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ORIGINAL ARTICLE

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Hypoxia-induced resistance to cisplatin and doxorubicin in non-small cell lung cancer is inhibited by silencing of HIF-1α gene

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Abstract *Objectives*: Hypoxia is associated with human non-small cell lung cancers (NSCLC), which are highly resistant to chemotherapy. The hypoxia inducible factor (HIF) as a transcription factor in response to hypoxia indicates that it could be a novel, tumor-specific target for anticancer therapy. We hypothesized that disruption of HIF pathway through lentiviral vector-mediated HIF-1α RNA interference (RNAi) could reverse the hypoxia-induced resistance to chemotherapy. *Methods*: We transfected Human NSCLC cell lines, SPCA1 and A549 with HIF-1α specific RNAi lentiviral vectors as well as controls. HIF-1α silenced cells [SPCA1/HIF-1α(-) and A549/HIF-1α(-)] were screened by blasticidin. They were incubated in 19 or 0.5% O_2 for 16 h followed by the assessment of chemosensitivity to cisplatin and doxorubicin with MTT and clonogenic assays. Quantitative RT-PCR and Western blot analysis were used to detect the expressions of HIF-1α mRNA and protein, respectively. Moreover, flow cytometry was used to monitor the expression of *P*-glycoprotein. *Results*: Exposure of SPCA1 and A549 cells to 0.5% O_2 significantly increased resistance to cisplatin and doxorubicin, in contrast to cells incubated in normoxia. Transduction of SPCA1 with HIF-1α RNAi vector resulted in sequence specific silencing with 87.2 and 84.6% decreases of HIF-1α mRNA transcription and 97.3 and 94.8% of protein expressions in normoxia and hypoxia, respectively. Correspondingly, they are 89.2, 89.9% and 97.2, 88.4% decreases in A549 cells. Hypoxia-induced resistance to cisplatin and doxorubicin were reversed in SPCA1/HIF-1α(-) and A549/HIF-1α(-) cells. There was no significant *P*-glycoprotein increase induced by hypoxia in NSCLC cells. *Conclusions*: Our studies demonstrated that hypoxia-induced chemoresistance to cisplatin and doxorubicin in NSCLC cells is through the HIF pathway. MDR1 regulation may not be involved in hypoxia-induced chemoresistance. Combining delivery of HIF-1α RNAi lentiviral vector with cisplatin-related chemotherapy regimens may enable us to develop more effective strategy for NSCLC therapy.

Keywords Non-small cell lung cancer · RNA interference · Hypoxia inducible factor-1 alpha · Drug resistance

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Introduction

Lung cancer is currently the leading cause of cancer deaths worldwide. Non-small cell lung cancer (NSCLC) constitutes approximately 85% of all lung cancers. The treatment of advanced NSCLC is based on the combination of cisplatin and one of the following agents: taxanes, gemcitabine, vinorelbine or irinotecan [1, 2]. Although chemotherapeutic regimens with greater efficacy continue to be developed, the best regimens can only give an overall response rate of 30–50%. Lack of response to chemotherapy in some individuals is attributed to the development of multidrug resistance (MDR). The mechanisms involved in chemotherapy resistance remain unknown.

Hypoxia, a reduction in the normal level of tissue oxygen tension, occurs in several pathophysiological processes including tumorigenesis. It also occurs in NSCLC as it does in most solid tumors. Although hypoxia induces a situation unfavorable for cell growth,

cancer cells may undergo a series of genetic and metabolic changes that allow them to survive and even proliferate [3, 4]. Hypoxia-inducible transcription factor (HIF-1) is a major transcription factor that plays a central role in hypoxic expressions of a variety of genes [5]. HIF-1 is a heterodimer HLH-PAS protein, which consists of α, and β subunits [6]. HIF-1β (ARNT) is constitutively expressed (in some cells) while cellular oxygen levels precisely regulate HIF-1α. Under hypoxic conditions, HIF-1α is induced, dimerizes with β subunits, translocates to the nucleus, and initiates gene transcription of the targeting genes [5]. It has been reported that HIF-1 has induced more than 40 genes under hypoxic conditions. For example, HIF-1 activates the transcription of glycolytic enzymes such as aldolases A and C, enolase 1, lacrate dehydrogenase A, phosphoglycerate kinase (PGK)1 [7]; glucose transporters such as glucose transporter 1 and 3 (GLUT1 and GLUT3) [7]; angiogenic molecules such as vascular endothelial growth factor (VEGF) and angiogenin [8]; survival and growth factors such as platelet-derived growth factor-B(PDGF-B), transforming growth factors-β(TGF-β) and insulin-like growth factors such as platelet-derived growth factor-II (IGF-II) [7]; enzymes and proteins involved in tumor invasiveness, metastasis and chemotherapeutic resistance, and some apoptosis-related proteins [9, 10].

Tumor hypoxia had been shown to be an independent prognostic indicator of poorly clinical outcome for cancer patients and to correlate with increase of tumor invasion and metastasis [11, 12], as well as to resist to certain chemotherapeutic agents [13]. This resistance to anticancer drugs has been attributed to the lack of O_2 available for anti-tumor drugs to act, the DNA over-replication, the increased genetic instability, the anti-proliferative effects of hypoxia [14], increase in the MDR transporter *P*-glycoprotein [15, 16], and mostly to the increases of gene transcription induced by HIF-1.The importance of HIF-1 as a transcription factor suggests that it could be a novel, tumor-specific target for anticancer therapy [17]. Chen et al. have demonstrated that dominant-negative HIF-1α rendered pancreatic cancer cells sensitive to apoptosis and growth inhibition induced by hypoxia and glucose deprivation, and reduced their tumorigenicity [18].

In the light of the previous studies showing that down-regulation of HIF-1 could reduce its HIF-1-induced transcriptional activation of genes, inhibit cell proliferation, reduce angiogenesis and xenograft tumorigenesis, we hypothesized that the interference of HIF-1α could not only disrupt the pathway of hypoxic NSCLC cell's response to hypoxia, but also sensitize cancer cells to chemotherapeutic agents. The combination of cytotoxic drugs and HIF-1 inhibition may have a synergistic anticancer effect. In this study, we established two stable HIF-1α silencing lung adenocarcinoma cell lines, SPCA1/HIF1α(-) and A549/HIF1α(-), with lentiviral vector mediated RNAi technology, and determined whether hypoxia increases the resistance of lung adenocarcinoma cells to cisplatin and doxorubicin and whether this hypoxia-induced blocking of the HIF-1 pathway could attenuate drug resistance.

Materials and methods

Cell lines and cell culture

The human lung adenocarcinoma cell lines SPCA1 (China Centre for Type Culture Collection, CCTCC, Wuhan, China) and A549 (American Type Culture Collection, Manassas, Virginia), were maintained in Dulbecco's modified Eagle's medium (DMEM, GIBCO, USA) containing 10% heat-inactivated fetal bovine serum (FBS, GIBCO), 100 U/ml penicillin, 100 U/ml streptomycin and 2 mM L-glutamine at 37°C in a humidified atmosphere of 5% CO_2. Cells were passaged every 2–3 days to maintain exponential growth.

Production of lentiviral vector

The BLOCK-iT Lentiviral RNAi Expression System (Catalog no: K4944-00) (Invitrogen corporation, USA) produced lentiviral vector expressing human HIF-1α short hairpin RNA(shRNA) following manufacture's instruction. In brief, a transfer plasmid pLenti6-HIF1α harbouring the human HIF-1α shRNA expressing cassette and a non-specific control plasmid were firstly constructed. The target sequence was GATACA AGTAGCCTCTTTG, which was selected according to HIF-1α mRNA sequence (Gene bank: BC012527) and tested to be efficacious to induce a strong RNAi previously. Lentiviral vector supernatant pseudotyped with the vesicular stomatitis virus glycoproteins (VSVG) was produced by four-plasmid cotransfection in the HEK 293FT transient system. 9 μg of the ViralPower™ Packaging Mix and 3 μg of pLenti6-HIF1α or control plasmid (12 μg total) were used to cotransfect 6×10^6 293FT cells with 36 μl of Lipofectamine™ 2000 in a 10 cm cell culture dish. The medium containing DNA-LF 2000 complexes was replaced with complete culture medium containing sodium pyruvate. Viral supernatant was harvested 48 and 72 h after transfection and filtered through a 0.45 μm cellulose acetate filter, then ultracentrifuged at 50,000*g* 4°C for 2 h. The viral pellets were resuspended in serum-free DMEM, divided into aliquots and frozen at −70°C. The functional titer of virus in SPCA1 and A549 cells was determined by in vitro transduction and blasticidin selection.

Construction of stable silencing lines

SPCA1 and A549 cells were transduced with specific or control lentiviral vectors at five MOI and were selected for stable integrants by culturing in complete medium containing blasticidin (10 μg/ml). After 10–12 days of selection, there are no viable cells in the mock well and discrete blasticidin-resistance colonies. The survival cell

colonies were indicated as SPCA1/HIF-1α(-), SPCA1/NS, A549/HIF-1α(-) and A549/NS respectively. Ten to 20 passages were used in the experiments. There were no significant changes of morphological characterization from passage 10–20.

Exposure to hypoxia

Cells were plated in 60 mm dishes, 96-well plates or 12-well plates according to the experiment requirements and cultured at 37°C in a 19% O_2, 5% CO_2 previously. On the day of experiment, the medium was replaced with a thin layer of fresh medium with 10% FCS to decrease the diffusion distance of the ambient gas. Culture dishes were then placed in a humidified airtight O_2-control incubator (Galaxy R, RS Biotech) and set the incubator at 37°C, 0.5% O_2 and 5% CO_2. The incubator was kept at set-point for 4–16 h. At the same time, normoxia cells were placed at 37°C in a 19% O_2 and 5% CO_2 incubator (BBD6220, Heraeus) until harvest.

MTT cell viability assay

Cells were seeded in 96-well plates (Costar; Corning Inc., Corning, NY, USA) for 8 h. Following exposure to 19 or 0.5% O_2, they were exposed to various concentrations of cisplatin or doxorubicin for 48 h in a CO_2 incubator. 10 μl of MTT (5 mg/ml in PBS) was added to each well for 4 h at 37°C. Subsequently the formazan crystals were solubilized with 100 μl of 10% sodium dodecyl sulfate (SDS) in 0.01 M HCl for 24 h. Absorbance at 550 nm relative to a reference wavelength of 630 nm was determined with a microplate reader (Bio-rad 680, Bio-rad, USA). Absorbance values were expressed as percentages relative to untreated controls, and the concentrations resulting in 50% inhibition of cell growth (IC_{50} values) were calculated.

Clonogenic assay

Following exposure to 19 or 0.5% O_2, cells in 12-well plates were incubated with cisplatin or doxorubicin (Sigma) for 1 h in a standard CO_2 incubator with 19% O_2. Cultures were then washed with drug-free PBS, harvested with 0.25% trypsin-EDTA in PBS, resuspended in10% FBS DMEM and counted. The cells were then seeded in 6-well plates triplicate with the cell number of 100, 200, 500, 1,000 and 2,000 corresponding to five different concentrations of the drugs. After 10–12 days of incubation under the condition of 37°C, 5% CO_2, colonies were fixed with acetic acid–methanol (1:4) and stained with dilute crystal violet (1:30) prior to being counted. Colonies containing 50 cells or more were counted as survivors. The triplicate colonies were averaged and divided by initial seeded cells to yield survival rate of clones for each concentration. Surviving fractions were determined by dividing the clonic survival rate of drug-treated groups by that of corresponding control groups.

Protein Western blot analysis

After exposure to hypoxia for 12 or 24 h, cells were washed once with pre-cold PBS quickly and homogenized in extraction buffer containing 0.15 M NaCl, 50 mM Tris–Cl (pH 7.4), 2 mM EDTA (pH 8.0), 2 mM EGTA (pH 8.0), 0.5% Triton-100, 5 mM DTT, 0.2 mM PMSF and 1 μg/ml aprotinin. The lysates were incubated on ice for 4 h with intermittent vortexing followed by centrifugation at 10,000*g* for 10 min at 4°C. The supernatant was collected. The total cellular protein concentration was determined with the method of Bradford. For immunoblot assays, 50 μg of protein samples were fractionated by 7.5% resolving SDS-PAGE and electroblotted onto PVDF membrane using an electronic Bio-Rad transfer apparatus. The membranes were blocked overnight at room temperature in 1× TBS-0.05%Tween 20 (PBS-T) containing 5% dry milk powder. The blots were subsequently incubated for 2 h in 1% BSA TBS-T buffer containing 1 μg/ml mouse anti-human HIF-1α antibody (BD Biosciences) followed by three 10-min washes with TBST. Membranes were then incubated for 1 h with horseradish peroxidase labeled rabbit antimouse IgG secondary antibody (Amersham Biosciences) (1:1,000). The amount of each protein sample was controlled by β-actin. After extensive washing with 1× PBS-T, the immunocomplexes on the membranes were reacted with ECL-plus reagent (Amersham Biosciences, Buckinghamshire, UK) for one min and exposed onto Kodak Imaging film. X-rays were scanned and analyzed using Multiimage light cabinet and software (Alpha Innotech Corporation).

RNA preparation and real-time RT-PCR analysis

Total RNA was isolated from culture cells using Trizol reagent (Invitrogen). Reverse transcription was performed at 42°C for 15 min in a volume of 10 μl of 1×M-MLV buffer, 0.5 μl of random 6-mer primers, 0.5 μM of dNTP mix, 0.25 μl of M-MLV RTase and 0.25 μl of RNase inhibitor (TaKaRa RT kit). The enzyme was inactivated at 95°C for 5 min. The cDNA products were used for real-time PCR. Quantitative determination of HIF-1α mRNA was performed by real-time RT-PCR with rRNA cDNA as an internal control. Primers and probes for HIF-1α were designed using Primer Express software (Applied Biosystems): U22431, forward primer: 5′-CTG AGG TTG GTT ACT GTT GGT ATC-3′, reverse primer: 5′-AGT GTA CCC TAA CTA GCC GAG GAA-3′ Probe: FAM 5′-TGC ACT GCA CAG GCC ACA TTC ACG-3′ TAMRA . The rRNA primers and probes with the reporter dye VIC were purchased from PE Applied Biosystems. Reactions were performed in triplicate for each sample and for NTC and NAC controls. Briefly, the multiple reactions were performed in a volume of 25 μl containing 12.5 μl of 2× PCR master mix, 5 μM of specific primers and probes, 2.5 μM of rRNA primers and probes, 200 ng of cDNA template (5 μl), and QuantiTect Probe RT mix (0.5 μl, Qiagen).

Amplifications were performed in an ABI Prism 7000 Sequence Analyzer using the following cycle program: 25°C for 2 min, 50°C for 30 min, then 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The relative expression level of HIF-1α gene was calculated by comparing threshold cycle (Ct) values of samples to that of the reference. All data was normalized to the standard internal rRNA. Accordingly, ΔCt = (mean Ct value of HIF-1α) – (mean Ct value of rRNA), and ΔΔCt = ΔCt (selected cells) – ΔCt (parental cells). The relative gene expression in a particular sample was then given by the following: $2^{-\Delta\Delta Ct}$ value.

Flow cytometric analysis of *P*-glycoprotein

Following 4–24 h exposure to hypoxia or normoxia, cells were harvested by trypsinization, washed twice with 1× PBS, and incubated with PE-conjugated mouse monoclonal antibody (BD PharMingen) against human *P*-glycoprotein at room temperature for 1 h in dark. The cells were subsequently washed twice and fixed in 0.5 ml 4% paraformaldehyde. *P*-glycoprotein expression on cell surface was analyzed with a Becton-Dickinson FACS Calibur Flow Cytometer.

Calculations and statistical analysis

Plating efficiency was calculated according to the number of surviving colonies expressed as a proportion of the total number of cells seeded. Surviving fractions were determined by dividing the plating efficiency of drug-treated groups by the plating efficiency of their control groups. The drug concentrations for 1% survival were determined according to the surviving fraction curve. Density values of Western blot experiments were normalized using β-actin bands and results were expressed as relative to control cells incubated in 19% O_2.

Results

Exposure to hypoxia and drug resistance

The effects of cisplatin and doxorubicin on the growth of NSCLC cells were evaluated by determining cell viability using MTT assay. The IC_{50} values of anticancer drugs in SPCA1 and A549 cells were shown in Fig. 1. Exposure of both cells to 0.5% O_2 for 16 h increased resistance to various concentrations of cisplatin and doxorubicin significantly, in contrast to cells incubated in normoxia condition (19% O_2) (Fig. 1). Compared with cells exposed to 19% O_2, the IC_{50} value of SPCA1 exposed to 0.5% O_2 was 1.81-fold higher for doxorubicin and 2.38-fold higher for cisplatin. Similarly, A549 cells exposed to 0.5% O_2 induced a 1.68-fold higher IC_{50} for doxorubicin, a 1.66-fold higher IC_{50} for cisplatin.

Hypoxia-induced chemoresistance was also observed in a clonogenic assay. SPCA1 and A549 cells were incubated with 19% O_2 or 0.5% O_2 for 16 h and then exposed to different concentrations of doxorubicin or cisplatin for 1 h. Statistically higher levels of survival were observed in both SPCA1 and A549 cells pre-incubated in hypoxic condition, in contrast to cells incubated in the standard condition of 19% O_2 (Fig. 2).

Down-regulation of hypoxia-induced HIF-1α accumulation by RNAi

Both SPCA1 and A549 cells demonstrated resistance to anticancer agents under the condition of hypoxia. In order to determine whether the accumulation of HIF-1α induced by hypoxia was a key factor in this process, we constructed HIF-1α gene silenced cells (SPCA1/HIF-1α(-) and A549/HIF-1α(-) with lentiviral vector-based RNAi technology. The mRNA levels of HIF-1α were quantitatively analysed by real time RT-PCR. As expected, in both SPCA1/HIF-1α(-) and A549/HIF-1α(-) cells, HIF-1α expression was virtually attenuated whether they grew in 19% O_2 or 0.5% O_2 compared to their parental cells. The mean relative expression levels decreased to 10.8 and 11.1% for A549/HIF-1α(-) cells, 13.8 and 15.4% for SPCA1/HIF-1α(-) cells in normoxia and hypoxia condition.

To confirm our findings of mRNA expression, protein levels of HIF-1α were determined by Western blot analysis. Since non-specific lentivirus transduced cells showed no significant influence on HIF-1α expression at mRNA level, only parental cells were used as controls in the subsequent experiments. During normoxia, HIF-1α was at a very lower level in both parental control cells, and almost undetectable in SPCA1/HIF-1α(-) and A549/HIF-1α(-) cells. Exposure to hypoxia (0.5% O_2) for 12 or 24 h induced sharp HIF-1α protein accumulation in parental cells but slight in gene silenced cells (Fig. 3). The relative HIF-1α protein level of SPCA1/HIF-1α(-) versus SPCA1 was 2.7 and 5.2% in 19 or 0.5% O_2 for 24 h, and 2.8 and 11.6% of A549/HIF-1α(-) versus A549 cells. The results showed that lentiviral vector-mediated RNAi could stably block the induction of HIF-1α by hypoxia in post-transcriptional stage, but not in post-translational changes.

Hypoxia-induced drug resistance was inhibited by HIF-1α interference

The increased resistance of SPCA1 and A549 cells to doxorubicin and cisplatin under hypoxia could be contributed to the accumulation of HIF-1α and its subsequent regulation of gene transcription. To verify this hypothesis, we observed the sensitivity of HIF-1α gene silenced cells to doxorubicin and cisplatin. It was proved that the HIF-1α mRNA of SPCA1/HIF-1α(-) and A549/HIF-1α(-) were at a relatively lower level and the protein accumulation under hypoxia was also blocked. Because the resistance of cancer cells to alkaline chemotherapeutic agents was partly due to low pH environment, to

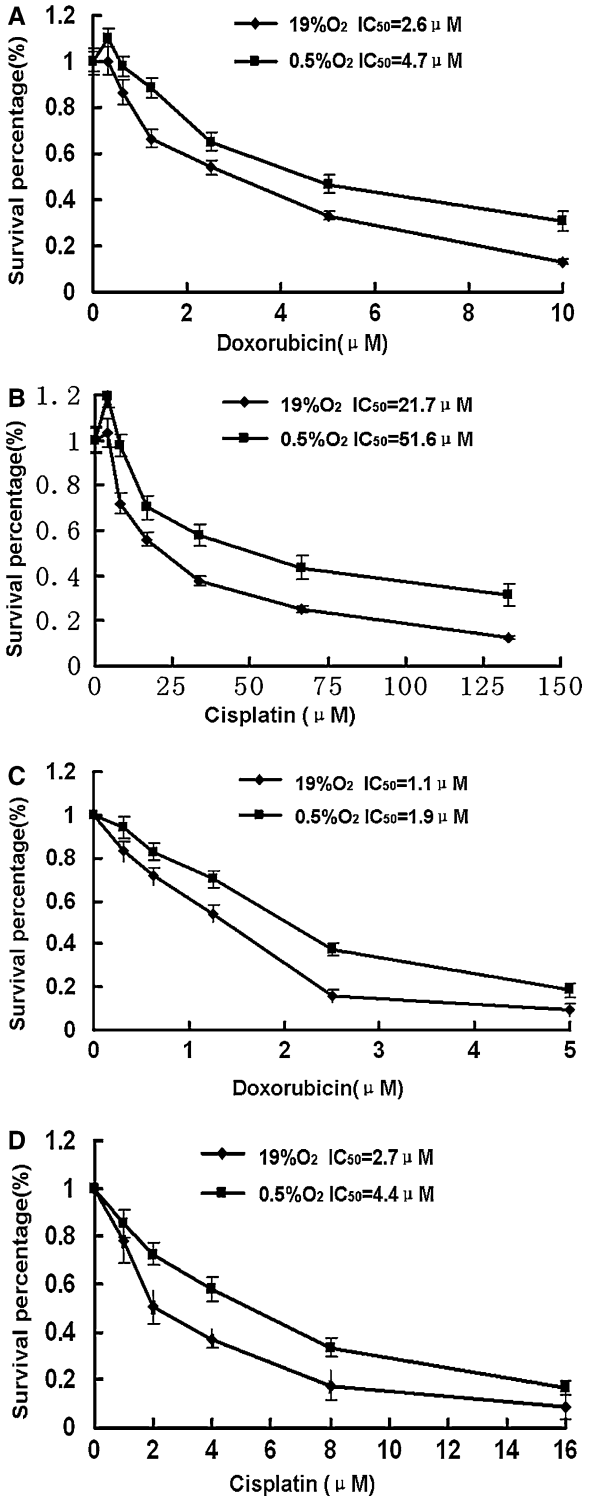


Fig. 1 Effects of O_2 concentration on chemosensitivity of NSCLC cells to cisplatin and doxorubicin. Cells were incubated in 19% O_2 (*squares*) or 0.5% O_2 (*diamond*), then exposed to various concentrations of cisplatin or doxorubicin for 48 h, and the viability was accessed. The percentage of cell growth was calculated by comparison of the A570 reading from treated cells versus control cells. The IC_{50}value of SPCA1 cells (**a**, **b**) to cisplatin (**a**) and doxorubicin (**b**) under normoxia or hypoxia condition was 21.7, 51.6, 2.6, and 4.7 μM, respectively. The IC_{50} values of A549 cells (**c**, **d**) to cisplatin (**c**) and doxorubicin (**d**) under normoxia or hypoxia condition were 2.7, 4.4, 1.1, and 1.8 μM, respectively

separate cell physiologic and chemical factors, we determined the sensitivity of cells first kept under hypoxia and reoxygenated while exposed to drugs. Whether in hypoxia or normoxia, the sensitivity of SPCA1/HIF-1α(-) cells to doxorubicin or cisplatin was similar. Hypoxia-induced resistance disappeared in HIF-1α gene silencing cells. Compared to normoxia, the 1% survival concentration for cisplatin was 1.92,1.09,1.96 and 1.11-fold increase under 0.5% O_2 for SCPA1, SPCA1/HIF-1α(-), A549 and A549/HIF-1α(-), respectively. For doxorubicin, it was 1.39, 1.23, 1.39 and 1.05 respectively (Fig. 3).

MDR1 gene expression of HIF-1α gene silenced cells

Flow cytometry analysis showed that Pgp expressions of SPCA1 and A549 cells were at very low level, the positive rates were less than 6%. Incubation in 0.5% O_2 for 16 h did not significantly increase the levels of Pgp in both cell lines (Fig. 4). Similarly, low levels of O2 while compared to their controls did not affect Pgp expressions of HIF-1α gene silencing NSCLC cells. However, there was an observed trend for increased Pgp expression in A549 cells incubated in 0.5% O_2 versus 19% O_2 ($P=0.083$).

Discussion

Hypoxia, a reduction in the normal level of tissue oxygen tension, occurs during several pathophysiological processes including tumorigenesis. Hypoxic cancer cells may undergo a series of genetic and metabolic changes that allow them not only to survive and proliferate but also to become more resistance to conventional therapies including ionizing radiation and chemical agents. Hypoxia is associated with poor treatment outcome regardless of the modality, suggesting that it should be considered in the development of optimum treatment strategies. The key role of hypoxia-inducible factors in the mechanism through which tumor cells respond to reduced oxygen levels has made it as a target for the development of anticancer therapeutics. Supportive data has arisen through the observations that disruption of HIF-1 transactivation was a strategy to reduce tumor growth [18–20]. HIF-1 has an influential role in modulating chemotherapeutic responses [21–23]. If HIF-1 targeted approaches could be rationally applied in the context of standard chemotherapy, there is potential for a greater therapeutic efficacy than could be achieved through HIF-1 targeting alone. In this study we provided evidence of hypoxia-induced chemoresistance in NSCLC cells. Specifically, the results showed that hypoxia increased resistance to cisplatin and doxorubicin. In an effort to develop new strategies to disrupt hypoxia-induced HIF-1α accumulation, we have examined the potential of a lentiviral vector mediated specific RNAi approach for silencing the HIF-1α in NSCLC cell lines. A significant inhibition of HIF-1α is being identified not only under normoxia but also hypoxia condition. We

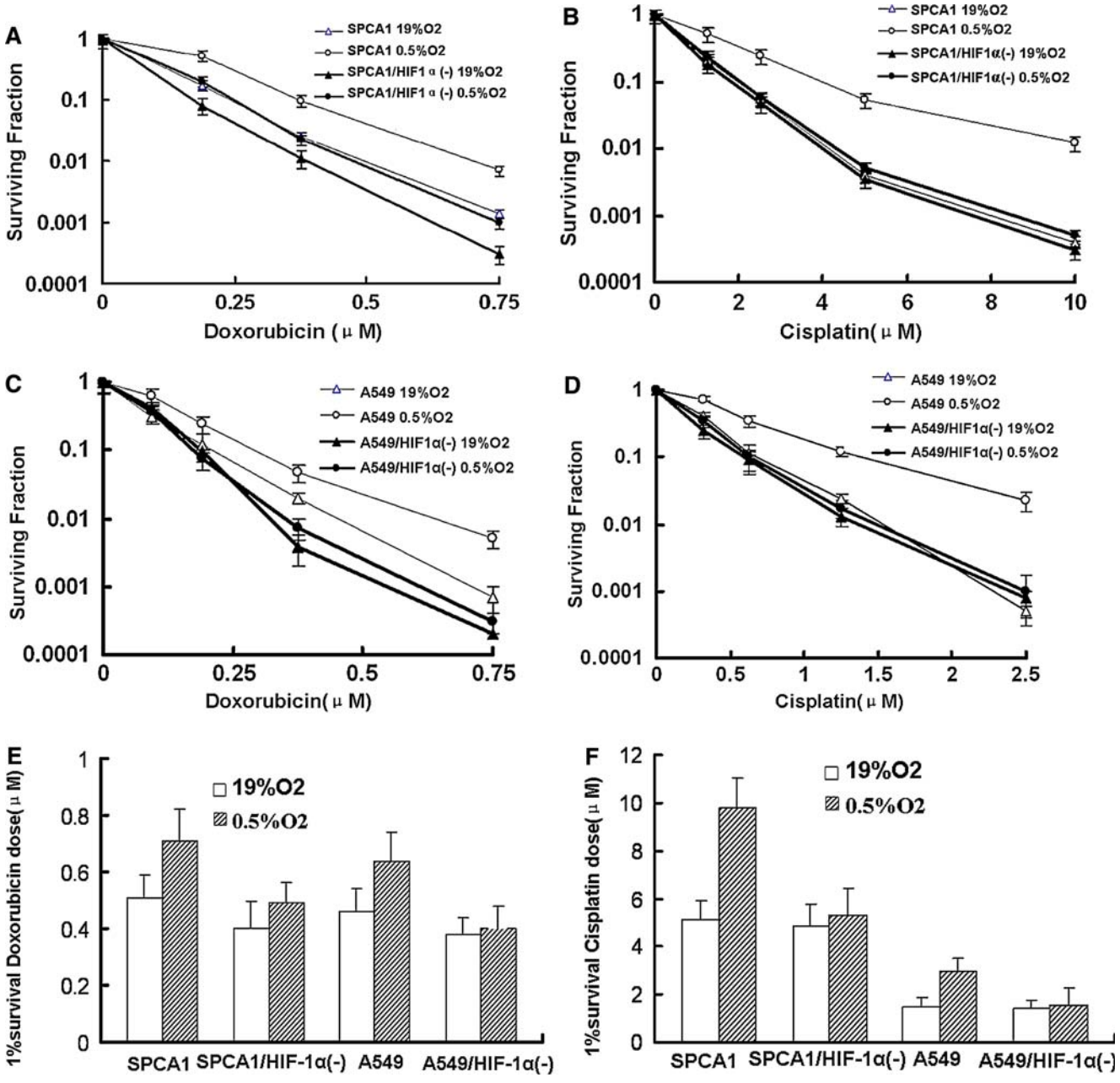
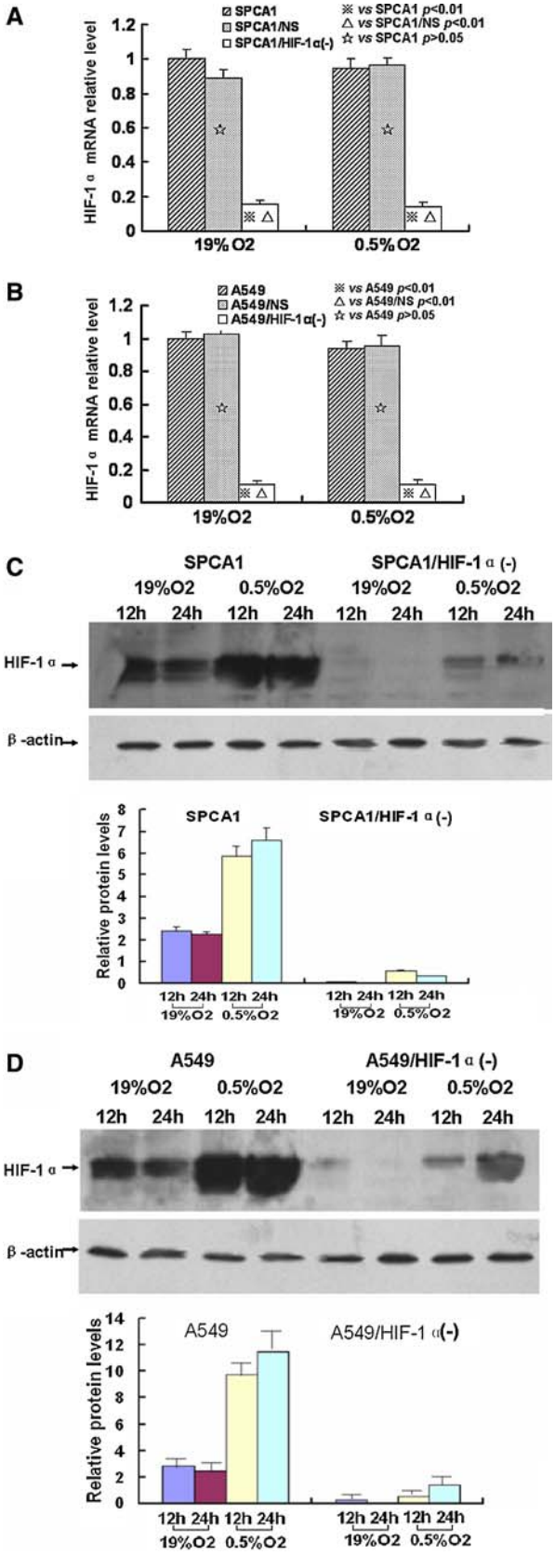


Fig. 2 Effect of O_2 concentration on survival of HIF-1α gene silencing NSCLC cells and the control cells to cisplatin and doxorubicin. Cells were incubated in 19% O_2 (*triangle*) or 0.5% O_2 (*circle*), then exposed to various concentrations of cisplatin or doxorubicin for 1 h and clonogenic assay was performed. Higher surviving fractions were observed in SPCA1 and A549 cultures incubated in 0.5% O_2 versus 19% O_2 for 16 h, and subsequently, they were exposed to cisplatin (**a**, **c**) and doxorubicin (**b**, **d**). HIF-1α gene silencing NSCLC cells revealed no chemoresistance induced by hypoxia. According to the surviving fraction curve, the 1% survival concentrations were calculated. For cisplatin, it was 1.9, 1.1, 2.0 and 1.1-fold increase in 0.5% O_2 versus 19% O_2 for SCPA1, SPCA1/HIF-1α(-), A549 and A549/H, respectively (**e**). Correspondingly, it was 1.4, 1.2, 1.4 and 1.1 for doxorubicin (**f**)

have provided additional evidence for the application of HIF-1 targeted approaches, especially the combination with cisplatin chemotherapy to reverse hypoxia-mediated drug resistance.

The development of transgenic animal models in which specific gene(s) have been ablated, overexpressed, or conditionally expressed has provided unique insights into their physiological significance and roles in various diseases including cancer. Analogous approaches have been used for studies in mammalian cells using transiently or stably transfected expression plasmids for specific genes or their antisense/dominant-negative counterparts. RNA interference associated with double-stranded RNA that is rapidly processed into siRNAs has been identified in many eukaryotes [24], and this approach has numerous applications [25]. For example, research in our laboratory [26] has shown that siRNA of EGFR decreases EGFR protein expression in SPCA1



and A549 cells. Moreover, silencing of EGFR sensitizes SPCA1 and A549 cells to cisplatin and inhibits the growth of cancer cells in vivo.

Fig. 3 Lentiviral vector-mediated RNAi effect on SPCA1 and A549 cells. SPCA1 and A549 cells were transduced with specific or control lentiviral vectors at 5 MOI and selected for stable integrants by culturing in complete medium containing 10 μg/ml blasticidin for 10–12 days. **a**, **b** HIF-1α mRNA levels were quantified in control and transduced cells by real-time RT-PCR. The mean relative expression levels were only 10.8 and 11.1% for A549/HIF-1α(-), 13.8 and 15.4% for SPCA1/HIF-1α(-) under normoxia and hypoxia condition compared to their control cells. **c**, **d** HIF-1α protein level in control and transduced cells was analyzed by Western blot. Relative density was analyzed using Multiimage light cabinet and software

The predominant mode of HIF-1α regulation occurs post-transcriptionally [27]. During normoxia, HIF prolyl hydroxylase-1–3 hydroxylates two proline residues of the oxygen-dependent degradation domain located in the central region of the protein [28–30]. The von Hippel-Lindau (VHL) tumor suppressor protein, a component of a multi-subunit ubiquitin ligase protein complex, binds to the hydroxylated oxygen dependent degradation domain of the α-subunit, resulting in the ubiquitination and proteasomal degradation of HIF-1α [31, 32]. Under hypoxic conditions, the VHL tumor suppressor protein fails to recognize the HIF-1α subunit, allowing HIF-1α to accumulate [33]. Due to degradation being blocked under hypoxic conditions, we suspected if HIF-1α could be effectively down-regulated by RNAi as other genes. As a matter of fact, we have successfully decreased the HIF-1α mRNA level and prevented the accumulation of HIF-1α protein under hypoxia in NSCLC cells. It provided convincing evidence for RNAi. We achieved significant effects of RNAi using Lentiviral vector (Fig. 2) and co-related with shRNA express sequence integrated into host DNA (unpublished data).

It has been proposed that a mechanism of chemotherapeutic agents is involved in the generation of cytotoxic radicals via a process that depends on the presence of O_2. In this study all incubations with cisplatin and doxorubicin were performed in the standard CO_2 incubator. This approach ensured that the lack of radical formation resulting from decreased O_2 would not be a confounding variable in explaining the increased chemoresistance observed in cells pre-exposed to hypoxia. In addition, the uptake of chemotherapeutic agents is highly influenced by the extracellular pH value. To avoid this influence, culture medium was refreshed before being exposed to drugs in MTT assay and clonogenic assay. Exposure of tumor cells to cisplatin or doxorubicin was also limited to only 1 h to avoid genetic alternations, and cells were maintained in 19% O_2 during colony formation to allow normal proliferative abilities of the cells.

Another mechanism by which hypoxia could increase chemoresistance was to enhance the expression of multidrug resistance (MDR1) gene via a HIF-1-dependent regulation [16, 34]. In our flow cytometry analysis, *P*-glycoprotein level was not significantly increased after 16 h incubation of hypoxia. Therefore, the induction of MDR1 gene expression may not be involved in the chemoresistance effect to hypoxia.

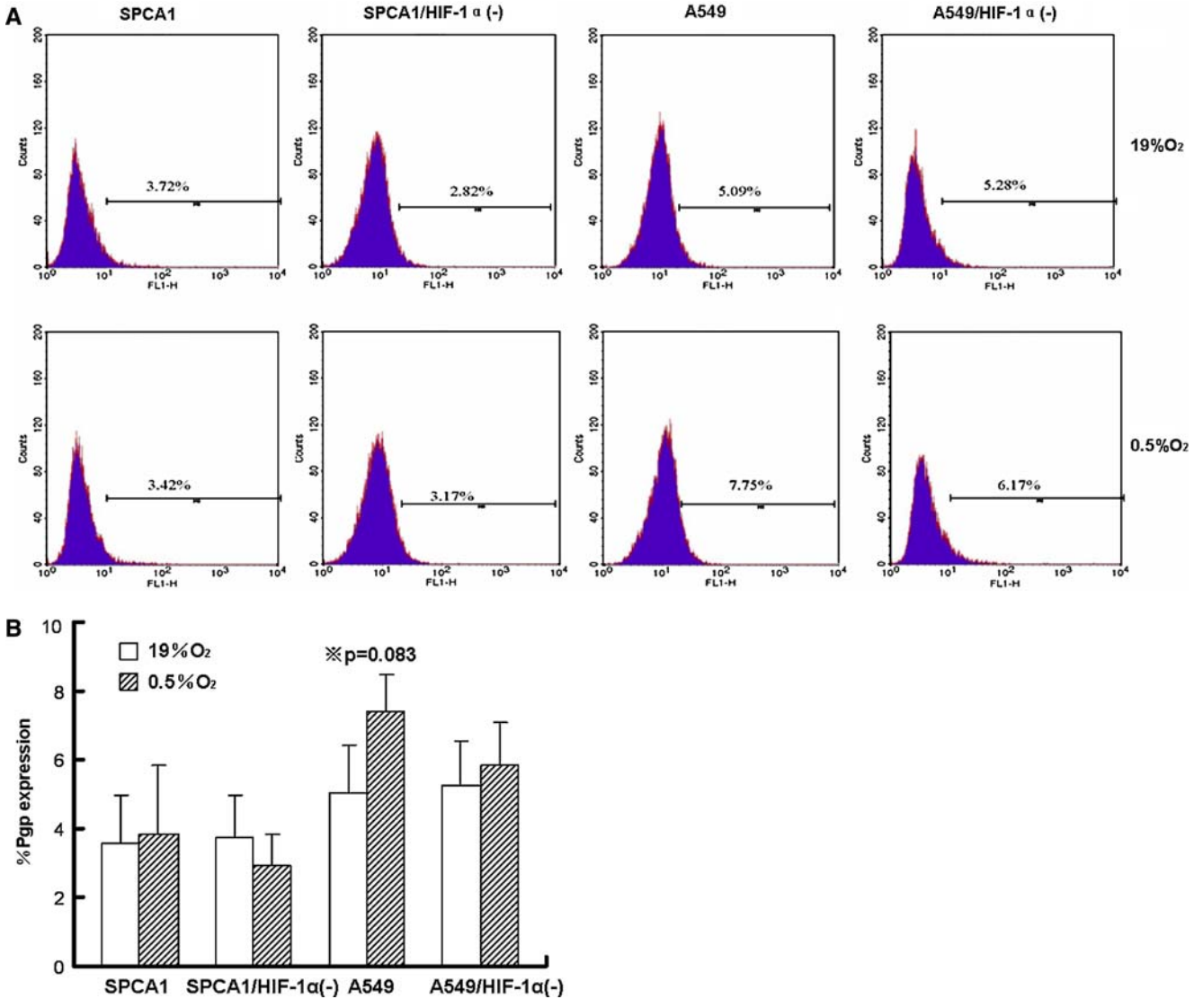


Fig. 4 Effect of O_2 concentration on expression of *P*-glycoprotein in HIF-1α gene silencing SPCA1 and A549 cells and the controls. Cultures were incubated in 19 or 0.5% O_2 for 16 h, and then stained with FITC labeled anti-Pgp monoclonal antibody and analyzed with a FACS calibur. **a** Representative result of two treatments is shown. **b** Mean ± SD of three experiments is shown

Cisplatin, a DNA-damaging anticancer agent, was one of the commonly used drugs in NSCLC chemotherapy. Most chemotherapy panels consisted of cisplatin and one of the following agents: taxanes, gemcitabine, vinorelbine or irinotecan. Our study found that the disruption of HIF-1 pathway resulted in enhanced twofold chemo-sensitivity to cisplatin in both A549 and SPCA1 cells. This could represent a clinical significance if transferred into clinical setting because cisplatin resistance of hypoxic NSCLC cells could be potentially overcome by this approach. Clearly, for the development of such a therapeutic strategy for clinical use, a suitable vector system is necessary. We have showed the lentiviral vector system was capable of inducing a significant silencing effect.

Recent reports demonstrated that hypoxic cells, in addition to being more chemoresistance, become resistant to apoptosis (programmed cell death), and are more likely to migrate to less hypoxic areas of the body (metastasis). Hypoxic cells also produce pro-angiogenic factors, such as VEGF, which stimulates the formation of new blood vessel from existing vasculature, increasing tumor oxygenation and, ultimately, tumor growth. For this reason, hypoxic tumors are the most pro-angiogenic and aggressive tumors. Therefore, it is a presumption that the disruption of HIF-1 pathway could help to overcome the chemoresistance as well as reverse the adaptive response of hypoxic NSCLC cells to low oxygen. When combined with anticancer agents such as cisplatin, this multi-effect of HIF-1α interference enable synergistic effect practically. To verify this hypothesis, we are now examining the sensitivity of HIF-1α silencing NSCLC cells to cisplatin and doxorubicin in xenograft mice models.

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