

Construction of pETNF-P16 plasmid and its expression properties in EC9706 cell line induced by X-ray irradiation

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Abstract

AIM: Recombined plasmid pETNF-P16 was constructed to investigate its expression properties in esophageal squamous carcinoma cell line EC9706 induced by X-ray irradiation and the feasibility of gene-radiotherapy for esophageal carcinoma.

METHODS: Recombined plasmid pETNF-P16 was constructed and transfected into EC9706 cells with lipofectamine. ELISA, Western blot, and immunocytochemistry were performed to determine the expression properties of pETNF-P16 in EC9706 after transfection induced by X-ray irradiation.

RESULTS: Eukaryotic expression vector pETNF-P16 was successfully constructed and transfected into EC9706 cells. TNF α expressions were significantly increased in the transfected cells after different doses of X-ray irradiation than in those after 0Gy irradiation (1 192.330-2 026.518 pg/mL, $P < 0.05-0.01$), and the TNF α expressions and P16 were significantly higher 6-48 h after 2 Gy X-ray irradiation (358.963-585.571 pg/mL, $P < 0.05-0.001$). No P16 expression was detected in normal EC9706 cells. However, there was strong expression in the transfected and irradiation groups.

CONCLUSION: X-ray irradiation induction could significantly enhance TNF α and P16 expression in EC9706 cells transfected with pETNF-P16 plasmid. These results may provide important experimental data and therapeutic potential for gene-radiotherapy of esophageal carcinoma.

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INTRODUCTION

Esophageal carcinoma is one of the most frequent malignant tumors. Its treatment choices include surgical resection,

radiation, chemotherapy and biological therapy. However, their limitations and adverse effects influence the therapeutic results. In 1992, Wechselbaum proposed the theory of gene-radiation combination treatment, that is, to ligate the promoter with irradiation-induced function and treatment gene so as to take advantages of the combined therapeutic functions of both modalities^[1].

In this study, pETNF-P16 plasmid was constructed and transfected into human esophageal cancer cell line EC9706. The expressions of TNF α and P16 in the transfected cells exposed to different doses of X-ray irradiation and the time course of the expressions after 2Gy X-ray irradiation 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 fetal bovine serum (Hyclone Laboratories), penicillin, streptomycin and nonessential amino acids (Life Technologies). PIRES1 vector was bought from Promega-Biotech (Shanghai, Promega Corporation).

Construction of pETNF-P16 plasmid

The expression vector for TNF α and P16 was constructed as Figure 1.

Transfection

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 μ g of pETNF-P16 or PIRES1 to 100 μ L serum-free medium (SFM), and solution B by addition of 10 μ L liposome to 100 μ L SFM. Solutions A and B were combined at room temperature for 30 min then 0.8 mL SFM was added to the tube containing the above solutions, and then the mixture was added to rinsed cells. The medium was replaced with a fresh and complete medium after 6 h in transfection. The cells were exposed to irradiation after 36 h in transfection.

Protein determination

TNF α protein was detected using ELISA kit (Genzyme). P16 protein was studied using Western blot analysis and immunocytochemistry (P16 antibody, Boster).

Ionizing irradiation

X-rays of 180 kV and 12 mA with 0.25 mmCu and 1.08 mm Al as filter were given at a dose-rate of 0.8639 Gy/min for doses of 2-20Gy.

Statistical analysis

Student's *t* test was used to determine the difference between groups. *P* values of less than 0.05 were considered statistically significant.

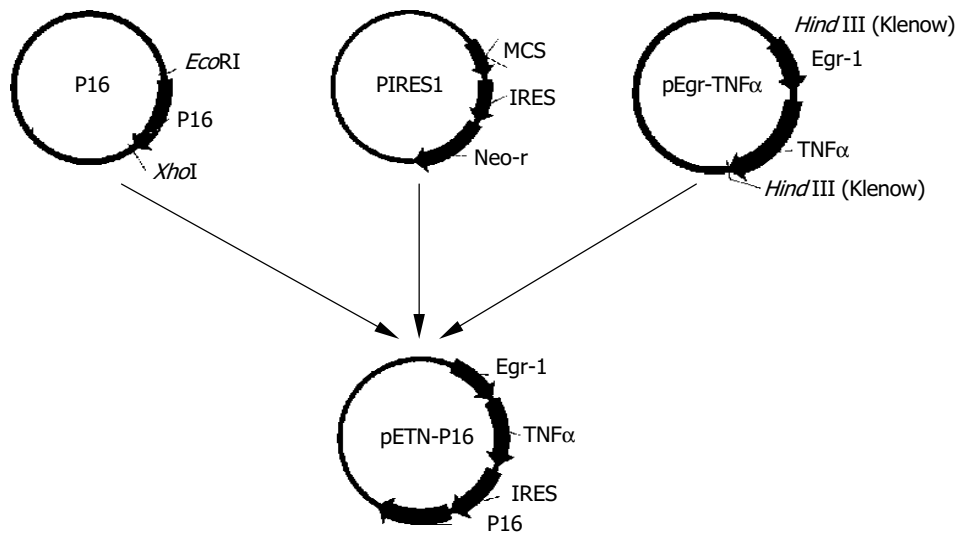


Figure 1 Construction of plasmid pETNF-P16.

RESULTS

TNFα expressions in EC9706 cells transfected with pETNF-P16 after different doses of X-irradiation

EC9706 cells transfected with pETNF-P16 received different doses of X-ray irradiation. The cells of control group were transfected with PIRES1. Eight hours after irradiation, the protein was extracted and TNFα expression was detected by ELISA.

The results showed that TNFα expression in the 2, 4, 10 Gy groups was significantly higher than that in 0 Gy group ($P < 0.05-0.01$) (Figure 2).

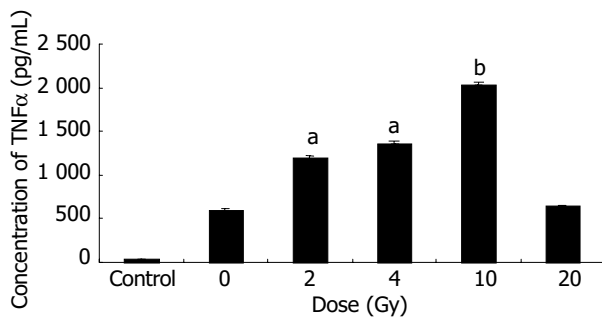


Figure 2 TNFα expression in EC9706 cells after irradiation with different doses of X-ray (mean±SD, $n = 3$). ^a $P < 0.05$, ^b $P < 0.01$ vs 0 Gy group.

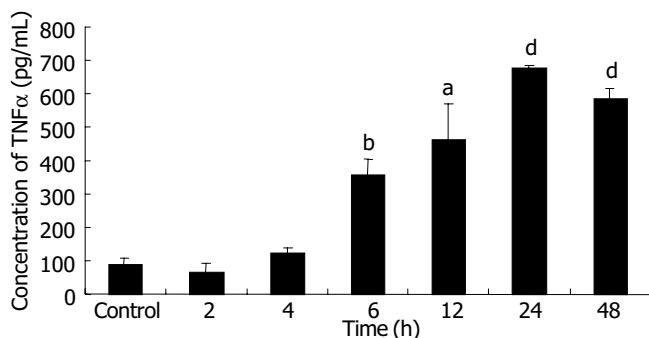


Figure 3 Expression time course of TNFα in EC9706 cells after 2 Gy of X-ray irradiation (mean±SD, $n = 3$). ^a $P < 0.05$, ^b $P < 0.01$, ^d $P < 0.001$ vs control group.

TNFα and P16 expressions in EC9706 cells transfected with pETNF-P16 at different time points after 2 Gy irradiation

EC9706 cells transfected with pETNF-P16 received 2 Gy of

X-ray irradiation while the control group did not receive. Proteins of TNFα and P16 were isolated at different time points after irradiation and detected by ELISA and Western blot.

ELISA results showed that TNFα expression increased from 2 to 24h, and reached the peak level at the 24th h, about 7.5 times of control group ($P < 0.01$). TNFα expression in 48 h group was significantly higher than that in control group ($P < 0.001$), but lower than that in 24 h group ($P > 0.05$) (Figure 3).

The results of Western blot analysis showed no P16 expression in cells transfected with PIRES1 plasmid. After 2Gy X-ray irradiation, P16 expression in the cells transfected with pETNF-P16 plasmid increased between 2 h and 48 h. The expression in the control group was lower than that in 4 h to 48 h groups (Figure 4).

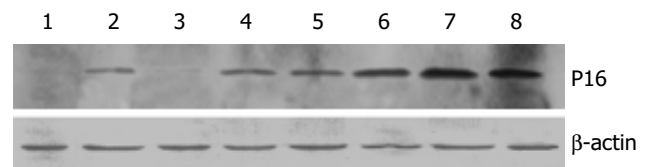


Figure 4 Expression time course of P16 in EC9706 cells after 2 Gy x-ray irradiation Lane 1: transfected with PIRES1 plasmid Lane 2: control group Lane 3: 2 h after irradiation Lane 4: 4 h after irradiation Lane 5: 6 h after irradiation Lane 6: 12 h after irradiation Lane 7: 24 h after irradiation Lane 8: 48 h after irradiation.

Immunocytochemistry

Thirty-six hours after transfected with pETNF-P16 plasmids, EC9706 cells received 2 Gy X-ray irradiation while the control group did not receive. After 24 h, anti-P16 IgG and Biotin-labeled secondary antibody were used to detect the P16 expression in treated cells. P16 protein was positive in the nucleus, cytoplasm and cellular membrane as judged by the brown color.

Immunocytochemical analysis demonstrated that all the normal EC9706 cells had no P16 expression. After transfected with pETNF-P16 plasmid, positive P16 expression in EC9706 cells was detected. Then, the transfected cells received 2 Gy X-ray irradiation and P16 expression remained positive, but morphological changes of EC9706 cells occurred.

DISCUSSION

It was reported that Egr-1 was transcriptionally induced

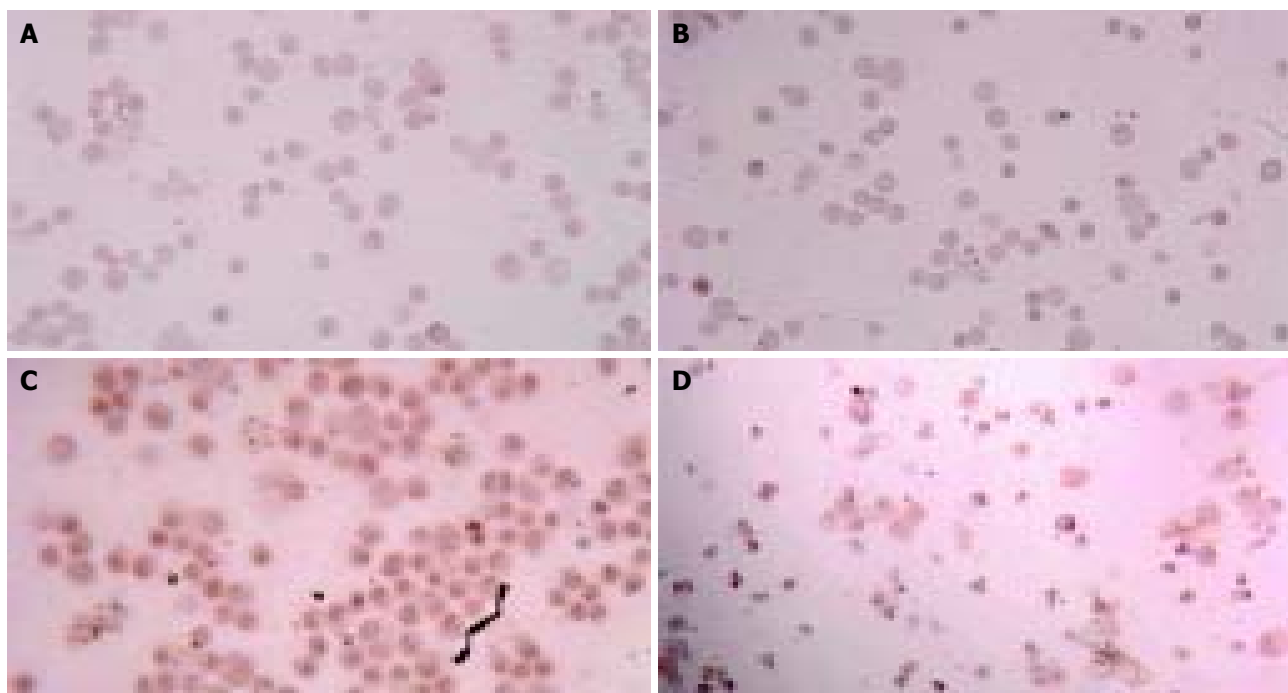


Figure 5 P16 expression in EC9706 cells transfected with pETNF-P16 plasmid after 2Gy X-ray irradiation. A: Negative control group; B: Untransfected group; C: Transfected group; D: Transfected and irradiated group.

following exposure to irradiation^[2]. Sequences responsive to ionizing radiation-induced signals were determined by deletion analysis of the Egr-1 promoter. The results demonstrated that induction of Egr-1 by X-rays was conferred by serum response or CC (A/T) rGG elements. Further analysis confirmed that the region encompassing the three distal or upstream CC(A/T)rGG elements was functional in X-ray response^[3-5].

Weichselbaum *et al.*^[6] were forerunners in tumor gene-radiotherapy. In 1992 they put forward that ionizing radiation could be used to activate the transcription of exogenous genes encoding cytotoxic proteins, and established the techniques that might be used to target gene therapy during the treatment of human neoplasms.

Combination of gene therapy with radiation therapy could overcome some problems and side effects of either radiation or gene therapy alone, including radioresistance of some tumors and toxicities to normal tissues. Weichselbaum *et al.*^[7] linked DNA sequences from the promoter region of Egr-1 to a cDNA sequence that encoded human tumor necrosis factor (TNF) alpha. Egr-TNF construction 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 improvement compared with those treated with radiation or injections of clone 2 alone. Thereafter, a variety of downstream genes were introduced to Egr-1 promoter to treat different tumors and similar results were obtained^[8-10].

TNF has complex *in-vivo* anti-tumor actions and involves a series of biochemical reactions, and varies according to host metabolism. It could not only kill tumor cells directly, but also act indirectly by occluding the feeding vessels of tumors, promoting inflammation reaction of the host, stimulating cytotoxic activities of megakaryocytes and producing tumor-specific cytotoxic antibody. Up to now, TNF has been found to have the strongest direct tumor-killing action. However, severe side effects made the effects of systemic treatment unsatisfactory. By contrast, regional administration into the tumor had better therapeutic effects. It has been widely reported that intra-tumor TNF injection had no severe side effects on patients with malignant tumors^[11-12]. We constructed pEgr-TNF α plasmid

and injected it into mouse melanoma locally to induce its expression by X-ray irradiation. The results showed that pEgr-TNF α gene in combination with radiotherapy could significantly inhibit tumor and had no side effects^[13].

P16 is a tumor suppressor gene product. Serrano *et al.*^[14] demonstrated that P16 bound to CDK4 and inhibited the catalytic activity of CDK4/cyclin D enzymes. P16 seemed to act in a regulatory feedback circuit with CDK4, D-type cyclins and retinoblastoma protein. Overexpression of P16 gene could block cell cycle progression through the G₁-to-S phase boundary in a pRB-dependent manner^[15,16]. Many P16 mutants identified from human tumors have been shown to have defects in this activity^[17-19]. These suggested that the CDK4-inhibitory activity of p16 was involved in regulating cell cycle progression through the G₁/S boundary.

On the basis of anti-tumor function of TNF α and P16, we constructed pETNF-P16 plasmid and transfected EC9706 cells to investigate the expression properties of plasmid induced by X-ray irradiation. The results showed that TNF α expression in pETNF-P16-transfected cells induced by irradiation was higher than that in control group ($P < 0.05-0.01$). Time-course studies between 6 h and 48 h after 2Gy irradiation revealed that TNF α expression in X-ray induced groups was higher than that in the control group ($P < 0.05-0.001$). It gradually increased and peaked at the 24th h with the expression level of 7.5 times of control group ($P < 0.01$). Immunocytochemistry showed no P16 expression in the control group, but strong expressions in the transfected and irradiated group. However, the cellular morphology was altered in the latter group and the mechanism is to be clarified.

Esophageal carcinoma still has high morbidity and mortality in China^[20-22], and its treatment is still difficult^[23-30]. Our work might have laid some theoretical basis for further study on esophageal cancer gene-radiotherapy, which should have promising therapeutic potential.

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