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Dual-sensitive HRE/Egr1 promoter regulates Smac overexpression and enhances radiation-induced A549 human lung adenocarcinoma cell death under hypoxia

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Abstract. The aim of this study was to construct an expression vector carrying the hypoxia/radiation dual-sensitive chimeric hypoxia response element (HRE)/early growth response 1 (Egr-1) promoter in order to overexpress the therapeutic second mitochondria-derived activator of caspases (Smac). Using this expression vector, the present study aimed to explore the molecular mechanism underlying radiotherapy-induced A549 human lung adenocarcinoma cell death and apoptosis under hypoxia. The plasmids, pcDNA3.1-Egr1-Smac (pE-Smac) and pcDNA3.1-HRE/Egr-1-Smac (pH/E-Smac), were constructed and transfected into A549 human lung adenocarcinoma cells using the liposome method. $CoCl_2$ was used to chemically simulate hypoxia, followed by the administration of 2 Gy X-ray irradiation. An MTT assay was performed to detect cell proliferation and an Annexin V-fluorescein isothiocyanate apoptosis detection kit was used to detect apoptosis. Quantitative polymerase chain reaction and western blot analyses were used for the detection of mRNA and protein expression, respectively. Infection with the pE-Smac and pH/E-Smac plasmids in combination with radiation and/or hypoxia was observed to enhance the expression of Smac. Furthermore, Smac overexpression was found to enhance the radiation-induced inhibition of cell proliferation and promotion of cycle arrest and apoptosis. The cytochrome *c*/caspase-9/caspase-3 pathway was identified to be involved in this regulation of apoptosis. Plasmid infection in combination with X-ray irradiation was found to markedly induce cell death under hypoxia. In conclusion, the hypoxia/radiation dual-sensitive chimeric HRE/Egr-1 promoter was observed to enhance the expression of the therapeutic Smac, as well as enhance the radiation-induced inhibition of cell proliferation and promotion of cycle arrest and apoptosis under hypoxia. This apoptosis was found to involve the mitochondrial pathway.

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Introduction

Radiotherapy is frequently used for the clinical treatment of lung cancer; however, its effectiveness is often impaired by radiation resistance (1). Combining gene therapy with radiotherapy, known as gene-radiotherapy, is a novel method for cancer treatment, and has attracted particular interest (2,3). At present, gene-radiotherapy focuses on the radiation-inducible early growth response 1 (Egr1) promoter. This promoter contains six serum response elements, CC(A+T-rich)6GG motifs, which are sensitive to ionizing radiation (4,5). Numerous studies have demonstrated that, under irradiation, the Egr1 promoter is capable of regulating the overexpression of downstream genes, including TNF-α, IFN-γ, endostatin and TRAIL (6-8).

Solid tumor hypoxia occurs when the growth rate of the tumor cells exceeds that of the tumor blood vessels, resulting in insufficient tumor blood supply. Hypoxic tumor cells account for between 10 and 50% of the cells in a solid tumor and cause radio- and chemotherapy resistance, leading to local tumor recurrence and distant metastasis (9). Hypoxic tumor cells have unique biological features that confer radioresistance and make tumor cells more aggressive. Under hypoxia, certain protective stress proteins are upregulated in tumor cells, including hypoxia-inducible factor-1, which specifically binds to hypoxia response elements (HREs), inducing the expression of downstream genes. HRE is a hypoxic enhancer with a core sequence of 5'-(A/G) COT (G/C) (G/C)-3'. Previous studies have shown that placing the HRE sequence upstream of the AFP, KDR, CMV and SV40 promoters, to constitute the chimeric promoters, markedly increases their transcriptional activities under hypoxia (10-12). Furthermore, placing the HRE sequence upstream of the Egr1 promoter has been found to enhance the radiation-induced overexpression of therapeutic genes in hypoxic tumor cells (13).

Ionizing radiation is capable of inducing tumor cell apoptosis and cell cycle arrest. Following cell cycle arrest, ionizing radiation-induced DNA damage is repaired, with

cells undergoing apoptosis if repair cannot be completed (14). Pro-apoptotic genes can be used as targets in gene-radiotherapy. Therefore, hypoxia/radiation dual-sensitive HRE/Egr-1 promoter-mediated gene-radiotherapy may enhance hypoxic tumor cell apoptosis, increasing cell death.

The second mitochondria-derived activator of caspases (Smac) is located in the mitochondria and is released into the cytosol to exert its pro-apoptotic activity. Smac specifically binds to anti-apoptotic proteins, abolishing the suppression of the pro-apoptotic proteins caspase-9 and -3, which have a key role in cell apoptosis. Furthermore, Smac is capable of exerting direct pro-apoptotic effects by binding to apoptotic protease activating factor-1, cytochrome *c* (Cyt *c*) and casapse-9 (15,16). The overexpression of Smac has also been utilized for cancer treatment (17,18).

The aim of the present study was to investigate the molecular mechanisms underlying radiation-induced A549 cell death upon the induction of hypoxia and the overexpression of Smac. A human Smac expression vector, pcDNA3.1-HRE/Egr-1-Smac (pH/E-Smac), containing the double-sensitive HRE/Egr1 promoter, was constructed. The effect of Smac overexpression on the inhibition of cell proliferation and the promotion of cell cycle arrest and apoptosis was then assessed in A549 human lung adenocarcinoma cells subjected to hypoxia and 2 Gy X-ray irradiation.

Materials and methods

Cell line. A549 human lung adenocarcinoma cells were purchased from Peking Union Medical College Cell Bank (Beijing, China) and were cultured in high-glucose Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS; Gibco-BRL, Grand Island, NY, USA) and 100 U/ml penicillin and streptomycin (Gibco-BRL) at 37˚C in 5% CO_2. A549 cells were grown to 80% confluence in six-, 24- or 96-well culture plates prior to treatment.

Plasmids. The plasmids pcDNA3.1-Egr1-TRAIL and pcDNA3.1-HRE-Egr1-TRAIL were obtained from Dr Yanming Yang (Jilin University, Changchun, China). The pcDNA3.1-CMV-Smac plasmid was constructed as described previously (19) and obtained from Dr Caixia Guo (Jilin University). Plasmids were digested using *Hin*dIII and *Bam*HI, and the vector backbones and *Smac* fragments were subsequently recycled and ligated using the T4 DNA ligase to obtain the recombinant pcDNA3.1-Egr1-Smac (pE-Smac) and pH/E-Smac plasmids. Plasmids were identified using sequencing and restriction digestion.

Cell transfection, hypoxia simulation and X-ray irradiation. A549 cells were transfected using the Lipofectamine 2000 reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Cells were cultured at 37˚C in 5% CO_2 for a further 6 h with fresh medium containing 10% FBS. Twenty-four hours after transfection, $CoCl_2$ (Sigma-Aldrich) was added at a final concentration of 150 μmol/l to simulate hypoxia, as described previously (20). X-ray irradiation was then performed 24 h subsequent to the addition of $CoCl_2$, using the Philips Deep Therapy machine at 200 kV and 10 mA, using 0.5-mm Cu and 1-mm Al plates at 50 cm skin distance. A dose of 2 Gy was delivered at a rate of 0.287 Gy/min. The dose and dose rate were selected using the 1986 report from the United Nation Scientific Committee on the Effects of Atomic Radiation (8,21,22).

MTT assay to detect cell proliferation. A549 cells were divided into six groups: Control, pE-Smac, pH/E-Smac, 2 Gy, pE-Smac+2 Gy and pH/E-Smac+2 Gy. Briefly, A549 cells were seeded on 96-well plates at $2x10^4$ cells/well, with six replicates for each treatment. After 12 h, transfection was performed. Twenty-four hours after transfection, cells were cultured in normoxic and hypoxic conditions simulated by the addition of $CoCl_2$. After 24 h, 0 or 2 Gy X-ray radiation was administered. A total of 10 μl MTT (Sigma-Aldrich) was added 0, 4, 12, 24 and 48 h after irradiation, to form a final concentration of 5 mg/ml. Following 4 h of incubation, the supernatant was discarded and 100 μl dimethylsulfoxide (Sigma-Aldrich) was added to dissolve the crystals. The absorbance (A) value was measured at 570 nm using a microplate reader (Bio Rad, Hercules, CA, USA) (8). The experiment was repeated three times.

Flow cytometry (FCM) for the analysis of the cell cycle and apoptosis. FCM (Becton Dickinson Co., Franklin Lakes, NJ, USA) was performed to detect cell cycle arrest and apoptosis using propidium iodide (PI; Sigma-Aldrich) and the Annexin V-fluorescein isothiocyanate (FITC) double-staining kit (Nanjing KGI Biological Technology Development Co., Ltd., Nanjing, China). Briefly, A549 cells were seeded on 24-well plates at $3x10^5$ cells/well and subjected to transfection, hypoxia simulation and irradiation according to the aforementioned methods. Twenty-four hours after irradiation, cells were collected in glass centrifuge tubes and washed twice with phosphate-buffered saline (PBS). The supernatant was discarded. To detect cell cycle phase, 50 μl RNase A and 200 μl PI were added and mixed in the dark at room temperature for 20 min. To detect the cell apoptosis, cells were resuspended in 500 μl PBS, and 5 μl Annexin V-FITC and 5 μl PI were added and mixed in the dark at room temperature for 15 min. The cell samples were then assessed using FCM. The BD CellQuest™ (Becton Dickinson Co.) software was used to acquire and analyze the data.

Quantitative polymerase chain reaction (qPCR) analysis of mRNA expression. A549 cells were seeded on six-well plates at $5x10^5$ cells/well, prior to transfection, hypoxia simulation and irradiation as described above. Cells were collected 24 h after irradiation and total RNA was extracted using TRIzol® Reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. The extracted RNA was quantified by detection of the A_{260}/A_{280} ratio. cDNA was synthesized using a reverse transcription kit (MBI Fermentas Inc., Burlington, ON, Canada) according to the manufacturer's instructions (200 ng RNA, 20 μl reaction system). The reaction conditions were 42˚C for 60 min, followed by 70˚C for 2 min. The PrimeScript® RT-PCR kit (Takara Bio, Inc., Dalian, China) and an Mx3000P™ Real-Time PCR System (Stratagene, La Jolla, CA, USA) were used for the qPCR and the subsequent analysis of results. Primer3 software (23) was used to design

the GAPDH, Smac, Cyt c, caspase-9 and -3 primers, which were synthesized by Takara Bio, Inc. and are shown in Table I. The reaction conditions were as follows: 95˚C for 30 sec, one cycle; 95˚C for 20 sec and 60˚C for 20 sec, 40 cycles; 95˚C for 1 min, one cycle; 55˚C for 30 sec and 95˚C for 30 sec. Relative mRNA expression was calculated as the ratio of the gene of interest mRNA/GAPDH mRNA.

Western blot analysis to detect protein expression. A549 cells were seeded on six-well plates at $1x10^6$ cells/well, and subjected to transfection, hypoxia simulation and irradiation according to the aforementioned methods. Cells were collected 24 h after irradiation, and lysis buffer (10 mmol/l Tris-HCl, pH 7.4; 1 mmol/l EDTA, pH 8.0; 0.1 mol/l NaCl; 1 μg/ml aprotinin; 100 μg/ml phenylmethanesulfonyl fluoride) was used to extract the total protein. The Coomassie Brilliant Blue protein quantification kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) was used to quantify the total protein. Proteins were separated using 12% SDS-PAGE with a sample loading of 50 μg/lane. Proteins were then transferred to a nitrocellulose membrane. The membrane was blocked using 5% non-fat, dry milk for 1 h, prior to incubation with primary antibodies against β-actin, Smac, Cyt c, caspase-9 and -3, respectively, overnight at 4˚C. Membranes were then washed twice using Tris-buffered saline containing 0.05% Tween 20, prior to incubation with horseradish peroxidase-conjugated secondary antibodies at 37˚C for 1 h (Pierce Biotechnology, Inc., Rockford, IL, USA). The enhanced chemical luminescence light system method (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was used to visualize the immunoreactive bands. Images were captured for analysis.

Statistical analysis. Experimental data are presented as the mean ± standard deviation. Results were analyzed using one-way analysis of variance with the SPSS 12.0 statistical software (SPSS, Inc., Chicago, IL, USA). A value of P<0.05 was considered to indicate a statistically significant difference.

Results

Sequencing and restriction digestion of pE-Smac and pH/E-Smac. The pE-Smac plasmid was digested by *Sma*I, *Xba*I and *Xho*I enzymes. The fragments generated were 2,292 and 3,772 bp (*Sma*I); 828 and 5,236 bp (*Xba*I) and 908 and 5,156 bp (*Xho*I) (Fig. 1A). The pH/E-Smac plasmid was digested using *Bam*HI and *Sma*I enzymes. The digested fragments were 1,284 and 4,926 bp (*Bam*HI) and 2,292 and 3,918 bp (*Sma*I) (Fig. 1B). All fragments were as expected, and sequencing revealed that fragment sequences were consistent with those expected (data not shown).

A549 cell proliferation. The results of the MTT assay used to assess A549 cell proliferation are shown in Table II. Under normoxic conditions, no significant differences were observed in A549 cell proliferation among the control, pE-Smac and pH/E-Smac groups (P>0.05). However, a significant decrease in A549 cell proliferation was observed in the 2 Gy (P<0.05), pE-Smac+2 Gy and pH/E-Smac+2 Gy groups (both P<0.001), compared with the control group at each time-point. Compared with normoxia, hypoxic treatment was found to significantly reduce A549 cell proliferation (P<0.05 or P<0.001), particularly in the pH/E-Smac+2 Gy group (P<0.001).

Table I. Primer sequences.

Gene	Sequence
GAPDH	
Sense	5'-ACCACAGTCCATGCCATCAC-3'
Antisense	5'-TCCACCACCCTGTTGCTGTA-3'
Smac	
Sense	5'-CTGTCGCGCAGCGTAACTTC-3'
Antisense	5'-GGTTACTCCAAAGCCAATCGTCA-3'
Cyt *c*	
Sense	5'-GGGCGAGAGCTATGTAATGCAAG-3'
Antisense	5'-TACAGCCAAAGCAGCAGCTCA-3'
Caspase-9	
Sense	5'-GGACATCCAGCGGGCAGG-3'
Antisense	5'-TCTAAGCAGGAGATGAACAAAGG-3'
Caspase-3	
Sense	5'-TTCAGGCCTGCCGTGGTACA-3'
Antisense	5'-CCAAGAATAATAACCAGGTGCT-3'

Smac, second mitochondria-derived activator of caspases; Cyt *c*, cytochrome *c*.

A549 cell cycle phase. Table III and Fig. 2 show A549 cell cycle phase under normoxia and hypoxia, detected using FCM with PI staining. Under normoxia, in the 2 Gy, pE-Smac +2 Gy and pH/E-Smac+2 Gy groups, the proportion of cells in S phase was significantly decreased compared with the control, while the proportion of cells in G_2/M phase was significantly increased compared with the control (P<0.05). No significant differences were observed in the other treatment groups under normoxia. Under hypoxia, in the pH/E-Smac, 2 Gy, pE-Smac+2 Gy and pH/E-Smac+2 Gy groups, the proportion of cells in the G_0/G_1 and G_2/M phases was observed to be significantly increased compared with the control, while the proportion in S phase was found to be significantly reduced (P<0.05 or P<0.001). With the exception of the G_2/M phase in the control and pE-Smac groups, the changes in the percentage of cells in G_0/G_1, S and G_2/M phases under hypoxia were significantly greater than those under normoxia (P<0.05 or P<0.001). These findings suggest that 2 Gy X-ray irradiation can induce G_2/M-phase arrest and that hypoxia induces G_0/G_1 arrest. The greatest cell cycle arrest was achieved in the pH/E-Smac+2 Gy group under hypoxia.

A549 cell apoptosis. Table IV and Fig. 3 show the results of the FCM used to detect A549 cell apoptosis under normoxia and hypoxia. Under normoxia, the percentage of apoptotic A549 cells in the 2 Gy, pE-Smac+2 Gy and pH/E-Smac+2 Gy groups was observed to be significantly increased compared with that in the control group (P<0.05 or P<0.01), particularly in the pE-Smac+2 Gy and pH/E-Smac+2 Gy groups. No significant difference was observed between the pE-Smac+2 Gy and pH/E-Smac+2 Gy groups. Under hypoxia, the percentage of

Table II. Absorbance value of A549 cells under normoxia and hypoxia.

Groups	Time (h)				
	0	4	12	24	48
Normoxia					
Control	0.416±0.002	0.423±0.008	0.489±0.023	0.665±0.058	0.787±0.035
pE-Smac	0.424±0.008	0.415±0.011	0.490±0.021	0.663±0.017	0.758±0.060
pH/E-Smac	0.414±0.016	0.414±0.005	0.452±0.027	0.628±0.056	0.775±0.037
2 Gy	0.432±0.010[a]	0.410±0.003[a]	0.421±0.003[a]	0.431±0.004[a]	0.469±0.009[b]
pE-Smac+2 Gy	0.435±0.011[a]	0.385±0.006[b]	0.361±0.012[b]	0.367±0.008[a]	0.381±0.005[b]
pH/E-Smac+2 Gy	0.442±0.013[a]	0.381±0.009[b]	0.361±0.009[b]	0.368±0.014[b]	0.378±0.021[b]
Hypoxia					
Control	0.383±0.050	0.419±0.018	0.387±0.009[c]	0.460±0.041[c]	0.495±0.034[d]
pE-Smac	0.402±0.011[c]	0.412±0.010	0.393±0.005[c]	0.485±0.023[d]	0.459±0.028[c]
pH/E-Smac	0.347±0.008[d]	0.339±0.006[b,d]	0.385±0.007[a,c]	0.424±0.008[c]	0.422±0.009[a,d]
2 Gy	0.408±0.006[c]	0.399±0.007	0.353±0.015[b,c]	0.397±0.010[c]	0.410±0.009[a,d]
pE-Smac+2 Gy	0.413±0.009[d]	0.394±0.006[b]	0.323±0.013[b,c]	0.343±0.006[a,c]	0.350±0.008[a,c]
pH/E-Smac+2 Gy	0.339±0.004[c]	0.329±0.020[c]	0.290±0.016[d]	0.254±0.032[b,c]	0.153±0.026[b,d]

Data are presented as the mean ± standard deviation, n=6. [a]P<0.05 and [b]P<0.001 versus control group; [c]P<0.05 and [d]P<0.001 versus normoxia. pE-Smac, pcDNA3.1-Egr1-Smac; pH/E-Smac, pcDNA3.1-HRE/Egr1-Smac.

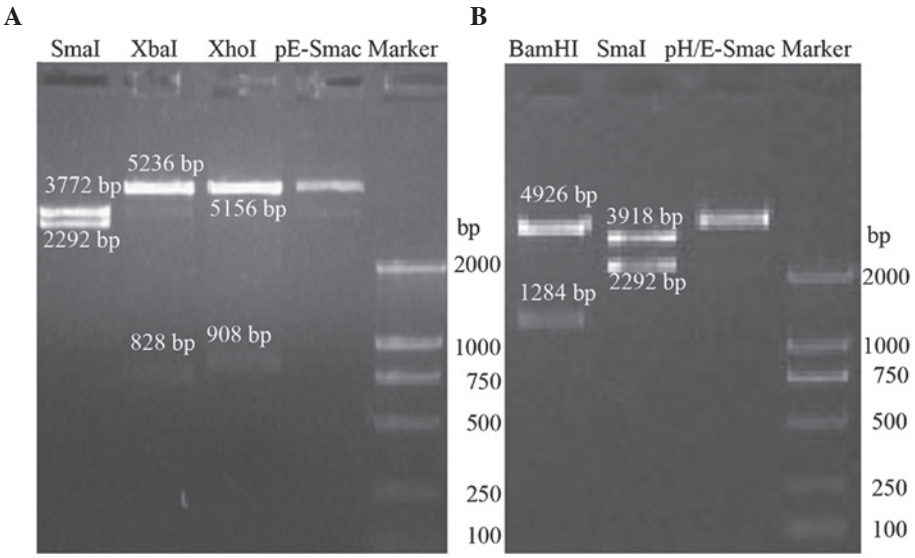

Figure 1. Identification of (A) pE-Smac and (B) pH/E-Smac using restriction digestion. pE-Smac, pcDNA3.1-Egr1-Smac; pH/E-Smac, pcDNA3.1-HRE/Egr1-Smac.

apoptotic A549 cells in the pH/E-Smac, 2 Gy, pE-Smac+2 Gy and pH/E-Smac+2 Gy groups was increased significantly compared with that in the control group (P<0.05 or P<0.01), particularly in the pH/E-Smac+2 Gy group. Furthermore, the percentage of apoptotic cells in each hypoxic group was found to be increased significantly compared with each normoxic group (P<0.05 or P<0.01).

Smac, Cyt c and caspase-9 and -3 mRNA expression in A549 cells. qPCR analysis was performed to detect Smac, Cyt *c*, and caspase-9 and -3 mRNA expression. The relative mRNA levels were calculated as the ratio of experimental/control mRNA expression, as shown in Table V. With the exception of Cyt *c* expression in the 2 Gy group, under normoxia Smac, Cyt *c*, and caspase-9 and -3 mRNA levels in the 2 Gy, pE-Smac+2 Gy and pH/E-Smac+2 Gy groups were significantly increased compared with those in the control group (P<0.05 or P<0.01), with no significant difference observed in the mRNA expression between the pE-Smac+2 Gy and pH/E-Smac+2 Gy groups. With the exception of Cyt *c* and caspase-3 in the pH/E-Smac group, the mRNA levels of Smac, Cyt *c*, and caspase-9 and -3 in the pH/E-Smac, 2 Gy, pE-Smac+2 Gy and pH/E-Smac+2 Gy groups under hypoxia were found to be significantly increased compared with those in the control

Table III. Percentage of A549 cells in each cell cycle phase under normoxia and hypoxia.

Groups	Cell percentage (%)		
	G_0/G_1	S	G_2/M
Normoxia			
Control	54.17±1.59	42.69±4.55	3.13±1.18
pE-Smac	53.78±0.69	42.77±0.53	3.45±0.49
pH/E-Smac	54.04±2.34	39.44±1.99	2.86±0.68
2 Gy	61.55±1.42	31.92±1.23[a]	6.53±0.21[a]
pE-Smac+2 Gy	61.85±2.33	31.95±1.51[a]	6.23±0.89[a]
pH/E-Smac+2 Gy	60.89±2.09	32.91±1.86[a]	6.20±0.26[a]
Hypoxia			
Control	65.47±0.62[b]	31.40±0.60[b]	3.13±0.21
pE-Smac	65.55±1.48[b]	30.91±1.66[c]	3.54±0.92
pH/E-Smac	70.72±0.86[a,b]	11.79±1.33[c,d]	17.49±1.21[a,c]
2 Gy	71.51±0.89[a,b]	10.82±1.26[c,d]	17.67±0.53[c,d]
pE-Smac+2 Gy	71.74±1.20[a,b]	6.85±1.29[c,d]	21.41±0.62[c,d]
pH/E-Smac+2 Gy	70.74±0.27[a,b]	7.12±1.54[c,d]	22.15±1.29[a,b]

Data are presented as the mean ± standard deviation, n=3. [a]P<0.05 and [d]P<0.01 versus control group; [b]P<0.05 and [c]P<0.001 versus normoxia. pE-Smac, pcDNA3.1-Egr1-Smac; pH/E-Smac, pcDNA3.1-HRE/Egr1-Smac.

group (P<0.05 or P<0.001), particularly in the pH/E-Smac+2 Gy group. Overall, with the exception of Cyt *c* in the control, pE-Smac and pE-Smac+2 Gy groups, hypoxia was found to significantly increase Smac, Cyt *c*, caspase-9 and -3 mRNA levels compared with normoxia (P<0.05 or P<0.001).

Smac, Cyt c, casapase-9 and -3 protein expression in A549 cells. As shown in Fig. 4, western blot analysis was used to detect Smac, Cyt *c*, and caspase-9 and -3 protein expression. Under normoxia, the expression of the housekeeping protein β-actin (molecular weight, 42 kDa) was consistent in each group. The protein expression of Smac (25 kDa) and Cyt c (12 kDa) was observed to increase in the 2 Gy, pE-Smac+2 Gy and pH/E-Smac+2 Gy groups, while that of the caspase-9 and -3 precursors (42 kDa and 27 kDa, respectively) were consistent among all six groups. Expression of the activated caspase-9 protein (35 kDa) was observed to increase in the 2 Gy, pE-Smac+2 Gy and pH/E-Smac+2 Gy groups, and that of the activated caspase-3 protein (21 kDa) was found to increase in the pE-Smac+2 Gy and pH/E-Smac+2 Gy groups.

Under hypoxia, expression of the housekeeping protein β-actin was consistent in each group. However, the protein expression of Smac and Cyt *c* was observed to increase in the pH/E-Smac, 2 Gy, pE-Smac+2 Gy and pH/E-Smac+2 Gy groups, with the highest expression observed in the pH/E-Smac+2 Gy group. The expression of the precursor and activated caspase-9 proteins was found to increase under hypoxia, particularly in the pE-Smac+2 Gy and pH/E-Smac+2 Gy groups. No difference was observed in the expression of the caspase-3 precursor protein under hypoxia; however, the expression of the activated protein was found to increase in the 2 Gy, pE-Smac+2 Gy and pH/E-Smac+2 Gy groups, particularly in the pH/E-Smac+2 Gy group. When compared with normoxia, hypoxic treatment was found to increase the expression of the Smac, Cyt c and caspase-9 and -3 precursor and activated proteins.

Table IV. Percentage of apoptotic A549 cells under normoxia and hypoxia.

Groups	Apoptotic percentage (%)	
	Normoxia	Hypoxia
Control	0.60±0.10	2.90±0.20[a]
pE-Smac	0.67±0.15	2.53±0.47[b]
pH/E-Smac	0.73±0.06	5.07±0.15[a,c]
2 Gy	2.87±0.15[c]	7.27±0.25[a,c]
pE-Smac+2 Gy	4.50±0.30[d]	8.27±0.15[a,c]
pH/E-Smac+2 Gy	4.80±0.30[d]	17.8±0.83[b,c]

Data are presented as the mean ± standard deviation, n=3. [d]P<0.05 and [c]P<0.01 versus control group; [b]P<0.05 and [a]P<0.001 versus normoxia. pE-Smac, pcDNA3.1-Egr1-Smac; pH/E-Smac, pcDNA3.1-HRE/Egr1-Smac.

Discussion

Cells in solid tumors cause a hypoxic microenvironment, which results in radiation resistance (24). Based on the hypoxia-inducible nature of the HRE promoter (10-12), the present study aimed to use the HRE promoter in lung cancer gene therapy to promote the downstream expression of the therapeutic Smac and increase the tumor cell death. Furthermore, the radiation-inducible Egr-1 promoter, which is activated

Table V. Relative mRNA expression of Cyt *c*, caspase-9 and -3 in A549 cells under normoxia and hypoxia.

Groups	Relative mRNA expression			
	Smac	Cyt *c*	Caspase-9	Caspase-3
Normoxia				
Control	1	1	1	1
pE-Smac	1.09±0.05	1.06±0.05	1.03±0.03	1.09±0.06
pH/E-Smac	1.09±0.06	1.08±0.06	1.06±0.06	1.05±0.04
2 Gy	4.42±0.51[a]	1.73±0.32	5.89±0.19[a]	5.72±0.29[a]
pE-Smac+2 Gy	20.51±1.67[a]	7.22±0.27[a]	5.76±0.10[b]	32.03±2.49[a]
pH/E-Smac+2 Gy	20.23±0.75[a]	7.41±0.42[a]	6.91±0.28[a]	32.08±3.60[a]
Hypoxia				
Control	2.82±0.13[c]	1.23±0.10	2.34±0.06[c]	3.06±0.16[c]
pE-Smac	2.75±0.09[d]	1.27±0.15	2.56±0.07[d]	3.18±0.17[c]
pH/E-Smac	6.34±0.26[b,d]	1.20±0.04[c]	6.04±0.13[b,d]	3.12±0.04[d]
2 Gy	9.75±0.72[a,c]	2.83±0.34[a,c]	12.73±1.21[a,c]	11.14±0.23[b,d]
pE-Smac+2 Gy	10.66±0.68[a,c]	7.64±0.43[a]	13.12±1.53[a,c]	41.82±1.57[b,c]
pH/E-Smac+2 Gy	39.71±2.69[a,c]	16.8±0.80[a,d]	19.56±0.99[a,c]	59.09±1.59[b,d]

Data are presented as the mean ± standard deviation, n=3. [a]P<0.05 and [b]P<0.01 versus control group; [c]P<0.05 and [d]P<0.001 versus normoxia. Smac, second mitochondria-derived activator of caspases; Cyt *c*, cytochrome *c*; pE-Smac, pcDNA3.1-Egr1-Smac; pH/E-Smac, pcDNA3.1-HRE/Egr1-Smac.

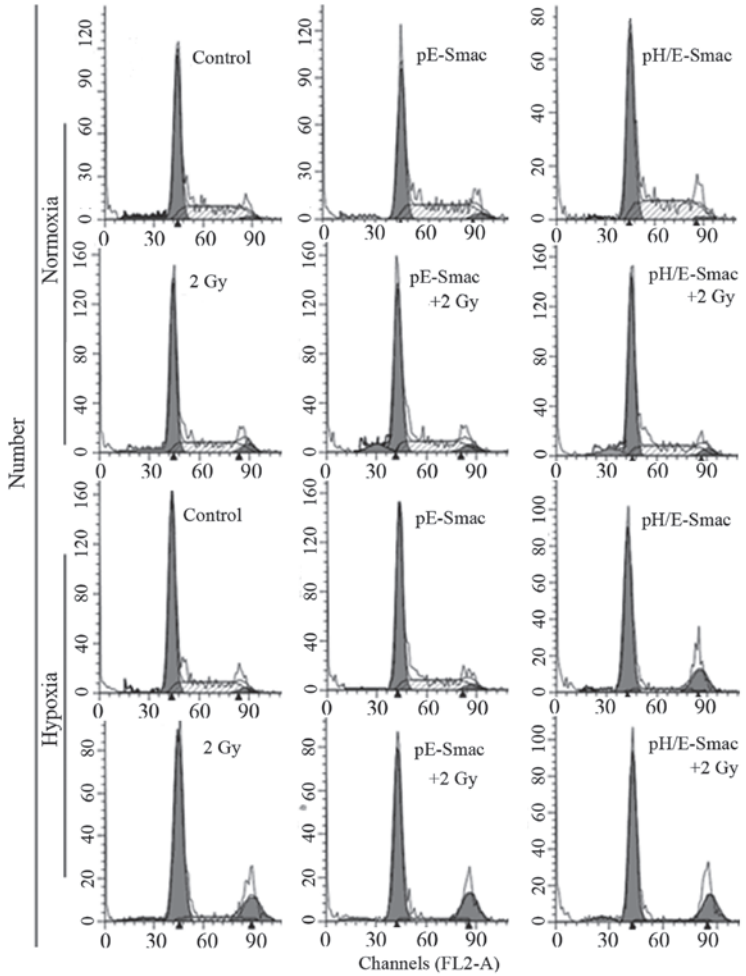

Figure 2. Percentage of A549 cells in each cycle phase, as determined by flow cytometry. pE-Smac, pcDNA3.1-Egr1-Smac; pH/E-Smac, pcDNA3.1-HRE/Egr1-Smac.

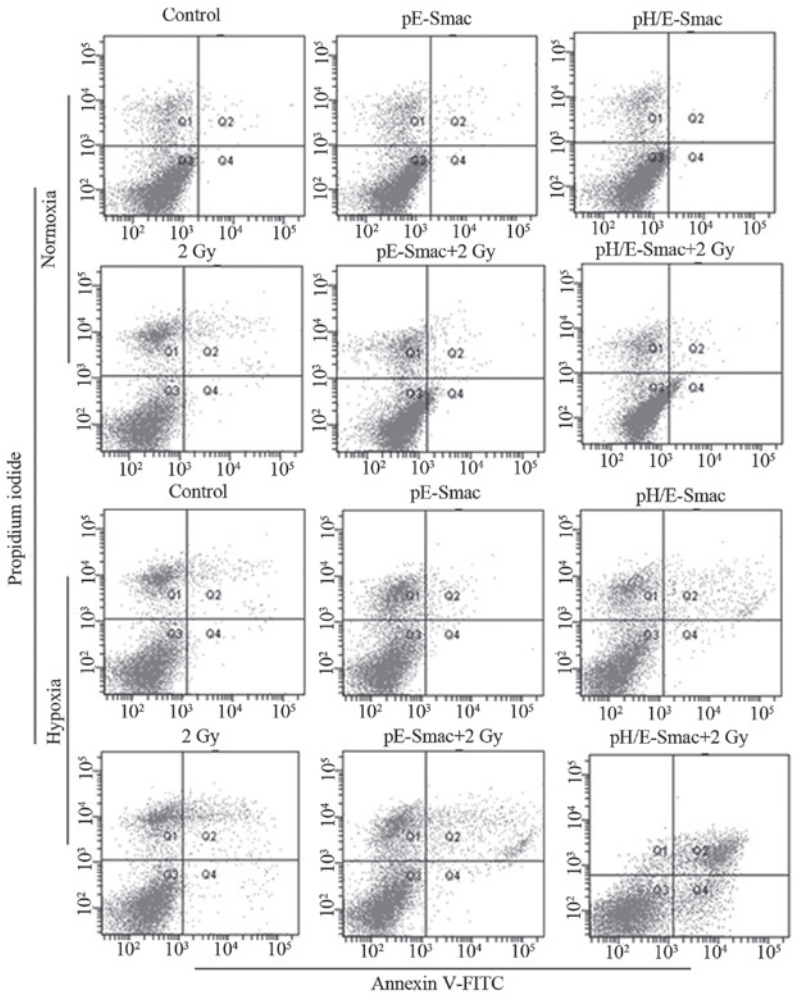

Figure 3. Percentage of apoptotic A549 cells, as determined by flow cytometry. FITC, fluorescein isothiocyanate; pE-Smac, pcDNA3.1-Egr1-Smac; pH/E-Smac, pcDNA3.1-HRE/Egr1-Smac.

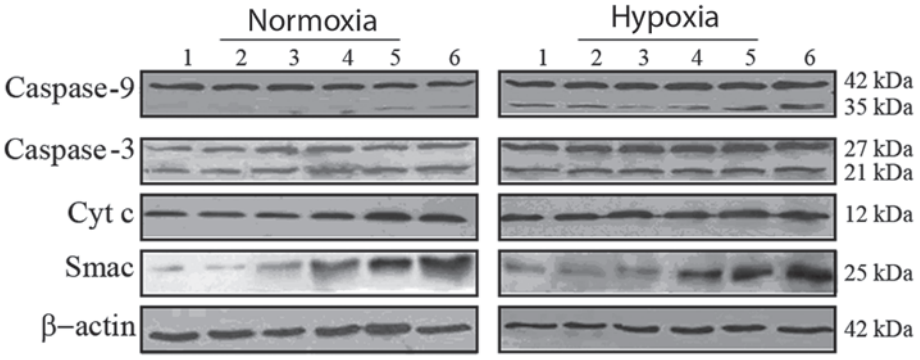

Figure 4. Protein expression of Smac, Cyt c, caspase-9 and -3 in A549 cells, as determined using western blot analysis. Lane 1, control; lane 2, pE-Smac; lane 3, pH/E-Smac; lane 4, 2 Gy; lane 5, pE-Smac+2 Gy; lane 6, pH/E-Smac+2 Gy. pE-Smac, pcDNA3.1-Egr1-Smac; pH/E-Smac, pcDNA3.1-HRE/Egr1-Smac; Cyt *c*, cytochrome *c*; Smac, second mitochondria-derived activator of caspases.

and regulated by ionizing radiation in a temporal and spatial dose-dependent manner (6,8,25), was used to enhance the expression of the *Smac* gene. The present study combined the HRE and Egr-1 promoters, forming a dual-sensitive chimeric HRE/Egr-1 promoter to induce the expression of the pro-apoptotic *Smac* gene in A549 human lung adenocarcinoma cells subjected to hypoxia and X-ray irradiation. The results showed that 2 Gy X-ray radiation alone was capable of inducing the Egr-1 promoter to enhance the expression of Smac. The HRE promoter is inactive under normoxia. Under hypoxia, 2 Gy X-ray radiation was observed to markedly enhance Smac expression in A549 cells. Therefore, the radiation-induced Egr-1 promoter and the hypoxia-induced HRE promoter may synergize to improve the efficacy of radiotherapy.

The overexpression of Smac has been reported to promote tumor cell apoptosis through the mitochondrial pathway

and enhance the sensitivity of tumor cells to radio- and chemotherapy (26-32). The present study found that the overexpression of Smac in A549 cells under normoxia enhanced X-ray radiation-induced G_2/M arrest and apoptosis, and increased the protein and mRNA expression of Cyt *c*, and caspase-9 and -3. These findings indicate that Smac overexpression is capable of inducing A549 cell apoptosis by activating caspase-9 and -3 and increasing Cyt *c*, suggesting that the Cyt *c*/caspase-9/caspase-3 pathway is involved in the regulation of apoptosis.

In accordance with a study by Zeng *et al* (33), $CoCl_2$-simulated hypoxia in the present study was observed to induce G_0/G_1 arrest. G_0/G_1 arrest renders cells sensitive to radiation, which enhances the efficacy of radiotherapy (34). Hypoxia-induced G_0/G_1 arrest also enhances radiation-induced apoptosis without radiation resistance. In the present study, the HRE/Egr-1 promoter was found to mediate the overexpression of Smac, leading to enhanced G_2/M arrest in A549 cells. G_2/M arrest also renders cells sensitive to radiation. In this study, compared with cells under normoxia, hypoxic cells were observed to exhibit enhanced apoptosis, G_2/M arrest and expression of Cyt *c*, as well as caspase-9 and -3. These findings indicate that the constructed hypoxia/radiation dual-sensitive chimeric HRE/Egr-1 promoter has a role in gene therapy under hypoxia.

In conclusion, the hypoxia/radiation dual-sensitive HRE/Egr-1 promoter-mediated Smac overexpression vector was successfully constructed in the present study. Following transfection of A549 human lung adenocarcinoma cells, radiation and hypoxia were observed to induce the overexpression of Smac, leading to proliferation inhibition, enhanced apoptosis and G_2/M-phase arrest. Furthermore, cell apoptosis was found to involve the mitochondrial Cyt *c*/caspase-9/caspase-3 pathway. HRE/Egr-1 promoter-mediated gene-radiotherapy achieved enhanced efficacy and synergized radiotherapy and gene therapy, providing an experimental basis for gene-radiotherapy in lung cancer.

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