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Upregulation of HIF-2α induced by sorafenib contributes to the resistance by activating the TGF-α/EGFR pathway in hepatocellular carcinoma cells

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ABSTRACT

Sorafenib, the first-line systemic drug for advanced hepatocellular carcinoma (HCC), has demonstrated limited benefits with very low response rates. Thus it is essential to investigate the underlying mechanisms for the resistance to sorafenib and seek potential strategy to enhance its efficacy. Hypoxic cells inside solid tumors are extremely resistant to therapies as their survival ability is increased due to the cellular adaptive response to hypoxia, which is controlled by hypoxia-inducible factor (HIF)-1 and HIF-2. Sorafenib inhibits HIF-1α synthesis, making the hypoxic response switch from HIF-1α- to HIF-2α-dependent pathways and providing a mechanism for more aggressive growth of tumors. The present study has demonstrated that upregulation of HIF-2α induced by sorafenib contributes to the resistance of hypoxic HCC cells by activating the transforming growth factor (TGF)-α/epidermal growth factor receptor (EGFR) pathway. Blocking the TGF-α/EGFR pathway by gefitinib, a specific EGFR inhibitor, reduced the activation of STAT (signal transducer and activator of transcription) 3, AKT and ERK (extracellular signal-regulated kinase), and synergized with sorafenib to inhibit proliferation and induce apoptosis of hypoxic HCC cells. Transfection of HIF-2α siRNA into HCC cells downregulated the expression of VEGF (vascular endothelial growth factor), cyclin D1, HIF-2α and TGF-α, and inhibited the activation of EGFR. HIF-2α siRNA inhibited the proliferation and promoted the apoptosis of HCC cells *in vitro*, and synergized with sorafenib to suppress the growth of HCC tumors *in vivo*. The results indicate that targeting HIF-2α-mediated activation of the TGF-α/EGFR pathway warrants further investigation as a potential strategy to enhance the efficacy of sorafenib for treating HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is the second most frequent cause of cancer death in men worldwide [1] and is resistant to systemic chemotherapy [2]. Sorafenib, a multi-targeted kinase inhibitor, targets the Raf/mitogen-activated protein kinase (MAPK)/extracellular signaling-regulated kinase (ERK) pathway and tyrosine kinase receptors including vascular endothelial growth factor (VEGF) receptor (VEGFR), platelet-derived growth factor receptor (PDFR) and c-Kit, thus inducing cell apoptosis and inhibiting cell proliferation as well as tumor angiogenesis [3]. Sorafenib has been approved as the first-line systemic drug for advanced HCC [4], but the promising treatment has demonstrated limited survival benefits with very low response rates [3,4]. Therefore, it is essential to investigate the underlying mechanisms for the resistance to sorafenib and seek potential strategy to enhance its efficacy to combat HCC.

Hypoxic cancer cells inside solid tumors are extremely resistant to therapies [5,6]. The discovery of hypoxia-inducible factor (HIF)-1 and HIF-2 as master driving forces of the cellular adaption to hypoxia has provided a fundamental molecular link to the clinical dilemma [6]. Each HIF is composed of an α-subunit and a β-subunit and binds to hypoxia-response elements in the promoters of the targeted genes [5,6]. HIF-1α and HIF-2α individually complex with HIF-1β (also known as ARNT, aryl hydrocarbon receptor nuclear translocator) to form a heterodimer, and are degradable in an oxygen-dependent manner [7]. HIFs regulate a vast array of genes encoding proteins involved in angiogenesis, glycolysis, cell growth, metastasis and anti-apoptosis of cancer cells [8]. Both HIF-1α and HIF-2α have been

Abbreviations: ANOVA, analysis of variance; ARNT, aryl hydrocarbon receptor nuclear translocator; CCK-8, Cell Counting Kit-8; CHX, cycloheximide; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; FITC, fluorescein isothiocyanate; HCC, hepatocellular carcinoma; HIF-1α, hypoxia-inducible factor-1α; HIF-2α, hypoxia-inducible factor-2α; MAPK, mitogen-activated protein kinase; PDFR, platelet-derived growth factor receptor; p-EGFR, phosphorylated; p-STAT3, phosphorylated STAT3; p-AKT, phosphorylated AKT; p-ERK1/2, phosphorylated ERK1/2; PHD, prolyl hydroxylase; qRT-PCR, Quantitative Reverse-Transcription Polymerase Chain Reaction; SD, standard deviation; STAT3, signal transducer and activator of transcription 3; TGF-α, transforming growth factor-α; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

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shown to be upregulated in HCC, and control tumor progression and therapy sensitivity [9,10]. HIF-1α and HIF-2α share 48% amino acid identity and a similar protein structure. While HIF-1α is ubiquitously expressed, HIF-2α is only expressed by certain types of cells including hepatocytes [8]. The hepatic erythropoietin is preferentially regulated by HIF-2 [11], and the expression of angiogenic genes in livers is predominantly regulated by HIF-2 [12], indicating that HIF-2α may be a preferential therapeutic target for HCC [13].

Inside solid tumors, the cells are subjected to a range of oxygen tensions and experience various hypoxic intensities based on the diffusion range of oxygen [5]. HIF-1α seems to play the dominant role in the response to acute hypoxia, whereas HIF-2α drives the response to chronic hypoxia, under which the regulatory feedback of HIF-1α may be responsible for the selectivity [14,15]. Importantly, depletion of one HIF-α subunit provokes a compensatory increase of the other α-subunit, such that knockdown of HIF-1α increases the expression of HIF-2α in HepG2 cells via the reciprocal regulatory mechanism [10]. The switch from HIF-1α- to HIF-2α-dependent ways provides a mechanism for more aggressive growth of tumors under hypoxia [16,17]. It has been reported that sorafenib executes its antitumor effects particularly for its anti-angiogenic activity against HCC by inhibiting HIF-1α synthesis, and sequentially downregulating the expression of VEGF [18]. These findings suggest that sorafenib may upregulate HIF-2α through a compensatory mechanism by inhibiting HIF-1α, contributing to the resistance to sorafenib and providing a potential target to overcome this resistance.

We have previously reported that downregulation of HIF-2α enhanced the efficacy of doxorubicin in the treatment of HCC by inhibiting cell proliferation and tumor angiogenesis [19]. Transfection of HIF-2α siRNA downregulated the expression of transforming growth factor (TGF)-α and cyclin D1 in human HCC cells [19]. TGF-α is one of the key ligands for epidermal growth factor receptor (EGFR) [20], and overexpressed in human HCC cells [21]. Overexpression of EGFR, a common molecular event in the pathogenesis of HCC [22], is also regulated by HIF-2α [23]. The above results suggest that the TGF-α/EGFR pathway may be involved in the mechanisms for the therapeutic effects of HIF-2α siRNA against HCC. Therefore, we designed the present study to investigate whether downregulating HIF-2α could enhance the efficacy of sorafenib to treat HCC by inhibiting the TGF-α/EGFR pathway.

2. Materials and methods

2.1. Cell culture, reagents and antibodies

Human HCC HepG2 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), and Huh7 cells from the Chinese Academy of Sciences Cell Bank (Shanghai, China). Cells were cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum. Hypoxic cells were induced by incubating the cells in a hypoxia chamber containing 1% O_2, 5% CO_2, and 95% N_2 at 37 °C for 48 h. Sorafenib and gefitinib were purchased from Jinan Trio Pharmatech Co., Ltd. (Jinan, China), and dissolved in dimethyl sulfoxide (DMSO) to make a stock solution of 100 mM individually for in vitro assays. For animal experiments, sorafenib was suspended in the vehicle solution containing Cremophor (Sigma-Aldrich), 95% ethanol and water in a ratio of 1:1:6 [24]. Antibodies (Abs) against EGFR, phosphorylated EGFR (p-EGFR, Tyr^{1068}), signal transducer and activator of transcription 3 (STAT3), phosphorylated STAT3 (p-STAT3, Tyr^{705}), AKT, phosphorylated AKT (p-AKT, Ser^{473}), ERK1/2, and phosphorylated ERK1/2 (p-ERK1/2, Thr^{202}/$Thyr^{204}$) were purchased from Cell Signaling Technology, USA. Abs against HIF-1α, HIF-2α, ARNT, TGF-α, VEGF, cyclin D1, β-actin and Ki-67 were from Santa Cruz Biotechnology, USA. An Ab against CD31 was from Thermo Scientific, Beijing, China. A fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse Ab and an alexa fluor 568-conjugated goat anti-rabbit Ab were from Zhongshan Golden Bridge Biotechnology, Beijing, China. A Cell Counting Kit-8 (CCK-8) kit was purchased from Dojindo Molecular Technologies, Gaithersburg, MD, USA.

2.2. Transfection of siRNAs

The methods of siRNA transfection have been described in details previously [19,25]. Briefly, cells were grown to 60–70% confluence, and incubated with siRNAs at a final concentration of 0.1 μM by using Lipofectamine™ 2000 (Invitrogen, Beijing, China) in a serum-free medium for 24 h and then subjected to the assays. The siRNAs produced by GenePharma (Shanghai, China) and their targeted genes are shown in Table 1.

2.3. Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR)

The methods have been described in details previously [19,26]. Briefly, total RNA was extracted from the cells, and cDNA was synthesized. The reaction mixtures for qRT-PCR were prepared with the primers (Table 2), and analyzed by MX3000P Real-time PCR systems (Stratagen, USA). Experiments were performed as triplicate, and the data were calculated by ΔΔCt methods.

2.4. Animal experimental protocols

Male nude BALB/ c-nu/nu mice (6–8 weeks old) were obtained from Vital River Laboratory Animal Technology, Beijing, China. All surgical procedures and care administered to the animals were in accordance with institutional ethic guidelines. The subcutaneous tumors were established by inoculation of 5×10^6 Huh7 cells suspended in PBS into the mice. Tumor volumes were estimated according to the formula: $\pi / 6 \times a^2 \times b$, where a is the short axis, and b is the long axis. When tumors reached ~100 mm^3 approximately 2 weeks later, the mice were assigned to 4 groups (each group had 8 mice), namely control, HIF-2α

Table 1
The siRNAs and their targeted genes used in this study.

Genes	GenBank no.	Strands	References
HIF-1α	NM_181054.2	Forward: 5′-GGGUAAAGAACAAAACACA-3′ Reverse: 5′-UGUGUUUUGUUCUUUACCC-3′	[23]
HIF-2α	NM_001430.4	Forward: 5′-GGUUUUGUUGCUAGCCCUU-3′ Reverse: 5′-AAGGGCUAGCAACAAAACC-3′	[23]
EGFR	NM_005228.3	Forward: 5′-CUAUGCCUUAGCAGUCUUAUCUA A-3′ Reverse: 5′-UUAGAUAAGACUGCUAAGGCAUAGG-3′	[25]
TGF-α	NM_003236	Forward: 5′-CAUUUUAAUGACUGCCCAGA-3′ Reverse: 5′-UCUGGGCAGUCAUUAAAAUG-3′	[19]
Control	–	Forward: 5′-UUCUCCGAACGUGUCACGU-3′ Reverse: 5′-ACGUGACACGUUCGGAGAA-3′	[19]

Abbreviations: EGFR, epidermal growth factor receptor; HIF-1α, hypoxia-inducible factor-1α; HIF-2α, hypoxia-inducible factor-2α; TGF-α, transforming growth factor-α.