

Original Article

Effect of hypoxia inducible factor-1 antisense oligonucleotide on liver cancer

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Abstract: Hepatocellular carcinoma (HCC) is one of the most frequent primary malignancies of the liver and is resistant to anticancer drugs. Hypoxia is a master cause of tumor resistance to chemotherapy. Hypoxia-inducible factor-one alpha (HIF-1 α) plays a key role in the adaptive responses to hypoxic environments. HIF-1 α is constitutively up-regulated in several tumor types might thus be implicated in tumor therapy resistance. We hypothesized that disruption of HIF-1 α pathway could reverse the hypoxia-induced resistance to chemotherapy. In this report, we prepared DOTAP (a liposome formulation of a mono-cationic lipid N-[1-(2,3-Dioleoyloxy)]-N,N,N-trimethylammonium propane methylsulfate in sterile water) cationic liposomes containing an antisense oligonucleotide (AsODN) against HIF-1 α . Gene transfer of antisense HIF-1 α was effective in suppressing tumor growth, angiogenesis, and cell proliferation, and inducing cell apoptosis. Our results suggested that antisense HIF-1 α therapy could be a therapeutic strategy for treating HCC.

Keywords: Antisense oligonucleotide, hypoxia inducible factor 1 α , hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) is the most frequent primary malignancy of the liver [1]. The incidence of HCC is rising while the prognosis is very poor [1]. Currently available therapeutic modalities are largely inadequate for HCC [1]. Only a few patients are suitable for surgical resection, and the utility of transplantation is limited by organ availability [1, 2]. Chemotherapy reaches unsatisfactory response rates, no chemotherapeutic agent was particularly effective against HCC [2]. Doxorubicin is probably the most widely used agent to treat HCC up to now [1, 2]. However, in a recent study involving a large number of patients, doxorubicin showed response rates only ranging from 4 to 10.5% in HCC patients, and had significant side effects [3]. Therefore, new strategies should be developed to improve the therapeutic efficacy.

Hypoxic microenvironments are frequent in solid tumors. Rapid cell proliferation associated with deficient vascularization leads to areas of hypoxia [4]. Hypoxia is a major cause of tumor resistance to radiotherapy and chemo-

therapy [5, 6]. Tumor hypoxia has direct consequences on clinical and prognostic parameters and is a potential therapeutic target. The hypoxic response depends critically on hypoxia inducible factor-1 α (HIF-1 α) in pathological as well as physiological processes [7]. HIF-1 α mediates the adaptation of cancer cells to the hypoxic environment by controlling the expression of hundreds of genes, including VEGF, glycolytic enzymes, and glucose transporters [8, 9]. HIF-1 α , formed by the assembly of HIF-1 α and HIF-1 β , binds HRE in the promoters of the above genes [10]. HIF-1 α is degraded rapidly during normoxia but activated during hypoxia by oxygen-sensing signaling processes [11, 12]. Oligonucleotide could target any complementary nucleic acid sequence and result the degradation of target gene in principle and was broadly used in anticancer research [13, 14]. HIF-1 α was found to be highly expressed in HCC [15], therefore, we hypothesize that inhibition on HIF-1 α expression would be a possible approach for anti-HCC strategy. In this report, the effect of oligonucleotide targeting HIF-1 α was evaluated both in the HepG2 cell line and HepG2 inoculated mice tumor model.

Materials and methods

Mice and cell line

Male nude BALB/c mice (H-2b), 6-8 weeks old, were obtained from the Animal Research Center, Zhejiang University, China. The human HCC cell line HepG2 cells were obtained from the American Type Culture Collection (Rockville, MD). The cells were maintained in RPMI-1640 medium containing 10% Fetal Bovine Serum (FBS) and 4 mM L-glutamine, adjusted to contain 3.7 g/l sodium bicarbonate and 4.5 g/l glucose (Hyclone, Logan, UT). Cell cultures were maintained as a monolayer in 75 cm² tissue-culture treated flasks (Bedford, MA) at 37°C in a humidified atmosphere of 5% CO₂. Cells were passaged every 2-3 days to maintain exponential growth.

Animal experimental protocols

Tumors were established by subcutaneous injection of 1×10^6 HepG2 cells into the flanks of mice. Tumor volumes were estimated according to the formula: $\pi/6 \times a^2 \times b$, where "a" is the short axis and b the long axis. When tumors reached approximately 100 mm³, the mice were assigned randomly to two treatment groups to receive pcDNA3.1 or aHIF-pcDNA3.1. Mice received intraperitoneal injection of 200 µL PBS, and intratumoral injection of 200 µg pcDNA3.1 or aHIF-pcDNA3.1. All experiments included 12 mice per group. The animal experimental protocols were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal study was also approved by the Committee on the Ethics of Animal Experiments of Sanmen People's Hospital.

Colony formation assay

Colony formation test was performed in HepG2 cells following transfection with pcDNA3.1 or aHIF-pcDNA3.1. Twenty-four hours after transfection, cells were trypsinized and plated at a density of 800 cells/well in 6-well plates and then cultured for 14 days at 37°C. Culture medium was changed at regular time intervals. Colonies were fixed with paraformaldehyde, stained with Giemsa (ECM550 Chemicon), and counted at last.

Immunohistochemistry

Paraffin sections of human HCC (n = 52) and normal live tissue (n = 20) were prepared. The sections were dewaxed in xylene and hydrated in a graded ethanol continuously. Following the antigen retrieval, the tissue sections were placed in 3% H₂O₂ in distilled water for 30 min to block endogenous peroxidase activity. Then, the sections were blocked with 5% BSA for 20 min at room temperature and then probed primary antibody at 4°C overnight. After rinsing with PBS, the sections were incubated with biotinylated goat antimouse immunoglobulins at room temperature for 1 h and visualized using the peroxidase conjugated streptavidin and diaminobenzidine (DAB), followed by counterstaining with Mayer's haematoxylin. Immunopositivity was scored according to the percentage of positive cells in four distinct categories: 0 for 5%, 1 for 5-10%, 2 for 10-50% and 3 for 50%. The staining intensity was then scored where 0 stands for negative, 1 for weak staining, 2 for intermediate staining, and 3 for strong staining. Both scores were added together, resulting in a maximum staining score of 6 for any tissue score. 0-1 and 2 were considered as negative and positive staining, respectively.

Western blot analysis

tumor tissues were minced and homogenized in protein lysate buffer (0.1 M Tris buffer (pH 7.4, 0.1 mM EDTA)), in the presence of 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (Roche, IN). Debris was removed by centrifugation. The equal amount of sample was loaded in each well of a 10% gels and subjected to SDS-PAGE. Gels were transferred to nitrocellulose membrane. The membrane was then incubated with primary antibodies for 4°C overnight. After washing with TBST, the membrane was incubated with secondary antibody and the signals were visualized using ECL plus western blotting system. Blots were stained with an antitubulin antibody to confirm that each lane contained similar amounts of tumor homogenate. All experiments were performed in triplicate.

In situ detection of apoptotic cells

Serial 6-µm tumor sections were stained with the TUNEL agent (Roche) and adjacent sections

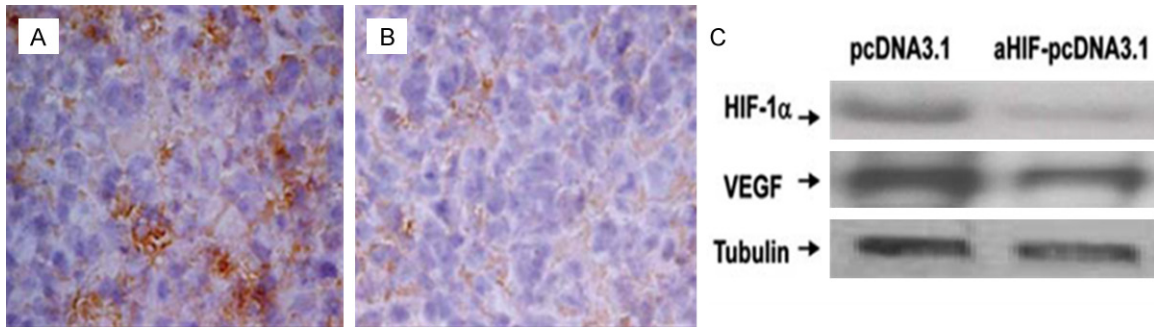


Figure 1. Antisense HIF-1 gene transfer downregulates HIF-1 α expression. Tumors of 100 mm³ in volume were injected with either (A) pcDNA3.1, or (B) aHIF-pcDNA3.1 plasmids. Tumor sections were prepared 4 days after gene transfer, stained with antibodies against HIF-1 (400 magnification). (C) Homogenates of tumors were prepared 4 days after intratumoral injection of the pcDNA3.1 or aHIF-pcDNA3.1 plasmids, and were western blotted with antibodies against HIF-1 α , VEGF and tubulin.

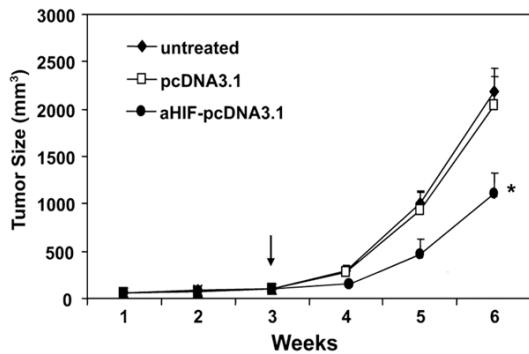


Figure 2. Antisense HIF-1 gene therapy suppresses tumor growth. HepG2 hepatomas were established subcutaneously in the flanks of mice. When the tumors reached approximately 100 mm³ in volume, they were injected with the pcDNA3.1, or aHIF-pcDNA3.1 plasmids. Untreated tumors served as controls. The sizes of tumors were recorded. **P* < 0.05 compared with pcDNA3.1-treated controls.

were counterstained with hematoxylin, and examined by fluorescence microscopy. The total number of apoptotic cells in 10 randomly selected fields was counted. The apoptosis index was calculated as the percentage of positive-staining cells.

In vitro assay

To detect cell viability, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay was performed. Briefly, HepG2 cells were transfected with 4 μ g aHIF-pcDNA3.1 or empty pcDNA3.1 plasmid using Lipofectamine Plus (Life Technologies, Shanghai, China). Cells were washed with PBS and suspended at a final concentration of 2-9 \times 10⁴ per ml in an assay medium in the presence or

absence of CoCl₂ and dispensed into 96-well plates (5 \times 10³ cells per well). The plates were incubated at 37°C in a humidified CO₂ incubator. After 6 h, 20 μ L of MTT (5 mg/ml) was added to each well, the plates were incubated at 37°C for 4 h, and then 10% dimethylsulfoxide (100 μ L) was added to each well. The absorbance at 490 nm was measured. Experiments were performed in triplicate.

Statistical analysis

Results are presented as the mean \pm SD and were analyzed via the Student's t-test or one-way ANOVA. A *P*-value of less than 0.05 was used for statistical significance. Statistical analysis was performed using the PRISM software (GraphPad, CA).

Results

Antisense HIF-1 α gene transfer inhibits HIF-1 α expression

Firstly, we checked whether transfection of antisense HIF-1 α downregulated expression of HIF-1 α in mice model. HepG2 tumors of approximately 100 mm³ in volume were injected with a DNA-FuGENE 6 transfection vehicle containing either aHIF-pcDNA3.1 or empty pcDNA3.1 vector. Representative tumor sections prepared 4 days after gene transfer, stained brown with antibodies against HIF-1. Immunohistochemistry and western blot analysis showed that transfer of a HIF-pcDNA3.1 significantly downregulated HIF-1 α expression in tumor cells, compared with vector-injected controls (**Figure 1**).

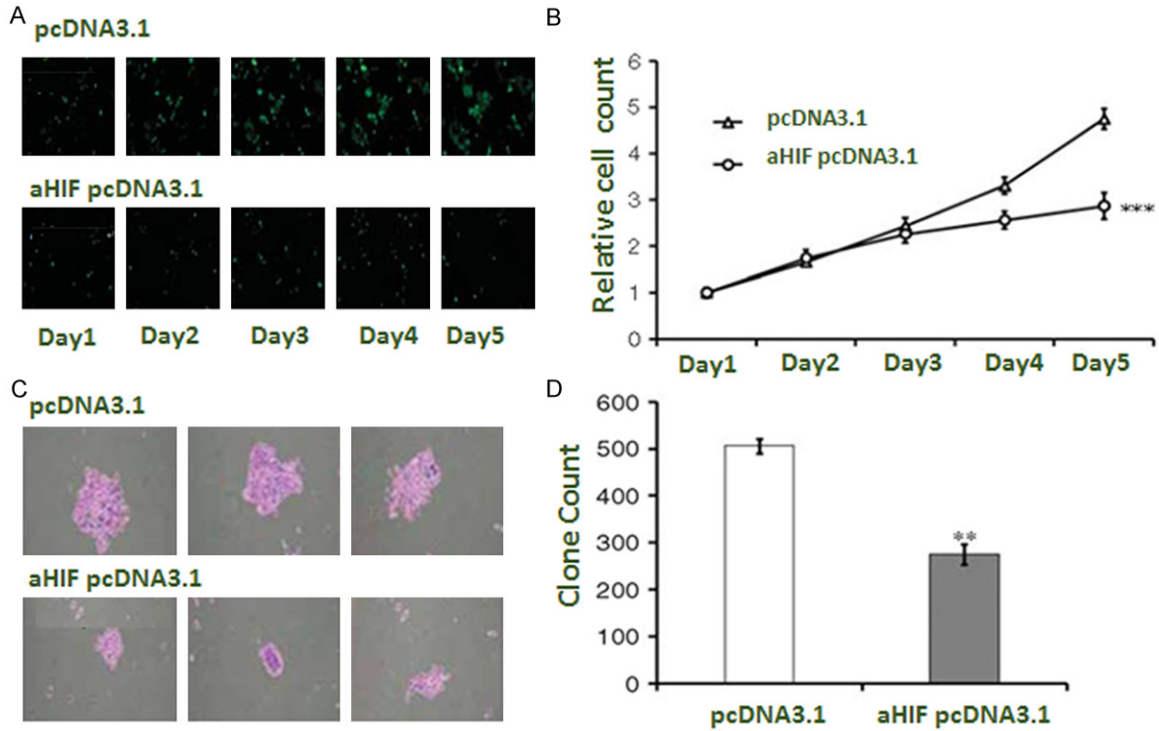


Figure 3. Downregulation of HIF-1 α expression effects the proliferation of HepG2 cells. A, B. The cell counting assay exhibited aHIF-pcDNA3.1-infected cancer cells were characterized by reducing growth ability compared with those infected pcDNA3.1. C, D. Colony formation test showed that aHIF-pcDNA3.1-infected cancer cells were characterized by reducing growth ability compared with those infected pcDNA3.1. Results represented the means \pm SD of three independent experiments (** $P < 0.01$, *** $P < 0.001$).

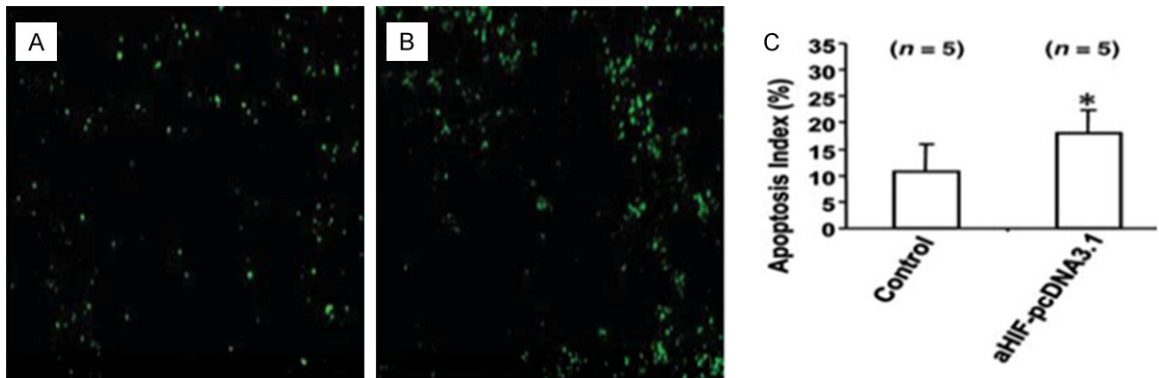


Figure 4. Antisense HIF-1 induces cell apoptosis. Representative tumor sections prepared 2 weeks after treatment from mice receiving (A) pcDNA3.1 or (B) aHIF-pcDNA3.1 treatment. Tumor sections were stained with the terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling agent to view apoptotic cells. (C) Cells stained by the TUNEL agent were counted to calculate the apoptosis index. *Significant difference in the apoptosis index for tumors treated with aHIF-pcDNA3.1 versus control.

Antisense HIF-1 α gene significantly suppresses hepatomas

HepG2 tumors were established in mice. Three weeks later, when the tumors reached approximately 100 mm³, the mice were assigned ran-

domly to two treatment groups, which received aHIF-pcDNA3.1 or pcDNA3.1 vector, respectively. Untreated mice served as an additional control. As shown in **Figure 2**, tumors treated with empty vector pcDNA3.1 grew remarkably fast, reaching 2014 \pm 287 mm³ in volume 6

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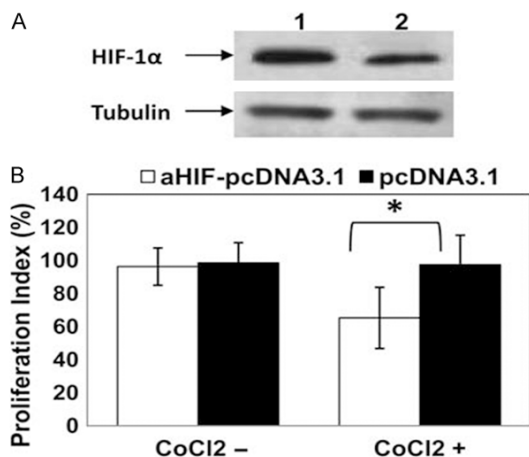


Figure 5. Antisense HIF-1 gene transfection down regulates HIF-1 expression and inhibits cell proliferation *in vitro*. A. Lysates of HepG2 cells transfected with aHIF-pcDNA3.1 (lane 2) and pcDNA3.1 (lane 1) were western blotted with antibodies against HIF-1 and tubulin. B. HepG2 cells transfected with aHIF-pcDNA3.1 or pcDNA3.1 were cultured in the absence or presence of CoCl₂ to mimic hypoxia. Untreated cells served as controls. Cell proliferation was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide method to calculate the proliferation index (% inhibition of cell proliferation). *Significant difference in the proliferation index.

weeks after implantation. The size was not significantly different from the growth of the untreated tumors ($2121 \pm 267 \text{ mm}^3$, $P > 0.05$). In contrast, the tumors of mice treated with antisense HIF-1 were significantly smaller than control tumors, reaching only $1041 \pm 204 \text{ mm}^3$ in volume 6 weeks after implantation ($P < 0.01$).

Antisense HIF-1 α inhibits cell proliferation *in situ*

In order to investigate whether downregulation of HIF-1 α expression effects proliferation of HepG2 cells, we analyzed the correlation between HIF-1 α expression and the growth and colony formation ability in HepG2 cells. We found that aHIF-pcDNA3.1-infected HepG2 cells exhibited less cell number ($P < 0.001$, **Figure 3**) compared with those infected with pcDNA3.1, suggesting that expression of HIF-1 α might be related to HepG2 cell proliferation. Additionally, the effect was observed with a time-dependent manner. Significant inhibition was found after 3 days (**Figure 3A**). Further investigation was applied to assess the colony formation capacity of aHIF-pcDNA3.1-infected HepG2 cells. Control cells infected by pcDNA3.1

were grown in the media to form colonies. Colony counting results showed that there were fewer colonies of HIF-1 α knock-down HepG2 cells (**Figure 3C**), indicating that there were fewer cells in each colony after downregulation of HIF-1 α . Taken together, knockdown of HIF-1 α could impair colony formation capacity of human HCC cells.

Antisense HIF-1 α induces cell apoptosis *in situ*

Tumor sections from the above experiments were stained with the TUNEL agent and examined by fluorescence microscopy. A small number of apoptotic cells were detected in tumors injected with pcDNA3.1 (**Figure 4A**), whereas a greater number of apoptotic cells were detected in tumors treated with aHIF-pcDNA3.1 (**Figure 4B**). The apoptotic cells in sections were counted to record the apoptosis index. The apoptosis index for tumors treated with aHIF-pcDNA3.1 was significantly higher than that of tumors treated with pcDNA3.1 (**Figure 4C**; both $P < 0.05$).

Antisense HIF-1 α down regulates HIF-1 α expression and inhibits the proliferation of HepG2 cells subjected to hypoxia

Transfection of HepG2 cells with aHIF-pcDNA3.1 induced downregulation of HIF-1 α expression in HepG2 cells cultured in the presence of CoCl₂ to induce hypoxia (**Figure 5A**). There was no significant difference in the rate of proliferation of HepG2 cells transfected with aHIF-pcDNA3.1 and pcDNA3.1 when the cells were cultured under normoxic conditions (**Figure 5B**). However, when the latter cells were exposed to hypoxia induced by CoCl₂, the cells transfected with aHIF-pcDNA3.1 grew significantly more slowly than those transfected with pcDNA3.1 (**Figure 5B**).

Discussion

HCC is extremely insensitive to chemotherapy. Hypoxia is a major cause of tumor resistance to radiotherapy and chemotherapy. HIF-1 is central to the hypoxia response of tumors as it regulates a wide range of hypoxia-related molecules [8]. HIF-1 overexpression induces angiogenesis in hypoxic tissues and it can lead to increased oxygenation of the organ [15]. Normoxic basal levels of HIF-1 α are sufficient to confer increased target gene expression as well as increased resistance to chemotherapy [12].

The present study has demonstrated that antisense HIF-1 α was effective in suppressing tumor growth, cell proliferation, and inducing cell apoptosis. Antisense HIF-1 α was shown to induce the apoptosis of HepG2 cells in situ, and Antisense HIF-1 α transfection also inhibited the proliferation of HepG2 cells in vitro under hypoxic conditions. HIF-1 also controls the expression of the two key glycolysis factors: glucose transporter 1 and lactate dehydrogenase A [15]. There is mounting evidence that HIF-1 α , the oxygen sensitive subunit of HIF-1, provides protection against cell death and stimulates tumor growth by upregulating genes that are involved in cellular energy metabolism [9].

In summary, our study has demonstrated that antisense HIF-1 α was effective in suppressing tumor growth, cell proliferation, and inducing cell apoptosis.

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Disclosure of conflict of interest

None.

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