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Original Article

Correlation between regulation of Cox-2 gene expression and radiosensitivity mechanism of esophageal cancer

Xiao-Xu Lu, Hui Wu, Xue-Ming Sun, Yan-Ling Wang, Rong Huang, Jing Xu

Affiliated Tumor Hospital of Zhengzhou University, Zhengzhou 450008, China

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Abstract: The aim of the present study was to investigate the mechanism of the effect of Cox-2 gene expression on the radiosensitivity of esophageal cancer. Cox-2-specific small interfering (si)RNA was constructed and transfected into EC9706 cells to downregulate intracellular Cox-2 expression. The expression of matrix metalloproteinase-2 (MMP2) and B-cell lymphoma 2 (Bcl-2) mRNA, Akt protein and phosphorylated (p)Akt was determined following exposure to radiation of various doses. Colony formation, cell proliferation, apoptosis and cell invasion were examined in vitro. One-way analysis of variance was used to analyze the results. Following radiation doses of 0, 2 and 4 Gy, a significant increase in the mRNA expression of Bcl-2 was observed in upregulation group ($P < 0.05$). In the Cox-2 downregulation group, MMP2 mRNA expression was significantly reduced ($P < 0.05$) following radiation doses of 2 and 4 Gy. Following doses of 0, 2 and 4 Gy, a significant decrease in Bcl-2 mRNA ($P < 0.01$) and a significant increase in Bcl-2-associated X protein (Bax) mRNA ($P < 0.05$) was detected. Following treatment with radiation doses of 0, 2 and 4 Gy, the levels of total Akt and pAkt protein was highest in the upregulation group and lowest in the downregulation group. The increase in apoptosis tended to be steepest with increasing radiation in the downregulation group ($P < 0.01$). The proportion of cells in the G_0/G_1 phase increased gradually and the proportion of cells in the S and G_2/M phases decreased gradually, concomitant with evident suppression of cell proliferation. A reduction in cell invasion was observed by an invasion assay in vitro. Downregulation of intracellular Cox-2 mRNA expression, concomitant with subsequent downregulation of MMP2 and Bcl-2 mRNA expressions and upregulation of Bax expression, resulted in reduced invasion and metastatic abilities of the tumor cells, and induction of G_0/G_1 phase arrest and apoptosis. These findings contribute to our understanding of the mechanism of the effect of Cox-2 gene expression on radiosensitivity. In addition, downregulation of Akt and pAkt protein expression may also interfere with the capability of the phosphatidylinositol-3-kinase/Akt signal transduction pathway to resist radiotherapy.

Keywords: Cox-2 gene, esophageal cancer, radiosensitivity, mechanism

Introduction

Esophageal cancer is a malignant tumor that threatens human health and life, with the sixth highest mortality rates of all malignant neoplasms. China is a high incidence area for esophageal cancer, accounting for > 50% of the total world cases [12]. Overall, > 60% of esophageal patients are in the intermediate and advanced stages of disease at the time of treatment. These patients are administered a combination treatment focused on radiation therapy, which has become one of the main therapies for intermediate and advanced esophageal cancer [13]. The main pathological type of esophageal cancer in China is squamous cell carcinoma, which is classed as a moderate radiation-sensitive tumor. The five-year survival rate of esophageal squamous cell carcinoma patients treated with pure radiation is only 10-20%, the majority succumbing to tumor local failure and recurrence [14]. Accurate prediction of the radiation sensitivity of esophageal cancer, the determination of methods and mechanisms to increase the radiation sensitivity, and improvements to the tumor local control rate are becoming key points for domestic and overseas studies.

Previous studies have revealed that Cox-2 can be used as an indicator to evaluate the resistance of a tumor to radiation therapy, as high Cox-2 protein expression in tumor tissue results in the radiation resistance being stronger. Nu-

Table 1. Primers and the probe sequence

Gene	Sequence type	Sequence
Cox-2	Forward Primer	5'-AATTCCAGTACCAAAATCGTATTGC-3'
	Reverse Primer	5'-ACTGTTGATAGTTGTATTTCTGGTCATGA-3'
	Probe	5'-FAM-TTTAACACCCTCTATCACTGGCATCCCCTT-TAMMR-3'
MMP2	Forward Primer	5'-TGTGACGCCACGTGACAAG-3'
	Reverse Primer	5'-GCCTCGTATACCGCATCAATC-3'
	Probe	5'-FAM-CCACATTCTGGCCTGAGCTCCCG-TAMMR-3'
Bcl-2	Forward Primer	5'-GTTCCGCGTGATTGAAGACA-3'
	Reverse Primer	5'-CCAGAGAAAGAAGAGGAGTTATAATCCA-3'
	Probe	5'-FAM-CCCCTCGTCCAAGAATGCAAAGCA-TAMMR-3'
Bax	Forward Primer	5'- GTTGTCGCCCTTTTCTACTTTG-3'
	Reverse Primer	5'-AGCCCATGATGGTTCTGATCA-3'
	Probe	5'-FAM-CAGCAAACTGGTGCTCAAGGCCC-TAMMR-3'
β-actin	Forward Primer	5'-TTGCCGACAGGATGCAGAAG-3'
	Reverse Primer	5'-GCGCTCAGGAGGAGCAATGA-3'
	Probe	5'-FAM-AGATCACTGCCCTGGCACCCAGCA-TAMMR-3'

Cox-2, cyclooxygenase-2; MMP2, matrix metalloproteinase-2; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein.

merous studies [15-17] into the association between the level of Cox-2 gene expression and the curative effect of radiation therapy have investigated the treatment of head and neck cancer [18], but few have investigated esophageal researches.

The vector pRNA-U6.1-siCox214, carrying small interfering (si)RNA against the Cox-2 gene, was constructed in our laboratory (the department of Pathophysiology, Basic Medical Sciences of Zhengzhou University, China) and was successfully transfected into esophageal cancer EC9706 cells to effectively silence Cox-2 gene expression. The pcDNA™4-Cox-2 strategy was successfully applied to upregulate Cox-2 gene expression. Previously, a prominent improvement of the radiosensitivity of esophageal cancer EC9706 cells was achieved by siRNA-mediated silencing of Cox-2 gene expression, as validated by liposome technology for gene transfer [1]. In the present study, the mechanism of Cox-2 gene expression in regulating radiosensitivity was investigated from several aspects.

Materials and methods

Cell lines and reagents

The human esophageal cancer EC9706 cell lines were supplied by the Department of Pathophysiology, Basic Medical Sciences of Zhengzhou University (Zhengzhou, Henan, China). The siRNA expression vector pRNA-U6.1 and control plasmids containing an irrelevant siRNA sequence (pRNAT-U6.1-Con) were purchased from GenScript Co. (Piscataway, NJ, USA) Mouse anti human Cox-2 monoclonal antibody (1:800), Mouse anti human Akt monoclonal antibody (1:500), Mouse anti-human monoclonal antibody phospho pAkt (1:350) Horseradish peroxidase-labeled goat anti-mouse IgG monoclonal antibody (1:2000) were purchased from Santa Cru, Co. (Dallas, TX, USA).

Experimental grouping

The EC9706 cells stably transfected with pRNA-U6.1-siCox214 were designated the downregulation group. Efficient silencing of Cox-2 gene expression was confirmed by fluorescence quantitative reverse transcription-polymerase chain reaction (RT-PCR) and western blot analysis. The EC9706 cells stably transfected with pcDNA4-Cox-2 were designated the upregulation group. Highly efficient expression of Cox-2 gene was confirmed by fluorescence quantitative RT-PCR and western blot analysis. The EC9706 cells stably transfected with pRNAT-U6.1-Con were designated the siRNA control group, and the untransfected EC9706 cells were designated blank control groups.

Cell irradiation

All experimental cell groups were cultivated until logarithmic phase growth was reached and were then irradiated in a Siemens Oncor impression linear accelerator (Munich, Bavaria, Germany).

RT-PCR

Total RNA was extracted from the group of cells using the small amount of total RNA Extraction Kit (German Qiagen) according to the manufacturer's instructions; using the step reverse transcription Kit (German Qiagen) for reverse transcription according to the manufacturer's instructions. Assays to quantify mature miRNAS were performed using Taqman microRNA

Table 2. Expressions of Cox-2, MMP2, Bcl-2, and Bax mRNA in each cell group following irradiation at various doses

mRNA	Dose, Gy	Downregulation group	siRNA control group	Upregulation group	Vector control group	Blank control group
Cox-2	0 GY	0.239±0.022[b]	1.216±0.045	2.639±0.119[b]	1.235±0.0710	1.255±0.044
	2 GY	0.239±0.024[b]	1.217±0.046	2.604±0.093[b]	1.237±0.058	1.210±0.028
	4 GY	0.243±0.030[b]	1.242±0.042	2.653±0.143[b]	1.256±0.044	1.239±0.024
MMP2	0 GY	0.484±0.041	0.527±0.041	0.568±0.048[a]	0.499±0.035	0.500±0.042
	2 GY	0.399±0.036[b]	0.509±0.051	0.567±0.043	0.494±0.042	0.504±0.049
	4 GY	0.323±0.024[b]	0.517±0.048	0.564±0.052	0.492±0.041	0.498±0.042
Bcl-2	0 GY	0.626±0.053[b]	0.735±0.043	0.832±0.071[b]	0.71±0.053	0.723±0.046
	2 GY	0.552±0.064[b]	0.719±0.050	0.827±0.067[b]	0.682±0.056	0.695±0.057
	4 GY	0.515±0.045[b]	0.703±0.056	0.825±0.057[b]	0.675±0.049	0.681±0.048
Bax	0 GY	0.339±0.029[b]	0.236±0.019	0.212±0.014[a]	0.230±0.017	0.231±0.015
	2 GY	0.386±0.035[b]	0.246±0.023	0.210±0.024[a]	0.241±0.024	0.245±0.028
	4 GY	0.413±0.043[b]	0.254±0.031	0.219±0.018[a]	0.254±0.019	0.248±0.025

[a]*P* < 0.05 and [b]*P* < 0.01 VS the blank control group. Cox-2, cyclooxygenase-2; MMP2, matrix metalloproteinase-2; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; siRNA, small interfering RNA.

probes (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The respective primers and probes (**Table 1**) were used to detect the mRNA expression profiles of Cox-2, matrix meralloproteinase-2 (MMP2), B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein (Bax) [1]. The procedures were performed according to the instructions of the Premix Ex Taq™ (Perfect Real Time) TaqMan kit and ABI7500 fast amplification system supplied by Takara (Otsu, Japan). The relative expression level is determined by the ratio of Cox-2, MMP2, Bcl-2, Bax expression level and endogenous reference gene β-action expression level.

Western blot

Western blot analysis was performed as previously described [2]. Protein was extracted from the group of cells using Total Protein extraction kit (Applygen Technologies Inc., Beijing, China). The supernatant was collected, and the protein concentration was calculated with a BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). Proteins were separated by SDS-PAGE (Bio-Rad). After electrophoresis, the proteins were electrotransferred to PVDF membranes (Bio-Rad) and then blocked with 5% skim milk for 1 h. After three washed in TBST, the membranes were incubated with Mouse anti human Cox-2 monoclonal antibody from Santa Cru, Co. (Dallas, TX, USA) for 1 h at room temperature. After three washed, the membranes were incubated with Horseradish peroxidase-labeled goat anti-mouse IgG antibody from Santa Cru, Co. (Dallas, TX, USA) for 1 h at room temperature. The protein levels were normalized by probing the same blots with β tubulin action antibody from Santa Cru, Co. (Dallas, TX, USA).

Determination of apoptosis and cell cycle by flow cytometry

The flow cytometry instrument was obtained from the United State Beckman-coulter Co. Ltd. The cells in the downregulation, siRNA control, upregulation, vector control and blank control groups were irradiated at 0, 2 and 4 Gy. The number of cells was maintained at a density of 1×10^6/ml for each cell sample.

Colony formation assay

The survival colony count [19] was determined by a cross-line approach under an inverted microscope (Leica Dm ILM, www.leica com) using a soft agar colony formation assay. A surviving colony refers to the formation of a single cell colony following cultivation. Colonies containing ≥ 50 cells were counted as one survival colony.

Cell proliferation assay using cell counting kit-8 (CCK-8)

The number of cells was counted for each group following irradiation at the various doses, and

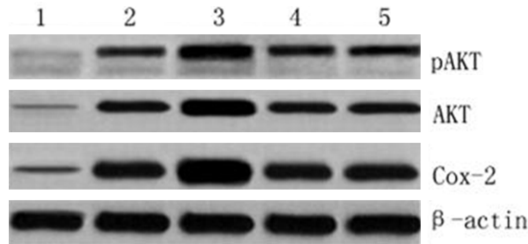

Figure 1. Western blot analysis for indicated proteins.

the cell density for each group was adjusted to 1×10^5 cells/ml. A 96-well culture plate was inoculated with 1×10^4 cells (100 μl/well) for each group, with five duplicates for each group. The absorbance value (A) of each well was measured using a CCK-8 cell proliferation assay kit (Japanese Dojindo Co., Ltd) 1-5 days after inoculation. The mean values of data were calculated and the cell growth curve of each group was determined.

In vitro invasion assay with transwell chamber

The cells of each group were cultivated to a density of 5×10^5 cells/ml prior to 200 μl of the cells being transferred to a Transwell chamber and cultivated under normal 5% CO_2 conditions for 48 h. Following crystal violet staining [19], the cells were observed and images were captured under an upright microscope (Olympus Bx61, www.olypus.com.cn) in 3-5 fields. Cell numbers were counted and the mean value was calculated.

Statistical analysis

All experimental data were analyzed using SPSS13.0 statistical software (SPSS, Inc., Chicago, IL, USA). All data, represented as the mean ± standard deviation, were analyzed by one-way analysis of variance and $P < 0.05$ was considered to indicate a statistically significant difference.

Results

RT-PCR assay

As shown in **Table 2**, following treatment with 0, 2 and 4 Gy of radiation, the downregulation group maintained decreased Cox-2 mRNA expression, while increased Cox-2 mRNA expression was observed in the upregulation group. There was no statistically intra-group difference in Cox-2 mRNA expression at 0, 2 and 4 Gy. RT-PCR result indicated that the 2, 4GY radiation to esophageal cancer EC9706 cells didn't exert visible effect on cell Cox-2 gene expression. Only in the EC9706 cell MMP2 at low Cox-2 expression level, the Bax mRNA gene expression increased along with the augment of exposure, and the Bcl-2 gene decreased along with the augment of exposure.

Following radiation doses of 0, 2 and 4 Gy, a significant increase in Bcl-2 mRNA expression was observed in the upregulation group compared with the blank control group ($P < 0.05$). Following 2 and 4 Gy treatments, a statistically significant reduction in MMP2 mRNA expression was observed in the downregulation group compared with the blank control group. Furthermore, a significant reduction in Bcl-2 mRNA expression in the upregulation group was observed compared with the blank control group following radiation of 0, 2 and 4 Gy ($P < 0.01$). There were no significant intergroup differences between the MMP2 and Bcl-2 mRNA expression profiles of blank control, siRNA control and vector groups following treatment with 0, 2 and 4 Gy of radiation ($P > 0.05$). However, the present study observed an intragroup difference between MMP2 mRNA and Bcl-2 mRNA in the upregulation group ($P < 0.01$). Subsequent to radiation doses of 0, 2 and 4 Gy, the expression in the downregulation group increased significantly compared with the blank control group ($P > 0.05$). There were no statistical differences in the expression profile of Bax mRNA among the blank control, siRNA control, vector control and upregulation groups following radiation doses of 0, 2 and 4 Gy ($P > 0.05$). Only the downregulation group demonstrated a significant tendency towards decreased Bax mRNA expression following 0, 2, and 4 Gy of radiation ($P < 0.01$) (**Table 1**).

Western blot analysis

The western blot analysis (**Figure 1**) of Cox-2 protein expression in each group revealed high Cox-2 expression levels in the upregulation group, moderate levels in the blank, siRNA and vector control groups, and low expression levels in the downregulation group. The level of total Akt and pAkt protein was found to be elevated in the upregulation group, moderate in the blank, siRNA and vector control groups, and low in the downregulation group. The result of

Table 3. Rate of apoptosis in each group following irradiation at various doses (%)

Group	N	Rate of apoptosis, %		
		0 Gy	2 Gy	4 Gy
Downregulation	5	5.98±0.573[b]	13.464±0.609[b]	19.158±0.720[b]
siRNA control	5	2.95±0.286	7.246±0.815	10.676±1.014
Upregulation	5	2.926±0.205	6.282±0.874	7.886±0.719[a]
Vector control	5	2.942±0.313	7.496±0.641	10.818±1.210
Blank control	5	3.03±0.129	7.224±0.672	10.748±1.242

[a]$P < 0.05$ and [b]$P < 0.01$ VS the blank control group. siRNA, small interfering RNA.

Table 4. Proportions of cells in different cellular phases in each group following irradiation at various doses

Group	N	Dose, Gy	Proportion of cells in each phase, %		
			G_0-G_1	S	G_2-M
Downregulation group	3	0	69.96±1.24[b]	22.06±0.52[b]	7.97±1.74[b]
		2	71.68±1.20[b]	20.44±0.97[b]	7.87±1.16[b]
		4	71.32±1.01[b]	21.59±2.02[b]	7.08±2.81[b]
siRNA control group	3	0	59.77±1.25	26.45±0.92	13.78±1.76
		2	62.66±1.06	24.46±0.58	12.87±1.05
		4	62.13±0.98	24.76±0.62	13.10±0.98
Upregulation group	3	0	54.22±1.97	29.68±1.45	16.09±0.69
		2	56.86±1.19[a]	28.47±1.33[a]	14.66±1.68
		4	58.54±0.51	27.96±1.125	13.5±1.26
Vector control group	3	0	62.42±1.34	26.16±1.85	11.41±1.45
		2	62.39±0.60	24.95±1.76	12.65±1.18
		4	61.01±1.16	25.63±1.37	13.34±0.92
Blank control group	3	0	57.82±1.53	27.38±2.03	14.79±0.81
		2	60.04±1.26	26.34±0.70	13.61±0.83
		4	59.72±1.02	26.66±1.10	13.61±0.86

[a]$P < 0.05$ and [b]$P < 0.01$ VS the blank control group. siRNA, small interfering RNA.

Western blot indicated the esophageal cancer Ec9706 cells showed high Akt protein expression level and high phosphorylation level and the intracellular Cox-2 expression level is positive correlated with the AKT and phosphorylated AKT.

Apoptosis assays

Following treatment with radiation doses of 0, 2 and 4 Gy, the rate of apoptosis in the downregulation group was increased compared with the blank control group ($P < 0.05$). In all experimental groups, the rate of apoptosis tended to increase with increasing doses (0, 2 and 4 Gy), with the steepest trend for apoptosis increase exhibited by the downregulation group ($P < 0.01$) (**Table 3**). The result of cell apoptosis indicated the apoptotic rate of esophageal cancer EC9706 cell at low Cox-2 expression level increased visibly in pace with the augment of exposure.

Cell cycle assays

The proportion of cells in the G_0/G_1 phase in the downregulation group was significantly increased compared with the control groups, concomitant with a significant decrease in the cells in the G_2/M compared with control groups. Each difference was statistically significant ($P < 0.01$). The proportions of cells in G_0/G_1, S and G_2/M phases was not distinctly altered in the blank control, siRNA control, vector control and upregulation groups following treatment with radiation doses of 0, 2 and 4 Gy. However, the proportion of cells in the G_0/G_1 phase tended to increase in the downregulation group, which was concomitant with a decrease of cells in the S and G_2/M phases (**Table 4**). The result of cell cycle analysis indicated the esophageal cancer Ec9706 cells at low Cox-2 expression level showed visible effect on cell cycle upon radiation exposure.

Colony formation assays

The ability of the downregulation group to form colonies was significantly decreased compared with the blank control, vector control and siRNA control groups following treatment with doses 0, 1, 2, 4, 6, 8 and 10 radiation ($P < 0.01$) (**Table 5**). The radiosensitization ratio was determined to be 1.18 quasi-threshold dose (D0) or 1.21 quasi-threshold dose (Dq) subsequent to fitting the data to a multi-target single hit model. The colony formation experiment indicated that the silence of Cox-2 expression could reduce the

Table 5. Colony formation in each group following irradiation at various doses

Group	N	Colony formation, %						
		0 Gy	1 Gy	2 Gy	4 Gy	6 Gy	8 Gy	10 Gy
Downregulation	5	24.2±2.61	9.6±1.14	6.5±1.65	3.7±1.03	2.5±1.54	0.9±0.74	0.3±0.44
siRNA control	5	37.8±4.89	18.1±3.64	13.5±2.85	11.7±1.82	9.5±1.45	3±1.27	1.6±1.29
Upregulation	5	42.7±4.13	22±4.75	17.2±3.68	13.8±2.56	10.6±2.48	5.2±1.44	2.6±1.08
Vector control	5	39.4±4.37	18.3±3.61	12.8±2.51	11.5±2.52	8.9±2.60	3±1.41	1.4±1.38
Blank control	5	38.4±3.34	18.1±3.30	13.5±1.96	11.7±1.82	9.4±2.13	2.9±1.29	1.6±1.43

siRNA, small interfering RNA.

Table 6. Absorbance values in each group following irradiation at various doses, as detected by cell counting kit 8. siRNA, small interfering RNA

Time, days	N	Dose, Gy	Absorbance				
			Down-regulation group	siRNA control group	Up-regulation group	Vector control group	Blank control group
1	3	0	0.227±0.018	0.232±0.021	0.243±0.023	0.238±0.028	0.223±0.023
		2y	0.223±0.017	0.232±0.025	0.241±0.023	0.234±0.028	0.22±0.023
		4	0.222±0.017	0.227±0.020	0.24±0.023	0.233±0.027	0.219±0.022
2	3	0	0.347±0.028	0.571±0.037	0.629±0.049	0.566±0.029	0.538±0.036
		2	0.321±0.024	0.563±0.037	0.624±0.048	0.555±0.034	0.531±0.051
		4	0.296±0.022	0.555±0.036	0.620±0.048	0.547±0.034	0.523±0.051
3	3	0	0.538±0.029	0.902±0.041	1.038±0.091	0.919±0.019	0.886±0.044
		2	0.49±0.026	0.890±0.04	1.029±0.088	0.903±0.015	0.875±0.051
		4	0.446±0.023	0.879±0.039	1.022±0.087	0.892±0.015	0.865±0.050
4	3	0	0.737±0.036	1.213±0.041	1.343±0.044	1.219±0.057	1.213±0.038
		2	0.604±0.031	1.223±0.081	1.354±0.065	1.212±0.094	1.183±0.056
		4	0.545±0.030	1.170±0.029	1.356±0.064	1.169±0.055	1.151±0.028
5	3	0	0.802±0.040	1.396±0.032	1.545±0.125	1.441±0.072	1.436±0.056
		2	0.627±0.020	1.383±0.030	1.538±0.066	1.395±0.093	1.337±0.128
		4	0.548±0.036	1.301±0.037	1.551±0.078	1.369±0.101	1.306±0.086

colony formation ability of esophageal cancer EC9706 cells.

Cell proliferation assays

CCK8 cell proliferation assays were performed for all experimental groups that were exposed to the various radiation doses (**Table 6**). A significant decrease in absorbance values was observed in the downregulation group on days 2, 3, 4 and 5 compared with the siRNA and blank control groups following 0, 2 and 4 Gy treatment ($P < 0.01$). The growth curve in **Table 7** was calculated according to the following formula: Rate of growth inhibition = [1-(DB-D blank)/(DA-D blank)] × 100 (DB: downregulation groups/upregulation groups; DA: control groups; D-Blank: Blank control group). The rates of growth inhibition for the downregulation group on days 3, 4 and 5 subsequent to treatment with 2 and 4 Gy radiation is shown in **Table 7**. However, the rate of growth inhibition was below zero on days 3, 4 and 5 for the upregulation group. Cell proliferation assays indicated the upregulated Cox-2 gene expression could exert resistance effect on radiation, while down-regulated Cox-2 gene expression could exert inhibition effect on cell growth.

In vitro invasion assays

The number of invading cells in the downregulation group was significantly decreased compared with the blank control group ($P < 0.01$). The number of invading cells in all experimental groups decreased with increasing irradiation doses, with the steepest decrease observed in the downregulation group (**Table 8**).

Table 7. Effects of various cyclooxygenase-2 expression levels on the inhibition of esophageal cancer EC9706 cell growth

Group	Dose, Gy	Rate of growth inhibition, %		
		Day 3	Day 4	Day 5
Downregulation	2	44.00±1.17	48.94±0.98	53.10±1.31
	4	48.44±1.35	52.65±1.81	58.04±1.67
Upregulation	2	-17.6±1.23	-14.46±0.87	-15.03±1.02
	4	-18.15±1.14	-17.81±1.13	-18.76±1.44

Table 8. Number of invading cells of each group following irradiation at various doses

Group	N	Number of invading cells		
		0 Gy	2 Gy	4 Gy
Down-regulation	5	72.4±12.17[b]	36.2±8.61[b]	30.2±7.12[b]
siRNA control	5	116±17.50	102.6±14.43	96±10.12
Up-regulation	5	119.4±16.56	110.8±14.13	106.6±11.28
Vector control	5	115.6±9.76	97.6±10.83	95.4±9.68
Blank control	5	118.2±12.19	100.2±8.64	99.6±10.23

[b]$P < 0.01$ VS the blank control group.

Discussion

Cox is a critical enzyme in the process of prostaglandin synthesis and is involved in the pathophysiology of numerous diseases. Previous studies have demonstrated high expression profiles of Cox-2 in a variety of tumor tissues, and the expression of Cox-2 has been found to be associated with the genesis and development of tumors, and the invasion and metastasis of malignant tumors [3, 4].

In the present study, the effect of the downregulation of Cox-2 gene expression on the radiosensitivity of EC9706 human esophageal cancer cells was investigated by in vitro clonogenic assays. The mean LD0 (lean lethal dose) and quasi-threshold dose (Dq) of the downregulation group was lower compared with pure radiation groups. The radiosensitization was higher compared with the mono radiation strategy. The radiosensitization ratios (SER) were 1.18 and 1.21 based on D0 and Dq data of each group, suggesting effects of downregulation of Cox-2 gene expression on the radiosensitization of EC9706 cells. The mechanism of enhanced radiosensitization was further investigated. The expression profiles of MMP2 and Bcl-2 mRNA were positively correlated with the expression profile of Cox-2 mRNA. The present study suggested that the downregulation of Cox-2 mRNA expression may reduce the proliferative capability and increase the proportion of cells in the G_0/G_1 phase, resulting in increased radiosensitivity.

MMP-2 is a critical enzyme that is responsible for extracellular matrix (ECM) decomposition and that plays a vital role in the process of angiogenesis, tumor growth and metastasis. John and Tuszynski [5] reported that high expression levels of MMP2 in tumor cells accelerated ECM and vascular basement membrane processes, resulting in the easy migration of tumor cells away from blood vessels, compromised adhesion and motility, and promotion of invasion and metastasis [5, 6]. The Bcl-2 protein is an important apoptosis regulatory factor involved in the mitochondrial apoptotic pathway, and its high expression is associated with the prevention of apoptosis induced by a variety of factors, including radiation and chemotherapy [7]. Bax promotes apoptosis, and while its expression may not be associated with apoptosis suppression, Bax alternatively suppresses apoptosis through reversing Bcl-2 protein activities [8-10]. Therefore, a decrease in Bcl-2 expression or an elevation in Bax expression may promote apoptosis and increase the radiosensitivity of tumor cells.

Esophageal cancer is characterized by local invasion and metastasis. Previous studies have revealed that in the process of invasion and metastasis in tumors, extracellular growth signals interact with phosphatidylinosital-3-kinase (PI3K) and activate Akt to initiate a series of processes associated with cell cycle regulation, apoptosis, telomerase activity, angiogenesis and cell migration [11]. However, the PI3K/Akt signaling pathway is also be activated by ionizing radiation, ultraviolet radiation or cytotoxic drugs, resulting in stress regulation of tumor cells, maintenance of cellular stability and resistance to radiation. As demonstrated in the present study, high Akt protein expression and phosphorylation levels were observed in esophageal cancer EC9706 cells. In addition, the expression level of intracellular Cox-2 was positively correlated to Akt and pAkt.

In conclusion, the downregulation of intracellular Cox-2 mRNA in EC9706 esophageal cells by Cox-2 specific siRNA was proposed to contribute to the improvement of sensitivity of EC9706 cells to radiation therapies. Downregulation of Cox-2 mRNA expression, followed by downregulation of MMP2 and Bcl-2 mRNA expression and upregulation of Bax expression, are associated with reduced capabilities of invasion and metastasis, enhanced accumulation in G_0-G_1 phases, and apoptosis induction. However, decreased expression of Akt and pAkt is likely to interfere with PI3K/Akt signaling pathway, resulting in reduced radiation resistance.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Hui Wu, Affiliated Tumor Hospital of Zhengzhou University, 127 Dongming Road, Zhengzhou, China. Tel: +86-371-6558-7762; E-mail: wuhui2_2016@126.com

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