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• ESOPHAGEAL CANCER •

Expression properties of recombinant pEgr-P16 plasmid in esophageal squamous cell carcinoma induced by ionizing irradiation

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Abstract

AIM: To construct the recombinant pEgr-P16 plasmid for the investigation of its expression properties in esophageal squamous cell carcinoma induced by ionizing irradiation and the feasibility of gene-radiotherapy for esophageal carcinoma.

METHODS: The recombinant pEgr-P16 plasmid was constructed and transfected into EC9706 cells with lipofectamine. Western blot, quantitative RT-PCR and flow cytometry were performed to study the expression of pEgr-P16 in EC9706 cells and the biological characteristics of EC9706 cell line after transfection induced by ionizing irradiation.

RESULTS: The eukaryotic expression vector pEgr-P16 was successfully constructed and transfected into EC9706 cells. The expression of P16 was significantly increased in the transfected cells after irradiation while the transfected cells were not induced by ionizing irradiation. The induction of apoptosis in transfection plus irradiation group was higher than that in plasmid alone or irradiation alone.

CONCLUSION: The combination of pEgr-P16 and irradiation could significantly enhance the P16 expression property and markedly induce apoptosis in EC9706 cells. These results may lay an important experimental basis for gene radiotherapy for esophageal carcinoma.

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INTRODUCTION

Early growth response gene-1 (Egr-1), also known as zif/268, NGFI-A, Krox-24 and TIS-8, encodes a nuclear phosphoprotein with a cysteine/hystidine zinc finger structure, which is partially homologous to the corresponding domain of the Wilm's tumor susceptibility gene[1-4] Zinc fingers are a protein structural motif that serves as DNA-binding domains in several transcriptional regulatory proteins. It was reported that Egr-1 was transcriptionally induced following exposure to irradiation. Promoter deletion analysis of Egr-1 promoter elements linked to the CAT reporter gene demonstrated that the first 5' CArG boxes (CC $(A/T)_6$GG elements) were of paramount importance for the induction of Egr-1 by irradiation or free radicals[5-7].

In this study, the pEgr-P16 plasmid was constructed and transfected into the human esophageal cancer cell line EC9706. The expression of P16 in the transfected cells exposed to different doses of γ-ray irradiation and its bioactivities were detected to explore the feasibility of gene- radiotherapy for esophageal carcinoma.

MATERIALS AND METHODS

Cell line and vectors

The EC9706 was maintained in Dulbecco's modified Eagle's medium (DMEM), high glucose media (Life Technologies) and generously supplemented with 100 ml· L^{-1} fetal bovine serum (Hyclone Laboratories), penicillin, streptomycin and nonessential amino acids (Life Technologies). The pcDNA3.1$^+$ vector was purchased from Invitrogen and pGL3-enhancer vector from Promega-Biotec.

Construction of pEgr-P16 plasmid

The expression vector for P16 was constructed as shown in Figure 1.

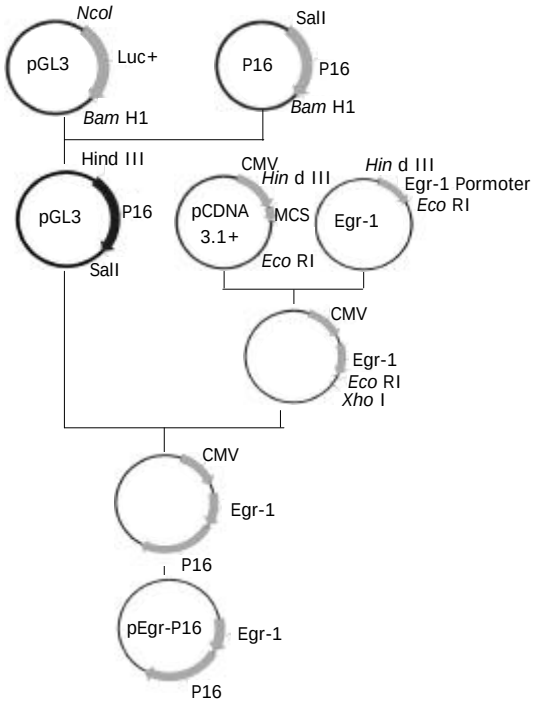

Figure 1 Diagram of the construction of the plasmid pEgr-P16.

Transfection

The transfection of EC9706 cells was carried out in a 6-well

plate. The transfection procedure began when the cells reached 70 % confluence on the surface of plate wells. Solution A was prepared by separate addition of 10 μg of pEgr-P16 and pcDNA3.1$^+$ to 100 μl serum-free medium (SFM), and solution B by addition of 10 μl Lipofectimine 2000 (Life Technologies) to 100 μl SFM. The two solutions were combined for 30 min at room temperature, 0.8 ml SFM was added to the tube containing the above solutions, and the mixture was added to the rinsed cells. The medium was replaced with fresh and complete one after 18 h in transfection. The cells were exposed to irradiation after 36 h in transfection.

Ionizing irradiation

The dose rate was 0.784 Gy/min for 0, 2, 4, 8, 10 and 20 Gy Co^{60} γ-ray irradiation, respectively.

Quantitative RT-PCR

Total RNAs of the transfection of EC9706 cells and control were obtained by extracting cells in Trizol (Invitrogen) and treated with heat-inactivated DNase I (Invitrogen). RNA quality and quantity were evaluated by UV spectrophotometry. Two μg total RNA was used for cDNA synthesis (25 μl) using M-MLV reverse transcriptase and random primers (Invitrogen).

A standard curve was constructed separately by the serial dilutions of PCR purified products of p16 and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). Template concentrations for reactions in the relative standard were 10^8, 10^7, 10^6 and 10^2 copies/μl. The cDNA (1:5 dilution) from the sample was analyzed as unknown. Real-time PCR was performed containing SYBRgreen I (1:20 000 QIAGEN), forward and reverse primers (50 nmol each), sample cDNA (1 μl) or standard sample (1 μl) under the following condition: denaturation at 95 ℃ (3 min); 40 cycles at 95 ℃ (45 s), at 59 ℃ (45 s), at 72 ℃ (40 s), at 80 ℃ (5 s). GAPDH was used as an internal reference in each PCR reaction. Primers were as follows: GAPDH, forward primer 5' -TGCACCACCAACTGCTTAGC-3' and reverse one 5' -GGCATGGACTGTGGTCATGAG-3' . P16, forward primer: 5' -GAATAGTTACGGTCGGAG-3' and reverse one 5' -CGGTGACTGATGATCTAA-3' . Amplification was followed by melting curve analysis using the program run at the step acquisition mode to verify the presence of a single amplification product in DNA contamination-free. For each set of primers, a non-cDNA template control was included to assess the overall specificity. Accumulation of PCR products was monitored and determined using the Icycler (Bio-Rad), and the crossing threshold (Ct) was determined using the Icycler software.

Flow cytometry analysis

Approximately 5×10^6 of centrifugally sedimented cells were immediately fixed in 700 ml· L^{-1} ethanol and stored at 4 ℃ in PBS in preparation for fluorescent-activated cell sorting. Flow cytometry analysis was performed on a FACStar flow cytometer (Becton Dicikinson). Histograms of cell number logarithmic fluorescence intensity were recorded for 10 000 cells per sample. The apoptotic cell rate was calculated.

Statistical analysis

Student' s *t* and correlation tests were used to determine the comparability of groups. Statistically significant *P* value was defined as <0.05.

RESULTS

Expression of P16 in EC9706 cells transfected with pEgr-P16 followed by different doses of γ-irradiation

EC9706 cells transfected with pEgr-P16 were irradiated by different doses of γ-rays. The cells of control group were transfected with pcDNA3.1$^+$. Eight hours after irradiation, the protein was extracted and the expression of P16 was detected by Western-blot. The results showed no P16 expression in the control and higher p16 expression in 2, 4, 8, 10 and 20 Gy groups than in 0Gy one.

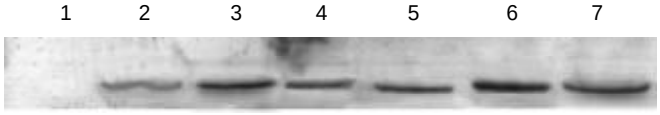

Figure 2 Expression of P16 in EC9706 cells after γ-irradiation. Lane 1: control; Lane 2: 0Gy; Lane 3: 2Gy; Lane 4: 4Gy; Lane 5: 8Gy; Lane 6: 10Gy; Lane 7: 20Gy.

P16 expression in EC9706 cells transfected with pEgr-P16 at different time points after 2Gy irradiation

EC9706 cells transfected with pEgr-P16 were irradiated by 2Gy irradiation. Total RNA was isolated at different time points after irradiation and the mRNA levels were detected by quantitative RT-PCR.

The results showed that P16 levels after 2Gy irradiation increased with time from 0 to 24 h. It reached the highest level at the 24th h and was about 4 times of that at the 2nd h (P<0.01).

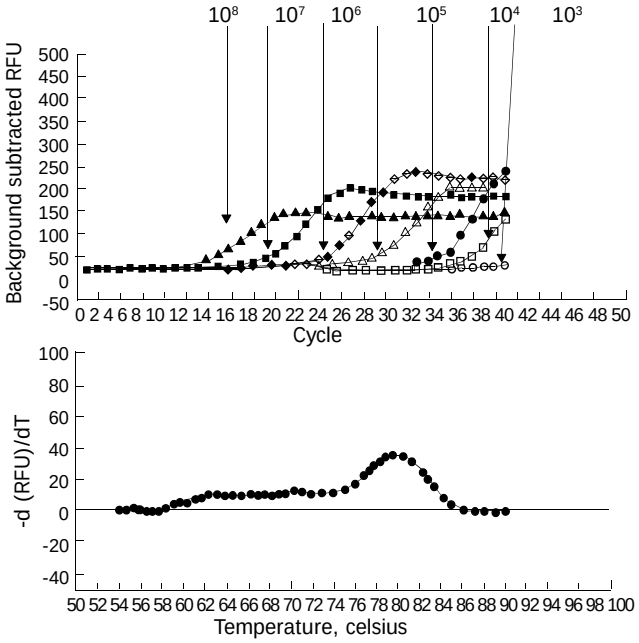

Figure 3 Amplification curves and post-amplification dissociation curves for P16 in EC9706 cells.

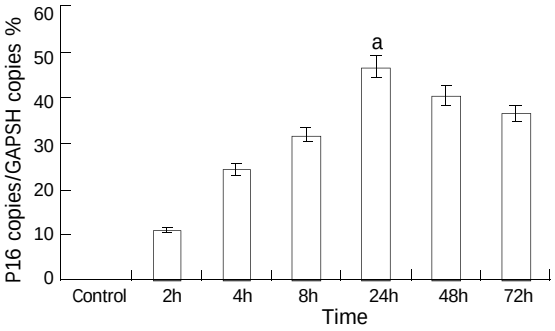

Figure 4 Expression of P16 in EC9706 cells at different time points after 2Gy γ-irradiation, ^aP<0.01 *vs.* 2 h group.

Apoptotic changes of transfected EC9706 cells after 2Gy g-irradiation

In P16 transfected EC9706 cells the apoptosis rate was 25.00, being higher than that of pcDNA3.1+ group (18.03, $P<0.05$). When exposed to 2Gy irradiation, the apoptosis rate was 33.23, higher than that in pcDNA3.1+ group (18.03, $P<0.01$) and P16 group (25.00). The differences were not significant between P16 and P16 plus irradiation groups.

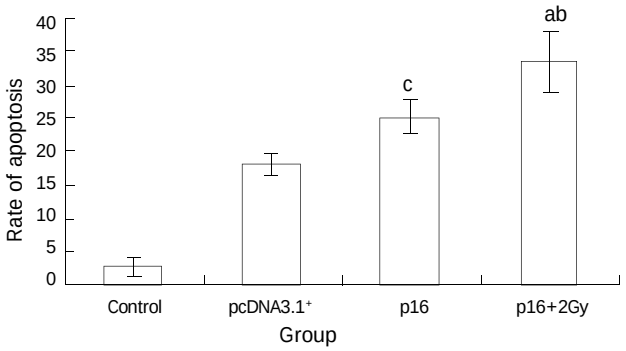

Figure 5 Apoptotic changes of transfected EC9706 cells. [a]$P<0.01$ *vs* pcDNA3.1$^+$group, [b]$P<0.001$ *vs* control group, [c]$P<0.05$ *vs* pcDNA3.1$^+$ group.

DISCUSSION

Radiotherapy is one of most important choices of the treatment for human tumors. Tumor destruction by radiation depends more on physical restriction of the radiation to a high-dose volume containing the tumor rather than a strict difference in radiosensitivity between tumor and normal cells. In fact, many tumor cells have lost the capacity for programmed cell death, resulting in radioresistance when compared with normal tissues. Vital structures are frequent within the radiotherapy volume restricting the amount of therapeutic radiation that can be safely delivered, thereby limiting tumor curability.

With the rapid development of molecular biology, gene radiotherapy has been considered as an effective way of cancer treatment. According to the mechanism that ionizing radiation could activate early Egr-1 gene promoter and induce the expression of downstream genes, Weichselbaum *et al* were the forerunners in tumor gene radiotherapy. They linked DNA sequences from the promotor region of Egr-1 with a cDNA sequence that encodes human tumor necrosis factor (TNF) alpha. The Egr-TNF construct was transfected into a human cell line of hematopoietic origin, HL525 (clone 2). The latter was injected into human xenografts of the radioresistant human squamous cell carcinoma cell line SQ-20B. Animals treated with radiation and clone 2 demonstrated an increase in tumor cures compared with animals treated with radiation alone or unirradiated animals given injections of clone 2 alone[6]. Thereafter, a variety of downstream genes were introduced to Egr-1 promoter to treat different tumors, and similar results were obtained[9-11].

The division cycle of eukaryotic cells is regulated by a family of protein kinases known as the cyclin-dependent kinases (CDKs). P16 is a tumor suppressor gene product. Serrano *et al* demonstrated that p16 could bind to CDK4 and inhibit the catalytic activity of the CDK4/cyclin D enzymes. P16 seemed to act in a regulatory feedback circuit with CDK4, D-type cyclins and retinoblastoma protein[12]. Overexpression of P16 gene could block cell cycle progression through the G_1-to-S phase boundary in a pRB-dependent manner[13-14]. Many P16 mutants identified from human tumors have been shown to have defects in this activity[15-17]. These data suggest that the CDK4-inhibitory activity of p16 is involved in regulating cell cycle progression through the G_1/S boundary.

On the basis of the antiangiogenic action of P16, we constructed pEgr-P16 plasmid and transfected EC9706 cells to study the expression properties of the plasmid induced by ionizing irradiation. The results revealed that no P16 expression in EC9706 cells transfected with pcDNA3.1$^+$ was detected and that the P16 expression in cells transfected with pEgr-P16 induced by irradiation was higher than that of sham-irradiation group. Time-course studies revealed that the P16 expression reached its peak at the 24th h after 2Gy irradiation, and the highest level was 4 times of that at the 2nd h ($P<0.01$). The combination of pEgr-P16 and radiation could induce markedly apoptosis of EC9706 cells although pEgr-P16 alone might induce transfected cells to undergo apoptosis. Our results suggested that pEgr-P16 could enhance expression property induced by radiation in EC9706 cells.

Esophageal carcinoma is still common in the world, especially in China[18-24], and the treatment remains a big problem up to date[25-31]. Gene radiotherapy may be of potential significance in the treatment of esophageal cancer. Our work will be a ground of further studies on esophageal cancer gene radiotherapy.

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