RESEARCH ARTICLE

Expression of bcl-2 and p53 in Induction of Esophageal Cancer Cell Apoptosis by ECRG2 in Combination with Cisplatin

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Abstract

Aim: To investigate the mechanisms of induction of apoptosis of esophageal cancer cells by esophageal cancer-related gene 2 (ECRG2) in combination with cisplatin (DDP). Methods: Hoechest staining was performed to analyze the effects of single ECRG2 and ECRG2 in combination with DDP on apoptosis of EC9706 cells. The expression levels of p53 and bcl-2 mRNA and protein were determined by RT-PCR and Western blotting, respectively. Results: The number of apoptotic cells after the treatment with ECRG2 in combination with DDP for 24 hours was more than that after the treatment with single ECRG2. RT-PCR and Western blotting showed that the expression levels of bcl-2 mRNA and protein were both down-regulated, while p53 mRNA and protein were both up-regulated in the cells treated with ECRG2 in combination with DDP compared with those given ECRG2 alone. Conclusion: ECRG2 in combination with DDP can enhance the apoptosis of EC9706 cells, possibly by down-regulating bcl-2 expression and up-regulating p53.

Keywords: Esophageal cancer related-gene 2 - cisplatin - apoptosis - p53 - bcl-2

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Introduction

Esophageal cancer, one of the common malignancies with the highest morbidity and mortality, is a major cause of death worldwide (Jemal et al., 2011). Chemotherapy is the main treatment for the patients with advanced esophageal cancers who cannot have the curative surgery (Ilson et al., 2008). Chemotherapy can improve the outcome of esophageal cancer (Sjoquist et al., 2011; van Hagen et al., 2012), but the curative effects of chemotherapy depend on the sensitivity of tumor cells to the chemotherapeutic agents. In addition, esophageal squamous cell carcinomas are often resistant against chemotherapeutic agents during chemotherapy, thus resulting in the failure of chemotherapy.

The clinical application and efficacy of cisplatin has been limited, owing to its side effects and the emergence of drug resistance (Ando et al., 2012), although DDP has been used as an important agent in patients with esophageal cancers. How to improve the therapeutic effects of DDP and how to reduce the drug resistance and side effects of DDP during the treatment has become a research focus. There was report (Su et al., 2012) showed that combination of nedaplatin and cisplatin at low concentrations were more effective for inhibition of proliferation and the induction of apoptosis in the Eca-109 (esophageal carcinoma cell line) and the expressions of bcl-2 mRNA and protein were decreased .

Esophageal cancer related gene2 (ECRG2) was separated and identified in normal esophageal tissues and esophageal cancer tissues of high cancer family from Lin county in 1998 by the mRNA differential display technique. RT-PCR analysis showed that ECRG2 mRNA is expressed in esophageal tissues and a variety of other normal tissues, but its expression level is lower in the esophageal cancer tissues (Su et al., 1998). It has been demonstrated that ECRG2 can inhibit tumor cell growth, and promote cell apoptosis in vivo and in vitro (Cui et al., 2003). However, the combined effects of DDP and ECRG2 in the treatment of esophageal cancer are rarely investigated. Accordingly, in the present study, we used esophageal squamous cell carcinoma (ESCC) cell line EC9706 cell as a model to explore effects of ECRG2 in combination with DDP on ESCC cells, we investigated the effect of ECRG2 in combination with DDP on cell apoptosis. Moreover, we observed that expression of p53 mRNA and protein were significantly up-regulation, but bcl-2 mRNA and protein were significantly down-regulation while ECRG2 in combination with DDP in EC9706. Our data demonstrated that ECRG2 in combination with DDP indeed enhanced cell apoptosis in human esophageal cancer cell line EC9706, comparing with ECRG2 alone. Moreover, this efficacy might result from the expression altering of apoptosis-related gene

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bcl-2 and p53. To our knowledge, the present work was the first study about the effect of ECRG2 in combination with DDP in esophageal cancer cells.

Materials and Methods

Materials

The human esophageal cancer cell line EC9706 was provided by the tumor cell library of Academy of Medical Sciences in China. ECRG2 protein was synthesized by Shanghai Sangon Biological Engineering Co., Ltd. Cisplatin was bought from Qilu Pharmaceutical Co., Ltd. RPMI 1640 medium and fetal bovine serum (FBS) were purchased from HyClone. Trizol were purchased from Sigma Co. Reverse transcription and amplification kit were purchased from Promega Co. Hoechst 33258 was provided by Beyotime Institute of Biotechnology. The rabbit anti-human p53 and bcl-2 antibody and goat anti-rabbit IgG was purchased from Abcam.

Methods

Cell culture

EC9706 cells were cultured in RPMI 1640 containing 10% FCS, 100 u/mL Penicillin and 100mg/L Streptomycin) in a humidified incubator at 37°C with 5% CO_2. Medium was replaced every two or three days. In all experiments, cells were in the logarithmic phase.

Cell apoptosis analysis

In order to investigate the cell apoptosis, morphological analysis was performed by Hoechst33258 staining. EC9706 cells (4×10^7/L) were seed into the 6 well plates with cover slips. For the ECRG2 groups, ECRG2 proteins at different concentrations (5.5 μg/L, 6.5μg/L, 7.5 μg/L, 8.5 μg/L) were respectively added to EC9706 cells. ECRG2 protein plus cisplatin groups were added to a final concentration of 3mg/L cisplatin, on the basis of each ECRG2 protein in the above groups. Then, the coverslips were removed for Hoechst33258 staining. Apoptotic cells were observed and counted under a light microscope. Nuclei of apoptotic cells were stained white. Apoptosis rate was calculated through the ratio of apoptotic cells number and the total cells number.

RT-PCR

The expression of p53 and bcl-2 mRNA was detected by RT-PCR. Total RNA was isolated from cells using the Trizol reagent according to the manufacturer's instructions. The primers were designed and synthesized by Shanghai Sangon Biological Engineering Co. Ltd. The primers for p53 were 5'- ATTTGCGTGTGGAGTATTTGG-3' (forward) and 5'-GCTGTTCCGTCCCAGTAGATTA-3' (reverse). The product size was about 209 bp. The primers for bcl-2 were 5'-CTGGTGGACAACATCGCTCTG-3' (forward) and 5'-GGTCTGCTGACCTCACTTGTG-3' (reverse). The product size was about 328 bp. The primers for GAPDH were 5'- TCATGGGTGTGAACCATGAGAA-3' (forward) and 5'-GGCATGGACTGTGGTCATGAG-3' (reverse). The product size was about 146bp. Each reaction system contained 12.5μl of GoTaq® GreenMaster Mix (Promega), 2.5μl of each primer and 5μl of cDNA. After the activation of Taq polymerase for 5 min at 95 °C, cDNA was amplified for 40s at 95°C, 40s at 55 °C, and 1min at 72°C, for 35 cycles, ending with a final extension for 5min at 72°C. To verify accuracy of the amplification, PCR products were further electrophoresed through a 1.5% agarose gel stained by ethidium bromid. The images were collected under the UV light. Data were analyzed with Light Cycler 480 software (Roche Applied Science). The mRNA expression was measured by densitometry. The target expressions were normalized using GAPDH and β-actin expression as reference.

Western blot

Western blot was used to detect p53 and bcl-2 protein expression. The total cell proteins were extracted from EC9706 cells treated with different concentrations of ECRG2 protein and ECRG2 protein plus CDD after for 48h. After the quantification of the total protein, the protein was separated on an 8% polyacrylamide gel (PAGE), and transferred to the PVDF membranes (Millipore). Then, the membranes were blocked with 5% non-fat dry milk in PBS-Tween 20. After incubation with a polyclonal rabbit anti-human bcl-2 and p53 antibody diluted 1:500 overnight at 4°C, the membranes were incubated with a secondary HRP-conjugated anti-rabbit antibody (1:1000). Blots were visualized by chemiluminescence.

Statistical analysis

Data were expressed as the mean±SEM. Statistical analysis was performed with one-way analysis of variance (ANOVA) using a SPSS17.0 software. $P< 0.05$ was considered statistically significant.

Results

The effects of ECRG2 protein and DDP on apoptosis of EC9706 cells

In Figure 1, ECRG2 protein alone significantly increased the rate of EC9706 cell apoptosis compared with

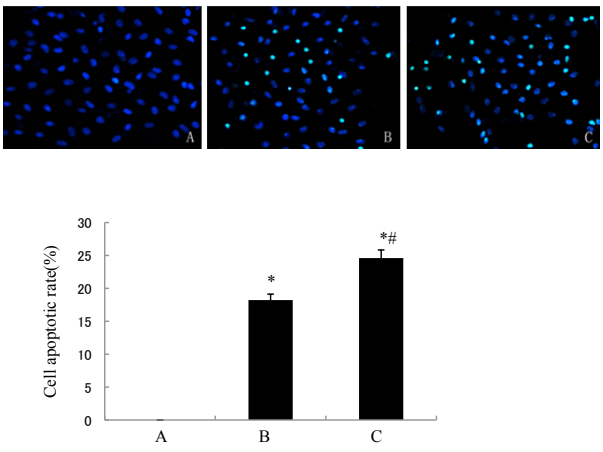

Figure 1. Effects of ECRG2 Protein and ECRG2 Protein in Combination with DDP on EC9706 Apoptosis for 24 Hours. A: Control group; B: ECRG2 protein (8.5 μg/L) group; C: ECRG2 protein (8.5μg/L) +DDP (3 mg/L)group. Data were expressed as the mean±SEM. n = 3. *P <0. 01 vs control; #P < 0.01 vs ECRG2 protein at the same dose

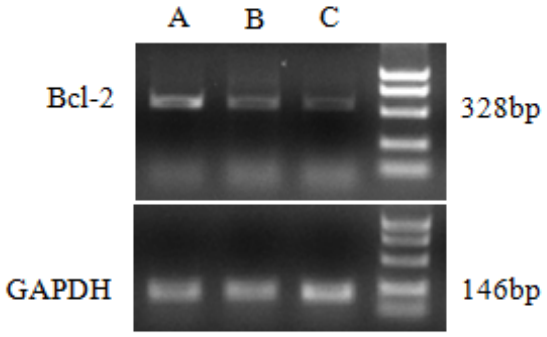

Figure 2. Expressions of bcl-2 mRNA Detected by RT-PCR. A: Control group; B: ECRG2 protein (8.5 μg/L) group; C: ECRG2 protein (8.5 μg/L) +DDP (3 mg/L)group. Data were expressed as the mean±SEM. n = 3

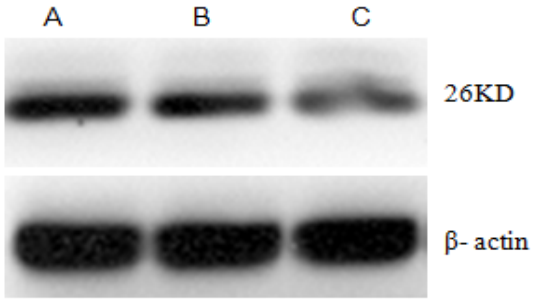

Figure 3. Expressions of bcl-2 Protein by Western Blot. A: Control group; B: ECRG2 protein (8.5 μg/L) group; C: ECRG2 protein (8.5 μg/L) +DDP (3 mg/L)group. Data were expressed as the mean±SEM. n = 3

the control group. When ECRG2 protein was combined with DDP, at a high concentration of ECRG2 protein, the number of apoptotic cells significantly increased in a dose-dependent manner. The bodies of apoptotic cells shrinked in volume and became round, and the concentration of cell nucleus was observed, and cell nucleus became white after stained by Hoechst33258 under a flourescent microscope.

The effects of ECRG2 protein and DDP on the expression of bcl-2 mRNA

To further elucidate the mechanisms involved in ECRG2 and ECRG2 in combination with DDP induced apoptosis, the expression of bcl-2 mRNA was evaluated in esophageal cancer cells by RT-PCR analysis. ECRG2 protein at a high concentration could significantly down-regulate the expressions of bcl-2 mRNA compared with control group. When ECRG2 protein was combined with DDP, the expressions of bcl-2 mRNA were significantly decreased compared with that when ECRG2 protein was used alone (Figure 2).

The effects of ECRG2 protein and DDP on the expression of bcl-2 protein

As shown in Figure 3, ECRG2 protein at a high concentration could significantly down-regulate the expressions of bcl-2 protein with compared with control group, When ECRG2 was combined with DDP, the bcl-2 protein expressions were significantly decreased compared with that when ECRG2 protein was used alone (Figure 3).

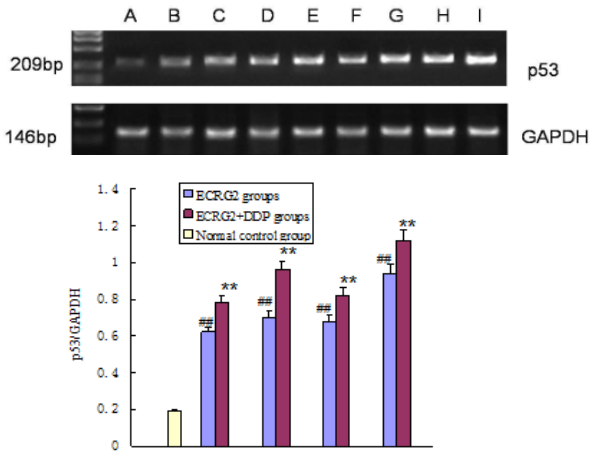

Figure 4. Expressions of p53 mRNA by RT-PCR. A: Control group; B:ECRG2 5.5μg/L; C: ECRG2 6.5μg/L; D: ECRG2 7.5μg/L; E: ECRG2 8.5μg/L; F: ECRG2 (5.5μg/L)+DDP (3mg/L); G: ECRG2 (6.5μg/L)+DDP (3mg/L); H: ECRG2 (7.5μg/L)+DDP (3mg/L); I: ECRG2 (8.5μg/L)+DDP (3mg/L). Data were expressed as the mean±SEM. n = 3. *##P* <0.01 vs control;** *P* < 0.01 vs ECRG2 protein at the same dose

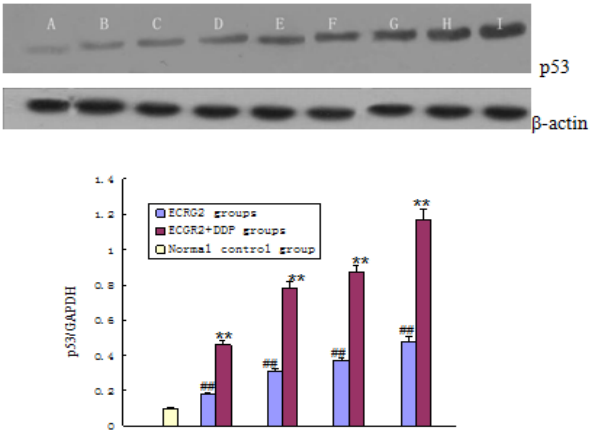

Figure 5. Expressions of p53 Protein by Western Blot. A: Normal control group; B: ECRG2 5.5 μg/L; C: ECRG2 6.5 μg/L; D: ECRG2 (7.5 μg/L); E:ECRG2 (8.5 μg/L); F: ECRG2 (5.5 μg/L)+DDP (3 mg/L); G: ECRG2 (6.5 μg/L)+DDP (3mg/L); H: ECRG2 (7.5 μg/L)+DDP (3 mg/L); I: ECRG2 (8.5 μg/L)+DDP (3 mg/L). Data were expressed as the mean±SEM. n = 3. ##*P*< 0. 01 vs control;**P < 0. 01 vs ECRG2 protein at

The effects of ECRG2 protein and DDP on the expressions of p53 mRNA

To further elucidate the mechanisms involved in ECRG2 and ECRG2 in combination with DDP induced apoptosis, the expression of p53 mRNA was evaluated in esophageal cancer cells by RT-PCR analysis. ECRG2 protein at different concentrations could significantly up-regulate the expressions of p53 mRNA with a dose-dependent relationship compared with control group (Figure 4). When ECRG2 protein was combined with DDP, with an increase in the concentration of ECRG2 protein, the expressions of p53 mRNA were significantly increased compared with that when ECRG2 protein was used alone (Figure 4).

The effects of ECRG2 protein and DDP on the expressions of p53 protein

As shown in Figure 5, ECRG2 protein at different

concentrations could significantly up-regulate the expressions of p53 protein with a dose-dependent relationship compared with control group, When ECRG2 was combined with DDP, with an increase in the concentration of ECRG2 protein, the p53 protein expressions were significantly increased compared with that when ECRG2 protein was used alone (Figure 5).

Discussion

Esophageal cancer is currently one of the malignancies with the highest morbidity and mortality as well as the poor prognosis worldwide with more than 480, 000 new cases and 400, 000 deaths annually (Jemal et al., 2011). Chemotherapy is an important treatment in the comprehensive therapies of esophageal cancer. Cisplatin, one of the chemotherapy drug commonly used in the treatment of esophageal cancer, kills tumor cells mainly through inducing DNA damage, which can activate the apoptosis-related signaling pathways and then lead to cell apoptosis. However, the clinical application of cisplatin has been limited just because of its serious toxic effects, such as bone marrow suppression, kidney damage and neurotoxicity. Therefore, a drug combination has been recommended for clinical application, which can not only improve the therapeutic effects, but also reduce the clinical doses of the platinum-based chemotherapy drugs and then alleviate its toxic side effects.

Human ECRG2 is located on the human chromsome 5q32-33, with four exons and three introns, and the total length is 3540 bp. The length of ECRG2 cDNA encoding a polypeptide with 85 amino acid residues is 569 bp, RT-PCR analysis showed ECRG2 mRNA is expressed in esophageal tissues and a variety of other normal tissues, but its expression level is significantly lower in esophageal cancer tissues (Su et al., 1998). ECRG2 has been demonstrated to be able to inhibit tumor cell growth, proliferation, and promote cell apoptosis in vivo and in vitro (Cui et al., 2003; Yue et al., 2004; Li et al., 2005; Song et al., 2012). ECRG2 gene participates in the control of centrosome duplication by regulating p53 transcriptional activity and centrosome positioning capability in cells. ECRG2 gene deletion caused abnormal centrosome duplication, multipolar spindle appearance, eventually leading to chromosomal instability and the appearance of aneuploid tumor cells (Cheng et al., 2008). The occurrence and development of tumor results from abnormal proliferation, differentiation and apoptosis of tumor cells. Most anticancer drugs can induce apoptosis in sensitive tumor cells, and the drug-induced apoptosis activity of tumor cells was associated with its anti-tumor efficacy. Thus, inducing apoptosis of tumor cells has been a new research focus for cancer therapy, and has also been used as a new index to evaluate the efficacy (Tan et al., 2008). Our Hoechest staining results showed that ECRG2 significantly could promote the apoptosis of EC9706 cells, ECRG2 protein alone significantly increased the rate of EC9706 cell apoptosis compared with the control group. When ECRG2 protein was combined with DDP, the number of apoptotic cells significantly increased in a dose-dependent manner.

In the apoptosis induced by a variety of death signals, the bcl-2 family proteins play an important role. The bcl-2 is an inhibitory factor can inhibit cell apoptosis. The bcl-2 mRNA and protein expression decreased, prompting cells apoptosis, when the bcl-2 mRNA and protein expression increased, inhibiting cell apoptosis. The combination of nedaplatin and cisplatin at low concentrations were more effective for inhibition of proliferation and the induction of apoptosis in the Eca-109 and the expressions of bcl-2 mRNA and protein were decreased (Su et al., 2012). Our result showed that ECRG2 protein at a high concentration could significantly down-regulate the expressions of bcl-2 mRNA and protein with compared with control group, When ECRG2 was combined with DDP, the bcl-2 mRNA and protein expressions were significantly decreased compared with that when ECRG2 protein was used alone

As a key transcription factor, p53 containing 393 amino acids is closely associated with the regulation of cell cycle, the repair of DNA damage, cell apoptosis and tumor suppression (Li et al., 2012; Mai et al., 2012; Umar et al., 2012; Vousden et al., 2009). As a tumor suppressor gene, p53 is the most widely mutated gene in human carcinogenesis, which plays an important role in the initiation and development of the tumor (Liu et al., 2010; Zhao et al., 2010). As a candidate biomarker, p53 is used to predict the efficacy of chemotherapy-based in patients with esophageal cancer (Zhang et al., 2013). Luteolin could induce apoptosis of Eca109 through upregulating the expression of p53 (Wang et al., 2012). It has been shown that ECRG2 could not only up-regulate p53 protein expression, but also exert some effects on transcriptional activity of p53. Transfection of ECRG2 gene could inhibit the normal growth of human esophageal cancer cells and suppress the proliferation of the cancer cells through regulation of p53 (Cui et al., 2008). In our study, RT-PCR and Western blot results demonstrated that different concentrations of ECRG2 protein could promote the expression of p53 mRNA and protein with a dose-dependent relationship. When ECRG2 was combined with DDP, p53 mRNA and protein expression were significantly increased with an increase in the concentration of ECRG2 protein compared with that when ECRG2 protein was used alone.

In this study, using ESCC cell line EC9706 cell as a model, we demonstrated that when ECRG2 was combined with DDP, the inhibitory effects of ECRG2 on induction of apoptosis and the increase in the expression of p53 were significantly stronger than that when ECRG2 protein was used alone, but bcl-2 were lower. These inhibitory effects were also increased accordingly with the increase in ECRG2 concentration. Our results suggest that the effects of ECRG2 in combination with DDP were stronger than that when ECRG2 protein was used alone. ECRG2 in combination with DDP could reduce the clinical dose of DDP, and then alleviate the toxic side effects of DDP and achieve the better curative effects.

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