phosphorylated ERK1/2 (p-ERK), ERK, and NF-κB subunit p65 were extracted from cells in different groups and concentrations of protein samples were determined using the BCA method. Western blotting assay was performed as described previously [21] with β-actin and Lamin A as reference: 20 μg of protein was subject to a 10% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Then targeted proteins were transferred onto polyvinylidene difluoride (PVDF) sheets. The membranes were washed with TBST for 5 min and then transferred into blocking buffer for incubation overnight at 4°C. After three cycles of 5 min washes with TBST, primary antibodies against indicated proteins were incubated with the membranes for 1 h at room temperature. After additional three washes, secondary HRP goat anti-rabbit IgG antibodies were added and incubated with the membranes for 40 min. After final three washes using TBST, the blots were developed using Beyo ECL Plus reagent and the results were detected in the Gel Imaging System. The relative expression levels of different proteins were calculated with Bio-Rad Quantity One.

Luciferase activity assay

Dual luciferase assay (Promega) were conducted by co-transfecting cells using vectors containing firefly luciferase under the control of SV40 promoter (pGL3-basic, Promega) and *Renilla* luciferase under the control of SV40 early enhancer/promoter region (pSV40, Promega). The firefly luciferase activity was measured using an AutoLumat LB953 luminometer (Berhold Analytical Instruments) and normalized to *Renilla* luciferase activity. A fragment of the 3'UTR of HIF-1α cDNA was inserted into pGL3 vector (pGL3-HIF-1α). Co-transfection of different combinations of pGL3-basic, pGL3-HIF-1α, pcDNA3, and pcDNA3-14-3-3ζ was conducted as described above.

Detection of activation of 14-3-3ζ gene on PI3K/Akt/NF-κB mediated HIF-1α expression

Several kinases, including PI3K, Akt, ERK1/2 have been reported to be involved in the regulation of HIF-1α. Moreover, transcription of HIF-1α depended on the activation of NF-κB. To further determine the molecular mechanism of 14-3-3ζ gene inducing the expression of HIF-1α, we assessed the effect of 14-3-3ζ gene overexpression on PI3K/Akt/NF-κB signal transduction pathway. 14-3-3ζ SMMC-772 cells were further treated with PI3K inhibitor LY294002 (5 μM), NF-κB inhibitor PDTC (100 μM), and ERK1/2 inhibitor PD98059 (30 μM) for 30 min, respectively. The effect of inhibitors on the relative mRNA expression of HIF-1α was determined using qPCR validation as described above. The production of 14-3-3ζ, HIF-1α, p-Akt, Akt, p-ERK1/2, ERK, and NF-κB subunit p65 was also determined using western blotting as described above. Given that results indicated the significant inhibition effect of LY294002 and PDTC on the expression of the HIF-1α protein, we set up a dose-dependent experiment to test the regulation of different concentration of LY294002 (0 μM, 2.5 μM, 5 μM, and 10 μM) and PDTC (0 μM, 50 μM, 100 μM, and 200 μM) on the synthesis of 14-3-3ζ, HIF-1α, p65, and phosphorylation of Akt. The results were illustrated by western blotting.

Statistical analysis

All the data were expressed in the form of mean ± SD. T-test and multiple comparisons with LSD method were conducted by using GLM model with a significant level of 0.05. All the statistical analysis were conducted using SPSS version 19.0 (IBM, Armonk, NY, USA).

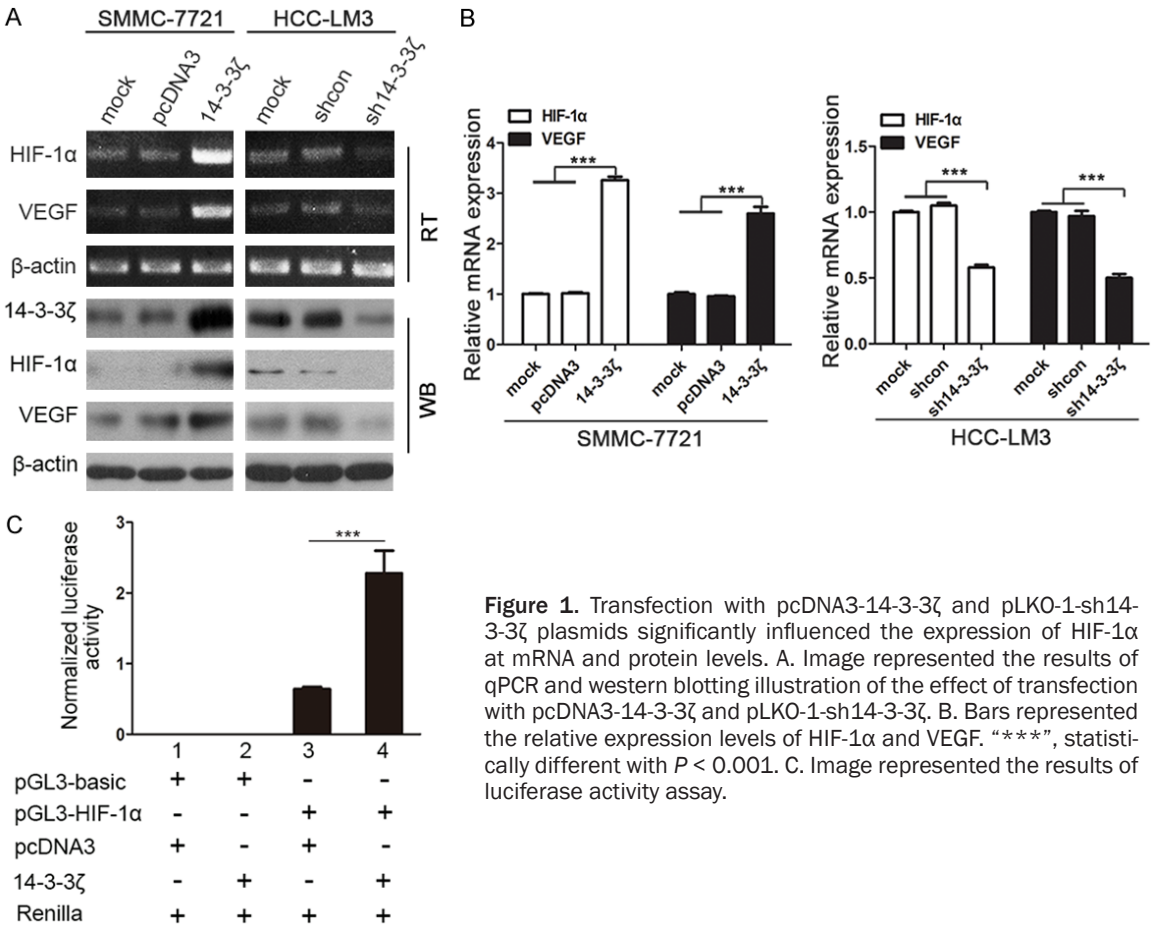

Figure 1. Transfection with pcDNA3-14-3-3ζ and pLKO-1-sh14-3-3ζ plasmids significantly influenced the expression of HIF-1α at mRNA and protein levels. A. Image represented the results of qPCR and western blotting illustration of the effect of transfection with pcDNA3-14-3-3ζ and pLKO-1-sh14-3-3ζ. B. Bars represented the relative expression levels of HIF-1α and VEGF. "***", statistically different with $P < 0.001$. C. Image represented the results of luciferase activity assay.

Results

Regulation of 14-3-3ζ influenced HIF-1α mRNA and protein levels

First, we investigated whether exposure to pcDNA3-14-3-3ζ would increase HIF-1α mRNA level. It was found when SMMC-772 cells were transfected with pcDNA3-14-3-3ζ vector, HIF-1α and VEGF mRNA levels were up-regulated (**Figure 1A** and **1B**) and the difference was statistically different. Next, HCC-LM3 cells were transfected with pLKO-1-sh14-3-3ζ plasmid, which resulted in dramatic reduction of HIF-1α and VEGF mRNA expression. Contrary to SMMC-772 cells, HCC-LM3 is a metastatic cell line, and expression of HIF-1α and VEGF in this cell line in innately high. Moreover, protein levels of HIF-1α, VEGF, and 14-3-3ζ were changed in response to exposure to transfection of 14-3-3ζ expression vector and shRNA as expected (**Figure 1A**). To further confirm the regulation of HIF-1α mRNA by 14-3-3ζ at the transcription level, cells were transfected with pGL3-HIF-1α in which HIF-1α promoter was cloned in front of luciferase gene. As shown in **Figure 3**, after co-transfection with pcDNA3-14-3-3ζ vector, the activity of luciferase was strengthened, and the difference was statistically significant ($P < 0.001$).

Regulation of 14-3-3ζ in HCC cell lines influenced PI3K/Akt/ERK and NF-κB pathways

HIF-1α has been proved to be regulated by hypoxia via PI3K/Akt/ERK and NF-κB pathways in HCC cells. And our previous studies showed that 14-3-3ζ could also be induced by hypoxia and bind to HIF-1α. Thus, we attempted to test whether the regulation of 14-3-3ζ on HIF-1α could also function through a pattern similar to that of hypoxia. First, we examined the effect of 14-3-3ζ on the activity of PI3K, Akt, ERK1/2, and NF-κB subunit p65. As illustrated by western blotting, it was found that all the molecules mentioned above were innately expressed in

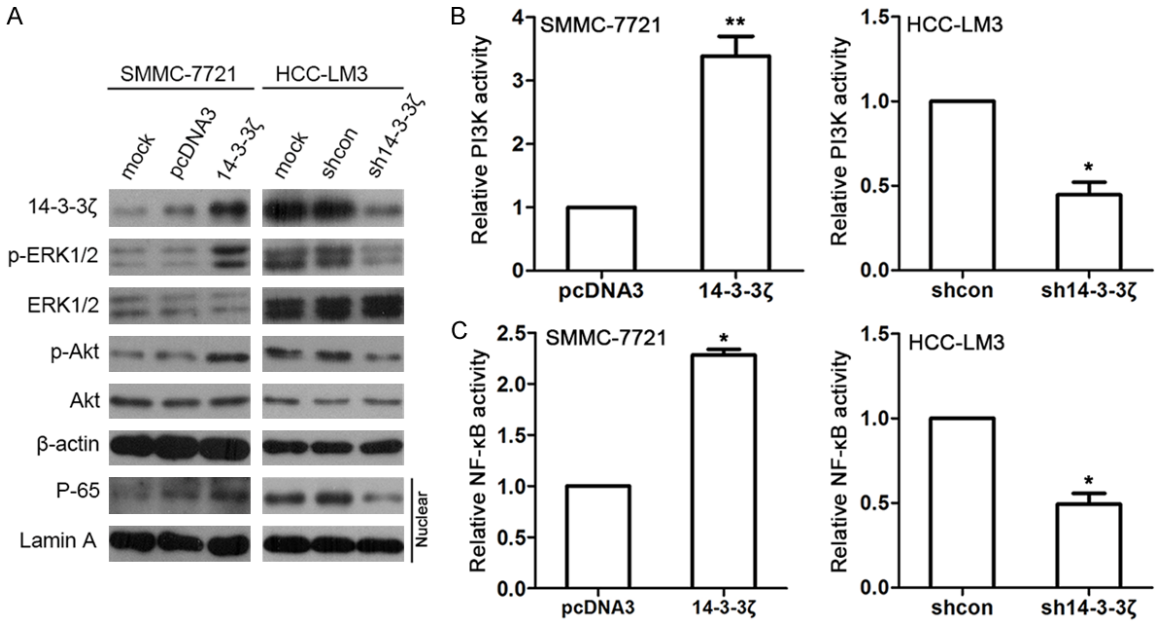

Figure 2. Transfection with pcDNA3-14-3-3ζ and pLKO-1-sh14-3-3ζ plasmids significantly influenced the expression of members of PI3K/Akt, ERK1/2, and NF-κB pathways. A. Image represented the results of western blotting illustration of the effect of transfection with pcDNA3-14-3-3ζ and pLKO-1-sh14-3-3ζ. B. Bars represented the effect of 14-3-3ζ regulation on the relativity activity of PI3K. C. Bars represented the effect of 14-3-3ζ regulation on the relativity activity of NF-κB. "**", statistically different with $P < 0.01$.

SMMC-7721 and HCC-LM3 cell lines (**Figure 2B**). After transfection with pcDNA3-14-3-3ζ and pLKO-1-sh14-3-3ζ vectors, the expressions levels of PI3K, p-ERK1/2, p-Akt, and p65 were altered (**Figure 2B** and **2C**). These results demonstrated the 14-3-3ζ could regulate the PI3K/Akt/ERK and NF-κB pathways in both positive and negative patterns.

14-3-3ζ increased HIF-1α expression via the activation of PI3K/Akt/NF-κB pathway

In the current study, SMMC-772 cells were transfected with pcDNA3-14-3-3ζ plasmid and incubated with PI3K inhibitor LY294002, NF-κB inhibitor PDTC and ERK1/2 inhibitor PD98059, respectively. We found that the increased phosphorylation of Akt was prevented by LY294002 combined with the down-regulation of HIF-1α both at mRNA and protein levels, while the expression of 14-3-3ζ was not influenced at all (**Figure 3A**). Similar results were observed for NF-κB subunit p65 inhibited by PDTC as well (**Figure 3B**). Moreover, LY294002 could also reduce the relative activity of NF-κB (**Figure 4**), which confirmed the upstream regulation of PI3K/Akt on NF-κB activation. 14-3-3ζ also enhanced the phosphorylation of ERK1/2, which was blocked by PD98059. However, treatment of PD98059 had no impact on the synthesis of HIF-1α, it seemed that ERK1/2 was not involved in the activation of HIF-1α regulated by 14-3-3ζ (**Figure 3C**). The inhibiting effects of LY294002 on PI3K and PDTC on NF-κB were dose-dependent as shown in **Figure 4D** and **4E**.

Discussion

HIF-1α plays critical roles in neovascularization in malignancies through the regulation of VEGF [7]. Thus, lots of studies have tested the potential of HIF-1α as a tumor diagnostic criteria and an anti-tumor therapy [7, 22]. 14-3-3ζ is a member of 14-3-3 protein family, which consists of 7 different isoforms in mammal. The 14-3-3 family is crucial in multiple cellular process, including cell cycle, protein trafficking, cell proliferation, and apoptosis [23]. Previous studies of us and other group have found that hypoxia treatment, which induced the transcription of HIF-1α mRNA via activation of PI3K/Akt and NF-κB [24], could up-regulate the expression of 14-3-3ζ in *in vitro* experiments. Considering that generation of 14-3-3 binding sites on diversity targeted protein depends on

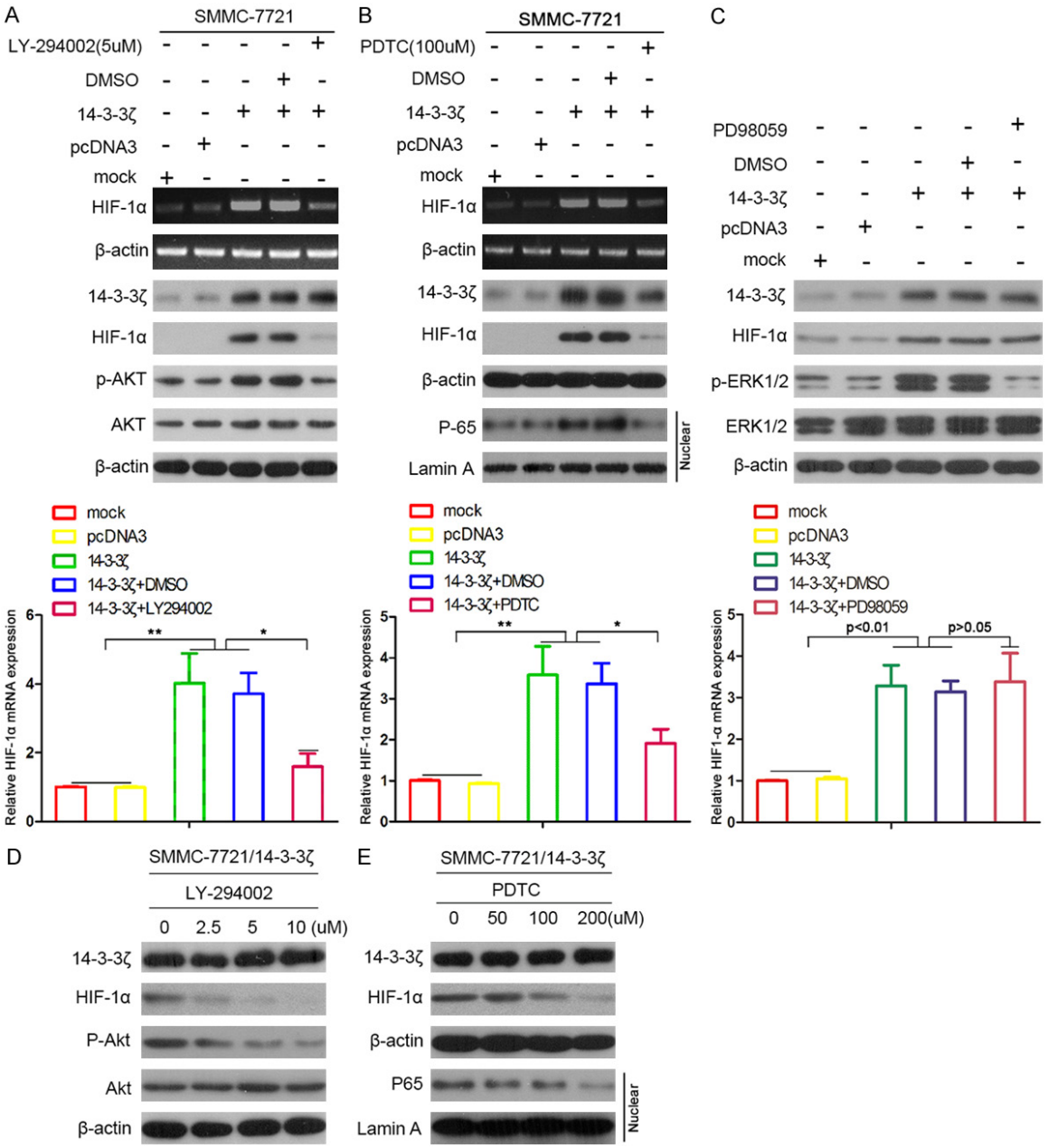

Figure 3. Overexpression of 14-3-3ζ induced the expression of HIF-1α via activation of PI3K/Akt/NF-κB signal transduction pathway. A. 14-3-3ζ overexpression increased the phosphorylation of Akt while LY294002 could block the activation of Akt and HIF-1α. B. 14-3-3ζ overexpression increased the production NF-κB subunit p65 and while PDTC could decrease the production of p65 and HIF-1α. C. 14-3-3ζ overexpression increased the phosphorylation of ERK1/2 while PD98059 could only inhibit the activation of ERK1/2. D. Dose-dependent test of the inhibiting effect LY294002 on the activation of Akt and HIF-1α. E. Dose-dependent test of the inhibiting effect PDTC on the activation of NF-κB and HIF-1α. "*", statistically different with $P < 0.05$. "**", statistically different with $P < 0.01$.

Akt [25], it inspired us with a possible mechanism by which 14-3-3ζ could induce the expression of HIF-1α.

To test our hypothesis, we stably regulated the expression of 14-3-3ζ in two human HCC cells and analyzed the effect of 14-3-3ζ on the expression of HIF-1α and other related molecules. Both overexpression and silencing of 14-3-3ζ in HCC cells influenced the activity of HIF-1α, VEGF, and members of PI3K/Akt, ERK1/2, and NF-κB pathways (**Figures 1** and

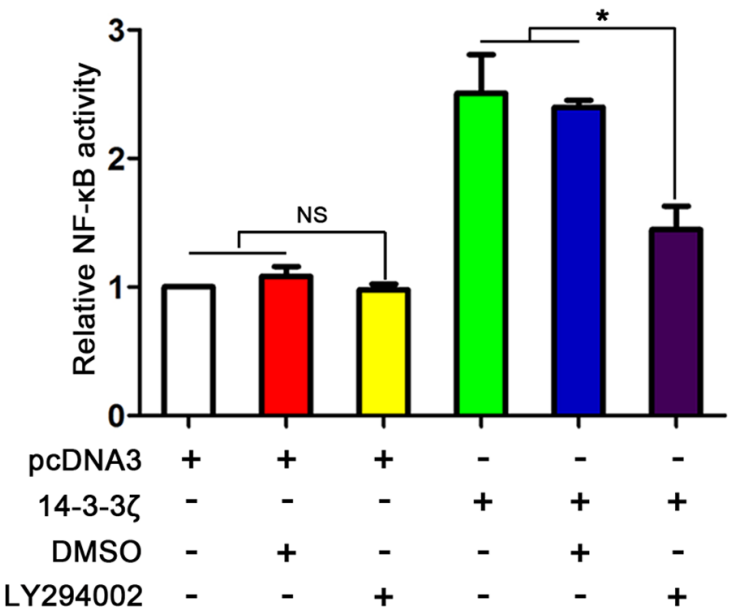

Figure 4. Effect of LY294002 on the activity of NF-κB. "*", statistically different with $P < 0.05$.

2). Overexpression of 14-3-3ζ in SMMC-772 cell line led to the up-regulation of HIF-1α both in mRNA and protein levels (**Figure 1**). This induced expression of HIF-1α was similar to the results of hypoxia treatment, which would further increase the expression of VEGF, MMP-2, MMP-9 and lead to the metastasis of tumors [7, 14]. In addition, we have previously confirmed the effect of hypoxia in stabilizing the expression of 14-3-3ζ by reducing its ubiquitination (unpublished data). Combining these findings, it was inspirative to demonstrate that our results revealed another mechanism by which hypoxia could influence the tumor cell proliferation [19, 20, 26]. Generally, hypoxia suppresses the proliferation of cells, but for HCC cells, hypoxia can stimulate the growth of the cells via suppression of apoptosis via HIF-1α related pathways [24, 27-30]. 14-3-3ζ is found to mechanistically bind to p85 subunit of phsphatidyl inositol 3-kinase (PI3K) and dramatically increases the phosphorylation of Akt [26]. All the information above reminded us of the close connection among hypoxia, HIF-1α, and 14-3-3ζ in HCC. Therefore, it was reasonable to test whether 14-3-3ζ regulated the expression of HIF-1α via the same mechanism of hypoxia treatment.

Several inhibitors targeted important members in hypoxia induced HIF-1α activity were employed to evaluate the function of 14-3-3ζ on HIF-1α expression. We found that overexpression of 14-3-3ζ led to transient increase in the phosphorylation of Akt in a PI3K-dependent manner and activated the translocation of NF-κB into cell nuclear (**Figure 3**). PI3K/Akt pathway is obligated for the activation of NF-κB subunit p65 and activation of both pathways result in the overexpression of cellular-FILCE inhibitory (c-FLIP), which further prohibits the apoptosis induced by death receptors such as tumor necrosis factor receptor-1 and Fas. Employment of inhibitors of PI3K and NF-κB in the present study prevented the transcription and production of HIF-1α, while inhibitors of ERK1/2 made no influence on the expression of HIF-1α either in mRNA or in protein levels. In addition, LY294002 inhibited the activation of NF-κB as well (**Figure 4**). All these results were exact the same as the study of BelAiba *et al.*, in which 14-3-3ζ overexpression was replaced by hypoxia treatment [24]. Given that expression of 14-3-3ζ was not influenced by these inhibitors, our data inferred that 14-3-3ζ regulated the activation of HIF-1α via PI3K/Akt/NF-κB pathway. Interestingly, in this regulation, NF-κB is capable of binding to the promoter of HIF-1α and serves as the director mediator of HIF-1α transcription [24]. In our unpublished studies, we found that 14-3-3ζ also possessed the ability to bind to HIF-1α and regulated its expression. Thus, we believed that the regulation of 14-3-3ζ on HIF-1α acts both in direct and indirect manners.

Together, our study for the first time highlighted the key role of 14-3-3ζ in the induction of HIF-1α production in HCC cells. Except for directly binding to HIF-1α, 14-3-3ζ could also exert its function via PI3K/Akt/NF-κB signal transduction pathway. This regulation is identical to that in hypoxia induced HIF-1α activation. However, our study only provided a preliminary illustration of the role of 14-3-3ζ in HCC or other cancer types. These findings merely brought in some putative explanations for hypoxia-related activities in cancers. More comprehensive

studies are demanding in the future to elucidate the role of 14-3-3ζ in cell hypoxia for design of 14-3-3ζ as a novel therapeutic target for hypoxia-related damage.

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Disclosure of conflict of interest

None.

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