

RESEARCH ARTICLE

RNAi-induced K-Ras Gene Silencing Suppresses Growth of EC9706 Cells and Enhances Chemotherapy Sensitivity of Esophageal Cancer

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Abstract

To analyze the growth, proliferation, apoptosis, invasiveness and chemotherapy sensitivity of EC9706 cells after K-Ras gene silencing, an expression carrier pSilencer-siK-Ras was constructed, and the EC9706 cell line was transfected using a liposome technique. Six groups were established: Control, siRNA NC (transfected with empty vector pSilencer2.1); Ras siRNA (transfected with pSilencer-siK-Ras2); Paclitaxel; Paclitaxel + siRNA NC; and Ras siRNA + Paclitaxel. After the treatment, RT-PCR, Western blotting, MTT assay, flow cytometry and the Transwell technique were used to assess expression of K-Ras mRNA and protein in EC9706 cells, as well as cell growth, proliferation, apoptosis and invasiveness. The effect of Paclitaxel chemotherapy was also tested. pSilencer-siK-Ras2 effectively down-regulated expression of K-Ras mRNA and protein in EC9706 cells, growth being significantly inhibited. Flow cytometry indicated obvious apoptosis of cells in the experimental group, with arrest in the G1 phase; cell migration ability was also reduced. After pSilencer-siK-Ras2 transfection or the addition of Paclitaxel, EC9706 cells were suppressed to different extents; the suppressive effect was strengthened by combined treatment. The results suggested that RNAi-induced K-Ras gene silencing could enhance chemotherapy sensitivity of esophageal cancer.

Keywords: RNAi - K-Ras - esophageal cancer - paclitaxel - chemotherapy sensitivity

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Introduction

Esophageal cancer is one of the most common malignant tumors, with mortality accounting for about a quarter of that of all malignant tumors (Tanaka et al., 2010). At present, surgery and chemoradiotherapy are the two major treatments for esophageal cancer. Multiple drug resistance, recurrence and serious adverse effects after chemoradiotherapy are the main reasons causing for the therapeutic side effect and high death rate of esophageal cancer. Identification of a target for gene therapy, which is a new technique for treating esophageal cancer, has been becoming more and more important. Extensive researches have shown that the K-Ras gene is abnormally expressed in esophageal cancer tissues. This had been confirmed before our research (Wang et al., 2011). Mutation of K-Ras gene is closely related to carcinogenesis (Tanaka et al., 2010). A lot of researches on the association of K-Ras gene expression with esophageal cancer had been carried out in esophageal cancer tissues and cells in in vitro and animal experiments. However, little had been reported about the application of RNAi to silence K-Ras gene expression in esophageal cancer.

In this study, we used RNAi to silence high expression of K-Ras gene in human esophageal cancer EC9706 cells.

Then, we studied the growth, proliferation, apoptosis and invasiveness of EC9706 cells, as well as its biological changes and chemotherapy sensitivity. We attempted to clarify the role of the K-Ras gene in chemotherapy resistance in esophageal cancer and look for new methods to reverse drug resistance, which might provide a new route for gene therapy of esophageal cancer.

Materials and Methods

Cells

Human esophageal cancer cells line EC9706 was stored in our laboratory (Henan Provincial Key Laboratory of Tumor Pathology, China), and was cultured at 37°C in an atmosphere consisting of 5% CO₂ in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, USA).

Plasmids

Silencers were also stored in our laboratory. Three shRNA sequences were designed on the Ambion web site. The shRNA oligonucleotides were used as following: siK-Ras1-Top: 5'-GATCCGAGTGCCTTGACGATACAGTTCAAGAGACTGTATCGTCAAGGCACTCTTTTTGGAAA-3', siK-Ras1-Bot: 5'-AGCTTTTCCAAA

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AAAGAGTGCCTTGACGATACAGTCTCTTGAAGTGTATCGTCAAGGCACTCG-3', siK-Ras2-Top:5'-GATCCACTTGTGGTAGTTGGAGCTTTCAAGAGAAGCTCCAACCTACCACAAGTTTTTTTTGGAAA-3', siK-Ras22-Bot:5'-AGCTTTTCCAAAAAACTTGTGGTAGTTGGAGCTTCTCTTGAAAGCTCCAACCTACCACAAGTG-3', siK-Ras3-Top:5'-GATCCGGACTCTGAAGATGTACCTTTCAAGAGAAGGTACATCTTCAGAGTCCTTTTTTGGAAA-3', siK-Ras33-Bot:5'-AGCTTTTCCAAAAAAGGACTCTGAAGATGTACCTTCTCTTGAAAGGTACATCTTCAGAGTCCG-3'.

The corresponding shRNA fragments were denatured, annealed and ligated with pSilencer2.1 vector digested by Bam HI and Hind III, which yielded pSilencer-siK-Ras1, pSilencer-siK-Ras2 and pSilencer-siK-Ras3. Double-enzyme digestion was performed using Bam HI and Hind III and sequenced to screen out the correct recombinant plasmids.

Transfection, RNA and protein extraction

2.5×10⁵ EC9706 cells were subscribed in 6 wells plates. Transfections were performed 24 h later. Briefly, 5µg plasmid DNA (pSilencer2.1, pSilencer-siK-Ras1, pSilencer-siK-Ras2 and pSilencer-siK-Ras3, respectively) was added to 240 µl Opti-MEM serum-free medium. Also, 10µl Lipofectamine 2000 was added to another 240µl of Opti-MEM serum-free medium, then stranded for 5 min at room temperature. The plasmid DNA and Lipofectamine were mixed together and left to stand for 20 min at room temperature. The medium were changed 6 h later. These cells were cultured for 72 h. Total RNA was extracted by using the trizol-based technique, and the protein were lysed by RIPA lysis buffer and PSMF.

K-Ras expression analysis by Real-time PCR and western blot

The total RNA was used for reversing transcription by using M-MLV reverse transcriptase (Promega) and other reagents in 20µl. 1.5µg RNA, 1µl 100µM Oligo (dT) 15, and 13µl DEPC double distilled water were added in at 65°C for 10 min and 25°C for 5 min, following by 2 min on ice; then 41µl AMV RT 5× buffer, 11µl 10 mM dNTPs, 11µl RNase Inhibitor and 11µl M-MLV were subsequently added in at 42°C for 30 min and 70°C for 10 min. RT product (0.5, 1µl) was used for real-time quantitative PCR using SYBR® Premix Ex Taq TM (TaKaRa). The Q-PCR primer was designed: RAS-qPCR-f, 5'-GTGCCCTTGACGATACAGC-3'; RAS-qPCR-r, 5'-CACAAAGAAAGCCCTCCC-3', β-actin-f, 5'-CGTGACATTAAGGAGAAGCTG-3', β-actin-r, 5'-CTAGAAGCATTGCGGTGGAC-3'. The real time PCR was performed: 94°C for 4 min, [94°C, 30 s; 50°C, 30 s; 72°C, 40s] for 40 cycles.

Relative expression level of mRNA was calculated by 2^{-ΔΔCt}. After the transfection, the cells were disrupted and protein was collected. Cell lysates were separated by 10% SDS-PAGE and then transferred to nitrocellulose membranes. The level of Ras and β-Tubulin was treated with t anti-RAS and anti-β-Tubulin (Santa Cruz, CA, USA) respectively, followed by incubation with a peroxidase conjugated secondary antibody (Santa Cruz,

CA, USA). Finally, reactivity was visualized by Western Lightning® Plus-ECL system.

Cell proliferation measurement by MTT assay

EC9706 cells were divided into 6 groups: control, siRNA NC (transfected with pSilencer2.1); Ras siRNA (transfected with pSilencer-siK-Ras2); Paclitaxel; Paclitaxel+siRNA NC; and Paclitaxel + Ras siRNA. Cells were subscribed into 6-well plates by 3×10⁵ cells/well. Before transfection, cells reached 60-80% confluence. 5µg of plasmid pSilencer-siK-Ras2 was added to 240 µl Opti-MEM serum-free medium. Also, 10µl Lipofectamine 2000 was added to another 240µl Opti-MEM serum-free medium; the mixtures were mixed well and stand for 5 min at room temperature. The plasmid DNA and Lipofectamine were mixed together and left to stand for 20 min at room temperature. 6 hours after the transfection, the medium were changed and the final concentration of 0.1 µg/ml Paclitaxel was added in. The cells of each group were spread into 96-well plates after 24 h. MTT assay was performed for each group of cells at 0, 24, 48, 72, 96 and 120 h after the treatment. The supernatant of each group culture medium was discarded, and 100 µl DMSO was added to each well to terminate the reaction. A spectrophotometer was used to measure OD490 for each well.

Cell cycle measurement by flow cytometry

48 h after the transfection and 24 h after addition of Paclitaxel (0.1 µg/ml), cells were trypsinized and transferred into a 1.5ml centrifuge tube. The cell suspension was added dropwisely into 3.5 ml pre-cooled 95% ethanol at -20°C while the tube was shaken, and then the solution was stored at 4°C. On the day of flow cytometry, 0.5 ml PI pigment (containing RNase) was added to each group of cells, which were transferred to a special detector tube for flow cytometry.

Transwell assay

The invasive potential of the cells was assessed by transwell assay after the transfection and treatment of Paclitaxel. Cells, transfected by the plasmid of pSilencer-K-Ras2 for 48 h and added Paclitaxel (0.1 µg/ml) for 24h, were harvested by trypsinization and 5 × 10⁴ cells were placed onto the top insert. One chamber consists of a cell insert and a well. The bottom of the cell insert is covered with a filter containing multiple 8-µm pores and is coated with a basement membrane matrix (Matrigel).

Cells in 500 µl of serum-free DMEM media were seeded in the cell insert and placed in the well, which was filled with 750 µl of DMEM supplemented with 10% fetal bovine serum. After 24 h of incubation at 37°C and 5% CO₂, the noninvasive cells presenting on the upper surface of the filter were removed by using a sterile cotton swab. The cells migrating through the Matrigel onto the lower surface of the filter were fixed and stained. The number of the invasion cells was counted.

Statistical analysis

SPSS version 10.0 was used for statistical analysis. A t test was performed on measurement data, and a χ² test on numerical data.

Table 1. Comparison of Cell Cycle and Apoptosis Rate after Transfection

Group	Diploid	%CV	G1/G0	S	G2/M	Apoptosis rate
Control	100%	4.54±0.23	56.91±3.50	38.81±2.10	4.28±0.17	2.57±0.21%
siRNA NC		4.02±0.21	57.10±3.12	39.55±2.11	3.35±0.20	2.73±0.15%
Ras siRNA		4.16±0.28	62.18±4.01	36.06±2.44	1.76±0.19	8.22±0.35%
Paclitaxel		3.86±0.30	60.56±3.81	30.17±1.97	9.27±1.66	69.8±4.75%
SiRNA NC/paclitaxel		4.01±0.26	61.36±3.22	36.17±2.31	2.47±0.28	71.6±5.77%
Ras siRNA /paclitaxel		3.88±0.26	64.20±3.45	28.42±1.66	7.38±1.10	87.0±3.97%

The control group was without any treatment, the siRNA NC was transfected with the plasmid of pSilencer, the RAS-siRNA group was transfected with the plasmid of pSilencer-siK-Ras2, paclitaxel group was treated with 0.1µg/ml paclitaxel, siRNA NC/paclitaxel group was treated with 0.1µg/ml paclitaxel after transfection of pSilence, Ras-siRNA/paclitaxel group was treated with 0.1µg/ml paclitaxel after transfection of pSilence-siK-Ras2

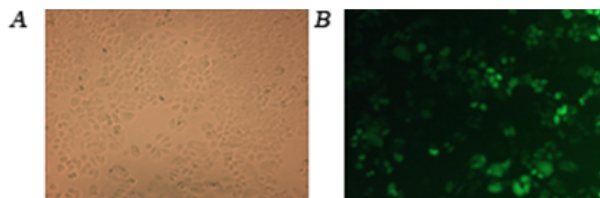


Figure 1. Images for Cell Transfection Efficiency Measurement. A: Bright field (×400), B: Fluorescence field (×400)

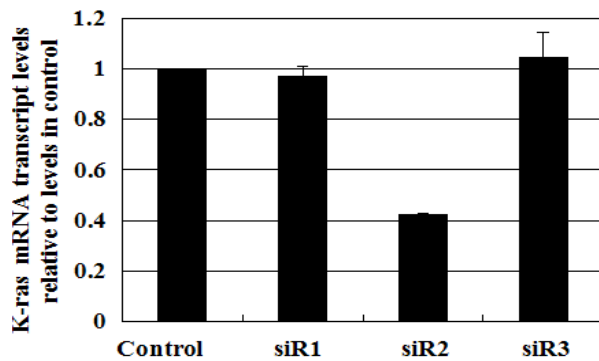


Figure 2. Real-time Quantitative PCR for mRNA of K-Ras. The control, siR1 siR2, and siR3 were transfected with the plasmid of pSilencer2.1, pSilencer-siK-Ras1, pSilencer-siK-Ras2, pSilencer-siK-Ras3, respectively. The mRNA of K-Ras were analysis by Q-PCR. The quantity of mRNA were quantificated with the relative of the mRNA of K-Ras and β-actin

Results

Transfection efficiency for EC9706 cells

48 h after the transfection EC9706 cells with pSilencer-siK-Ras, the cells were observed under a light microscope. The adherent cell population reached 80%, and was fluorescent green. Figure 1 shows that the transfection rate was >90%.

Measurement of RAS gene silencing efficiency

48 h after the transfection with pSilencer-siK-Ras, total RNA and total protein were extracted from transfected EC9706 cells for real-time PCR and Western blotting. Expression of RAS gene in the three groups of cells was suppressed to different extents.

Expression was most significantly suppressed in cells transfected with pSilencer-siK-Ras-2, and the suppression efficiency reached 60±5% (P<0.05) (Figure 2 and 3). Figure 3 showed that protein expression of the K-Ras in the Silencer-siK-Ras-2 group was significantly suppressed.

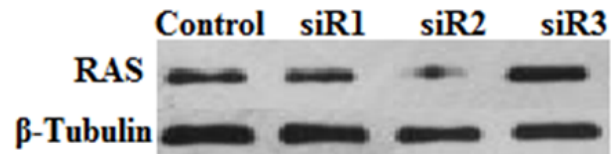


Figure 3. Western Blot Analysis of K-Ras Protein Expression after Transfection with Ras-siRNA Plasmid. The control, siR1 siR2, and siR3 were transfected with the plasmid of pSilencer2.1, pSilencer-siK-Ras1, pSilencer-siK-Ras2, pSilencer-siK-Ras3, respectively. The total protein was analysis by western bolt, the β-Tubulin is the internal control

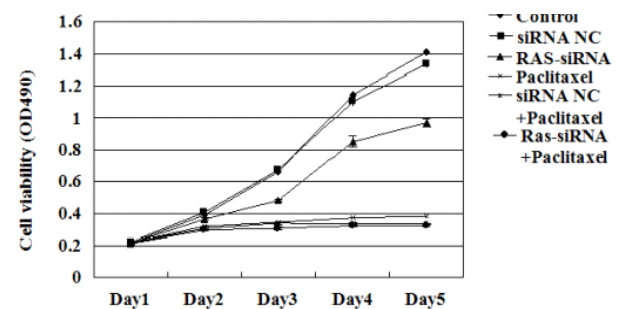


Figure 4. Proliferation of Each Group of Cells Transfection and Treated by Paclitaxel. The control group was cell without any treatment, the siRNA NC was transfected with the plasmid of pSilencer2.1, the RAS-siRNA group was transfected with the plasmid of pSilencer-siK-Ras2, paclitaxel group was treated with 0.1µg/ml paclitaxel, siRNA NC+ paclitaxel group was treated with 0.1µg/ml paclitaxel after transfection of pSilence2.1, Ras-siRNA+ paclitaxel group was treated with 0.1µg/ml paclitaxel after transfection of pSilence-siK-Ras2

Impact of siK-Ras and Paclitaxel on cell proliferation

Suppression of RAS gene expression led to significant cell growth inhibition. Combination of Paclitaxel and Ras siRNA suppressed cell growth more greater extent than the group of siRNA or Paclitaxel alone. Cell activity was significantly reduced compared with the control group (Figure 4).

Impact of Ras- siRNA transfection on apoptosis and cell cycle

Transfection of pSilencer-siK-Ras2 increased apoptosis in the RAS siRNA and Paclitaxel groups compared with the control group. After addition of Paclitaxel, the RAS siRNA transfection group had a higher rate of apoptosis than the other groups, and cell cycle was also affected. Compared with the control group and siRNA NC group, cells in G0/G1 phase constituted a higher proportion, whereas cells in S or G2 phase constituted a lower

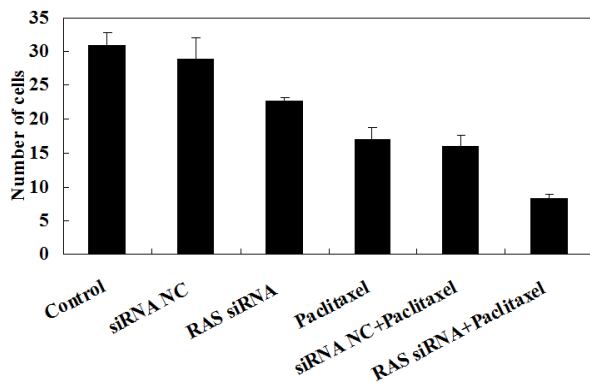


Figure 5. Comparison of in Vitro Invasiveness of Transfected Cells in Each Group. The control group was cell without any treatment, the siRNA NC was transfected with the plasmid of pSilencer, the RAS-siRNA group was transfected with the plasmid of pSilencer-siK-Ras2, paclitaxel group was treated with 0.1 μ g/ml paclitaxel, siRNA NC+ paclitaxel group was treated with 0.1 μ g/ml paclitaxel after transfection of pSilence, Ras-siRNA+ paclitaxel group was treated with 0.1 μ g/ml paclitaxel after transfection of pSilence-siK-Ras2

proportion. Cells in the RAS siRNA or Paclitaxel group were mostly arrested at G1 phase. With the combination of Paclitaxel and Ras-siRNA, more cells were arrested at G1 phase (Table 1).

Invasiveness of transfected cells

PSilencer-Ras transfection decreased the invasiveness of the RAS siRNA and Paclitaxel groups, compared with the negative control and blank groups. With the combination of RAS siRNA and Paclitaxel, the decrease in invasiveness was more significant (Figure 5).

Discussion

Mutation in the Ras gene is closely associated with tumorigenesis. The K-Ras gene in the Ras gene family is attracting particular attention because of its high mutation rate. The Ras pathway is a highly conservative signaling pathway, which mediates cell differentiation, proliferation, survival and oncogenic transformation by stimulating various signaling pathways under the action of effector molecules (Song et al., 2005; Cong et al., 2006; Yoo et al., 2006). As an oncogene, Ras induces cancer once it is activated. It has been demonstrated that K-Ras expression is higher in tissues of esophageal and gastric cancer than in paracancerous tissues (Cong et al., 2006; Lo et al., 2007; Zhang et al., 2007; Chen et al., 2008; Schimanski et al., 2010). Some researchers had reported that K-Ras gene mutation was usually identified in some endodermal tissues, such as pancreas, lung, colon and rectum (Quinlan et al., 2008; Li et al., 2009; Pu et al., 2011). Shen et al. (2008) had identified a point mutation at codon 12 (GGTGT) in the K-Ras gene. K-Ras antisense oligonucleotide could prevent proliferation and induce apoptosis of Patu8988 cells. In colorectal cancer, K-Ras gene mutation is more frequent than mutation of other genes in the Ras family (Akkiprik et al., 2008). Riely et al. (2009) had found that when K-Ras was positively expressed, chemotherapy sensitivity would increase, and a better clinical effect of adjuvant chemotherapy

and higher survival rate were obtained in patients with non-small cell lung cancer (Leung et al., 2005; Bivona et al., 2006; Wang et al., 2007; Stella et al., 2009; Zhong 2010). This convincingly demonstrates that the K-Ras gene is a molecular marker for various tumors, especially gastrointestinal cancer. This finding is of significance for the auxiliary diagnosis, treatment and prognosis of tumors.

Using RNAi, we transfected EC9706 esophageal cancer cells with K-Ras siRNA and treated with Paclitaxel, to silence the K-Ras gene and down-regulate its expression. Then, the role of the Ras gene in growth, apoptosis and chemotherapy sensitivity of esophageal cancer cells was investigated. It showed that, 48 h after pSilencer-siK-Ras2 transfection, EC9706 cell cycle was arrested; the cells grew at a slower rate, with reduced proliferation, increased apoptosis rate, and lowered in vitro invasiveness. These effects were especially obvious in the Ras siRNA + Paclitaxel group. The cell cycle of EC9706 cells was also affected. A greater proportion of cells were arrested at G0/G1 phase, and fewer at S/G2 phase. The effects were more obvious in the Ras siRNA/Paclitaxel group than the control group. In all the experimental groups, especially the Ras RNAi + Paclitaxel group, the effects were significantly changed after the addition of Paclitaxel. This indicated that, after RNAi-induced K-Ras gene silencing, the growth and proliferation of EC 9706 cells was inhibited, with greater apoptosis and reduced invasiveness. Moreover, chemotherapy sensitivity to Paclitaxel was improved, and the effect was more significant with the combined use of Paclitaxel. Our results indicated that Ras gene silencing not only significantly reduced cell activity but also resolved or reversed the problem of drug resistance in chemotherapy of esophageal cancer. Our results are useful for further improvements of chemotherapy.

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