A preliminary study of the effect of ECRG4 overexpression on the proliferation and apoptosis of human laryngeal cancer cells and the underlying mechanisms

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Abstract. Human esophageal cancer-related gene 4 (ECRG4) is a potential tumor suppressor gene isolated from human esophageal epithelial cells. Studies have shown that ECRG4 effectively inhibits the proliferation of tumor cells and induces apoptosis. However, the role of ECRG4 in laryngeal cancer has not yet been clearly defined. In this study, a human laryngeal cancer cell line stably overexpressing ECRG4 was established. The effect of ECRG4 on the proliferation and apoptosis of laryngeal cancer cells and the associated mechanisms were investigated. The Hep-2 human laryngeal carcinoma cell line exhibited a low basal level of ECRG4 expression and was selected for the present study. The eukaryotic expression plasmid pcDNA3.1-ECRG4 was constructed and introduced into Hep-2 cells by transfection reagents. Western blot analysis, reverse transcription-quantitative polymerase chain reaction and immunofluorescence staining confirmed high-level expression of ECRG4. The 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and colony formation assay showed that ECRG4 overexpression suppressed the proliferative capacity of laryngeal cancer cells in vitro. Cell cycle analysis showed that ECRG4 induced cell cycle arrest at the G0/G1 phase. Flow cytometric analysis and Hoechst staining demonstrated that overexpression of ECRG4 significantly induced apoptosis. Western blot analysis confirmed that Bcl-2-associated X protein, cleaved-caspase-3 and cleaved-poly (ADP-ribose) polymerase were upregulated in the apoptotic process, whereas B-cell lymphoma 2 was downregulated. In conclusion, overexpression of ECRG4 inhibited laryngeal cancer cell proliferation and induced cancer cell apoptosis. Therefore, ECRG4 exhibits potential as an effective target in gene therapy for laryngeal cancer.

Introduction

Laryngeal cancer is a type of malignancy that originates in the epithelial tissue of laryngeal mucosa. Laryngeal cancer constitutes 2.4% of all cancers. It is one of the most common malignant tumors of the head and neck region, ranking third after nasopharyngeal cancer and sinonasal cancer (1,2). With increasing industrialization and air pollution, the worldwide incidence of laryngeal cancer has shown a gradually increasing trend (3). In the last 30 years, novel surgical procedures, new chemotherapeutic agents, more advanced radiotherapy and targeted drugs have been applied in the treatment of laryngeal cancer. However, the overall survival rate of laryngeal cancer patients has shown little improvement (4). Therefore, investigation of the mechanisms underlying the proliferation and apoptosis of laryngeal cancer cells is of particular importance to the development of novel and more effective treatments for laryngeal cancer so as to reduce the rate of mortality.

Human esophageal cancer-related gene 4 (ECRG4) is a tumor suppressor gene that was initially identified and cloned from human esophageal epithelial cells in 1998. ECRG4 is widely expressed in normal human tissues (5). However, ECRG4 expression is downregulated or lost in esophageal squamous cell carcinoma tissues and cell lines (6). Studies have found that in patients with breast cancer, the ECRG4 mRNA expression level is positively correlated with the survival rate and overall survival time (7). Similar results have been obtained from patients with esophageal cancer (8) and prostate cancer (9). ECRG4 has been shown to inhibit the growth of colon cancer cells, esophageal cancer cells and glioma cells through the induction of cell cycle arrest (10-12). Knockout of the ECRG4 gene in zebrafish embryos using RNA interference technology results in an enhanced capacity
for cell proliferation (13). In addition, ECRG4 is closely associated with apoptosis in a variety of tumor cells (14-16). However, the effect of ECRG4 on the proliferation and apoptosis of laryngeal cancer cells and its mechanisms are not yet clear.

In the present study, an ECRG4-overexpressing laryngeal cancer cell line was obtained after G418 screening. The effect of ECRG4 overexpression on the proliferation and apoptosis of laryngeal carcinoma cells was assessed.

**Materials and methods**

*Cell lines.* Hep-2 and LSC-1 human laryngeal cancer cell lines (Wanleibio, Shenyang, China) were cultured in RPMI-1640 medium ( Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, HyClone, Logan, UT, USA). The cells were passaged by trypsinization when they reached 80-90% confluence.

**Construction of the ECRG4overexpression vector and selection of the stably transfected cell line.** The following primers were designed for amplification of the coding region of the ECRG4 gene: ECRG4-F, 5'-ATAAGAAGTCTGCCCCTCGCCCTC-3' (HindIII restriction site underlined); and ECRG4-R, 5'-CCGGATCTCCAGAAAACCGTACTG-3' (BamHI restriction site underlined). The human cDNA preserved in the laboratory was utilized as the template. The amplified ECRG4 gene was ligated into the pcDNA3.1 plasmid (Invitrogen Life Technologies, Carlsbad, CA, USA). The recombinant plasmid, pcDNA3.1-ECRG4, was digested with HindIII and BamHI, according to the manufacturer’s instructions (Fermentas, Ontario, Canada). The recombinant plasmid was sequence-analyzed by Sangon Biotech (Shanghai, China). After the double restriction analysis and sequence analysis, the correct recombinant plasmid was termed pcDNA3.1-ECRG4. Logarithmically growing Hep-2 cells were seeded into 6-well plates. Upon reaching ~80% confluence, the cells were transfected with pcDNA3.1-ECRG4 or negative control (pcDNA3.1 plasmid; Invitrogen Life Technologies) using Attractene Transfection Reagent (Qiagen, Shanghai, China) according to the manufacturer’s instructions. At 24 h after transfection, the cells were subjected to G418 selection (400 µg/ml, Invitrogen Life Technologies). After 7-14 days, positive clones were selected and ECRG4 expression was examined.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted from all groups of cells using the total RNA extraction kit (Tiangen Biotech, Beijing, China) according to the manufacturer’s instructions. Total RNA was reverse transcribed into cDNA and then subjected to quantitative fluorescence analysis on the Exicycler™ 96 quantitative fluorescence analyzer (Bioneer, Daejeon, Korea) using the SYBR Green MasterMix (Solarbio, Beijing, China). RT-qPCR was performed in a volume of 20 µl, containing 0.5 µl each primer (10 µM), 1 µl cDNA template, 10 µl SYBR GREEN master mix and 8 µl ddH2O. Thermal cycling conditions were as follows: Initial denaturation at 95°C for 10 min; amplification for 40 cycles of 95°C for 10 sec, 60°C for 20 sec and 72°C for 30 sec. The mixture was then cooled at 4°C for 5 min. The internal control was β-actin and each sample was tested in triplicate. The 2^ΔΔCt method (17) was used for relative quantification of gene expression. The sequences of the primers are listed in Table I.

**Western blot analysis.** The total protein was extracted from all groups of cells, and the protein concentration was determined using the Bichinonic Acid Protein Assay kit (Beyotime Institute of Biotechnology, Haimen, China). Equal quantities of protein were loaded onto each lane, separated using 10 or 13% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were incubated first with the following primary antibodies: Rabbit anti-human anti-ECRG4 polyclonal antibody (1:200; cat. no. sc-135139; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), rabbit anti-human anti-cleaved-caspase-3 polyclonal antibody (1:500; cat. no. bs-0081R; Bioss, Beijing, China), rabbit anti-human anti-cleaved-poly ADP-ribose polymerase (PARP) polyclonal antibody (1:200; cat. no. sc-23461-R; Santa Cruz Biotechnology, Inc.), rabbit anti-human anti-Bcl-2-associated X protein (Bax) polyclonal antibody (1:400; cat. no. BA0315; Boster, Wuhan, China) and rabbit anti-human anti-B-cell lymphoma 2 (Bcl-2) polyclonal antibody (1:400; cat. no. BA0412; Boster) at 4°C overnight. The membranes were washed four times with Tween-20 in Tris-buffered saline for 5 min. They were then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5,000 dilution, Beyotime Institute of Biotechnology) for 45 min at 37°C. After addition of the enhanced chemiluminescence (ECL) reagents (Qihai Biotec, Shanghai, China), the target proteins were visualized and scanned using Gel-Pro Analyzer software 4.0 (Media, Cybernetics, Inc., Bethesda, MD, USA). The detected proteins were normalized to β-actin.

**Immunofluorescence staining.** Cells grown on coverslips were fixed in 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 for 30 min and incubated with rabbit anti-human anti-ECRG4 polyclonal antibody (1:100; cat. no. sc-135139; Santa Cruz Biotechnology, Inc.) at 4°C overnight. The cells were then incubated with Cy3-labeled goat anti-rabbit secondary antibody (1:100 dilution; Beyotime Institute of Biotechnology) for 1 h at room temperature. 4',6-Diamidino-2-phenylindole (Bioshop, Heifei, China) was added drop-wise to completely cover the cells for nuclei staining. The coverslip with cells was inversely placed and mounted on a slide with anti-fluorescence quenching agent

| Primer name | Sequence (5'-3') |
|-------------|-----------------|
| ECRG4-F     | AACGAGAAGCACCTGTCCAAA |
| ECRG4-R     | TCGCCATAGTTATCTGTCCA |
| β-actin-F   | CCATCGTCACCGCAAAAT |
| β-actin-R   | GCTGTACCTTCACCGTTCA |

ECRG4, esophageal cancer-related gene 4; F, forward; R, reverse.
Figure 1. Selection of cell lines. The expression levels of ECRG4 in Hep-2 and LSC-1 human laryngeal cancer cell lines were examined by western blot analysis. Grayscale analysis was performed using β-actin as the internal control. Experimental data are expressed as the mean ± standard deviation. *P<0.05, compared with Hep-2 cells. ECRG4, human esophageal cancer-related gene 4.

(Solarbio). The staining results were observed under a laser scanning confocal microscope and imaged (FV1000S-SIM/IX81; Olympus, Tokyo, Japan).

Examination of cell proliferation using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells from each experimental group were plated in 96-well plates at a density of 2x10^4 cells/well. Five replica wells were set up for each experimental group. Blank control wells were also included. At days 0, 1, 2, 3 and 4 after cell incubation, MTT solution (final concentration of 0.2 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) was added to each well containing cells. After incubation at 37°C for 5 h, the supernatant was removed, and 200 µl dimethylsulfoxide (Sigma-Aldrich) was added to each well to dissolve the purple crystals. The optical density at 490 nm (OD_{490}) was determined using a microplate reader (ELX-800; Bio-TEK Instruments Inc, Winooski, VT, USA), and cell growth curves were constructed.

Colony formation assay. Cells from each group were seeded at ~300 cells per 60 mm Petri dish. The Petri dishes were incubated at 37°C and 5% CO₂ until visible colonies were formed. The colonies were fixed in 4% paraformaldehyde, stained with Wright-Giemsa staining reagent (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and observed under a microscope (AE31; Motic Electric, Xiamen, China). A cell cluster containing at least 50 cells was counted as a colony. The colony formation rate was calculated based on the following formula: Colony formation rate (%) = (number of colonies/number of seeded cells) x 100.

Analysis of the cell cycle by flow cytometry. Cells were trypsinized, harvested and resuspended in 500 µl Binding Buffer according to the instructions of the apoptosis detection kit (WanleiBio). A total of 5 µl Annexin V-fluorescein isothiocyanate was added to the cell suspension and mixed thoroughly. Subsequently, 5 µl PI was added. After incubation for 15 min at room temperature in the dark, the cells were analyzed by flow cytometry (FACSCalibur; Becton, Dickinson and Company) for determination of apoptosis rate.

Statistical analysis. Experimental data are expressed as the mean ± standard deviation. Comparisons between the experimental groups were conducted using one-way analysis of variance. Multiple comparisons were conducted using the Bonferroni post hoc test. Data analysis and image processing were performed using the Graphpad Prism 5.0 software (GraphPad Software, Inc., San Diego, CA, USA). *P<0.05 was considered to indicate a statistically significant difference.

Results

Selection of cell lines. To select a suitable human laryngeal cancer cell line for stable transfection and subsequent ECRG4 gene-related experiment, the total protein from Hep-2 and LSC-1 cells was extracted. The expression levels of ECRG4 in the two cell lines were examined using western blot analysis. The results showed that the ECRG4 expression level in Hep-2 cells was significantly lower than that in LSC-1 cells (Fig. 1, P<0.05). To eliminate the effect of basal expression of ECRG4, Hep-2 cells were selected for subsequent experiments.

Establishment and identification of the cell line stably overexpressing ECRG4. To investigate the function of the ECRG4 gene, pcDNA3.1-ECRG4 was transfected into Hep-2 cells, and ECRG4 expression in positive cells was analyzed by western blot analysis and RT-qPCR. Cells transfected with empty pcDNA3.1 vector and parental cells were used as controls.
The results showed that the expression levels of ECRG4 protein and mRNA in the pcDNA3.1-ECRG4 group were increased by 3.05 (Fig. 2A, P<0.01) and 3.07-fold (Fig. 2B, P<0.01), respectively, compared with the pcDNA3.1 group. The immunofluorescence staining results showed that ECRG4 expression was obviously elevated in the pcDNA3.1-ECRG4 group compared with the other two groups (Fig. 2C), which were consistent with the western blot analysis and RT-qPCR results. Thus, a laryngeal cancer cell line stably overexpressing ECRG4 was established.

**ECRG4 overexpression inhibits the proliferation of laryngeal cancer cells.** To investigate the effect of ECRG4 overexpression on the proliferative capability of laryngeal cancer cells, cell proliferative capability was examined using the MTT assay and colony formation assay. The results of the MTT assay showed that at day 2, day 3 and day 4, the proliferative capability was severely impaired in the pcDNA3.1-ECRG4 group compared with the pcDNA3.1 group (Fig. 3A; day 2, P<0.05; days 3 and 4, P<0.01). The effect of ECRG4 on the clonogenic capacity of laryngeal cancer cells was further examined using the colony formation assay. The results showed that the colony formation rate in the pcDNA3.1-ECRG4 group was 45.27±7.19%, which was lower than that in the pcDNA3.1 group (83.07±7.51%). The results indicated that ECRG4 significantly reduced the colony formation ability of laryngeal cancer cells (Fig. 3B, P<0.01). This study further investigated the cell-cycle phase distribution in all three groups of cells using flow cytometry. As shown in Fig. 3C, the percentage of cells in the G0/G1 phase was significantly increased in the pcDNA3.1-ECRG4 group compared with that in the pcDNA3.1 group (71.7 vs. 39.41%, P<0.01). By contrast, the percentage of cells in the S phase and G2/M phase were decreased markedly in the pcDNA3.1-ECRG4 group (S phase, 20.14 vs. 44.83%, P<0.01; and G2/M phase, 8.17 vs. 15.76%, P<0.05, respectively). These results indicated that overexpression of ECRG4 inhibited laryngeal cancer cell proliferation and arrested cells in the G0/G1 phase of the cell cycle.

**Overexpression of ECRG4 effectively induces apoptosis in laryngeal cancer cells.** Cell apoptosis was measured by flow cytometry and fluorescence microscopy using Annexin V/PI and Hoechst staining, as well as the analysis of the expression of apoptosis-related factors. The results of flow cytometric analysis showed that the apoptotic rate was significantly elevated in the pcDNA3.1-ECRG4 group compared with the pcDNA3.1 group (19.37±0.67 vs. 0.66±0.09%; Fig. 4A, P<0.01). Hoechst staining showed that compared with cells in the parental group and the pcDNA3.1 group, cells in the pcDNA3.1-ECRG4 group exhibited significantly increased chromatin condensation and more densely stained nuclei (Fig. 4B). To determine whether ECRG4-induced apoptosis of laryngeal cancer cells affected the expression of apoptosis-related factors, western blot analysis was performed to examine the expression of Bax, Bcl-2, cleaved-caspase-3 and cleaved-PARP. The results showed that the expression levels of
cleaved-PARP, cleaved-caspase-3 and Bax were significantly elevated in cells from the pcDNA3.1-ECRG4 group compared with that from the pcDNA3.1 group (Fig. 4C, P<0.01). By contrast, the expression level of Bcl-2 was markedly decreased (P<0.01). These results demonstrated that overexpression of ECRG4 significantly induced apoptosis in laryngeal cancer cells.

Discussion

ECRG4 is expressed at low or undetectable levels in a variety of malignant tumor tissues and cell lines. The expression level of ECRG4 is closely associated with tumor proliferation and apoptosis. However, the role of ECRG4 in laryngeal cancer has not been reported. In the present study, a laryngeal cancer cell line stably overexpressing ECRG4 was established. This study found that upregulation of ECRG4 induced cell cycle arrest and inhibited laryngeal cancer cell proliferation. In addition, upregulation of ECRG4 accelerated apoptosis in laryngeal cancer cells by regulating apoptosis-related factor expression. This study preliminarily clarified the role of ECRG4 in the proliferation and apoptosis of laryngeal cancer cells and its mechanisms during apoptosis.

The Hep-2 and LSC-1 cells were selected from the available human laryngeal cancer cell lines and the expression levels of ECRG4 were compared in the two cell lines. The results showed that the ECRG4 expression level in Hep-2 cells was significantly lower than that in LSC-1 cells. As high basal ECRG4 expression would interfere with subsequent experiments, Hep-2 cells were selected for further experiments.

Attenuated ECRG4 expression levels have been confirmed in esophageal squamous cell carcinoma (8), prostate cancer (9), colon cancer and glioma (10), while enhanced expression levels of ECRG4 were detected in normal tissues. ECRG4
efficiently inhibits the growth of colon cancer cells, glioma cells and esophageal cancer cells. ECRG4 induces cell cycle arrest at the G0/G1 phase (10-12), which is hypothesized to be the key determinant that leads to proliferation inhibition in tumor cells (18-20). Based on the above findings, this study investigated the impact of ECRG4 on the proliferation and cell cycle of the human laryngeal cancer cells. The results showed that overexpression of ECRG4 significantly inhibited laryngeal cancer cells proliferation. Further cell cycle analyses by flow cytometry revealed that ECRG4 overexpression induced G0/G1 cell cycle arrest. The results demonstrated that ECRG4 inhibited the growth of laryngeal cancer cells through arresting cells in the G0/G1 phase and delaying cell cycle progression from the G0/G1 phase to the S phase and G2/M phase. The present results are consistent with the findings of previous studies (11,12).

Apoptosis can be initiated through the death receptor- or the mitochondria-dependent pathways (21). Stimulated by apoptotic signals, the proapoptotic member of the Bcl-2 family, Bax, undergoes a conformational change in the mitochondrial pathway of apoptosis. Bax translocates from the cytoplasm to the mitochondria and inserts into the mitochondrial membrane, which induces an increase in mitochondrial membrane permeability and results in the release of cytochrome c. Cytochrome c then activates caspase-3, thereby inducing apoptosis (22). The level of Bcl-2, an important antiapoptotic protein, is correlated with tumor cell apoptosis. Bcl-2 is overexpressed in a variety of tumor cells, which conveys a certain degree of resistance to apoptosis-inducing drugs (23-25). Additionally, downregulation of Bcl-2 abolishes the resistance and promotes apoptosis in tumor cells (26,27). The caspase family of proteases has been shown to exhibit a critical role in apoptosis (28). Caspase-3 is a member of the caspase family and a key protease in apoptosis. Once activated, caspase-3 triggers the activation of the downstream proteins and inevitably leads to apoptosis. Therefore, caspase-3 is known as the death protease (29). Caspase-3 is normally present in the cytoplasm in the form of an inactive zymogen. Apoptotic signals induce caspase-3 cleavage and activation through a variety of proteolytic enzymes, resulting in the generation of cleaved-caspase-3.

PARP, the substrate of caspase-3, is activated and subsequently induces apoptosis (30-32). Studies have shown that ECRG4 effectively induces apoptosis in esophageal squamous cell carcinoma cells (14), head and neck squamous cell carcinoma
cells (15) and gastric cancer cells (16); accompanied by upregulation of Bax and downregulation of Bcl-2 (15). This study further investigated whether ECRG4 overexpression induced apoptosis in human laryngeal cancer cells and examined the expression levels of a number of key apoptosis-related factors. The results of this study also demonstrated that overexpression of ECRG4 activated caspase-3 and PARP, and ultimately induced apoptosis through upregulating the expression of proapoptotic protein Bax and downregulating the expression of antiapoptotic protein Bcl-2.

In conclusion, ECRG4 suppresses the proliferation of laryngeal cancer cells through the induction of G0/G1 cell cycle arrest. In addition, ECRG4 induces apoptosis via regulation of the expression of Bax, Bcl-2, cleaved-caspase-3 and cleaved-PARP. Therefore, overexpression of ECRG4 may become an effective gene therapy strategy for the treatment of laryngeal cancer.

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