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# Effect of Chronic Intermittent Hypoxia on Biological Behavior and Hypoxia-Associated Gene Expression in Lung Cancer Cells

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## ABSTRACT

Hypoxia plays an important role in the development of solid tumors and is associated with their therapeutic resistance. There exist three major forms of hypoxia: acute, chronic, and intermittent hypoxia. Previous studies have shown that cancer cells could behave in the form of adaptation to hypoxia in tumor growth, which could result in their biological changes and determine their responses to the therapies. To investigate the tumor cells' adaptation to hypoxia, we recreated two models using two lung cancer cell lines in the presence of intermittent hypoxia, which is characterized by changes in oxygen pressure within the disorganized vascular network. We investigated biological behaviors such as cell cycle, proliferation, radiation sensitivity, apoptosis and migration, hypoxia signal pathway in the lung cancer cells treated with chronic intermittent hypoxia, as well as the role of hypoxia inducible factor 1 there, hypoxia-inducible genes analyzed by real-time RT-PCR chip in H446 cells treated with the model. The results indicated the changes of some hypoxia target gene expressions of those induced by hypoxia, some of which were confirmed by real-time RT-PCR. The cells mediated by irradiation induced resistance to radiation and

Abbreviations used: CIH, chronic intermittent hypoxia; HIF-1α, hypoxia-inducible factor 1, alpha subunit; HIF-2α, hypoxia-inducible factor 2, alpha subunit; MTT, 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide; MOI, multiplicity of infection; ADM, adrenomedullin; ANGPTL4, angiopoietin-like 4; BHLHB2, basic helix-loop-helix domain containing, class B,2; GP1, GTP-binding protein 1; HK2, hexokinase 2; HMOX1, heme oxygenase (decycling) 1; IGFBP1, insulin-like growth factor binding protein 1; MT3, metallothionein 3; PTX3, pentraxin-related gene, rapidly induced by IL-1 beta; SLC2A1, solute carrier family 2, member 1; VEGFA, vascular endothelial growth factor A; BIRC5, baculoviral IAP repeat-containing 5; CASP1, caspase 1 (apoptosis-related cysteine peptidase); CAT, catalase; CHGA, chromogranin A (parathyroid secretory protein 1); DR1, down-regulator of transcription 1, TBP-binding (negative cofactor 2); EP300, E1A binding protein p300; PRPF40A, PRP40 pre-mRNA processing factor 40 homolog A (*Saccharomyces cerevisiae*); GNA11, guanine nucleotide binding protein (G protein), alpha 11 (Gq class); HIF1A, hypoxia-inducible factor 1, alpha subunit; HIF3A, hypoxia-inducible factor 3, alpha subunit; KHSRP, KH-type splicing regulatory protein; MOCS3, molybdenum cofactor synthesis 3; PRKAA1, protein kinase, AMP-activated, alpha 1 catalytic subunit; SAE1, SUMO1 activating enzyme subunit 1; SLC2A4, solute carrier family 2, member 4; SNRP70, small nuclear ribonucleoprotein 70 kDa; TST, thiosulfate sulfurtransferase; UCP2, uncoupling protein 2; ARNT2, aryl-hydrocarbon receptor nuclear translocator 2; CA1, carbonic anhydrase I; COL1A1, collagen, type I, alpha 1; CREBBP, CREB-binding protein; CYGB, cytoglobin; DAPK3, death-associated protein kinase 3; HBB, hemoglobin, beta; HIF1AN, hypoxia-inducible factor 1, alpha subunit inhibitor; IGF2, insulin-like growth factor 2; IL1A, interleukin 1 alpha; IL6, interleukin 6 (interferon, beta 2); IL6ST, interleukin 6 signal transducer (gp130, oncostatin M receptor); LCT, lactase; LEP, leptin; NOS2A, nitric oxide synthase 2A; PEA15, phosphoprotein enriched in astrocytes 15; PPARA, peroxisome proliferator-activated receptor alpha; PSMB3, proteasome subunit, beta type, 3; RPL28, ribosomal protein L28; SSSCA1, Sjogren syndrome/scleroderma autoantigen 1; TH, tyrosine hydroxylase.

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apoptosis and increased metastasis in lung cancer cells. It was found that such changes were related to hypoxia inducible factor 1, alpha subunit (HIF-1α). J. Cell. Biochem. 111: 554–563, 2010. 

**KEY WORDS:** INTERMITTENT HYPOXIA; LUNG CANCER; MIGRATION; HYPOXIA-INDUCIBLE FACTOR; APOPTOSIS

Hypoxia, common in solid tumors, is associated with therapeutic resistance and malignant progression [Raghunand et al., 2003; Ahn and Brown, 2007]. Ample evidence indicate that the effect of hypoxia on malignant progression is mediated by a series of hypoxia-induced proteomic and genomic changes activating and promoting angiogenesis, anaerobic metabolism, and other processes facilitating cancer cells to survive or escape their low oxygen environment [Chaudary and Hill, 2007; Keith and Simon, 2007; Mabjeesh and Amir, 2007; Stadler, 2007]. In most tumor tissues, structurally and functionally compromised vasculature and disrupted diffusion conditions result in heterogeneous perfusion to tumors with an inadequate supply of oxygen (hypoxia) to regions [Kizaka-Kondoh et al., 2003; Zhou et al., 2006; Brahimi-Horn et al., 2007].

There are three major forms of hypoxia in solid tumors: chronic, acute, and intermittent hypoxia. The third case was characterized by cyclic periods of hypoxia and reoxygenation [Cooper et al., 2004; Weinmann et al., 2004, 2005; Okunieff et al., 2005; Yao et al., 2005; Liu et al., 2006; Martinive et al., 2006; Dewhirst, 2007]. The previous studies showed that the major phenotypic shift associated with chronic hypoxia led to cellular resistance to chemotherapy, radiotherapy, promoting the tumor cells to become more invasive and metastatic. Intermittent hypoxia increased tumor invasion [Yao et al., 2005; Martinive et al., 2006]. Hill and his co-workers found that exposure of tumor-bearing rodent hosts to cyclical (not chronic) hypoxia produced a significant increase in metastasis to the lungs [Cairns et al., 2001]. Many previous studies focused on acute or chronic hypoxia, but intermittent hypoxia also played an important role in solid tumors, whose exact biological responses, remained unknown, needing further investigations.

In this study, we constructed the cell models with lung cancer cells induced by chronic intermittent hypoxia (CIH) so as to study their biological behaviors such as radiation sensitivity, migration, and apoptosis, and analyze hypoxia's influence on the gene expression profile in the cells treated with CIH. Consequently, we confirmed some hypoxia-inducible gene expressions such as HIF-1α, VEGFA, and IGFBP1. To further investigate the molecular mechanism of the cell behaviors in the microenvironment of intermittent hypoxia, we examined the role of HIF-1α in migration, proliferation, radiosensitivity, and apoptosis.

## MATERIALS AND METHODS

### CELL CULTURE AND HYPOXIA TREATMENT

A549 and NCI-H446 (H446) human lung cancer cell lines, obtained from the American Type Culture Collection (Manassas, VA), were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin, pH 6.7–7.0. For all the experiments, the cells were maintained in a humidified incubator at 37°C and 5% $CO_2$. Approximately, the beginning passages of A549 and H446 cells were determined as the tenth.

For one cycle of hypoxia and reoxygenation, those growing exponentially at a density of $10^5$ cells/ml were placed into a hypoxic chamber to be cultured for 24 h before they were reoxygenated and the medium was changed. The cells were recovered under the normoxia condition for 72 h, which experienced 20 cycles of CIH. The culturing of the cells in hypoxia was performed in a chamber equilibrated with a water-saturated gas mixture of 0.1% oxygen, 5% carbon dioxide, and 94.5% nitrogen (Galaxy R $CO_2$ incubator, RS Biotech) at 37°C, and that of normoxic control cells was performed using a water-saturated incubator supplemented with 20% oxygen and 5% carbon dioxide at 37°C. The cells were not with more than 50 passages.

### CELL GROWTH ASSAY

The cell growth was analyzed via 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT; Roche) assay, the seeded cells at a concentration of $1 \times 10^4$ cells/well (a 96-well plate) in 100 μl culture medium, to which, at the intervals, 10 μl MTT labeling solution was added for continuous culture. Following 4 h, SDS containing 0.01 N HCl was added to solute the purple formazan crystals and to measure the spectrophotometrical absorbance at a wavelength of 570 nm.

### CELL-CYCLE ANALYSIS

In 2 ml culture medium $2 \times 10^5$ cells/well (6-well plate) were seeded, and cultured in hypoxia (0.1% oxygen) or normoxia for 16 h before collection. The cells were stabilized with 75% ethanol for 24 h, and dyed with PI, and analyzed with ModFit of flow cytometry.

### HYPOXIA SIGNALING PATHWAY ANALYSIS

Hypoxia Signaling Pathway Microarray (SuperArray Bioscience Corporation, catalog number: OHS-032, human), representing 113 genes related to the hypoxia signaling pathways, was used to analyze the effect of CIH on signaling-related gene expressions in the lung cancer cells. Total RNA was extracted with Trizol reagent in accordance with the manufacturer's manual, was treated with DNase I, and the first-strand cDNA synthesis was performed as per the following procedure: the mix containing 1 μl of 500 ng of oligo$(dT)_{18}$; 2 μg total RNA, 1 μl 10 mM dNTP mix; and distilled water to 13 μl; was heated to 65°C for 5 min and incubated on ice for at least 1 min; the contents of the tube were collected with centrifugation and added with 5× first-strand buffer 4 μl, 0.1 M DTT 1 μl, RNase inhibitor 1 μl, SuperScript III RT 1 μl; was mixed and incubated at 50°C for 60 min; the reaction was inactivated by heating at 70°C for 15 min; 91 μl of $ddH_2O$ was added to each 20 μl of cDNA synthesis reaction; the finished first-strand cDNA synthesis

reaction was held on ice or stored overnight at −20°C until the next step. The real-time PCR was performed as follows: the components were mixed in a 5-ml tube of 2× SuperArray PCR master mix 1275 μl, diluted first-strand cDNA synthesis reaction 102 μl, and $ddH_2O$ 1,173 μl, to the volume 2,550 μl; the real-time PCR detection was performed as cycles duration temperature for 10 min at 95°C for one cycle, and 15 s at 95°C, 1 min 60°C for 40 cycles. The data were analyzed by $\Delta\Delta C_t$ method: the $\Delta C_t$ was calculated for each pathway-focused gene in each treatment group, $\Delta C_t$ for group 1 (=average $C_t$ − average of HK genes' $C_t$), $\Delta C_t$ for group 2 (=average $C_t$ − average of HK genes' $C_t$); the $\Delta\Delta C_t$ was calculated for each gene across two PCR arrays (or groups) ($\Delta\Delta C_t = \Delta C_t$ for group 2 as the experimental − $\Delta C_t$ for group 1 as the control); the fold-change was calculated for each gene from group 1 to group 2 as $2^{-\Delta\Delta C_t}$.

### RNA ISOLATION AND REAL-TIME RT-PCR

Total RNA, following the manufacturer's instructions, was isolated from the cells under the normoxic or hypoxic condition using Trizol reagent (Invitrogen). Briefly, the cells were lysed in TRIzol and then mixed with chloroform. The lysate was centrifuged to separate RNA, DNA, and protein, total RNA was recovered, precipitated with isopropanol, and washed in 75% ethanol to remove impurities before dissolved in water. After that, 1.5 μg of RNA was taken and treated with DNase to remove contaminating DNA prior to the reverse transcription to cDNA using SYBR® RT-PCR Kit (Takara, cat. no. DRR066A, Japan). To measure human HIF-1α, HIF-2α, VEGF, CASP1, TH, and IGFBP mRNA expression, real-time RT-PCR was performed using a sequence detector (ABI-Prism, model 7000, Applied Biosystems) as the following primers: HIF-1α, forward: 5′-AGT GTA CCC TAA CTA GCC GAG GAA-3′, reverse: 5′-CTG AGG TTG GTT ACT GTT GGT ATC A-3′; HIF-2α, forward: 5′-GAC CAG CAG ATG GAC AAC TTG TAC-3′, reverse: 5′-CAG AAA GAT CAT GTC GCC ATC TT-3′; VEGFA, forward: 5′-GAA GTG GTG AAG TTC ATG GAT GTC-3′, reverse: 5′-TCA GGG TAC TCC TGG AAG ATG TC-3′; IGFBP1, forward: 5′-TGT GTC CAG CGA GCA TCG GC-3′, reverse: 5′-ACC AGC CAG ACG CGA GCA AC-3′; TH, forward: 5′-CAG CCC TAC CAA GAC CAG ACG T-3′, reverse: 5′-ACT CAC GGT AAC CGA TCC ACG-3′; CASP1, forward: 5′-CTC AGG CTC AGA AGG GAA TG-3′, reverse: 5′-TTT GTG AGA CTC GTT CAG GGT C-3′. The relative expression levels were calculated by comparing $C_t$ values of the samples with those of the reference, all data were normalized to the internal control β-actin.

### RNAi LENTIVIRAL VECTOR CONSTRUCT AND LENTIVIRUS PRODUCTION

The RNAi target gene sequence was chosen with siRNA converter software (www.ambion.com), the oligonucleotide DNA fragments were synthesized. The target gene sequence of HIF-1α (NM_001530) went as GAT ACA AGT AGC CTC TTT G. Double-stranded oligonucleotides targeting the endogenous HIF-1α gene were generated and cloned into pENTR™/U6 using the BLOCK-iT™ U6 RNAi Entry Vector Kit (Invitrogen). The U6-HIF-1αRNAi cassette was transferred into the pLenti6/BLOCK-iT™-DEST vector using the LR recombination reaction to generate the pLenti6-GW/U6-HIF-1αshRNA expression construct. The lentivirus was produced according to the manufacturer's instructions: DNA–Lipofectamine 2000 complexes were prepared containing 3 μg of pLenti6/BLOCK-it-DEST expression plasmid DNA and 9 μg of the ViraPower Packaging Mix in 1.5 ml of Opti-MEM I medium without serum; the packaging cell 293FT was trypsinized and counted and the cells were resuspended at a density of $1.2 \times 10^6$ cells/ml in growth medium containing serum; the DNA–Lipofectamine 2000 complexes were added to a 10-cm culture plate containing 5 ml of growth medium with serum; 5 ml 293FT cells were added to the plate and mixed gently by rocking. On the next day, the media containing the DNA–Lipofectamine 2000 complexes were removed and replaced with complete culture medium, and virus-containing supernatants 48 h post-transfection were harvested and centrifuged at 3,000 rpm for 5 min at 4°C to pellet debris, and the virus was concentrated at 20,000 rpm and stored at −70°C, lentiviral vectors were titered in A549 and H446 cells based on the protocols.

### TRANSDUCTION OF TARGET CELLS AND SELECTION

$2 \times 10^5$ A549 and H446 cells were seeded in each well of a six-well plate and transducted by lentiviral vectors at a MOI 5 in the presence of polybrene (6 μg/ml). The medium was removed and replaced with fresh complete medium under blasticidin selection (10 μg/ml), which was then replaced with fresh one containing blasticidin every 3–4 days until blasticidin-resistant colonies were identified.

### WESTERN BLOTTING

The normoxic or hypoxic cells were scraped from the dishes, cellular protein extracts were prepared by homogenization in an ice-cold buffer of 0.15 M NaCl, 50 mM Tris–Cl, pH 7.4, 2 mM EDTA, 5 mM DTT, 0.5% Triton 100, 0.2 mM PMSF, 1 μg/ml apoptinin, and their lysates were obtained by centrifugation at 12,000*g* for 20 min, and the total protein concentration was determined using Bio-Rad Protein Assay. Equal amounts of protein, separated by SDS–PAGE, were electrophoretically transferred to a PVDF membrane at 100 V for 2 h at a low temperature and the membrane was blocked with 5% fat-free milk with 0.05% Tween-20 in TBS. Subsequently, the membrane was probed with the primary antibodies of HIF-1α, HIF-1β (BD Biosciences, San Diego, CA) at 1:500, and anti-β-actin monoclonal antibody (Biovision) at 1:2,000. The blots were washed in TBST and then incubated in anti-mouse IgG secondary antibody (Amersham Biosciences) at 1:2,000 for about 2 h at RT. Washed in TBST (×4, 5 min each wash), the proteins were finally visualized using ECL plus (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

### DETERMINATION OF APOPTOSIS

For apoptosis assay, the Annexin V staining was quantified by flow cytometry. The normoxic and hypoxic cells were plated in a six-well plate in hypoxia (0.1% $O_2$) for 16 h, irradiated with 6-MV X-ray from the accelerator before they, with the growth medium changed, were cultured in normoxia, and collected 48 h later, washed in cold PBS twice, and resuspended in 1× binding buffer at a concentration of $1 \times 10^6$ cells/ml. After that, the cells in 100 μl solution were transferred to a 5-ml culture tube, added with 5 μl Annexin V-FITC and 5 μl PI (BD Biosciences), and gently vortexed and incubated for

15 min at RT in the dark. Finally, 400 μl 1× binding buffer was added to each tube to be analyzed by flow cytometry within 1 h.

### COLONY FORMATION ASSAY

The cells, pretreated with hypoxia or normoxia, were harvested, sparsely plated, and then exposed to the specified doses of radiation, and with the medium immediately replaced with a fresh one, were cultured under normoxic condition. The medium was replaced at 3-day intervals. And then the cells were fixed in 90% ethanol, stained with crystal violet and colonies consisting of at least 50 cells were counted 7 days later.

### MIGRATION ASSAY

The migration assay was conducted as follows: $2 \times 10^4$ cells in 0.1 ml of media added to the upper chamber (Costar Transwell Plastic Inserts, Cambridge, MA), followed by the addition of 0.6 ml medium in lower chamber; non-invading cells on the surface of the polycarbonate membrane were removed 16 h later by scraping with a cotton swab; those migrated to the pores of the underlying membrane were fixed in 90% ethanol and stained with 0.1% crystal violet and counted under fluorescence microscopy (100×); the mean and SD were calculated from three independent experiments. Migrated cells = cells migrated through the membrane × 100%/cells seeded in the well.

### STATISTICAL ANALYSIS

Each experiment was repeated at least three times; Student's *t*-tests were performed to determine the statistical significance for the assay of migration, FACS, and colony formation; error bars representing ±SE.

## RESULTS

### PROLIFERATION AND CELL CYCLE OF LUNG CANCER CELLS TREATED WITH CIH

CIH models established using lung cancer cells A549 and H446 were cultured in hypoxia at 0.1% $O_2$ for 24 h, and in reoxygenation at 20% $O_2$, intermittent hypoxia for 72 h, which was repeated for 20 cycles. As indicated by their proliferation, A549 and H446 cells treated with CIH were more proliferative than their parent cells (Fig. 1A). In A549 cells, the cell-cycle analysis showed that both S phase and G2/M phase increased C20 compared with C0 cells, G0/G1 phase of C20 decreased compared with C0. The results of cell cycle of C0 and C20 treated with hypoxia showed that S phase increased while G2/M phase decreased (Fig. 1B). In H446 cells, C20 compared with C0 showed that G0/G1 phase decreased, S phase increased, and G2/M phase did not change, and in both H446 C0 and C20 treated with hypoxia, compared with C0, it was found that S phase increased, G2/M phase decreased. The fact that the proliferation of A549 C20 and H446 C20 increased, and the S phase increased when compared with their parent cells, and suggested that CIH could promote cellular proliferation.

### RADIOSENSITIVITY OF LUNG CANCER CELLS TREATED WITH CIH

Colony formation was applied to investigate the radiosensitivity of lung cancer cells, which were irradiated with 0, 4, 8, and 12 Gy. The

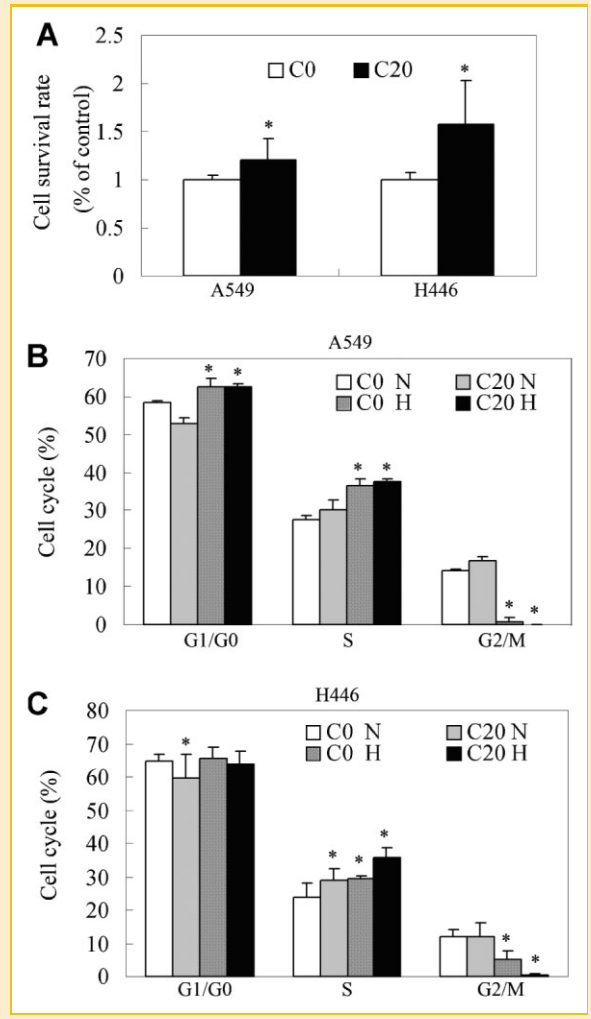


Fig. 1. Proliferation and cell cycle of cells treated with CIH. Cells were cultured in normoxia and hypoxia, the proliferation was analyzed by MTT assay, and cell cycle was analyzed by flow cytometry. A: Proliferation of A549 and H446 cells, C0: A549 or H446 in normoxia; C20: A549 or H446 with 20 cycles of hypoxia and reoxygenation. B: Cell cycle of A549 cells. C: Cell cycle of H446 cells. Error bars represent ±SD. $^*P < 0.05$, versus lung cancer cells C0, n = 3. C0N: cells in normoxia for 16 h; C0H: cells in hypoxia for 16 h; C20N: cells treated with 20 cycles of hypoxia and reoxygenation in normoxia for 16 h; C20H: cells treated with 20 cycles of hypoxia and reoxygenation in hypoxia for 16 h.

results showed that in both A549 and H446 cells, C20 was more resistant than C0 in prolonged hypoxia of 16 h. Moreover, prolonged hypoxia induced radiation resistance in A549 C0 cells, but made H446 C0 cells more sensitive to radiation than those in normoxia, which might be explained by the fact that 0.1% $O_2$ was so low that the cells were sensitive to radiation (Fig. 2).

### HYPOXIA SIGNAL PATHWAY AND VERIFICATION OF SOME GENES

The hypoxia signal pathway in H446 cells treated with CIH was investigated through microarrays to gain an insight into its molecular mechanism, which covered 113 genes, including hypoxic target and hypoxia-inducible ones related to biological behaviors such as response to hypoxia, oxidative stress, immune stress,

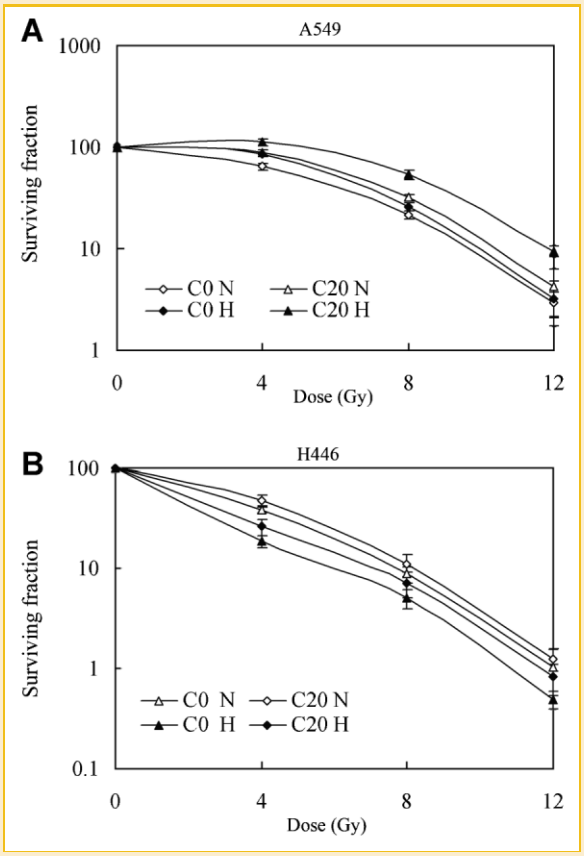


Fig. 2. Radiosensitivity of lung cancer cells treated with CIH. Cells were cultured in hypoxia or normoxia for 16 h, and irradiated with a single dose of 0, 4, 8, and 12 Gy and their colonies were counted after 10 days. A: Radiosensitivity of A549 cells. B: Radiosensitivity of H446 cells, n = 3. Error bars represent ±SD. C0N: cells in normoxia for 16 h; C0H: cells in hypoxia for 16 h; C20N: cells treated with 20 cycles of hypoxia and reoxygenation in normoxia for 16 h; C20H: cells treated with 20 cycles of hypoxia and reoxygenation in hypoxia for 16 h.

hemoglobin complex, transcription factors and regulators, apoptosis, extracellular matrix-related molecules, cell growth, and metabolism. The results showed that 11 genes, ADM, ANGPTL4, GP1, HK2, HMOX1, IGFBP1, BHLHB2, PTX3, SLC2A1, VEGFA, and MT3, were up-regulated (Table IA); 18 genes, BIRC5, CASP1, CAT, CHGA, DR1, EP300, PRPF40A, GNA11, HIF1A, HIF3A, KHSRP, MOCS3, PRKAA1, SAE1, SLC2A4, SNRP70, TST, and UCP2, were down-regulated (Table IB); and others in hypoxia signal pathway, ARNT2, CA1, CREBBP, CYGB, HBB, IL1A, IL6, IL6ST, LCT, NOS2A PSMB3, SSSCA1, TH, LEP, HIF1AN, IGF2, PPARA, PSMB3, RPL28, SSSCA1, and TH, were up-regulated or down-regulated under different hypoxic conditions (Table IC). In addition, several anti-apoptosis genes, BAX, BIRC5, PRKAA1, were found to have been down-regulated, and the apoptosis gene, CASP1, was found to have been down-regulated.

Some hypoxia-inducible genes such as hypoxia-inducible factor (HIF-1α and HIF-2α), vascular endothelial growth factor A (VEGFA), caspase 1 (CASP1), tyrosine hydroxylase (TH), and insulin-like growth factor binding protein-1 (IGFBP1) were confirmed by real-time RT-PCR, and the results were consistent with those of microarrays (Fig. 3). HIF-1α and HIF-2α are the major hypoxia-inducible factors, and they were found to have changed in hypoxia or intermittent hypoxia, but the change was not much. VEGFA, a known hypoxia response gene, could promote tumor vasculargenesis; and IGFBP1, insulin-like growth factors (IGFs) bind to the receptor IGF-1R and transduct a variety of signals to stimulate proliferation and promote cell survival [Koga et al., 2008]. TH is another up-regulated gene from the array, and we confirmed that the gene by real-time RT-PCR. VEGFA, IGFBP1, and TH mRNA of C20N, C0H, and C20H were up-regulated much more significantly compared with C0N. Those anti-apoptosis genes such as CASP1 were down-regulated at C20H, C0H, and C20H, respectively, when compared with C0N.

## ROLE OF HIF-1α IN APOPTOSIS, RADIOSENSITIVITY, AND MIGRATION OF CELLS TREATED WITH CIH

It was found that many hypoxic genes were changed in CIH. HIF-1α is a known important transcriptional factor in tumor hypoxic microenvironment. And it was examined to decide whether it was related to apoptosis induced by radiation and radiosensitivity in CIH. The results showed that HIF-1α was knocked down by lentivirus-mediated RNA interference technology and its protein expression was down-regulated in normoxia, hypoxia, and intermittent hypoxia, and that HIF-1β, a universe HIF-1 subunit, its protein had no change (Fig. 4A). In addition, CIH increased HIF-1α protein level in both cell lines.

The apoptosis of the cells was detected by Annexin V using FACS. The findings indicated that A549/C20 cells presented less apoptosis in comparison with those in prolonged hypoxia or normoxia. Following 10 Gy radiation, the apoptosis increased when compared with that in the un-irradiated cells, but in those treated with CIH, a decreased apoptosis in the irradiated cells was shown (Fig. 4B), which was true of C0 in both A549 and H446 cells following CIH treatment (Fig. 4C). The apoptosis-resistant phenotype was not found to have been reversed under the normoxic condition 2 weeks later. However, in H446 cells, C0 cells in prolonged hypoxia at 0.1% $O_2$ for 16 h produced a higher apoptosis rate than those in normoxia (Fig. 4C), which was consistent with the results of radiosensitivity.

The radiation sensitivity of A549 and H446 was studied, and the results showed that after HIF-1α down-regulation, A549 cells irradiated by 8 Gy were more sensitive to radiation than their control, which meant that the surviving factions were decreased. In addition, the results indicated that the intermittent hypoxia could make cells more resistant to radiation than those in prolonged hypoxia once again (Fig. 5A), as in the case of H446 cells (Fig. 5B).

From the investigation on the migratory ability of the cells in intermittent hypoxia using transwell chambers, the results showed that A549 and H446 cells treated with CIH produced a significantly higher invasion compared with the parental ones of A549 and H446, respectively (Fig. 5C,D). HIF-1α gene down-regulated, the cellular migration was reduced in comparison with that of the controls.

TABLE I. Gene Expression Profile of Hypoxia Signal Pathway in H446 Cells

| Gene symbol | Gene name | GenBank (accession number) | Fold change C20N/C0N | C0H/C0N | C20H/C0N | C20H/C20N | C20H/C0H |
|---|---|---|---|---|---|---|---|
| (A) Up-regulated genes in hypoxia signal pathway | | | | | | | |
| ADM | Adrenomedullin | NM_001124 | – | 37.01 | 37.01 | 52.47 | – |
| ANGPTL4 | Angiopoietin-like 4 | NM_139314 | – | 6.45 | 5.54 | 5.04 | – |
| BHLHB2 | Basic helix-loop-helix domain containing, class B,2 | NM_003670 | – | 5.78 | 5.82 | 6.16 | – |
| GP1 | GTP binding protein 1 | NM_004286 | – | 2.81 | 2.51 | 2.63 | – |
| HK2 | Hexokinase 2 | NM_000189 | – | 7.73 | 6.92 | 7.53 | – |
| HMOX1 | Heme oxygenase (decycling) 1 | NM_002133 | – | 2.48 | 2.5 | 2.33 | – |
| IGFBP1 | Insulin-like growth factor binding protein 1 | NM_000596 | – | 20.39 | 16.45 | 29.93 | – |
| MT3 | Metallothionein 3 | NM_005954 | – | 4.59 | 4.82 | 4.27 | – |
| PTX3 | Pentraxin-related gene, rapidly induced by IL-1 beta | NM_002852 | – | 3.61 | 4.5 | 4.77 | – |
| SLC2A1 | Solute carrier family 2, member 1 | NM_006516 | – | 5.35 | 6.73 | 5.36 | – |
| VEGFA | Vascular endothelial growth factor A | NM_001025366 | – | 5.06 | 5.62 | 4.7 | – |
| (B) Down-regulated genes in hypoxia signal pathway | | | | | | | |
| BIRC5 | Baculoviral IAP repeat-containing 5 | NM_001168 | – | −2.31 | −3.05 | −3.45 | – |
| CASP1 | Caspase 1, apoptosis-related cysteine peptidase | NM_033293 | – | −9.06 | −6.15 | −6.62 | – |
| CAT | Catalase | NM_001752 | – | −2.6 | −2.46 | −2.71 | – |
| CHGA | Chromogranin A (parathyroid secretory protein 1) | NM_001275 | – | −5.66 | −3.46 | −4.37 | – |
| DR1 | Down-regulator of transcription 1, TBP-binding (negative cofactor 2) | NM_001938 | – | −2.57 | −2.5 | −2.28 | – |
| EP300 | E1A binding protein p300 (EP300) | NM_001429 | – | −2.13 | −2.89 | −2.61 | – |
| PRPF40A | PRP40 pre-mRNA processing factor 40 homolog A (*S. cerevisiae*) | NM_017892 | – | −4.99 | −4.92 | −3.62 | – |
| GNA11 | Guanine nucleotide binding protein (G protein), alpha 11 (Gq class) | NM_002067 | – | −2.35 | −2.53 | −2.09 | – |
| HIF1A | Hypoxia-inducible factor 1, alpha subunit | NM_001530 | – | −3.29 | −4.06 | −2.86 | – |
| HIF3A | Hypoxia-inducible factor 3, alpha subunit | NM_152794 | – | −5.43 | −6.73 | −4.75 | – |
| KHSRP | KH-type splicing regulatory protein | NM_003685 | – | −2.36 | −2.14 | −2.17 | – |
| MOCS3 | Molybdenum cofactor synthesis 3 | NM_014484 | – | −8.75 | −6.45 | −7.34 | – |
| PRKAA1 | Protein kinase, AMP-activated, alpha 1 catalytic subunit | NM_006251 | – | −2.19 | −2.45 | −2.04 | – |
| SAE1 | SUMO1 activating enzyme subunit 1 | NM_005500 | – | −3.29 | −3.39 | −2.9 | – |
| SLC2A4 | Solute carrier family 2, member 4 | NM_001042 | – | −7.78 | −5.58 | −10.03 | – |
| SNRP70 | Small nuclear ribonucleoprotein 70 kDa | NM_003089 | – | −2.51 | −2.69 | −3.08 | – |
| TST | Thiosulfate sulfurtransferase (rhodanese) | NM_003312 | – | −3.66 | −4.29 | −4.49 | – |
| UCP2 | Uncoupling protein 2 | NM_003355 | – | −2.62 | −2.45 | −2.34 | – |
| (C) Changes of other genes in hypoxia signal pathway | | | | | | | |
| ARNT2 | Aryl-hydrocarbon receptor nuclear translocator 2 | NM_014862 | – | – | −4.06 | −3.65 | −2.07 |
| CA1 | Carbonic anhydrase I | NM_001738 | −2.21 | −2.81 | −2.53 | – | – |
| COL1A1 | Collagen, type I, alpha 1 | NM_000088 | – | – | −2.19 | – | – |
| CREBBP | CREB-binding protein | NM_004380 | – | −4.89 | 5.5 | −5.12 | – |
| CYGB | Cytoglobin | NM_134268 | 3.29 | – | 2.35 | – | 2.03 |
| DAPK3 | Death-associated protein kinase 3 | NM_001348 | – | – | −2.51 | −2.12 | – |
| HBB | Hemoglobin, beta | NM_000518 | −2.54 | 4.63 | 3.58 | 9.08 | – |
| HIF1AN | Hypoxia-inducible factor 1, alpha subunit inhibitor | NM_017902 | – | −2.1 | – | – | – |
| IGF2 | Insulin-like growth factor 2 (somatomedin A) | NM_000612 | – | – | – | – | 2.36 |
| IL1A | Interleukin 1, alpha | NM_000575 | −3.82 | 6.73 | 2.91 | 11.11 | 2.31 |
| IL6 | Interleukin 6 (interferon, beta 2) | NM_000600 | 2.58 | – | – | – | 2.13 |
| IL6ST | Interleukin 6 signal transducer (gp130, oncostatin M receptor) | NM_002184 | – | – | −2.07 | – | – |
| LCT | Lactase | NM_002299 | 2.01 | – | – | −3.02 | – |
| LEP | Leptin | NM_000230 | 3.13 | −2.33 | −2.1 | −6.57 | – |
| NOS2A | Nitric oxide synthase 2A (inducible, hepatocytes) | NM_000625 | −2.15 | −3.36 | −4.86 | −2.26 | – |
| PEA15 | Phosphoprotein enriched in astrocytes 15 | NM_003768 | – | 2.28 | – | – | – |
| PPARA | Peroxisome proliferator-activated receptor alpha | NM_005036 | – | – | −2.25 | −2.2 | – |
| PSMB3 | Proteasome (prosome, macropain) subunit, beta type, 3 | NM_002795 | – | −2.33 | – | −2.08 | – |
| RPL28 | Ribosomal protein L28 | NM_000991 | – | – | 2.04 | – | – |
| SSSCA1 | Sjogren syndrome/scleroderma autoantigen 1 | NM_006396 | – | −2.48 | – | −2.11 | – |
| TH | Tyrosine hydroxylase | NM_199292 | 5.84 | 6.63 | 13.45 | 2.3 | 2.03 |

C0N, cells in normoxia for 16 h; C0H, cells in hypoxia for 16 h; C20N, cells treated with 20 cycles of hypoxia and reoxygenation in normoxia for 16 h; C20H, cells treated with 20 cycles of hypoxia and reoxygenation in hypoxia for 16 h.
Cells were treated with CIH 1 week later, and cells were seeded in 10 cm plates in hypoxia or normoxia for 16 h, and then total RNA was isolated from H446 cells. Gene expression was studied by microarray.
"–" indicates that the gene expression has no change.
Positive number denotes up-regulation fold.
Negative number denotes down-regulation fold.
Fold >2 is listed in the table.

## DISCUSSION

Hypoxia has reportedly been associated with tumor's therapeutic resistance, recurrence, poor outcome, and metastatic potential [Wouters et al., 2007]. Previous studies have focused on prolonged or acute hypoxia, but neglected intermittent hypoxia, which is nowadays recognized as a hallmark of many tumors [Dewhirst, 2007]. It is not clear, however, how intermittent hypoxia affects radiotherapy. Therefore, we studied the effect of intermittent hypoxia on radiotherapy, apoptosis, and the signal pathway of lung cancer cells induced by CIH.

The cell cycle and proliferation were detected based on the established CIH models with A549 and H446 induced by 20 cycles of hypoxia and reoxgenation. The results showed that CIH promoted

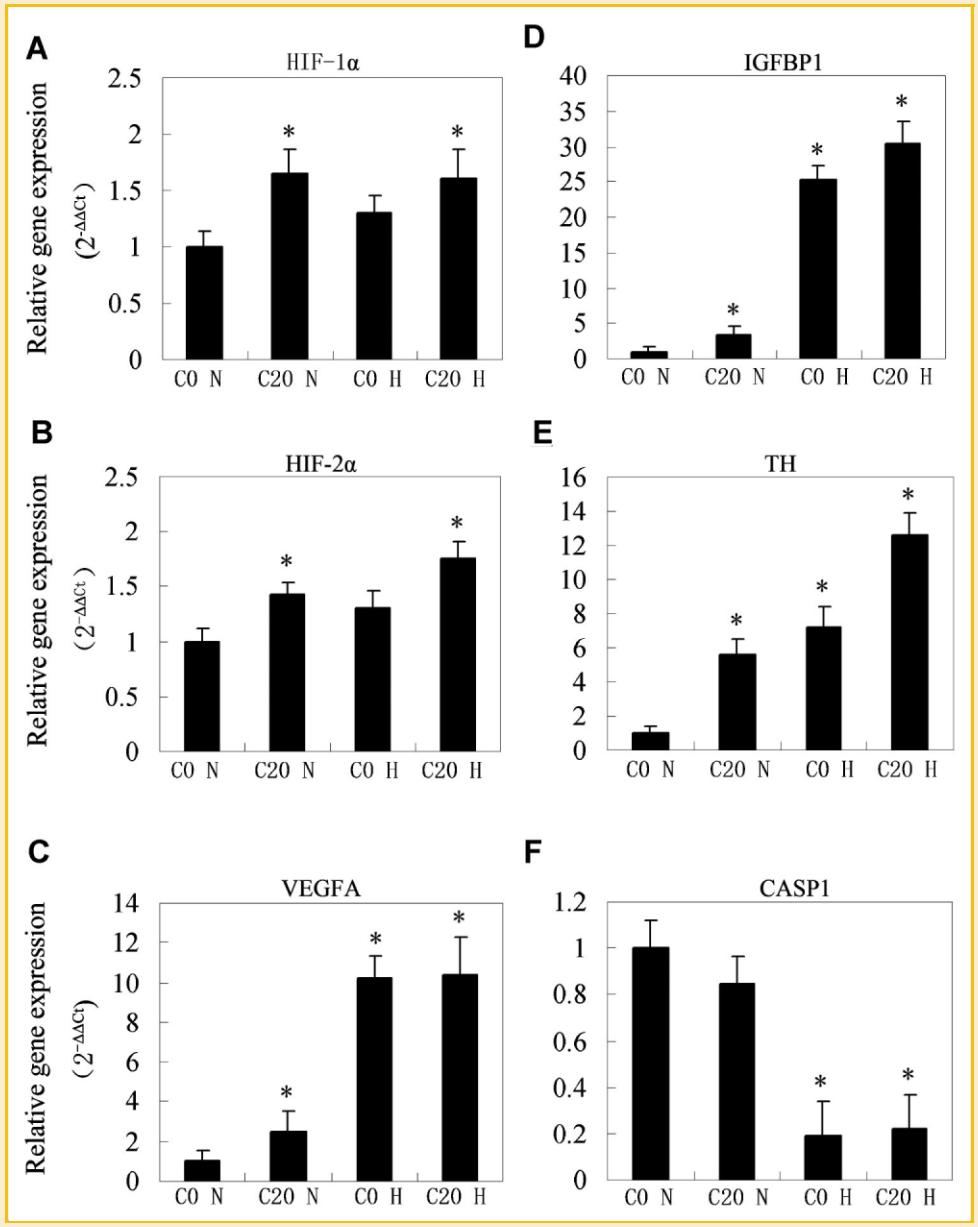


Fig. 3. Confirmation of gene expression from microarray analysis by real-time RT-PCR in H446 cell gene expression analyzed in H446 cells by real-time RT-PCR. A: HIF-1α mRNA. B: HIF-2α mRNA. C: VEGFA mRNA. D: IGFBP1 mRNA. E: TH mRNA. F: CASP1 mRNA. N, normoxia; H, hypoxia. C0N, cells in normoxia for 16 h; C0H, cells in hypoxia for 16 h; C20N, cells treated with 20 cycles of hypoxia and reoxygenation in normoxia for 16 h; C20H, cells treated with 20 cycles of hypoxia and reoxygenation in hypoxia for 16 h.

proliferation, and shortened doubling time, which was consistent with the previous studies [Hunter et al., 2006; Fukumura and Jain, 2007]. From the radiosensitivity performed, the results showed that CIH led to radiation resistance under different hypoxic conditions. Thus, radiotherapy resistance may be associated with CIH, and the cells resistant to radiation may be the origin of CIH selection.

We speculated that biological changes of CIH due to those of gene expression were associated with hypoxia. The pattern of gene expression in hypoxia signal pathway was analyzed by array assay to elucidate the cellular changes induced by CIH. The data revealed that CIH was accompanied by a series of altered gene expressions, that is, 18 genes were down-regulated, 11 genes up-regulated, and other genes were up-regulated or down-regulated (Table I). In the hypoxic context, anti-apoptosis genes were not up-regulated, and apoptosis genes were up-regulated in H446 cells in chronic hypoxia and CIH. CIH or chronic hypoxia was not associated with the changes of an apoptosis gene, but with the alterations in a wide variety of genes, from which we concluded that hypoxia response genes were not induced to increase, such as CA1 and HIF1A. Such genes were strongly induced as ADM and IGFBP1, which could promote angiogenesis, suggesting that intermittent hypoxia was potentially capable of inducing up-regulation of some

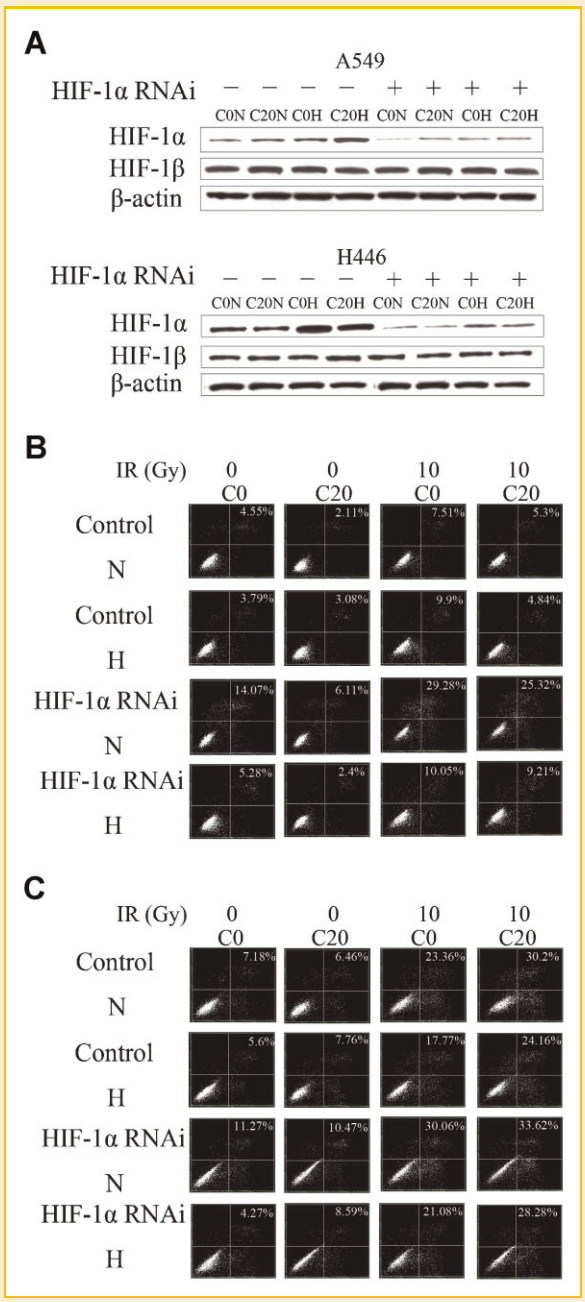


Fig. 4. Response to apoptosis in lung cancer cells treated with CIH and role of HIF-1α in CIH. A: HIF-1α and HIF-1β protein expression. B: Apoptosis was induced by radiation in A549 cells. C: Apoptosis was induced by radiation in H446 cells. One week after the cells were treated with CIH, $10^5$ cells were seeded in six-well plates in hypoxia or normoxia for 16 h and irradiated with a single dose of 10 Gy, Annexin V were analyzed by flow cytometry after 48 h, the rates in the figures indicate up-right quadrant and down-right quadrant (total apoptotic cells) of coordinate axis. C0N: cells in normoxia for 16 h; C0H: cells in hypoxia for 16 h; C20N: cells treated with 20 cycles of hypoxia and reoxygenation in normoxia for 16 h; C20H: cells treated with 20 cycles of hypoxia and reoxygenation in hypoxia for 16 h.

genes such as IGFBP1 and down-regulation of some genes such as CASP1. The profile of changed gene expressions in those treated with CIH may be adaptable to the tumor's microenvironment.

From the above details, we knew that CIH induced radiation resistance and changes of many hypoxic genes. It has been well recognized that HIF-1α is related to many tumor biological behaviors such as apoptosis, and metastasis in chronic hypoxia [Mekhail et al., 2004; Vaupel, 2004; Cairns et al., 2006; Gatenby et al., 2007; Thews et al., 2007; Gillies and Gatenby, 2007]. Previous studies have shown that chronic hypoxia could lead to radiotherapy resistance [Chan and Giaccia, 2007; Moeller et al., 2007; Pettersen et al., 2007], which was found to be consistent with the results of the study. More importantly, we found that CIH protected tumor cells against radiation-induced cellular death (Fig. 4). The data suggested that CIH was a trigger of survival pathways to tumor cells, and that intermittent hypoxia could lead to more resistance to radiation than prolonged hypoxia and normoxia in A549 and H446 cells. However, H446 cells in prolonged hypoxia were more sensitive than those in normoxia, which could be explained in terms of the difference of genotype in comparison with A549 cells. Upon the down-regulation of HIF-1α, the resistance to radiation and its induced apoptosis were reversed in the two lines of lung cancer cells in both CIH and prolonged hypoxia, hence the cells were becoming sensitive. The results indicated that CIH induced more tolerance in CIH than in prolonged hypoxia, which confirmed the cellular adaptation to the hypoxic microenvironment. Thus, we could draw a conclusion that tumor is resistant to radiotherapy in the clinic, which might be associated with the cellular adoption to hypoxia, and that the increasing proliferation of CIH might be related to radiation resistance.

It was previously reported that hypoxia induced tumor to be more aggressive [Chan and Giaccia, 2007]. The study showed that those treated with CIH could migrate increasingly; those treated with CIH in hypoxia migrated in a greater number than those in prolonged hypoxia, suggesting that CIH could strengthen the metastatic potential. Moreover, migration decreased in those with a down-regulation of HIF-1α expression (Fig. 5C,D), suggesting that HIF-1α played a role in lung cancer metastasis.

Taken together, our findings unraveled that CIH created a hypoxia-tolerant cellular phenotype in lung cancer cells such as A549 cells, which could be associated with radiation and apoptosis resistance, and increasing migration, and that those who could adapt themselves to their microenvironment, that is, CIH could survive in the unfavorable condition, and in other words, they could achieve their growth advantage following many cycles of selection. In others, however, CIH also promoted apoptosis or radiation resistance, although they were sensitive to radiation in chronic hypoxia, whose mechanism might be explained by the fact that those who had acquired resistant phenotype upon the treatment of CIH were companied by a series of changes in genes expression. The phenomenon could partially explain the fact that the strategies aiming at the improvement of tumor oxygenation during radiotherapy often failed to reverse the negative outcome of hypoxic cells. It could be that the hypoxic cells in tumor tissues, upon reoxygenation, gain more aggressive or resistant phenotype in some patients.

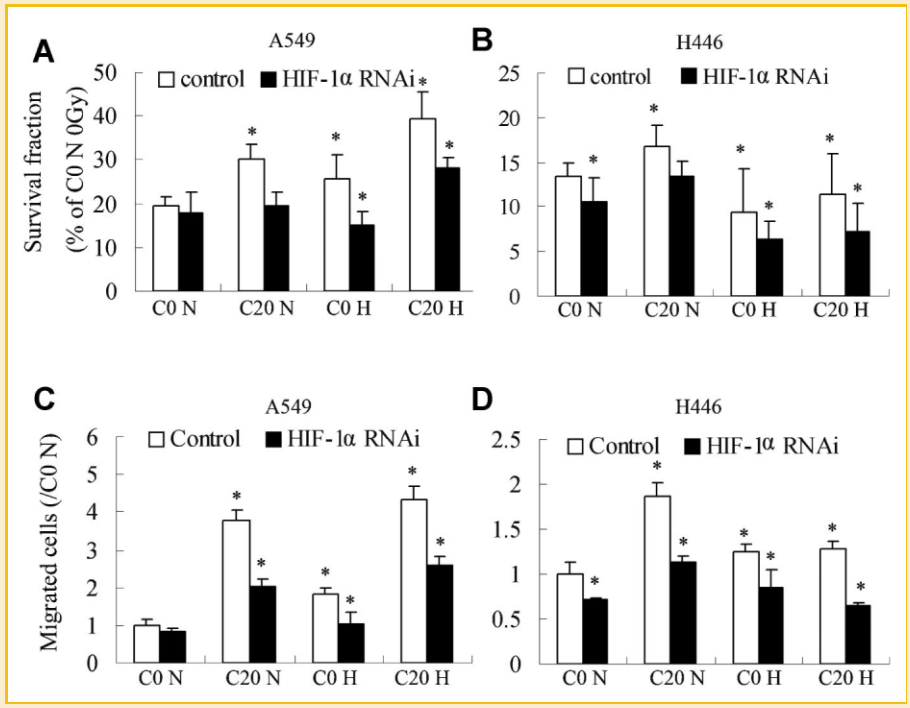


Fig. 5. Role of HIF-1α in radiosensitivity and migration of A549 and H446 cells treated with CIH. A: Radiosensitivity of A549 cells. B: Radiosensitivity of H446 cells. Cells were treated with intermittent hypoxia or normoxia, and then irradiated with the dose of 8 Gy, and after 1 week, colonies were counted (more than 50 cells per colony), the survival fraction (% C0N 0 Gy) indicated the percent of colony formation versus the normal un-irradiated cells, the method was referred to previous report [Zölzer and Streffer, 2002]. C: Migration ability of A549 cells. D: Migration ability of H446. Migration ability was analyzed by transwell chamber and migrated cells were counted after hypoxia or normoxia for 16 h. N, normoxia; H, hypoxia. $^{*}P < 0.05$, versus cells in normoxia, n = 3, error bars represent ±SD, IR: irradiation. C0N, cells in normoxia for 16 h; C0H, cells in hypoxia for 16 h; C20N, cells treated with 20 cycles of hypoxia and reoxygenation in normoxia for 16 h; C20H, cells treated with 20 cycles of hypoxia and reoxygenation in hypoxia for 16 h.

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## REFERENCES

Ahn GO, Brown M. 2007. Targeting tumors with hypoxia-activated cytotoxins. Front Biosci 12:3483–3501.

Brahimi-Horn MC, Chiche J, Pouyssegur J. 2007. Hypoxia signalling controls metabolic demand. Curr Opin Cell Biol 19:223–229.

Cairns RA, Kalliomaki T, Hill RP. 2001. Acute (cyclic) hypoxia enhances spontaneous metastasis of KHT murine tumors. Cancer Res 61:8903–8908.

Cairns R, Papandreou I, Denko N. 2006. Overcoming physiologic barriers to cancer treatment by molecularly targeting the tumor microenvironment. Mol Cancer Res 4:61–70.

Chan DA, Giaccia AJ. 2007. Hypoxia, gene expression, and metastasis. Cancer Metastasis Rev 26:333–339.

Chaudary N, Hill RP. 2007. Hypoxia and metastasis. Clin Cancer Res 13: 1947–1949.

Cooper C, Liu GY, Niu YL, Santos S, Murphy LC, Watson PH. 2004. Intermittent hypoxia induces proteasome-dependent down-regulation of estrogen receptor alpha in human breast carcinoma. Clin Cancer Res 10: 8720–8727.

Dewhirst MW. 2007. Intermittent hypoxia furthers the rationale for hypoxia-inducible factor-1 targeting. Cancer Res 67:854–855.

Fukumura D, Jain RK. 2007. Tumor microenvironment abnormalities: Causes, consequences, and strategies to normalize. J Cell Biochem 101: 937–949.

Gatenby RA, Smallbone K, Maini PK, Rose F, Averill J, Nagle RB, Worrall L, Gillies RJ. 2007. Cellular adaptations to hypoxia and acidosis during somatic evolution of breast cancer. Br J Cancer 97:646–653.

Gillies RJ, Gatenby RA. 2007. Hypoxia and adaptive landscapes in the evolution of carcinogenesis. Cancer Metastasis Rev 26:311–317.

Hunter A, Hendrikse A, Renan M, Abratt R. 2006. Does the tumor microenvironment influence radiation-induced apoptosis? Apoptosis 11:1727–1735.

Keith B, Simon MC. 2007. Hypoxia-inducible factors, stem cells, and cancer. Cell 129:465–472.

Kizaka-Kondoh S, Inoue M, Harada H, Hiraoka M. 2003. Tumor hypoxia: A target for selective cancer therapy. Cancer Sci 94:1021–1028.

Koga T, Endo H, Miyamoto Y, Mukai M, Akira S, Inoue M. 2008. IGFBPs contribute to survival of pancreatic cancer cells under severely hypoxic conditions. Cancer Lett 268:82–88.

Liu W, Guo M, Xu YB, Li D, Zhou ZN, Wu YL, Chen Z, Kogan SC, Chen GQ. 2006. Induction of tumor arrest and differentiation with prolonged survival by intermittent hypoxia in a mouse model of acute myeloid leukemia. Blood 107:698–707.

Mabjeesh NJ, Amir S. 2007. Hypoxia-inducible factor (HIF) in human tumorigenesis. Histol Histopathol 22:559–572.

Martinive P, Defresne F, Bouzin C, Saliez J, Lair F, Grégoire V, Michiels C, Dessy C, Feron O. 2006. Preconditioning of the tumor vasculature and tumor cells by intermittent hypoxia: Implications for anticancer therapies. Cancer Res 66:11736–11744.

Mekhail K, Khacho M, Gunaratnam L, Lee S. 2004. Oxygen sensing by H+: Implications for HIF and hypoxic cell memory. Cell Cycle 3:1027–1029.

Moeller BJ, Richardson RA, Dewhirst MW. 2007. Hypoxia and radiotherapy: Opportunities for improved outcomes in cancer treatment. Cancer Metastasis Rev 26:241–248.

Okunieff P, Fenton B, Chen Y. 2005. Past, present, and future of oxygen in cancer research. Adv Exp Med Biol 566:213–222.

Pettersen EO, Bjørhovde I, Søvik A, Edin NF, Zachar V, Hole EO, Sandvik JA, Ebbesen P. 2007. Response of chronic hypoxic cells to low dose-rate irradiation. Int J Radiat Biol 83:331–345.

Raghunand N, Gatenby RA, Gillies RJ. 2003. Microenvironmental and cellular consequences of altered blood flow in tumours. Br J Radiol 76: S11–S22.

Stadler W. 2007. Chromosomes, hypoxia, angiogenesis, and trial design: A brief history of renal cancer drug development. Clin Cancer Res 13:1630–1633.

Thews O, Gassner B, Kelleher DK, Schwerdt G, Gekle M. 2007. Impact of hypoxic and acidic extracellular conditions on cytotoxicity of chemotherapeutic drugs. Adv Exp Med Biol 599:155–161.

Vaupel P. 2004. Tumor microenvironmental physiology and its implications for radiation oncology. Semin Radiat Oncol 14:198–206.

Weinmann M, Jendrossek V, Güner D, Goecke B, Belka C. 2004. Cyclic exposure to hypoxia and reoxygenation selects for tumor cells with defects in mitochondrial apoptotic pathways. FASEB J 18:1906–1908.

Weinmann M, Belka C, Güner D, Goecke B, Müller I, Bamberg M, Jendrossek V. 2005. Array-based comparative gene expression analysis of tumor cells with increased apoptosis resistance after hypoxic selection. Oncogene 24:5914–5922.

Wouters A, Pauwels B, Lardon F, Vermorken JB. 2007. Review: Implications of in vitro research on the effect of radiotherapy and chemotherapy under hypoxic conditions. Oncologist 12:690–712.

Yao K, Gietema JA, Shida S, Selvakumaran M, Fonrose X, Haas NB, Testa J, O'Dwyer PJ. 2005. In vitro hypoxia-conditioned colon cancer cell lines derived from HCT116 and HT29 exhibit altered apoptosis susceptibility and a more angiogenic profile in vivo. Br J Cancer 93:1356–1363.

Zhou J, Schmid T, Schnitzer S, Brüne B. 2006. Tumor hypoxia and cancer progression. Cancer Lett 23:710–721.

Zölzer F, Streffer C. 2002. Increased radiosensitivity with chronic hypoxia in four human tumor cell lines. Int J Radiat Oncol Biol Phys 54:910–920.