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Influence of Dual Control Cox-2 Expressions on Radiation Sensitivity to Transplanted Tumor of Human Esophageal Cancer EC9706 in Nude Mice

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The present study was to explore the influence of up-regulated and down-regulated Cox-2 gene expression on the growth and the radiation sensitivity to transplanted tumor of esophageal cancer EC9706 in nude mice. Constructing siRNA vector for Cox-2 gene and Cox-2 gene eukaryotic expression vector, shifting to esophageal cancer EC9706 cells by the technology of lipofectamine, and obtaining stable transfected cell line by G418 screening. Taqman real-time RT-PCR and Western blot separately detect Cox-2mRNA and Cox-2 protein expression level; the experiment on transplanted tumor in nude mice detects the growth inhibiting role of up-regulated and down-regulated Cox-2 expression combined with X-radiation on esophageal cancer. The sequencing confirms the construction of siRNA vector pRNA-U6.1-siCox214 for Cox-2 gene, the results of RT-PCR and Western blot reflect that Cox-2 gene expression of transfected esophageal cancer EC9706 cells is an efficient silence. The average volume of transplanted tumor for nude mice in Cox-2 down-regulated group is significantly less than that in control group; and while the average volume in Cox-2 up-regulated group is significantly more than that in control group. Down-regulating Cox-2 expression can inhibit the growth of transplanted tumor for human esophageal cancer EC9706 in nude mice, enhance the sensitivity of tumor volume to radiotherapy, and up-regulating Cox-2 expression will make tumor radiation produce the resistance.

Key words: Esophageal Cancer; EC9706; Cox-2; Transplanted tumor; Nude mice.

Radiotherapy is one of main methods to treat esophageal cancer, the studies find that the curative effect of radiotherapy is closely related to the radiosensitivity of esophageal cancer cells. Clinical studies show that the radiotherapy combined with radiation sensitizer or a small dose of chemotherapeutic drugs, to some extent, which boosts the local control rate of esophageal cancer. This study explores the silence or up-regulated Cox-2 protein expression of esophageal cancer cells. Through models of transplanted tumors in nude mice, it surveys the influence of regulating Cox-2 gene expression on the radiosensitivity to tumors, and even still provide a theoretical basis on searching an evaluation index to curative effect of radiotherapy for esophageal cancer.

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MATERIALS AND METHODS

Cell Lines and Main Reagents

Human esophageal cancer cells EC9706 are preserved in the Department of Pathophysiology, Basic Medical College, Zhengzhou University; siRNA expression vector pRNA - U6.1, siRNA irrelative sequence controlled plasmid (pRNAT-U6.1-Con) are purchased from GenScript Company; eukaryotic expression vector pcDNA™4/HisMax C and LipofectAmineTM2000

are purchased from Invitrogen Company. Fetal bovine serum and RPMI1640 are purchased from Gibco Company.

EXPERIMENTAL

Animals

25 male BALB/c nude mice of 4-6 weeks old (purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd.), and the weights are from 15.2g to 18.5g.

Construction of Cox-2 siRNA Vector

Coded sequence of human Cox-2 cDNA (NM_000963) is analyzed by BLAST homology, located 214-232 as siRNA target sequence, synthetized a couple of DNA single strand for encoding short hairpin RNA sequence, separately added BamHI and HindIII incision enzyme residue at both ends, Si214-F5'-gatcc GTCAAAA CCGAGGTGTATGTTCAAG AGACATACA CCTCGGTTTTGACTTTTTTa-3' and Si214-R5'-agcttAAAAAAGTCAAAACCGAGGTGTAT GTCTCTTGAACATACACCTCGGTTTTGACg-3', connected with linear double strands siRNA vector pRNA-U6.1 after annealing into double strands, transformed into competence cells DH5±, selected positive transformed bacteria, extracted plasmid, and sent to detect the sequence in Shanghai Sangon Biotech Co., Ltd.; compared inserting sequence with designing one, and then obtained pRNA-U6.1-siCox214.

Construction of Cox-2 Gene Eukaryotic Expression Vector

Apply the full-length amplimer of Cox-2 gene cDNA encoding region- Forward primer 5'TTAGGATCCATGCTCGCCCGCGCCCTGCTGCTG 3' and Reverse primer 5'CCCGAATTCCTACA GTTCA GTCGAACGTTCTTT 3' to make PCR amplification for plasmid pCMV-SPORT6-Cox-2 containing full-length Cox-2 gene (purchased from OriGene Technologies Company); Cox-2 gene ORF fragments after PCR amplification recombine with pcDNA™4/HisMax C which passes through BamHI and EcoRI double enzyme, screen the transformed recon by PCR amplification, determine DNA sequence, and obtain Cox-2 expression vector.

Cell groups and transfection

Cox-2 silence group: transfected pRNA-siC U6.1ox214; irrelative siRNA control group: transfected pRNAT-U6.1-Con; expression vector control group: transfected pcDNA™4/HisMax C; Cox-2 up-regulated group: transfected pcDNA™4-Cox-2; blank control group: untransfected EC9706 cells. EC9706 cells in logarithmic phase are inoculated in 6-well culture plate in accordance with 5×10^5 cells/wells, transfected by LipofectAmineTM-2000, screened positive clones by G418, obtained stable transfected cell lines, and make an experiment after a large amount of amplification.

Detecting Cox-2mRNA Expression in Each Group by RT-PCR

Using a small amount of total RNA extraction kit from Qiagen Company to extract mRNA of cells in each group, adopting TaqMan probe fluorescence detection method, separately detecting cell Cox-2 mRNA expression level with internal reference of ²-actin. The primer and the probe sequence of Cox-2: Forward Primer 5'AATTCCAGTACCAAAATCGTATTGC 3'; Reverse Primer 5'ACTGTTGATAGTTG TATTTCTGGTCATGA 3' Probe 5' FAM-TTTAACACCCTCTATCACTGGCAT CCCCTT-TAMMR 3'.

Detecting Protein Expression by Western-blot

With reference to References[1]. Collect each group of cells, prepare cell total protein to take sodium lauryl sulfate-polyacrylamide gel electrophoresis. Transfer the protein onto the nitrocellulose membrane by the method of Electron Transfer, detect Cox-2 protein expression as primary antibody of Cox-2 protein antibody and secondary antibody of goat anti-mouse IgG antibody labeled at horse radish peroxidase.

Establishing the Model of transplanted tumor for EC9706 cell line in nude mice

Nude mice are randomly divided into five groups, each group has 5 mice, and injected cell suspension of stable transfected EC9706 for each group into subcutaneous neck. On the next day, observe tumor formation of nude mice, measure the line of apsides for tumors by a vernier caliper, and then make a record. End in 15 days after the injections.

Irradiating on nude mice

Take a radiotherapy after three weeks of inoculation. Apply special postural fixation of mice to fix the arms and the heads, 6MV-X Ray (Siemens Linear Accelerator, Germany), irradiation field of 2 cm ×2 cm, and source skin distance (SSD)=100cm,

the dose rate of 200cGy/min, 2Gy/time/day, 5 times/week, the total dose is 20Gy. Cushion 2cm of solid water on tumor surface as a compensation, so as to improve the percentage depth dose of tumors.
2.10 Observing the reaction to tumor radiotherapy
During the period of radiotherapy, observe the reaction to radiotherapy of subcutaneous transplanted tumor on the next day. Euthanize the mice after 3 days of radiotherapy, wring the tumors, weigh by photoelectric scales (G), calculate the tumor volume (V) and inhibiting tumor rate in accordance with the following formula: tumor volume=L(long diameter)×l(short diameter)^2 ×0.52[2.3]; inhabiting tumor rate (%)=(control group V-experimental group V)/control group V×100%; or inhabiting tumor rate (%)=(control group G-experimental group G)/control group G×100%.

Statistical Treatment

Make statistical analysis by SPSS13.0 statistical software package, all measurement data adopt by($\bar{x}$ ±s), the results analyze by one-way ANOVA, there are significant differences in P<0.05.

RESULTS

Results of constructing Cox-2 siRNA vector

si214-F and si214-R hairpin single-stranded DNA oligonucleotide, bright stripes are visible in forming double-stranded DNA electrophoresis by annealing, which is located below 100 bp and close to 100 bp, consistent with the design, as shown in figure 1; Annealing product connects with linear double sticky siRNA vector pRNA-U6.1, obtains multiple positive bacterium after the transformation, randomly selects 1 strain of extracted plasmid, detects the insertion sequence (see figure 2), the results are in complete accord with design sequence, and obtains pRNA-U6.1-siCox214.

Results of constructing Cox-2 expression vector

PCR amplification products electrophoresis, observing under 256nm UV lamp, 1815bp band is visible, consistent with the length of cox-2 gene ORF as shown in figure 3. The

Table 1. RT-PCR Detects Relative Expression Level of Cox-2 mRNA in Each Group of Cells

Group	Transfected Plasmid	n	Expression Level ($\bar{x}$±S)
Cox-2 Silence Group	pRNA-U6.1-siCox214	5	0.239±0.022**
siRNA Irrelative Sequence Control Group	pRNAT-U6.1-Con	5	1.216±0.045
Cox-2 Expression Group	pcDNA™4-Cox-2	5	2.639±0.119**
Expression Vector Control Group	pcDNA™4	5	1.235±0.071
Blank Control Group	-	5	1.255±0.044

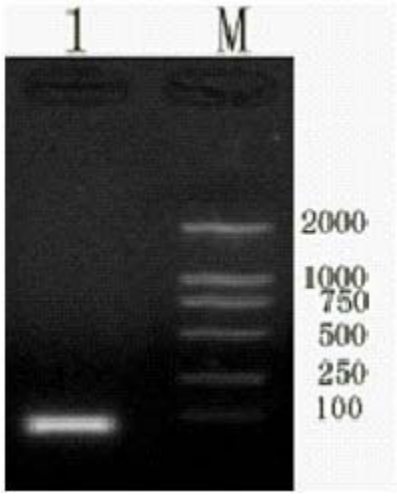
Compared with control group, **p<0.01

Table 2. Volume of Subcutaneous Implanted Tumors between Different Treatment Groups in 3 weeks after Inoculation($\bar{x}$ ±s)

Group	Volume (mm^3)	Anti-tumor Rate(%)
Down-regulated Group	715.2±383.2	25.7
Up-regulated Group	1178.5±336.4	-22.5
Empty Vector Control Group	953.4±323.6	0.9
Irrelative siRNA Sequence Group	946.6±487.2	1.6
Blank Control Group	962.1±300.2	

Table 3. Comparison of the Qualities after Transplanted Tumors Finished the Radiotherapy in Each Experimental Group of Nude Mice (n=5, ±s)

Group	Quality of Tumor (G)
Down-regulated Group	0.612±0.061
Up-regulated Group	1.314±0.105
Irrelative siRNA Sequence Group	1.015±0.087*
Empty Vector Control Group	1.007±0.092*
Blank Control Group	0.997±0.045*



1: si214-F and si214-R Hairpin Single-stranded DNA Annealing Product; M: DNA Molecular Weight Marker DL2000

Fig. 1. Results of Annealing Electrophoresis for Cox-2 Gene siRNA Hairpin DNA

detected sequence of recon pcDNA™4-Cox-2 takes a homology comparison analysis on Cox-2 sequence in GenBank, the results show that the insertion sequence is complete consistent with cox-2 sequence in GenBank.

RT-PCR results

Relative expression level of Cox-2 mRNA in each group of cells is as shown in table 1, relative expression level of Cox-2 mRNA in blank control group is compared with that in siRNA irrelevant sequence control group, and expression vector control group, the differences has no significance ($p>0.05$). The relative expression level of Cox-2 mRNA in silence group improves much more significantly than that in blank control group, the differences have extraordinary significance($p<0.01$, $F=918.84$), which indicates it can inhibit Cox-2 mRNA expression after constructed siRNA vector transfects EC9706 cells; the relative expression level of Cox-2 mRNA in up-regulated group improves

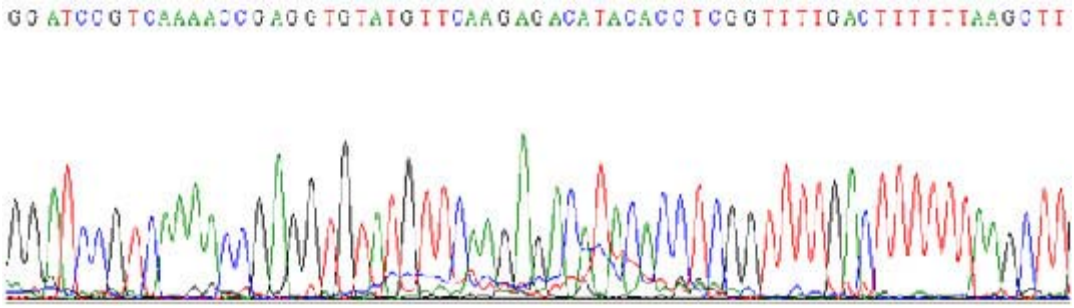
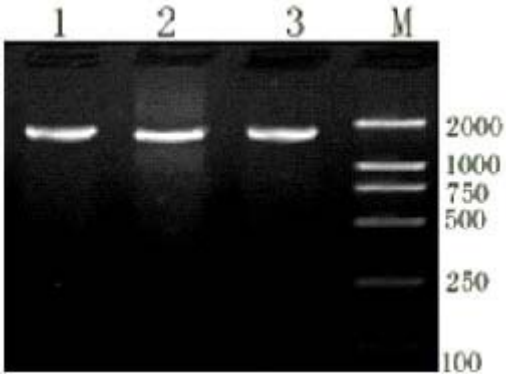
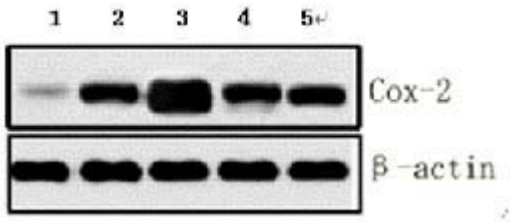


Fig. 2. Results of pRNA-U6.1-siCox214 Insertion Sequence



1: PCR amplification stripe of target gene Cox-2;
2-3: recon amplification stripe;
M: molecular weight Marker DL2000

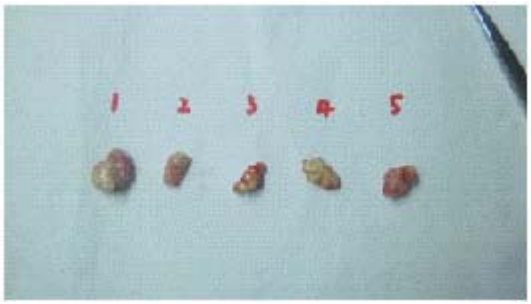
Fig. 3. Results of PCR amplification product electrophoresis



1: down-regulated group;
2: siRNA irrelative sequence control group;
3: up-regulated group;
4: expression vector control group;
5: blank control group

Fig. 4. Western blot results

Fig. 5.



1: up-regulated group;
2: down-regulated group;
3: siRNA irrelative sequence control group;
4: expression vector control group;
5: blank control group

Fig. 6.

much more significantly than that in blank control group, the differences have an extraordinary significance ($p<0.01$, $F=1481.71$), which indicates it can express Cox-2 mRNA high-efficientlyafter constructed Cox-2 expression vector transfects EC9706 cells.

Western blot results

The cells of Blank control group, control group with siRNA irrelative sequence, control group with expression vector have stronger Cox-2 western blotting stripes; Cox-2 western blotting stripes in silence group weaken much more significantly than those in three control groups, in accordance with RT-PCR results, as shown in figure 4. Cox-2 blotting in up-regulated group is even deeper than that in three contrl groups, which indicate they can express Cox-2 mRNA and protein high-efficiently after constructed Cox-2 expression vector tranfects EC9706 cells.

Growth situation of subcutaneous implanted tumor

In 1 week after the inoculation, newborn tumor nodules are visible in subcutaneous neck of nude mice, add upto about 5~10 mm×5~10mm, the volumes of tumor nodules for different tumor-bearing mice are different. The tumor formation rate of subcutaneous implanted tumors for 25 nude mice is up to 100%, without natural death, the tumor formation rates between groups have no significant differences. In three weeks after inoculation, compared long diameter and short diameter measured tumors (figure 5) in blank control group, those in irrelative siRNA sequence group with those in empty vector control group, the average volume of subcutaneous implanted tumor in nude mice of down-regulated group reduces obviously, the difference has statistical significance ($p<0.05$); and while the average volume of subcutaneous implanted tumor in nude mice of up-regulated group increases obviously, the difference has statistical significance ($p<0.05$). As shown in table 2.

By observing radiation reaction of nude mice, when exposure dose is up to 2-4Gy, subcutaneous implanted tumor increases obviously, which is considered to be the result of local tissue edema after radiation therapy. Soon afterwards, with radiotherapy dose increasing, the tumors shrink significantly. Gray or reddish tumor tissue is visible in dissecting nude mice at the end of radiotherapy, of which the surface is nodular, the texture when touching is a little harder (see figure 6). Measuring long diameter and short diameter of tumors at the end of radiotherapy, the average volumes of tumors in blank control group, irrelative siRNA sequence group, Empty vector control group and silence group reduce to be smaller than those before radiotherapy ($p<0.05$), of which they reduce most obviously in down-regulated group ($p<0.01$); and while average volume of subcutaneous implanted tumor for nude mice in up-regulated group has no obvious changes than that before radiotherapy ($p>0.05$). As shown in figure 7.

Weigh transplanted tumor of nude mice after stripping in each experimental group

The results are shown in table 3, the quality of tumors in down-regulated group reduce much more significantly than that in blank control group, irrelative siRNA sequence group and empty vector control group, the difference has the significance ($p<0.01$); and while the quality of

tumors in up-regulated group increases much more significantly than that in blank control group, irrelative siRNA sequence group, empty vector control group, the difference has the significance ($p<0.05$).

DISCUSSIONS

Cyclooxygenase(Cox) is one of the main rate-limiting enzymes during the period of prostaglandin synthesis, which can metabolize arachidonic acid into various prostaglandin products, and accordingly participate in pathophysiological processes of a variety of diseases. The research shows that cyclooxygenase -2 (Cox-2) is high expression in such many tumor tissues as esophageal cancer, and also participates in the occurrence and development of tumors, even promotes the invasion and the metastasis of malignant tumors[4-5].

By observing the growth situation of transplanted tumors for esophageal cancer cells EC9706, the tumor formation rates of these 25 nude mice are up to100%, without natural death, the tumor formation rates between groups have no significant differences, which prompts transfected Cox-2 siRNA has no effect on the tumor formation of esophageal cancer cells EC9706. In down-regulated group, the average volumes of subcutaneous implanted tumors in down-regulated group of nude mice are significantly smaller than those in control group, the differences have the significance ($p<0.05$); the average volumes of subcutaneous implanted tumors in up-regulated group are significantly larger than those in cotrol group, the differences have the significance ($p<0.05$). Accordingly when cox-2 is higher expression, the growth and the invasion of tumors are stronger, the tumors grow more rapidly, and while when cox-2 is lower expression, the growth and the invasion are relatively weaker, the growth of tumors is relatively slower. To observe the influence of regulating Cox-2 gene expression on the radiosensitivity to esophageal cancer, we make further investigations as an evaluation index of the changes before and after the radiotherapy for tumors and the qualities after the radiotherapy, the results show reducing amplitude of tumor volume in down-regulated group is obviously higher than that in blank control group, empty vector control group, irrelative siRNA sequence group, and also there is significant difference ($p<0.05$). And while reducing amplitude of tumor volume in up-regulated group is obviously lower than that in blank control group, empty vector control group, irrelative siRNA sequence group, and also there is a significant difference ($p<0.05$). The quality of tumors in down-regulated group after radiotherapy is significantly lower than that in blank control group, empty control group, and irrelative siRNA sequence group. The quality of tumors in up-regulated group is significantly higher than that in blank control group, empty control group, and irrelative siRNA sequence group. Which illustrates that down-regulating Cox-2 expression plays a radiosensible role on transplanted tumors of esophageal cancer, and that up-regulating Cox-2 expression makes tumors resisting radiotherapy.

Thus we think down-regulating Cox-2 gene expression combined with X-ray irradiation plays the role of synergic enhancement on antitumor, and inhibits the repair ability of tumor cells to radiation damage, thereby enhances the sensitivity to radioactive rays, consequently the radiotherapy effect of Cox-2 tumor with low expression is better, and that of Cox-2 tumor with high expression tumors will resist the radiation, the radiotherapy effect is poorer.

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