

# Targeting hypoxia-inducible factor-2 $\alpha$ enhances sorafenib antitumor activity via $\beta$ -catenin/C-Myc-dependent pathways in hepatocellular carcinoma

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**Abstract.** Sorafenib is a type of multikinase inhibitor that exhibits antiangiogenic and antiproliferative effects; in addition, sorafenib is a unique first-line drug recommended for the treatment of advanced hepatocellular carcinoma (HCC). However, the effectiveness of HCC treatment remains poor due to acquired drug resistance. It has been suggested that hypoxia, induced as a results of the antiangiogenic effects of sustained sorafenib treatment, may be an important factor in sorafenib resistance. The transcription factor hypoxia-inducible factor (HIF)-2 $\alpha$  has been reported to be associated with cell proliferation under hypoxic conditions; therefore, it was hypothesized that hypoxia may enhance tumor cell proliferation via this mechanism. The present study aimed to evaluate whether the knock-down of HIF-2 $\alpha$  was able to enhance the therapeutic efficacy of sorafenib in order to effectively treat HCC. The results demonstrated that hypoxia protected HCC cells against sorafenib; however, short hairpin RNA-HIF-2 $\alpha$  transfection in combination with sorafenib treatment exhibited a significantly synergistic effect against HCC cell proliferation. In addition, HCC cells acquired increased  $\beta$ -catenin/C-Myc expression, which enhanced proliferation under hypoxic conditions; however, targeted knock-down of HIF-2 $\alpha$  or C-Myc markedly decreased cell proliferation in HCC cells. In conclusion, the results of the present study indicated that the targeted

knock-down of HIF-2 $\alpha$  in combination with sorafenib may be a promising strategy for the treatment of HCC.

## Introduction

Hepatocellular carcinoma (HCC) has the second highest rate of cancer-associated mortality in men worldwide (1); in addition, chemotherapeutic drug resistance is common in HCC patients (2). Previous studies have indicated that the adjuvant therapies currently available for HCC treatment following surgery are ineffective and inadequate to prevent recurrence (3). Large-scale randomized phase III clinical studies have demonstrated the survival benefits of sorafenib treatment, a unique first-line drug recommended for advanced HCC; however, sorafenib is not commonly used due to its low response rate and drug resistance (4). It is therefore important to elucidate the underlying mechanisms of sorafenib resistance in order to identify potential therapeutic strategies to enhance its efficacy for the treatment of HCC (5-7).

Solid tumors often have a hypoxic internal microenvironment that includes numerous molecular pathways, which provide challenges for therapeutic strategies; this microenvironment is the primary cause of drug resistance to antitumor therapies (8,9). Previous studies have reported that sustained sorafenib treatment may promote hypoxia within tumors, which has been associated with sorafenib-resistance in HCC patients as well as subcutaneous mice models of HCC (10). In addition, it was reported that short-term sorafenib therapy reduced vascularization, resulting in increased tumor hypoxia (11). Therefore, numerous previous studies have aimed to elucidate the potential mechanisms of hypoxia-induced sorafenib resistance mechanisms (5,7,9,10).

Hypoxia-inducible factors (HIFs), which are heterodimers composed of an  $\alpha$  and  $\beta$  subunit, have been implicated in the regulation of the hypoxic response (12,13). HIF-1 was reported to mediate the expression of hundreds of genes, including those for vascular endothelial growth factors, glycolytic enzymes and glucose transporters (14,15). In addition, HIF-2 was identified to be involved in implementing the hypoxic

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response (14,15). HIF-1 $\beta$  was reported to be able to form a heterodimer complex with either HIF-1 $\alpha$  or HIF-2 $\alpha$ , both of which undergo oxygen-dependent degradation via the von Hippel-Lindau protein pathway (14,15). In addition, it was reported that targeting HIF-1 $\alpha$  may significantly enhance sorafenib antitumor sensitivity under hypoxic conditions (10); however, the association between HIF-2 $\alpha$  and sorafenib sensitivity remains to be elucidated.

The present study aimed to investigate the potential association between HIF-2 $\alpha$  and sorafenib sensitivity under hypoxic conditions and the mechanisms by which this may proceed.

## Materials and methods

**Cell culture.** Human HepG2, Bel-7402 and Huh-7 HCC cell lines were purchased from the American Type Culture Collection (Rockville, MD, USA); in addition, SMMC-7402 HCC cells were obtained from the Type Culture Collection Cell Bank, Chinese Academy of Science (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (ThermoFisher Scientific, Shanghai, China), 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin (Beyotime Institute of Biotechnology, Shanghai, China) at 37°C in 95% air and 5% CO<sub>2</sub> or when hypoxic conditions were required, cells were stored at 37°C in a sealed MIC-101 hypoxia chamber (Billups-Rothenberg, Inc., Del Mar, CA, USA) equilibrated with certified gas containing 1% O<sub>2</sub>, 5% CO<sub>2</sub> and 94% N<sub>2</sub>.

**Antibodies (Abs) and reagents.** Abs against HIF-2 $\alpha$ ,  $\beta$ -catenin, C-Myc and proliferating cell nuclear antigen (PCNA), were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA); Ab against GAPDH was purchased from Beyotime Institute of Biotechnology (Jinan, China); and Abs against Ki-67, HIF-2 $\alpha$  small interfering (si) RNA and control siRNA were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Lipofectamine 2000 was purchased from Invitrogen Life Technologies (Beijing, China) and sorafenib tosylate was purchased from Bayer Pharmaceuticals Co. (West Haven, CT, USA).

**HIF-2 $\alpha$ /C-Myc silencing using lentiviral vector-mediated short hairpin (sh)RNA.** Silencing of HIF-2 $\alpha$ /C-Myc was achieved via lentiviral transduction of the following specific shRNA vectors (Santa Cruz Biotechnology, Inc.): HIF-2 $\alpha$ -specific shRNA, sc-35316-v; C-Myc-specific shRNA, sc-29226-v; and scramble shRNA control, sc-108080. The process of transduction was executed according to the product protocols. Briefly, HepG2 cells (5 $\times$ 10<sup>5</sup> cells/well) were seeded in 24-well plates. When the cell were ~50% confluent, the complete medium in the wells was replaced by complete medium with Polybrene (Santa Cruz Biotechnology, Inc., Dallas, TX, USA; sc-134220) at a final concentration of 3.5  $\mu$ g/ml. In the control shRNA group, 10  $\mu$ l scramble shRNA (sc-108080) was added to each well and in the HIF-2 $\alpha$  shRNA group, 10  $\mu$ l HIF-2 $\alpha$  shRNA (sc-35316-v) was added to each well. The cells were cultured for 8 h, then the mixture of complete medium with Polybrene was

replaced with complete medium, 12 h later, the cells were harvested for the following assays.

**Cell viability assay.** Cell viability was detected using the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Beijing, China). In brief, cells (5 $\times$ 10<sup>3</sup>/well) in the control (scramble shRNA-treated group) and the group treated with HIF-2 shRNA were seeded onto 96-well plates and cultured for 12, 24, 48 and 72 h. The culture medium was then replaced with fresh medium containing 10  $\mu$ l CCK-8 solution. Cells were incubated for a further 2 h at 37°C and the optical density was measured using a PowerWave HT Microplate Spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA) at 450 nm.

**Western blot assay.** Cells or tumor tissues were homogenized in protein lysate buffer and debris was removed by centrifugation. Protein concentrations in cell/tissue extracts were determined using the BCA Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, Inc., Richmond, CA, USA). In brief, equal amounts of protein fractions of lysates were resolved over SDS-PAGE gels (Beyotime Institute of Biotechnology) transferred to polyvinylidene fluoride membranes (Merck Millipore, Darmstadt, Germany) and immunoblotted as previously described (16,17). The primary antibodies used for western blotting included, monoclonal rabbit anti-human HIF-2 $\alpha$ , (1:1,000 dilution, #7096), polyclonal rabbit anti-human- $\beta$ -catenin (1:1,000, #9562), polyclonal rabbit anti-human c-Myc (1:1,000 dilution, #9402), monoclonal mouse anti-human PCNA (1:2,000 dilution, #2586) and monoclonal mouse anti-human GAPDH (1:2,000 dilution, AG019). The secondary antibodies included horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:1,000 dilution, #A0208; Beyotime Institute of Biotechnology) and (HRP)-conjugated goat anti-mouse IgG (1:1,000 dilution, A0216; Beyotime Institute of Biotechnology). Protein bands were developed using ECL reagent (Tiangen Biotech Co., Ltd., Beijing, China) and visualized using the AlphaImager HP System (Naturegene Corporation, Medford, NJ, USA). The protein band intensities were quantified by densitometric analysis using Image J software, version 2.0 (National Institutes of Health, Bethesda, MD, USA).

**Immunocytochemistry (ICC).** For ICC, the cells (2 $\times$ 10<sup>4</sup>) were plated on coverslips, fixed with 4% paraformaldehyde for 30 min at room temperature, and permeabilized with 0.2% Triton X-100 for 20 min at room temperature. Subsequently, the sections were incubated with primary anti- $\beta$ -catenin (1:200 dilution, #9562) overnight at 4°C, followed by incubation with HRP-conjugated goat anti-rabbit IgG secondary antibody (1:1,000 dilution, #A0208). Subsequently, immunoreactivity was developed with 3,3'-diaminobenzidine (DAB; ZSGB-BIO Biotechnology Co., Ltd., Beijing, China), followed by counterstaining with hematoxylin.

**Subcutaneous HCC experiments.** All surgical procedures and care administered to the animals were in accordance with institutional ethics guidelines. The study was approved by the ethics committee of Qianfoshan Hospital, Shandong

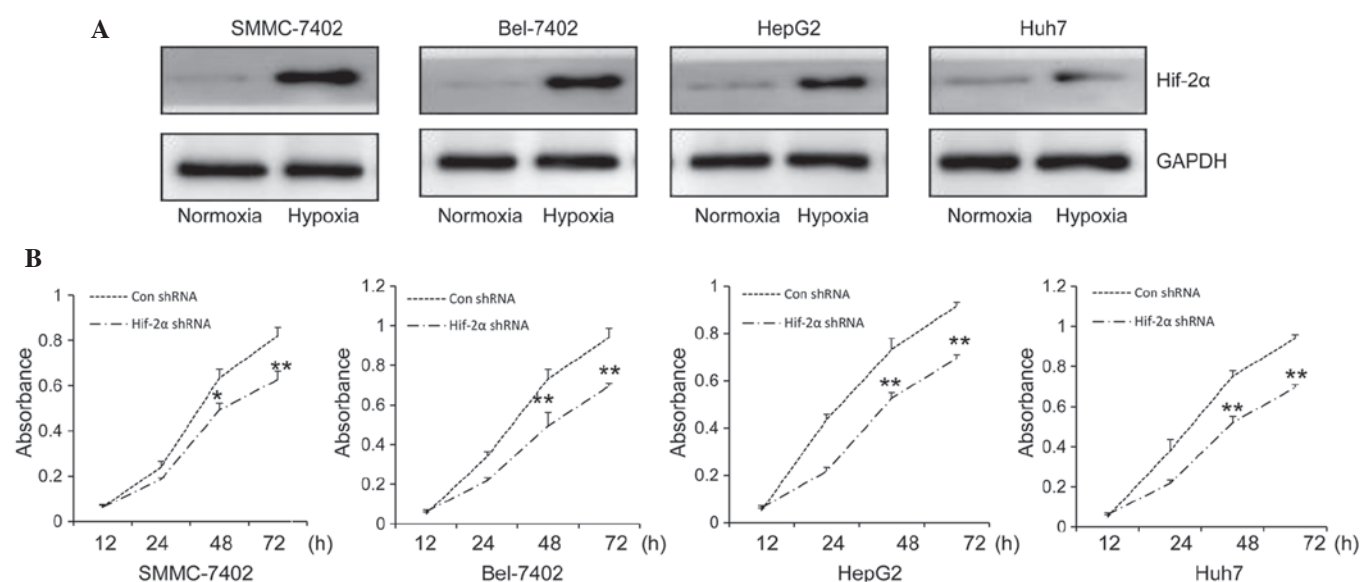


Figure 1. Targeting HIF-2 $\alpha$  enhances the antiproliferation activity of sorafenib under hypoxic conditions. (A) Expression of HIF-2 $\alpha$  in hepatocellular carcinoma cell lines SMMC-7402, Bel-7402, HepG2, Huh-7 was detected by western blot analysis following treatment for 24 h under hypoxic conditions. GAPDH served as an internal control. (B) SMMC-7402, Bel-7402, HepG2 and Huh-7 cells were treated with Con shRNA or HIF-2 $\alpha$  shRNA in combination with Sorafenib (10  $\mu$ M) under hypoxic conditions and cell viability was measured following 12, 24, 48 and 72 h of treatment. Values are presented as the mean  $\pm$  standard deviation of three independent experiments. \*P<0.05 and \*\*P<0.001 vs. Con shRNA. HIF-2 $\alpha$ , hypoxia-inducible factor 2 $\alpha$ ; Con, control; shRNA, short hairpin RNA.

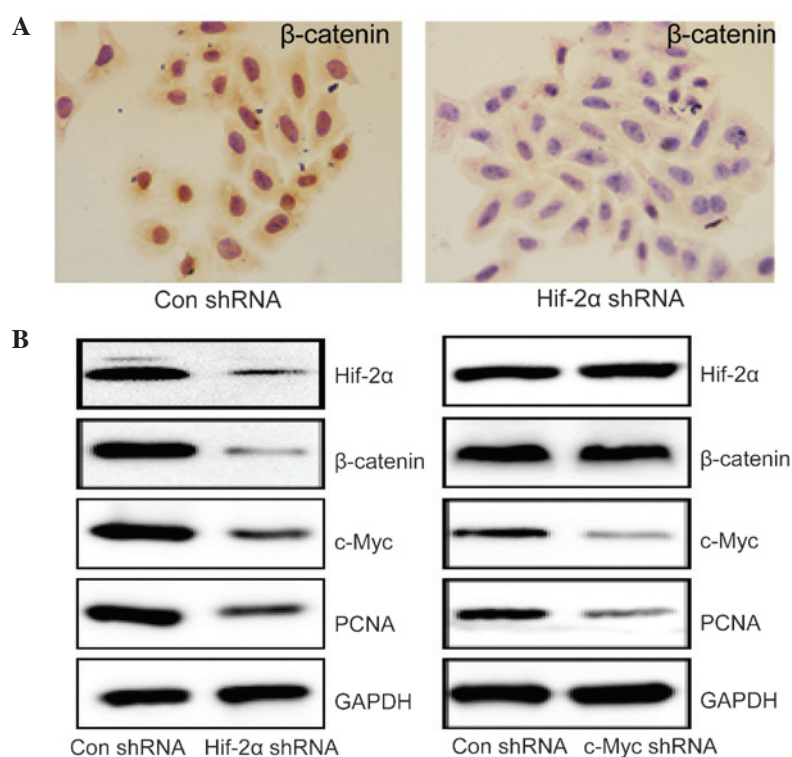


Figure 2. HIF-2 $\alpha$  regulates PCNA expression through  $\beta$ -catenin/C-Myc-dependent pathways under hypoxic conditions. (A) HepG2 cells were transfected with HIF-2 $\alpha$  shRNA or Con shRNA, then the expression of  $\beta$ -catenin was detected by immunocytochemistry. (B) HepG2 cells were treated with HIF-2 $\alpha$  shRNA, C-Myc shRNA or Con shRNA and cell lysates were analyzed by western blot analysis to detect expression of HIF-2 $\alpha$ ,  $\beta$ -catenin, C-Myc and PCNA. GAPDH served as an internal control. HIF-2 $\alpha$ , hypoxia-inducible factor 2 $\alpha$ ; PCNA, proliferating cell nuclear antigen; Con, control; shRNA, short hairpin RNA.

University (Jinan, China). Male BALB/c mice were purchased from the Model Animal Research Center (Nanjing, China). The mice (n=56) were housed in polycarbonate cages (5/cage), provided with food and water *ad libitum* and maintained on a 12-12 h light-dark cycle at  $22\pm 2^\circ\text{C}$  and  $55\pm 20\%$  relative

humidity. Male BALB/c mice were randomly assigned into four groups (12/group). Eight mice were excluded for different reasons (mortality or no tumor formed). Male BALB/c (5-6 weeks old) mice underwent subcutaneous inoculation in the flank with  $1.5\times 10^6$  HepG2 cells/mice suspended in

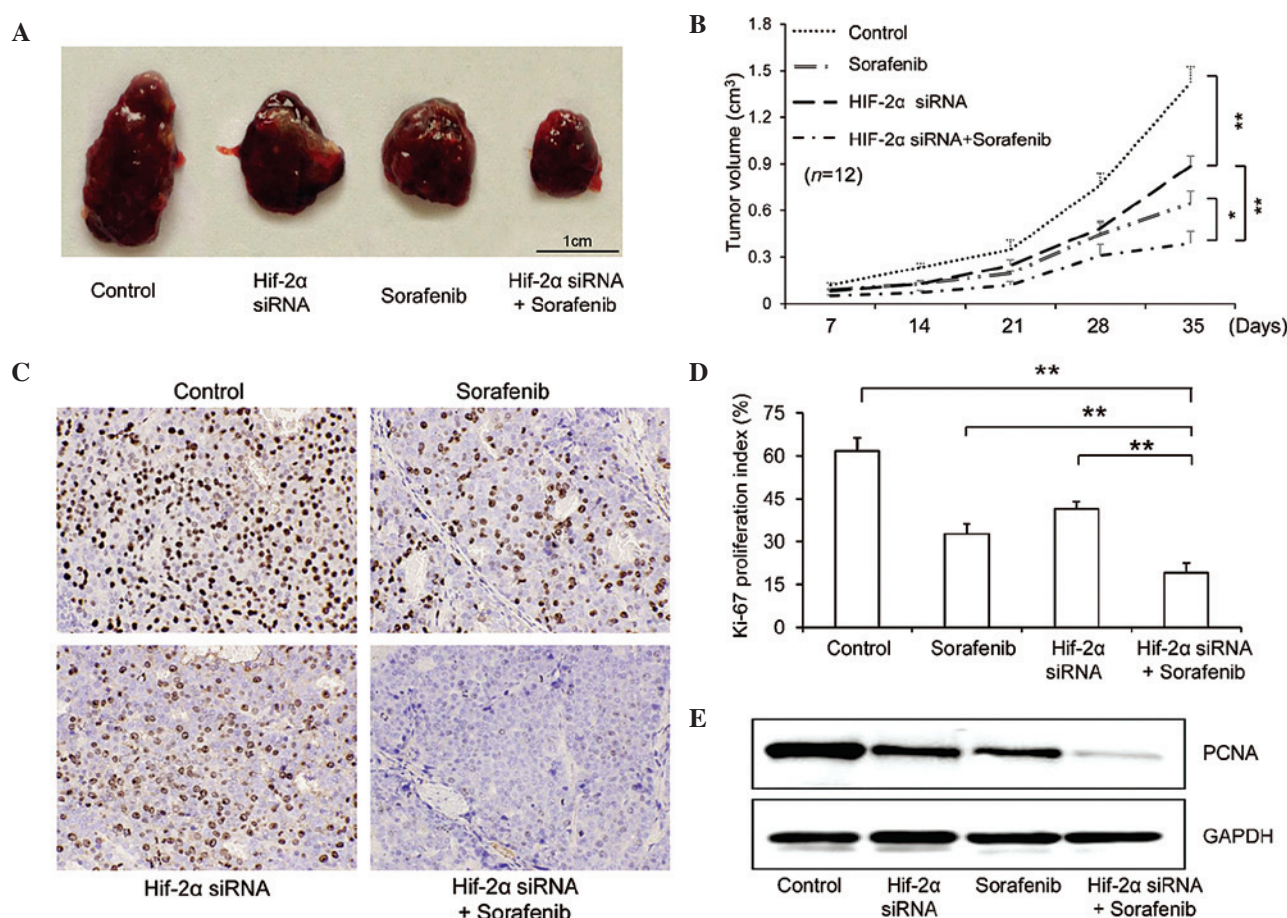


Figure 3. Targeting HIF-2 $\alpha$  potentiates the antitumor effects of sorafenib in HepG2 xenograft tumor models by inhibiting cell proliferation. HepG2 hepatomas were established. When the tumors reached  $\sim 100 \text{ mm}^3$  ( $\sim 7$  days post inoculation), they were administered sorafenib, HIF-2 $\alpha$  siRNA or a combined treatment ( $n=12/\text{group}$ ). (A) Representative images of tumors from each treatment group. (B) Tumor volume was measured every 7 days for 35 days following treatment. (C) Tumor sections were stained with an anti-Ki-67 antibody in order to detect proliferating cells (magnification,  $\times 100$ ). (D) Ki-67 positive cells were quantified in order to calculate the Ki-67 proliferation index. (E) Tumors from mice of each group were homogenized and subjected to western blot analysis in order to detect expression of PCNA. GAPDH served as an internal control. Values are presented as the mean  $\pm$  standard deviation. \* $P<0.05$  and \*\* $P<0.001$  vs. control. HIF-2 $\alpha$ , hypoxia-inducible factor 2 $\alpha$ ; siRNA, small interfering RNA; PCNA, proliferating cell nuclear antigen.

phosphate-buffered saline (PBS). Sorafenib tosylate was used *in vivo* experiments. When tumors of  $\sim 100 \text{ mm}^3$  were detected ( $\sim 7$  days post inoculation), mice were divided at random into the following treatment groups ( $n=12/\text{group}$ ): Control, sorafenib-treated group, HIF-2 $\alpha$  siRNA-treated and HIF-2 $\alpha$  siRNA + sorafenib-treated. Mice in each group were administered treatment five times/week either intratumorally or orally (p.o.). In the sorafenib-treated group, mice were administered once daily with sorafenib (p.o.;  $10 \text{ mg/kg}$ ; 5 times/week). Equal volumes of  $20 \text{ nM}$  siRNA and Lipofectamine 2000 were mixed and this solution was further mixed with an equal volume of serum-free medium;  $50 \mu\text{l}$  siRNA transfection solution, containing  $250 \text{ pmol}$  siRNA, was injected into tumors (5 times/week). Mice in the control group were treated with equal volume PBS (p.o.) and control siRNA (intratumoral injection). The tumor volumes were estimated twice a week according to the formula:  $\pi/6 \times a^2 \times b$ , where  $a$  is the short axis, and  $b$  the long axis. All mice were sacrificed by cervical dislocation on day 35.

**Quantification of Ki-67 proliferation index.** As previously described (18), tumor sections were immunostained with an

anti-Ki-67 Ab. Briefly, polyclonal rabbit anti-human Ki-67 (1:50 dilution, SC-15402) was applied overnight at  $4^\circ\text{C}$ . After washing, the sections were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:75 dilution, #A0208; Beyotime Institute of Biotechnology) for 1 h at  $37^\circ\text{C}$ . Immunoreactivity was developed using DAB and counterstained with hematoxylin. Ki-67 positive cells were counted under microscopy with a DM5500B equipped with HCX PL FLVOTAR 5/0.15 and HCX PL FLVOTAR 10/0.15 dry objective lenses (Leica, Solms, Germany) in ten randomly selected high-power fields (magnification,  $\times 400$ ). The Ki-67 proliferation index was calculated according to the following formula: Proliferation rate = (number of Ki-67 positive cells/total cell count)  $\times 100\%$ .

**Statistical analysis.** Values are presented as the mean  $\pm$  standard deviation and statistical analysis was performed using SPSS software version 16.0 (SPSS Inc., Chicago, IL, USA). Differences between groups were analyzed using Chi-Square test or Student's *t*-test according to the data type.  $P<0.05$  was considered to indicate a statistically significant difference between values.

## Results

**Targeting HIF-2 $\alpha$  enhances the antiproliferation activity of sorafenib under hypoxic conditions.** Western blot analysis was used to determine HIF-2 $\alpha$  protein expression levels in various HCC cell lines, SNNC-7402, Bel-7402, HepG2 and Huh7, exposed to normoxia or hypoxia for 24 h. All of the cell lines demonstrated low expression levels of HIF-2 $\alpha$  under normoxic conditions, while HIF-2 $\alpha$  expression was markedly increased under hypoxic conditions in all the selected HCC cell lines (Fig. 1A). Based on this data, these HCC cell lines were further used to detect the inhibitory effects of sorafenib on the proliferation viability of cells under hypoxic conditions. Cells were transfected with HIF-2 $\alpha$  shRNA or control shRNA and then treated with sorafenib (10  $\mu$ M) for 12, 24, 48 and 72 h, following which cell viability was assessed. Compared with the control shRNA, HIF-2 $\alpha$  shRNA further enhanced the antiproliferation activity of sorafenib in all of the four cell lines under hypoxic conditions (Fig. 1B).

**Regulation of cell proliferation-associated protein expression by HIF-2 $\alpha$ /C-Myc shRNA.**  $\beta$ -catenin is considered to be a key signaling protein in the regulation of cell proliferation (19-21). HepG2 cells were incubated in a sealed hypoxia chamber for 24 h, which resulted in significantly higher levels of  $\beta$ -catenin compared with cells in normoxic conditions (Fig. 2A). Transfection of HIF-2 $\alpha$  shRNA markedly reduced the expression of HIF-2 $\alpha$ ,  $\beta$ -catenin, C-Myc and PCNA in HepG2 cells compared with those transfected with control shRNA (Fig. 2B). As C-Myc is a key downstream protein in  $\beta$ -catenin-mediated cell proliferation (19-21), the effects of C-Myc shRNA transfection were investigated in HepG2 cells. C-Myc shRNA decreased C-Myc and PCNA expression with no effect on the expression of HIF-2 $\alpha$  and  $\beta$ -catenin (Fig. 2B). These results indicated that HIF-2 $\alpha$  regulated HCC cell proliferation in a  $\beta$ -catenin/C-Myc-dependent pathway under hypoxic conditions.

**Targeting HIF-2 $\alpha$  potentiates the antitumor effects of sorafenib in HepG2 xenograft tumor in mice through inhibiting cell proliferation.** A gradual increase in xenograft HepG2 tumor growth was observed in the control group compared with the experimental groups. In addition, mice that underwent combination therapy had tumors that were significantly smaller than those treated with sorafenib or HIF-2 $\alpha$  siRNA alone (Fig. 3A and B). Furthermore, a reduced number of Ki-67-positive cells were present in tumors treated with either sorafenib or HIF-2 $\alpha$  siRNA compared with the control group; while in the combination therapy group, the number of Ki-67-positive cells was significantly reduced compared with that of the independent treatment groups ( $P < 0.01$ ) (Fig. 3C and D). As shown in Fig. 3E, the reduced PCNA expression levels in HIF-2 $\alpha$ -transfected cells, as determined by western blot analysis of tumor homogenates, were comparable to the results observed *in vitro* (Fig. 3E).

## Discussion

In the present study it was demonstrated that HIF-2 $\alpha$  was involved in sorafenib resistance via the regulation of

$\beta$ -catenin/c-Myc-dependent pathways under hypoxic conditions in HCC. Although sorafenib is first choice of treatment for advanced HCC, it has been reported that this systemic drug has limited benefits and poor response rates (22,23). Solid tumors contain hypoxic cells, which exhibit resistance to antitumor treatments due to an enhanced cellular adaptive response to hypoxia, mediated by HIF-1 $\alpha$  and HIF-2 $\alpha$ , which promotes survival (14,24-26). HIF-1 $\alpha$  synthesis is suppressed following treatment with sorafenib, which results in the altered hypoxic response from HIF-1 $\alpha$  to HIF-2 $\alpha$ -dependent pathways; this mechanism was reported to result in more aggressive tumor growth (22,23). In the present study, it was demonstrated that targeted HIF-2 $\alpha$  knock-down inhibited HCC cell proliferation under hypoxic conditions *in vitro*; in addition, this acted synergistically with sorafenib to further suppress the growth of HCC tumors *in vivo*.

$\beta$ -catenin and C-Myc are important oncogenes, the over-expression of which were reported to be associated with the poorer prognosis of HCC patients (27-30). These oncogenes have numerous biological functions; however, data regarding the role of  $\beta$ -catenin/C-Myc in tumor proliferation are limited in HCC cells. Previous studies have indicated that  $\beta$ -catenin and HIF were frequently co-activated in rapidly growing tumors (31-34). In addition, it was reported that HIF-1 $\alpha$  expression was closely associated with Wnt/ $\beta$ -catenin signaling activity (35,36). However, limited studies have investigated the involvement of HIF-2 $\alpha$  in  $\beta$ -catenin regulation and cell proliferation (34,37). Furthermore, HIF-2 $\alpha$  was demonstrated to interact with  $\beta$ -catenin/T-cell factor (TCF) in order to promote gene transcription (34). Contrary to the results of previous studies (38,39), the present study suggested that HIF-2 $\alpha$  may regulate  $\beta$ -catenin/TCF activity through decreasing  $\beta$ -catenin expression under hypoxic conditions in HCC cells; this change may be attributed to different clonal variations.

C-Myc is an important target gene of  $\beta$ -catenin, the expression of which was reported to be significantly upregulated by  $\beta$ -catenin in tumor cells (40-42). In the present study, HIF-2 $\alpha$  positively regulated  $\beta$ -catenin/C-Myc expression and C-Myc directly upregulated PCNA expression under hypoxic conditions in HCC cells. However, the exact mechanism of C-Myc-mediated PCNA expression remains to be fully elucidated. Previous studies have indicated that C-Myc may enhance transcription factor accumulation in gene promoter regions, resulting in transcription factor amplification and increased levels of transcripts within the cells gene expression program (43-45). Thus, C-Myc may amplify the output of the PCNA gene expression program in HCC cells under hypoxic conditions.

In conclusion, the present study demonstrated that HIF-2 $\alpha$  gene transfer and sorafenib effectively inhibited the proliferation of HCC cells *in vivo* when administered independently; however, the combination of HIF-2 $\alpha$  shRNA and sorafenib treatment resulted in a synergistic effect on inhibiting cell proliferation. The mechanisms underlying these results were indicated to be primarily attributed to the decreased expression of PCNA. Given the distinct transcriptional targets of HIF-2 $\alpha$ , the results suggested that downregulating HIF-2 $\alpha$  may further enhance the antitumor activity of sorafenib in HCC via  $\beta$ -catenin/C-Myc-dependent pathways.

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## References

- Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D: Global cancer statistics. *CA Cancer J Clin* 61: 69-90, 2011.
- Bruix J and Sherman M; American Association for the Study of Liver Diseases: Management of hepatocellular carcinoma: An update. *Hepatology* 53: 1020-1022, 2011.
- Samuel M, Chow PK, Chan Shih-Yen E, Machin D and Soo KC: Neoadjuvant and adjuvant therapy for surgical resection of hepatocellular carcinoma. *Cochrane Database Syst Rev* (1): CD001199, 2009.
- Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, de Oliveira AC, Santoro A, Raoul JL, Forner A, *et al*; SHARP Investigators Study Group: Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 359: 378-390, 2008.
- Zhai B and Sun XY: Mechanisms of resistance to sorafenib and the corresponding strategies in hepatocellular carcinoma. *World J Hepatol* 5: 345-352, 2013.
- van Malenstein H, Dekervel J, Verslype C, Van Cutsem E, Windmolders P, Nevens F and van Pelt J: Long-term exposure to sorafenib of liver cancer cells induces resistance with epithelial-to-mesenchymal transition, increased invasion and risk of rebound growth. *Cancer Lett* 329: 74-83, 2013.
- Berasain C: Hepatocellular carcinoma and sorafenib: Too many resistance mechanisms? *Gut* 62: 1674-1675, 2013.
- Rankin EB and Giaccia AJ: The role of hypoxia-inducible factors in tumorigenesis. *Cell Death Differ* 15: 678-685, 2008.
- Rohwer N and Cramer T: Hypoxia-mediated drug resistance: Novel insights on the functional interaction of HIFs and cell death pathways. *Drug Resist Updat* 14: 191-201, 2011.
- Liang Y, Zheng T, Song R, Wang J, Yin D, Wang L, Liu H, Tian L, Fang X, Meng X, *et al*: Hypoxia-mediated sorafenib resistance can be overcome by EF24 through Von Hippel-Lindau tumor suppressor-dependent HIF-1 $\alpha$  inhibition in hepatocellular carcinoma. *Hepatology* 57: 1847-1857, 2013.
- Chang YS, Adnane J, Trail PA, Levy J, Henderson A, Xue D, Bortolon E, Ichetovkin M, Chen C, McNabola A, *et al*: Sorafenib (BAY 43-9006) inhibits tumor growth and vascularization and induces tumor apoptosis and hypoxia in RCC xenograft models. *Cancer Chemother Pharmacol* 59: 561-574, 2007.
- Yamashita T, Ohneda K, Nagano M, Miyoshi C, Kaneko N, Miwa Y, Yamamoto M, Ohneda O and Fujii-Kuriyama Y: Hypoxia-inducible transcription factor-2 $\alpha$  in endothelial cells regulates tumor neovascularization through activation of ephrin A1. *J Biol Chem* 283: 18926-18936, 2008.
- Menrad H, Werno C, Schmid T, Copanaki E, Deller T, Dehne N and Brüne B: Roles of hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) versus HIF-2 $\alpha$  in the survival of hepatocellular tumor spheroids. *Hepatology* 51: 2183-2192, 2010.
- Luo D, Wang Z, Wu J, Jiang C and Wu J: The role of hypoxia inducible factor-1 in hepatocellular carcinoma. *BioMed Res Int* 2014: 409272, 2014.
- Lin TY, Chou CF, Chung HY, Chiang CY, Li CH, Wu JL, Lin HJ, Pai TW, Hu CH and Tzou WS: Hypoxia-inducible factor 2  $\alpha$  is essential for hepatic outgrowth and functions via the regulation of *leg1* transcription in the zebrafish embryo. *PLoS One* 9: e101980, 2014.
- Dong X, Li R, Xiu P, Dong X, Xu Z, Zhai B, Liu F, Jiang H, Sun X, Li J, *et al*: Meloxicam executes its antitumor effects against hepatocellular carcinoma in COX-2-dependent and -independent pathways. *PLoS One* 9: e92864, 2014.
- Xiu P, Dong X, Dong X, Xu Z, Zhu H, Liu F, Wei Z, Zhai B, Kanwar JR, Jiang H, *et al*: Secretory clusterin contributes to oxaliplatin resistance by activating Akt pathway in hepatocellular carcinoma. *Cancer Sci* 104: 375-382, 2013.
- Chen H, Sun B, Pan S, Jiang H and Sun X: Dihydroartemisinin inhibits growth of pancreatic cancer cells in vitro and in vivo. *Anticancer Drugs* 20: 131-140, 2009.
- Xie C, Pan Y, Hao F, Gao Y, Liu Z, Zhang X, Xie L, Jiang G, Li Q and Wang E: C-Myc participates in  $\beta$ -catenin-mediated drug resistance in A549/DDP lung adenocarcinoma cells. *APMIS* 122: 1251-1258, 2014.
- Wu H, Li Z, Yang P, Zhang L, Fan Y and Li Z: PKM2 depletion induces the compensation of glutaminolysis through  $\beta$ -catenin/c-Myc pathway in tumor cells. *Cell Signal* 26: 2397-2405, 2014.
- Lee KB, Ye S, Park MH, Park BH, Lee JS and Kim SM: p63-Mediated activation of the  $\beta$ -catenin/c-Myc signaling pathway stimulates esophageal squamous carcinoma cell invasion and metastasis. *Cancer Lett* 353: 124-132, 2014.
- Zhao D, Zhai B, He C, Tan G, Jiang X, Pan S, Dong X, Wei Z, Ma L, Qiao H, *et al*: Upregulation of HIF-2 $\alpha$  induced by sorafenib contributes to the resistance by activating the TGF- $\alpha$ /EGFR pathway in hepatocellular carcinoma cells. *Cell Signal* 26: 1030-1039, 2014.
- Yang SL, Liu LP, Jiang JX, Xiong ZF, He QJ and Wu C: The correlation of expression levels of HIF-1 $\alpha$  and HIF-2 $\alpha$  in hepatocellular carcinoma with capsular invasion, portal vein tumor thrombi and patients' clinical outcome. *Jpn J Clin Oncol* 44: 159-167, 2014.
- Dong ZZ, Yao M, Wang L, Wu W, Gu X and Yao DF: Hypoxia-inducible factor-1 $\alpha$ : Molecular-targeted therapy for hepatocellular carcinoma. *Mini Rev Med Chem* 13: 1295-1304, 2013.
- Wong CC, Kai AK and Ng IO: The impact of hypoxia in hepatocellular carcinoma metastasis. *Front Med* 8: 33-41, 2014.
- He C, Sun XP, Qiao H, Jiang X, Wang D, Jin X, Dong X, Wang J, Jiang H and Sun X: Downregulating hypoxia-inducible factor-2 $\alpha$  improves the efficacy of doxorubicin in the treatment of hepatocellular carcinoma. *Cancer Sci* 103: 528-534, 2012.
- Lin CP, Liu CR, Lee CN, Chan TS and Liu HE: Targeting c-Myc as a novel approach for hepatocellular carcinoma. *World J Hepatol* 2: 16-20, 2010.
- Fang Y, Huang B, Liang Q, Li H and Huang C: Clinical significance of c-myc oncogene amplification in primary hepatocellular carcinoma by interphase fluorescence in situ hybridization. *Zhonghua Bing Li Xue Za Zhi* 30: 180-182, 2001 (In Chinese).
- Li P, Cao Y, Li Y, Zhou L, Liu X and Geng M: Expression of Wnt-5a and  $\beta$ -catenin in primary hepatocellular carcinoma. *Int J Clin Exp Pathol* 7: 3190-3195, 2014.
- Qu B, Liu BR, Du YJ, Chen J, Cheng YQ, Xu W and Wang XH: Wnt/ $\beta$ -catenin signaling pathway may regulate the expression of angiogenic growth factors in hepatocellular carcinoma. *Oncol Lett* 7: 1175-1178, 2014.
- Yoshida T, Hashimura M, Mastumoto T, Tazo Y, Inoue H, Kuwata T and Saegusa M: Transcriptional upregulation of HIF-1 $\alpha$  by NF- $\kappa$ B/p65 and its associations with  $\beta$ -catenin/p300 complexes in endometrial carcinoma cells. *Lab Invest* 93: 1184-1193, 2013.
- Jeong JK and Park SY: HIF-1 $\alpha$ -induced  $\beta$ -catenin activation prevents prion-mediated neurotoxicity. *Int J Mol Med* 32: 931-937, 2013.
- Yang YY, Lee PC, Huang YT, Lee WP, Kuo YJ, Lee KC, Hsieh YC, Lee TY and Lin HC: Involvement of the HIF-1 $\alpha$  and Wnt/ $\beta$ -catenin pathways in the protective effects of losartan on fatty liver graft with ischaemia/reperfusion injury. *Clin Sci (Lond)* 126: 163-174, 2014.
- Choi H, Chun YS, Kim TY and Park JW: HIF-2 $\alpha$  enhances  $\beta$ -catenin/TCF-driven transcription by interacting with  $\beta$ -catenin. *Cancer Res* 70: 10101-10111, 2010.
- Zhang Q, Bai X, Chen W, Ma T, Hu Q, Liang C, Xie S, Chen C, Hu L, Xu S, *et al*: Wnt/ $\beta$ -catenin signaling enhances hypoxia-induced epithelial-mesenchymal transition in hepatocellular carcinoma via crosstalk with hif-1 $\alpha$  signaling. *Carcinogenesis* 34: 962-973, 2013.
- Lee SH, Kim MH and Han HJ: Arachidonic acid potentiates hypoxia-induced VEGF expression in mouse embryonic stem cells: Involvement of Notch, Wnt, and HIF-1 $\alpha$ . *Am J Physiol Cell Physiol* 297: C207-C216, 2009.
- Wu L, Huang X, Li L, Huang H, Xu R and Luyten W: Insights on biology and pathology of HIF-1 $\alpha$ /2 $\alpha$ , TGF $\beta$ /BMP, Wnt/ $\beta$ -catenin, and NF- $\kappa$ B pathways in osteoarthritis. *Curr Pharm Des* 18: 3293-3312, 2012.
- Choi H, Chun YS, Kim TY and Park JW: HIF-2 $\alpha$  enhances  $\beta$ -catenin/TCF-driven transcription by interacting with  $\beta$ -catenin. *Cancer Res* 70: 10101-10111, 2010.

39. Park YK, Park B, Lee S, Choi K, Moon Y and Park H: Hypoxia-inducible factor-2 $\alpha$ -dependent hypoxic induction of Wnt10b expression in adipogenic cells. *J Biol Chem* 288: 26311-26322, 2013.
40. Li YJ, Wei ZM, Meng YX and Ji XR: Beta-catenin up-regulates the expression of cyclinD1, c-myc and MMP-7 in human pancreatic cancer: Relationships with carcinogenesis and metastasis. *World J Gastroenterol* 11: 2117-2123, 2005.
41. Anna CH, Iida M, Sills RC and Devereux TR: Expression of potential beta-catenin targets, cyclin D1, c-Jun, c-Myc, E-cadherin, and EGFR in chemically induced hepatocellular neoplasms from B6C3F1 mice. *Toxicol Appl Pharmacol* 190: 135-145, 2003.
42. Shiina H, Igawa M, Shigeno K, Terashima M, Deguchi M, Yamanaka M, Ribeiro-Filho L, Kane CJ and Dahiya R: Beta-catenin mutations correlate with over expression of C-myc and cyclin D1 Genes in bladder cancer. *J Urol* 168: 2220-2226, 2002.
43. Nie Z, Hu G, Wei G, Cui K, Yamane A, Resch W, Wang R, Green DR, Tessarollo L, Casellas R, *et al*: c-Myc is a universal amplifier of expressed genes in lymphocytes and embryonic stem cells. *Cell* 151: 68-79, 2012.
44. Lin CY, Lovén J, Rahl PB, Paranal RM, Burge CB, Bradner JE, Lee TI and Young RA: Transcriptional amplification in tumor cells with elevated c-Myc. *Cell* 151: 56-67, 2012.
45. Rahl PB, Lin CY, Seila AC, Flynn RA, McCuine S, Burge CB, Sharp PA and Young RA: c-Myc regulates transcriptional pause release. *Cell* 141: 432-445, 2010.