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# Hypoxia-Inducible Factor-1α Protects Cervical Carcinoma Cells from Apoptosis Induced by Radiation via Modulation of Vascular Endothelial Growth Factor and p53 under Hypoxia

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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**Background:** The aim of the study was to assess the role of irradiation in the expression of HIF-1α, VEGF, and P53 in human cervical carcinoma cells under a simulated hypoxia environment.

**Material/Methods:** The tetrazolium-based colorimetric cellular assay (MTT) and flow cytometry (FCM) were used to detect the growth inhibition rates of HeLa cells in different groups. Western blot and reverse transcription polymerase chain reaction (RT-PCR) were used to observe gene and protein expression of HIF-1α, VEGF, and P53. The effect of HIF-1α on radioresistance and expression of VEGF and P53 were confirmed with the HIF-1α siRNA *in vivo* and *in vitro*.

**Results:** Hypoxic conditions enhanced the radiation resistance dependent on HIF-1α by elevating the expression of VEGF and inhibiting the expression of p53. After transfection of HIF-1α siRNA, MTT assay showed the survival rates were increased in the cells receiving irradiation under hypoxia. The expression of VEGF decreased significantly more than that of cells transfected with sense oligodeoxynucleotides, while an opposite result was found in the expression of P53 protein in the same X-ray dose ($p<0.05$). *In vivo*, the radioresistance of HIF-1α was consistent with the results *in vitro*.

**Conclusions:** In the future, we might inhibit human cervical cancer progression and enhance the radiosensitivity by inhibiting HIF-1α to reduce VEGF and increase P53 expression. The challenge is how to use this information to optimize cancer therapy.

**MeSH Keywords:** **Abnormalities, Radiation-Induced • Apoptosis • Genes, p53 • Vascular Endothelial Growth Factor, Endocrine-Gland-Derived**

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## Background

Cervical cancer is the third most common cancer in developed countries but is the second in developing countries, despite the existence of effective screening methods. There are about 528,000 new cases every year in the world, and about a third of these are Chinese. The morbidity of advanced cervical cancer increased significantly, especially in remote regions. Radiotherapy and chemotherapy are the 2 main effective treatments for advanced cervical cancer. There are still more than one-third of patients who develop recurrence or metastatic disease, despite availability of modern advanced technology. Radioresistance of tumor cells remains a major therapeutic problem. The exact mechanism of the radiation resistance of cervical cancer remains unclear. Multiple factors, including tumor cell proliferation, hypoxia, and intrinsic radioresistance, are among the possible mechanisms. Among these, hypoxia is an important factor. The development of cervical cancer exhibits some unique differences compared to other solid tumors [1]. Normal cervical stratified epithelia have characteristics of hypoxic tissue.

The development of a hypoxic microenvironment induces the expression of a variety of genes, such as hypoxia-inducible factor (HIF) in tumor tissue, that act to promote tumor cell growth and survival [2]. HIF, an essential mediator of cellular response to hypoxia, regulates gene expression for tumor angiogenesis and resistance to oxidative stress. HIF-1α protein levels are increased in most solid tumors and correlate with patient prognosis. During the response to hypoxia in cells, HIF-1α plays an important mediating role via the control of its downstream genes, such as members of the vascular endothelial growth factor (VEGF) families [3].

As a tumor suppressor transcription factor, p53 regulates the expression of genes involved in cellular proliferation, cell death, and mutagenesis [4]. The loss of p53 functions may cause a further acquisition of malignant tumor phenotypes, which could be promoted by tumor hypoxia [5].

As described above, HIF-1α, VEGF, and p53 appear to play an important role in the radiation resistance of tumor cells and are potential therapeutic targets to enhance radiation effects in treating human cervical cancer. To test this hypothesis, we conducted experiments using HeLa cells as our *in vitro* tumor model. The relationship of these 3 genes in the radiation resistance was evaluated under both normoxic and hypoxic conditions. We investigated whether suppression of HIF-1α expression by HIF-1α siRNA might affect the radiosensitivity, angiogenesis, and tumor inhibition *in vitro* and *in vivo*.

## Material and Methods

### Ethics statement

All animal work was conducted according to relevant national and international guidelines. The animal use protocol was reviewed and approved by the institutional animal care and use committee of the Laboratory Animal Centre of Fuzhou General Hospital.

### Cell culture

The human cervical carcinoma cell line (HeLa) was purchased from the cell bank of the China Science Academy (Shanghai, China) and maintained in RPMI1640 medium (Gibco, Gaithersburg, MD, USA) with 10% fetal bovine serum (FBS; Gibco), and incubated at 37°C in a humidified incubator at 5% $CO_2$. Cells were harvested after reaching 80-90% confluence.

### Radiation and hypoxia

Hypoxia condition was obtained by adding cobalt chloride ($CoCl_2$) into the culture medium to a final concentration of 125 umol/L for the indicated time. Cells were irradiated using a Varian 2300C/D Medical Linear Accelerator at room temperature under both normoxic and hypoxic conditions. Radiation doses were given 8 Gy at a dose-rate of approximately 4 Gy/min. The cells were divided into 4 groups: the control group, the hypoxia group (H group: cells were maintained in hypoxic condition for 24 h and then cultured in fresh medium for 24 h), the hypoxia and irradiation group (H+R group: after cultured in hypoxic condition for 24 h, the cells were irradiated, and then were cultured continuously with fresh medium for 24 h), and the irradiation group (R group).

### Transfected with HIF-1α siRNA

Transfection of HIF-1α (Ambion) siRNA in HeLa cells was performed by using siPORT NeoFx transfection agent (Ambion) according to the manufacturer's instructions.

### Proliferation assays of HeLa Cells

The cell survival rate was determined by a standard spectrophotometric 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [6]. Cells in different groups were incubated for 4 h with the MTT solution (5 mg/mL) and the supernatant was discarded. The precipitate was dissolved in 200 ml of DMSO for 10 min and the absorbance values at 490 nm (A490 nm) were measured. The cell survival ratio of each group was calculated as follows: survival ratio – A490 nm of experiment group/A490 nm of control group.





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### Cell apoptosis analysis using flow cytometry (FCM)

Cells of the above groups were trypsinized using 0.25% trypsin and rinsed in phosphate-buffered saline (PBS). The cells were centrifuged at 800 g for 5 min and resuspended in up to 500-µl PBS. The cells were divided into 2 tubes, 1 for cell cycle and 1 for apoptosis. One was centrifuged and cells were collected and fixed in ice-cold 70% ethanol for 24 h and recentrifuged. Cells were then incubated with RNase (0.5 mg/ml) and stained with 1 ml of 50 u g/ml propidium iodide (PI, Sigma Chemical Co., St. Louis, MO) in the dark for 30 min at room temperature. The other received 5 ul FITC and 5ul PI, and was incubated for 15 min at 25°C. Cells were added to 400 ul buffer and kept in the dark for 1 h. Then the FAC scan analysis system was used to collect FCM data on the change in cell cycle and cell apoptosis. Each experiment was performed in triplicate.

### RNA extraction and reverse transcription (RT)-PCR analysis

Cells were harvested in TRIzol Reagent (Invitrogen) and total RNA was isolated following the manufacturer's instructions. The first-strand cDNA and the semi-quantitative RTPCRs were carried out essentially as described previously [13] by using gene-specific oligonucleotides under conditions of linear amplification. PCR products were run on a 2% agarose gel and visualized by ethidium bromide staining using UV light. The housekeeping glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA, used as an internal standard, was amplified from the same cDNA reaction mixture. Densitometric analysis was used to quantify mRNA levels. Data presented are representative of at least 3 independent experiments. Gene expression for HIF-1α, VEGF and P53 was detected by RT-PCR analysis. HIF-1α: (F)5'-CTTCTGGATGCTGGTGATTTG-3'; (R)5'-TATACGTGAATGTGGCCTGTG-3'. (2)VEGF: (F)5'-AGG AGGGCAGAATCATCACG-3'; (R)5'-TATGTGCTGGCCTTGGTGAG-3'. P53: (F) 5'-TTGGATCCATGTTTTGCCAACTGGCC-3', (R): 5'-TTG AATTCAGGCTCCCCTTTCTTGCG-3' GAPDH: (F)5'-TGAAGG TCGGTG TGAACGGATTTGGC-3'; (R)5'-CATGTAGGCCAT GAGG TCCACCAC-3'.

### Analysis of the expression of HIF-1α, VEGF and P53 protein by Western blotting

Western blotting was performed as described previously [7]. The HIF-1α VEGF and P53 and β-actin (as a control) protein in each group were assayed using a BAC protein quantitative kit (Wuhan Boster Biological Technology Co. Ltd., Wuhan China). Briefly, 80 µg of protein was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane (Millipore, Billerica, MA, USA). Skimmed milk (2%) was used as a blocking solution (room temperature, 1 h). The membrane was incubated with the primary antibody at room temperature for 3 h, and with the secondary antibody was incubated at room temperature for 1 h. The proteins were detected using an enhanced chemiluminescence system (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and were exposed to X-ray film. Protein expression was analyzed semi-quantitatively using the Kodak Gel Logic Analysis System.

### *In vivo* studies

This experiment performed was in accordance with institutional guidelines of the Second Military University and with appropriate institutional certification. Animal surgical and X-ray radiation were performed under general anesthesia with 50 mg/kg i.p. injection of pentobarbital sodium. Approximately $2\times10^7$ of HeLa cells were subcutaneously inoculated into the flanks of 4-week-old female athymic nude mice (BALB/c). Tumor growth rates were determined by measuring 2 orthogonal dimensional diameters of each tumor 3 times each week. Tumor volumes were calculated according to the formula V=1/2×a2×b (a=short axis, b=long axis. When tumors reached an average volume of about 150 $mm^3$, the tumor-bearing BALB/c-nu/nu mice were divided into 2 groups with assigned 6 nude mice in each group: (a) irradiation group, tumors were exposed to X-ray of 8 Gy alone for each time; and (b) combination group, 100 ug of HIF-1α siRNA was injected into the solid tumor 3 times (the interval time was 2 days) before 8 Gy X-ray exposure. BALB/c-nu/nu mice were killed 12 days later.

### Statistical analysis

All data are presented as mean ±SEM. Calculations were performed with SPSS version 13.0 (SPSS, Chicago, IL, USA). Differences among groups were assessed by unpaired Student's t test and 1-way ANOVA. A P value less than 0.05 was considered to be statistically significant.

## Results

### The Effect of HIF-1α on proliferation of HeLa cells after radiation

Before transfection, MTT showed that the proliferation of HeLa cells that received radiation alone was the lowest among the 4 groups. As shown in Figure 1, it was observed that the cell viability was markedly reduced in the irradiated cells without hypoxia–pretreatment ($p<0.05$). The cell survival ratio of the irradiation group was 16.47±3.56%. There has no significant difference in the cell viability between the control group and the hypoxia group ($p>0.05$).

After exposure to 8 Gy irradiation, under mimetic hypoxia, cellular viability was significantly reduced by treatment with HIF-1α siRNA compared with control cells under mimetic hypoxia; no





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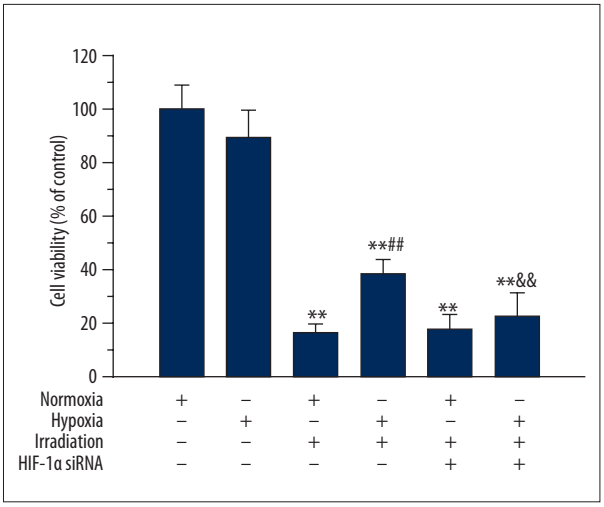


**Figure 1.** The proliferation of HeLa cells by MTT in different group. Before transfection, MTT assay showed that the proliferation of HeLa cells in the radiation group was the lowest among the 4 groups. Cell viability with or without HIF-1α siRNA was significantly increased at the same radiation dose compared with normoxic cells. Subsequent analysis showed that the viability of HIF-1α siRNA transfected cells irradiated with 8 Gy was reduced relative to untransfected cells. The data represent the mean ±S.E.M. from 4 independent experiments. ** P<0.01 *vs.* control group; ## P<0.01 *vs.* normoxia + irradiation group; && P<0.01 *vs.* hypoxia + irradiation.

change was observed under normoxic conditions. As shown in Figure 1, although the expression of HIF-1α was inhibited, cellular viability was higher in the hypoxia + HIF-1α group than that in the control group or normoxia + HIF-1α group, perhaps due to the effect of $CoCl_2$ on increasing the level of HIF-1α expression.

### The Effect of HIF-1α on cell apoptosis of HeLa cells

Before transfection, FCM showed that the apoptotic rates of HeLa cells were higher in the irradiation group than the other 3 groups ($p<0.01$). While the lowest apoptotic rates of HeLa cells were found in the irradiation + hypoxia group ($p<0.01$) (Figure 2A, 2B).

Consistent with the results of MTT assay, the apoptotic rates were increased significantly by treatment with HIF-1α siRNA compared with control cells under mimetic hypoxia in the same X-ray dose ($p<0.05$) (Figure 2C, 2D).

### The Effect of irradiation on HIF-1α under hypoxia

The application of $CoCl_2$, a chemical HIF-1α inducer, provides protection against UV induced corneal stromal cell apoptosis [8]. According to the above results, we postulated increased tumor hypoxia protect cell apoptosis induced by irradiation, in which HIF-1α might play an important role. To test this, we determined the level of HIF-1α mRNA in different cell groups. As shown in Figure 3A, the accumulation of HIF-1α increased more significantly in the hypoxia and irradiation group than that in the other 3 groups. No significant changes in the expression of HIF-1a were found in the control group or the irradiation group. Thus, we hypothesized that irradiation could increase the accumulation of HIF-1α during hypoxia.

Transfection with HIF-1α siRNA resulted in loss of HIF-1α expression in cells treated with $CoCl_2$. However, a higher expression of HIF-1α was still observed in the hypoxia + HIF-1α siRNA group. There was no statistical difference in levels of HIF-1α between cells treated with HIF-1α siRNA cultured in normoxic condition and control cells.

### Expression of VEGF and P53 in different groups

Genes prominently induced by hypoxia include growth factors like VEGF and EPO [9,10]. Among these growth factors, VEGF was found to be up-regulated in most cell types [11]. We tested here whether the transcription of VEGF is up-regulated in HeLa cells by hypoxia preconditioning. Figure 3B shows that VEGF is prominently up-regulated in the cells subjected to irradiation in addition to hypoxia ($p<0.05$).

Another factor that affects the stability of HIF-1α is the tumor suppressor, p53. P53 is known to participate in the degradation of HIF-1α under hypoxic conditions [7]. As shown in Figure 3C, the decrease in P53 levels was significantly lower in cells treated by irradiation in addition to hypoxia than in cells treated by irradiation alone. A higher expression of P53 protein was found in cells after irradiation; interestingly, a low expression of P53 was also observed in the hypoxia group.

For the above results, we hypothesized that VEGF and P53 might contribute to angiogenesis and apoptosis upon loss of HIF-1a. To verify this assumption, we transfected HeLa cells with the HIF-1α siRNA and detected the expression of VEGF and P53 protein. After transfection, VEGF protein decreased significantly in cells cultured in hypoxia with or without irradiation. The P53 protein gradually accumulated in cells pretreated with $CoCl_2$, an effect that was strikingly enhanced in HIF-1a-inhibited cells ($p<0.05$, Figure 4B, 4C).

### Effect of HIF-1α AS-ODNs on tumor growth *in vivo* after irradiation

The volumes of xenograft tumor after 8 Gy radiation plus transfection of HIF-1α AS-ODNs were significantly decreased when mice were sacrificed compared to 8 Gy radiation alone, reducing mean tumor volume >30% by 12 days after the initiation of treatment. When mice were killed, the mean weight of tumors





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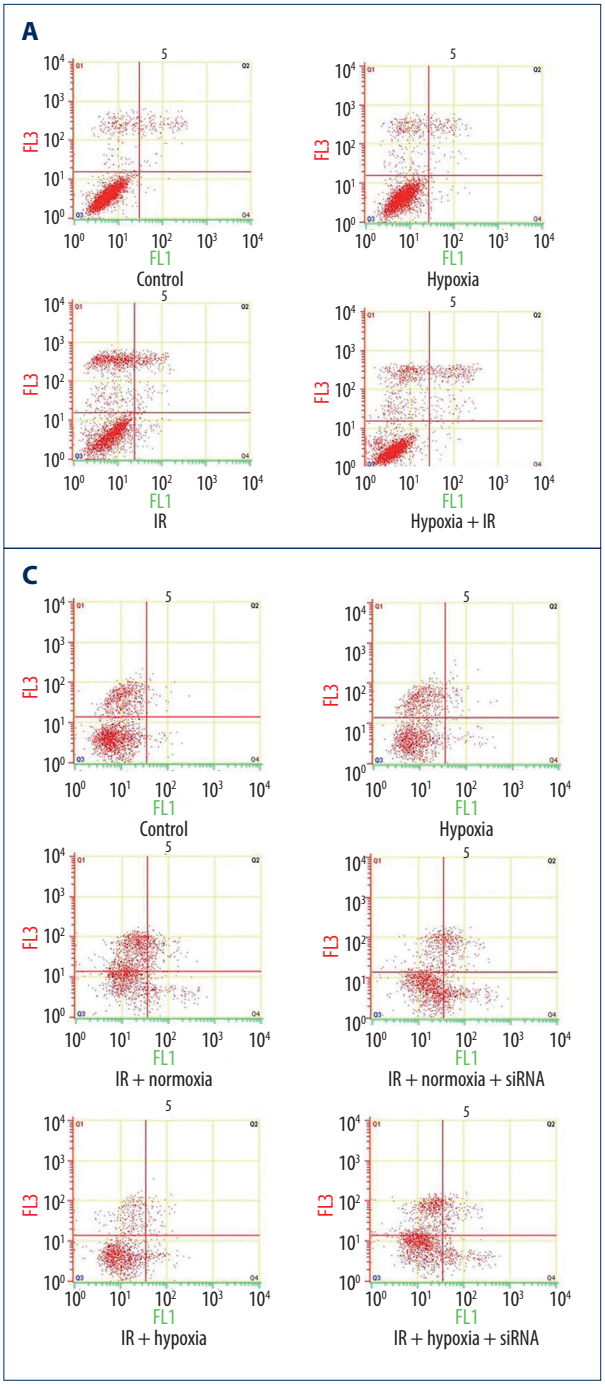


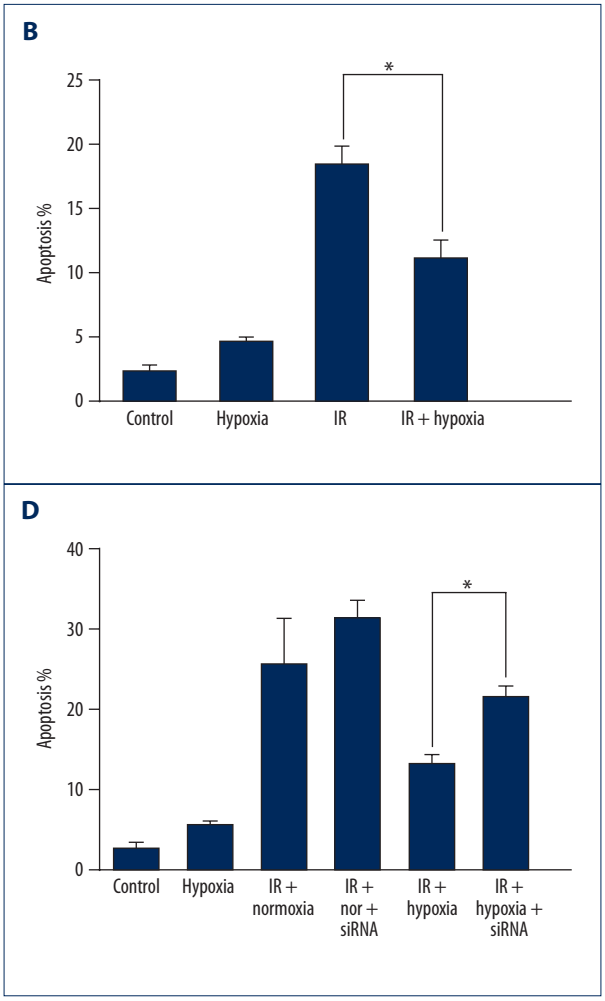


**Figure 2.** Apoptosis percent of HeLa cells was determined by flow cytometry. (**A**) Detection of apoptosis percent of HeLa cells analyzed by flow cytometry before transfection. (**B**) Compared with the cells pretreated by $CoCl_2$, the apoptosis rate of cells cultured in normoxia increased markedly in the same X-ray dose (* P<0.05). (**C**) Detection of apoptosis percent of HeLa cells analyzed by flow cytometry after transfection. (**D**) Compared with the normoxic cells, the apoptosis rate of cells with or without transfection cultured in hypoxic condition decreased markedly in the same X-ray dose. The apoptosis rate of cells with transfection was higher than that without transfection in hypoxia. (* P<0.05)

after 8 Gy radiation plus transfection of HIF-1α siRNA was significantly lower than that in mice after 8 Gy radiation alone (0.1625±0.0774 g *vs.* 1.1273±0.1909 g, p<0.001, Figure 5A).

Western blotting also showed that the expressions of HIF-1α and VEGF protein after 8 Gy radiation plus transfection of HIF-1α AS-ODNs were decreased compared to 8 Gy radiation alone (p<0.01, Figure 5B, 5C). The opposite result was observed in the expression of P53 protein (p<0.05, Figure 5B, 5C).

## Discussion

Radiotherapy is still one of the best cervical cancer therapies. Radiotherapy technology has evolved remarkably during the past decade, and radiation can be precisely delivered, thereby permitting higher doses to the tumor and reduced doses to surrounding normal tissues [12]. Tumor recurrence following a course of radiation therapy results from radiation resistance in





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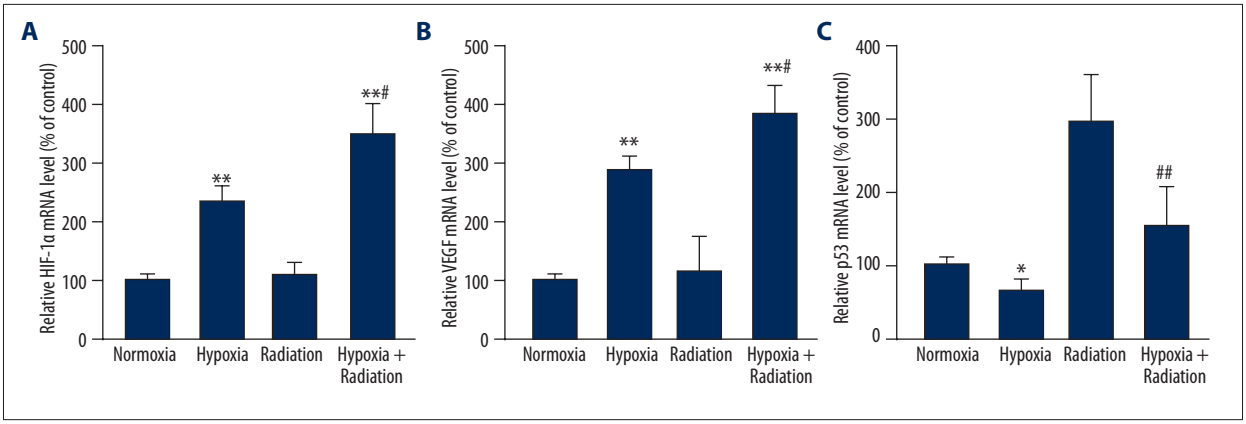


**Figure 3.** HIF-1α, VEGF and P53 mRNA expression level in different cell groups determined by RT-PCR. The mRNA levels were normalized to GAPDH expression. The data represent the mean ±S.E.M. from 4 independent experiments. (**A**) HIF-1α expression increased more significantly in the hypoxia and radiation group compared to the other 3 cell groups. Hypoxia-induced accumulation of HIF-1α was observed in the Hypoxia group. * P<0.05, ** P<0.01 as compared to Control group; # P<0.05 as compared to the hypoxia group. (**B**) Consistent to HIF-1α, the VEGF expression was observed. * P<0.05, ** P<0.01 as compared to control group; # P<0.05 as compared to hypoxia group. (**C**) Compared with the hypoxic and irradiated cell, the expression of p53 in the irradiated alone cell was increased significantly (** P<0.01). A low expression of p53 was observed in hypoxic cell (* P<0.05).

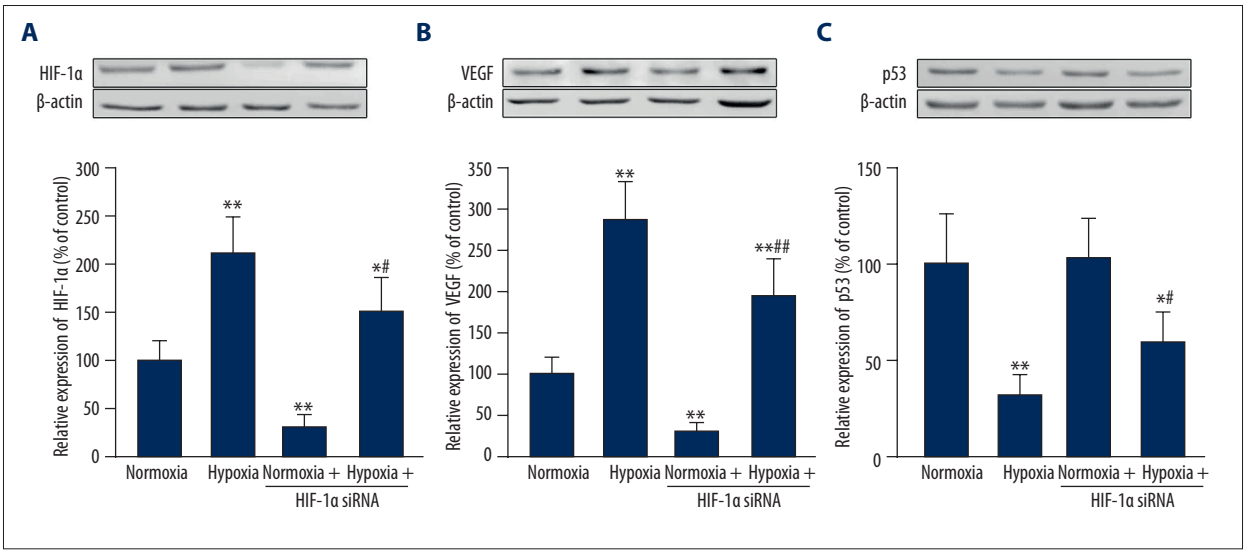


**Figure 4.** Changes of HIF-1α and its target gene expression after transfection. Expressions of HIF-1α, VEGF, and p53 protein analyzed by Western blotting experiment. * P<0.05; ** P<0.01 compared to normoxia group; # P<0.05 ## P<0.01 compared to the normoxia + HIF-1α siRNA group.

some cells which can survive and re-proliferate. It is well known that one of the major reasons for the failure of radiation therapy is hypoxia [2,13,14,]. Cervical cancer exhibits some unique differences compared to other solid tumors. Normal cervical stratified epithelia have characteristics of hypoxic tissue. The development of a hypoxic microenvironment induces the expression of a variety of genes in tumor tissue. One such gene is hypoxia-inducible factor (HIF-1), which is a suitable indicator of cells growing under hypoxic conditions. Several studies have investigated the role of HIF-1 in radiation sensitivity in cancer cells *in vivo* and *in vitro*, but the results are somewhat controversial. For example, Vordemark et al. have shown that the HIF-1 protein accumulation levels under hypoxia do not always correlate with cellular radiation resistance (i.e., a cell type-specific association was observed) [15]. To investigate whether HIF-1α is associated with cellular radiation resistance in human cervical carcinoma, we tested the relationship of cell apoptosis rate and the expression of HIF-1α. As reported previously [16,17], cobalt chloride ($CoCl_2$) can induce biochemical and molecular responses similar to those observed under hypoxic conditions. In our study, a hypoxic condition was mimicked with $CoCl_2$ in the concentration of 125 umol/L





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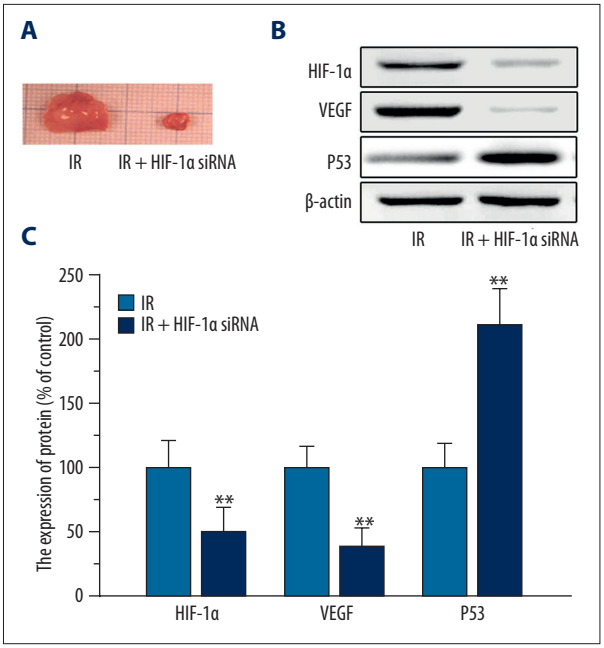


**Figure 5.** Effect of combined treatment with HIF-1α siRNA plus radiation on xenograft tumor growth *in vivo*. (**A**) Representative tumors from each group. HIF-1α siRNA plus radiation group had smaller volume than the control group. (**B**) Western blotting showed the different expression of HIF-1α, VEGF, and p53 in different groups. (**C**) A higher level of p53 and lower level of VEGF expression were observed with the inhibiting of HIF-1α in mice that received irradiation.

to promote HIF-1 expression and avoid the significant apoptosis induced by hypoxia. As our results show, the expression of HIF-1α was the highest in the cells treated by irradiation in addition to hypoxia, the cell apoptosis rate of which is the lowest of the 4 cell groups. Furthermore, the apoptosis rate increased markedly in cells transfected with HIF-1α siRNA cultured in hypoxia condition in the same dose of X-ray. Thus, we concluded that hypoxia-mediated radiation resistance is dependent on HIF-1α in human cervical cancer cells. In this study, we also thought that irradiation could increase the accumulation of HIF-1α during hypoxia.

VEGF protein plays an important role in tumor angiogenesis, showing a vital role in the metastasis, invasion, and growth of tumors. During the response to hypoxia in cells, HIF-1 plays an important mediating role via the control of its downstream genes, such as the members of the vascular endothelial growth factor (VEGF) families [18]. Several studies [19,20] have demonstrated that high HIF-1 and VEGF expression levels in tumor cells correlate with greater malignant potential and more aggressive growth characteristics, but whether the effect of VEGF on protection is dependent on HIF-1α is cell type-specific. Consistent with literature reports, our results indicate that VEGF expression is up-regulated by hypoxia.

Another factor that affects the stability of HIF-1a is the tumor suppressor p53. P53 is a pivotal cell fate determinant due to its role in regulating cell-cycle progression and apoptosis in response to cellular stress, and is the most commonly mutated gene in human cancers. Hypoxia is also known to modulate the p53 pathway, in a manner dependent or not on HIF-1 (hypoxia-inducible factor-1), the main transcription factor activated by hypoxia [7]. The correlations of p53 and HIF-1α in the hypoxia-protecting cell apoptosis induced by irradiation were examined. In the radiation group, the highest expression of P53 protein was found, perhaps because cell senescence is promoted by irradiation, depending on functional p53. The p53 levels in the cells treated by irradiation in addition to hypoxia were significantly lower than those in other cell groups. The expression of p53 protein increased following inhibition of HIF-1α. These results indicate that HIF-1a participated in protecting the human cervical cancer cell apoptosis induced by irradiation via inhibiting the expression of p53. We found a low level of p53 in cells cultured in hypoxia alone, possibly because HIF-1α up-regulation is not sufficient for p53 induction in low hypoxia.

To further verify the role of HIF-1α in radioresistance, we investigated whether targeted HIF-1α siRNA against HIF-1α expression can enhance the radiosensitivity of cervical cancer. In this study, we found that HIF-1α AS-ODNs had a synergy with X-ray radiation in reducing the proliferation of HeLa cells *in vitro*. A single radiation dose (8 Gy) on xenograft tumors dramatically reduced the growth rate of HeLa cells treated with HIF-1α siRNA. The same changes of VEGF and P53 were found *in vitro* and *in vivo*.

Except for HIF-1α, HIF-2α bearing some similar function to HIF-1α was also demonstrated as a poor prognosis factor in patients with cervical cancer undergoing radiotherapy [21]. However, in contrast to the ubiquitous expression of HIF-1α, the expression of HIF-2α had a more restricted pattern. Contrasting roles were found between HIF-1α and HIF-2α. HIF-1α stimulates p53 phosphorylation but HIF-2α inhibited p53 activity by regulating different genes [22–23]. Other study also showed that HIF-1α could be rapidly induced by acute exposure hypoxia but not prolonged hypoxia, whereas HIF-2α could be hypoxia-induced with prolonged hypoxia [24]. Moreover, HIF-2α would not substitute in regulating any of the hypoxia-inducible genes when HIF-1α was inactive in cells that HIF-1α and HIF-2α coexpressed. In our study, the cells were cultured with CoCl2, mimicking the acute hypoxia condition. Thus, we investigated only the role and mechanism of HIF-1α in radioresistance. HIF-2α will be a new research field for radioresistance in the future.

Overall, the results from this study show that hypoxia preconditioning protection requires induction of HIF-1α. HIF-1alpha increased the radiation resistance by elevating the expression of VEGF and inhibiting the expression of p53. In our study, a low





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hypoxia condition was mimicked. Further research is needed to determine if severe hypoxia is synergistic with X-ray radiation in tumor cell apoptosis, as well as the impossible mechanism.

## Conclusions

In cervical cancer cells, the mechanism of HIF-1alpha in radioresistance was by elevating the expression of VEGF and inhibiting the expression of p53. In the future, we might inhibit human cervical cancer progression and enhance the radiosensitivity by inhibiting HIF-1α. The challenge is how to use this information to optimize cancer therapy.

### Competing interests

The authors have declared that no competing interests exist.

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