JTCM 中医杂志 Journal of Traditional Chinese Medicine

Online Submissions: http://www.journaltcm.com
info@journaltcm.com

J Tradit Chin Med 2013 June 15; 33(3): 361-366
ISSN 0255-2922

EXPERIMENTAL STUDY

Effect of blocking Ras signaling pathway with K-Ras siRNA on apoptosis in esophageal squamous carcinoma cells

Xinjie Wang, Yuling Zheng, Qingxia Fan, Xudong Zhang

Xinjie Wang, Department of Integrated Chinese and Western Medicine, the First Affiliated Hospital, Zhengzhou University, Zhengzhou 450052, China

Yuling Zheng, Qingxia Fan, Xudong Zhang, Department of Tumor, the First Affiliated Hospital, Zhengzhou University, Zhengzhou 450052, China

Supported by the Natural Science Research Program of the Education Department of Henan Province (No. 2011B320012)

Correspondence to: Prof. Yuling Zheng, Department of Tumor, the First Affiliated Hospital, Zhengzhou University, Zhengzhou 450052, China. zhengyl@zzu.edu.cn

Telephone: +86-13503817159

Accepted: September 14, 2012

Abstract

OBJECTIVE: To study the effect of RNAi silencing of the K-Ras gene on Ras signal pathway activity in EC9706 esophageal cancer cells.

METHODS: EC9706 cells were treated in the following six groups: blank group (no transfection), negative control group (transfection no-carrier), transfection group (transfected with pSilencer-siK-ras), taxol chemotherapy group, taxol chemotherapy plus no-carrier group, taxol chemotherapy plus transfection group. Immunocytochemistry, Reverse transcription-polymerase chain reaction and western blotting were used to analyze the expression of MAPK1 (mitogen-activated protein kinases 1) and cyclin D1 in response to siRNA (small interfering RNA) transfection and taxol treatment.

RESULTS: K-Ras (K-Ras gene) siRNA transfection of EC9706 esophageal squamous carcinoma cells decreased the expression of K-Ras, MAPK1 and cyclin D1 at the mRNA and protein level. Reverse transcription-polymerase chain reaction indicated that the expression levels of MAPK1 and cyclin D1 mRNAs were significantly lower in the transfection group than in the blank group ($P<0.05$). Western blotting showed that 72 h after EC9706 cell transfection, the expression levels of MAPK1 and cyclin D1 proteins had decreased in all groups, and the expression levels in the transfection group were significantly inhibited as compared with the blank group. Apoptosis increased significantly in the transfection group or after addition of taxol as compared with the blank group and the no-carrier group. The degree of apoptosis in the taxol plus transfection group was more severe.

CONCLUSION: Apoptosis increased significantly in EC9706 esophageal carcinoma cells after siRNA-mediated inhibition of Ras signaling, with the most obvious increase observed in the transfection plus taxol chemotherapy group. Ras knockdown therefore increased cellular sensitivity to the chemotherapeutic agent, taxol. Ras knockdown also down-regulated the expression of the downstream genes, MAPK1 and cyclin D1, thus inhibiting the growth, proliferation and metabolism of esophageal cancer cells.

Key words: RNA, small interfering; Genes, ras; Ras signal pathway; Esophageal neoplasms; Taxol; Apoptosis; Chemotherapy sensitivity

INTRODUCTION

Carcinoma of the esophagus is a highly invasive, malig-

nant tumor responsible for approximately 25% of all malignant tumor deaths.[1] Carcinoma of the esophagus is clinically characterized by rapid advance, a tendency for metastasis and recurrence, and poor prognosis. Therefore, new techniques for treating carcinoma of the esophagus, including the development of effective drugs, are increasingly required.[2] Ras gene mutations are closely related to human tumors, particularly the K-ras gene because of its high mutation rate. The Ras (Ras gene) pathway is a highly conserved signal transduction pathway that activates many other signaling pathways to mediate differentiation, proliferation and survival of cells, and proto-oncogene mediated transformation.[3-5] Expression of the K-Ras gene in cancer tissues in the esophagus and stomach is higher than in the control tissues adjacent to the cancers,[6-10] and abnormal expression of the Ras gene is closely related to the development of esophageal cancer.[11,12] In the present study, the expression of MAPK1 (mitogen-activated protein kinases1) and cyclin D1 was investigated after Small interfering RNA (siRNA): silencing of the K-Ras gene in the EC9706 esophageal cancer cell line to investigate the value of targeting the K-ras gene for cancer treatment.

MATERIALS AND METHODS

Reagents

Antibodies against K-Ras, MAPK and cyclin D1 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The restriction endonucleases, BamHI and HindIII, were purchased from Bao Biological Company (Tokyo, Japan).

Cell culture

The EC9706 esophageal cancer cell line, the plasmid, pSilencer, and the bacterial strain, top10, were preserved at the Key Laboratory of Tumor Pathology of Henan Province (Zhengzhou, China). EC9706 cells were cultured in a constant temperature, humidified incubator at 37℃ with 5% CO_2. The cells were subcultured by digesting with 0.25% trypsin solution. For experiments, cells in the logarithmic growth phase were used.

Transfection

The cells were divided into six groups: blank group (no-transfection control), no-carrier group (transfection no-carrier), transfection group (transfected with pSilencer-siK-ras), taxol chemotherapy group, no-carrier + taxol chemotherapy group, and transfection + taxol chemotherapy group. The cells were inoculated in 6-well plates 1 day before transfection. On reaching 60%-80% confluence on the following day, the cells were transfected with 5 mg of pSilencer siRNA plasmid and 10 mL of Lipofectamine 2000 in 240 mL of Opti-MEM (Opti-MEM Reduced Serum Medium, powder) serum-free medium. Taxol was then added to the appropriate wells at a concentration of 0.1 μg/mL, following which the cells were incubated at 37℃ for 48 or 72 h before replacing the medium. Total RNA was extracted from the cells with Trizol, and the concentration and purity of the RNA were determined by ultraviolet spectrophotometry.

Immunocytochemical analysis of Ras, MAPK1 and cyclin D1 expression

Upon reaching confluence, the transfected cells were fixed with 4% paraformaldehyde for 30 min at 4℃, treated with 0.3% H_2O_2 at room temperature for 10 min to block endogenous peroxidase activity, and then blocked with 10% animal serum at room temperature for 30 min. The cells were incubated with primary antibody at 4℃ overnight and then with secondary antibody at room temperature for 1 h. Bound antibodies were detected by incubating the cells with SABC/DAB at room temperature for 30-60 min before stopping with tap-water, following which the cells were counter-stained with hematoxylin, dehydrated in a graded series of alcohols and xylene for 20 min, and then mounted in neutral resin.

Realtime Polymerase Chain Reaction (RT-PCR) analysis of MAPK and cyclin D1 expression

Upstream and downstream primers for MAPK1 and cyclin D1 were designed using Primer Premier 5.0 software (Primer company, Toronto, Canada). Five micrograms of RNA were reverse transcribed with Promega MMLV (Moloney murine leukemia virus) reverse transcriptase in a 20 μL reaction, and 0.5 μL of RT (Realtime) product was amplified using SYBR® Premix Ex Taq™ (TaKaRa).

Western blot analysis of MAPK and cyclin D1 expression

Seventy-two hours after transfection, the EC9706 cells were lysed with RIPA buffer and the lysates were stored at − 80℃ prior to use. The lysates were separated on 10% SDS (Sodium dodecyl sulfate)-polyacrylamide gels and transferred to nitrocellulose membranes (Millipore company, MA, USA). After blocking with Blotto blocking solution, the membranes were incubated with primary and secondary antibodies.

Detection of apoptosis by TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay) staining

Twenty-four hours after transfection, the EC9706 cells were trypsinized, counted and resuspended at 6×10^4/mL. The cells were inoculated in a 96-well plate (100 μL per well) and 0.1 μg/mL taxol was added before fixing the cells with 4% paraformaldehyde for 1 h at room temperature. Then, 50 μL of TUNEL reaction solution was added. After washing five times with 1 × PBS, the cells were observed under a fluorescence microscope and photographed.

Statistical analysis

Statistical analysis was performed using Student's *t*-test

for the measurement data and the χ^2 test for the enumeration data with SPSS10.0 software. (SPSS Cpmpany, Chicago, IL, USA), (P<0.05).

RESULTS

Immunocytochemical analysis of Ras, MAPK1 and cyclin D1 expression

The expression of Ras protein in the transfection group was significantly lower than in the blank group. After addition of the chemotherapeutical agent, taxol, the expression of Ras protein decreased in the chemotherapy group, the no-carrier + chemotherapy group and the transfection+chemotherapy group, with the most obvious decrease observed in the transfection+chemotherapy group (Figure 1).

The expression of MAPK protein in the transfection group was slightly lower than in the blank group. After addition of taxol, the expression of MAPK protein significantly decreased, with the most obvious decrease observed in the transfection + chemotherapy group (Figure 2).

The expression of cyclin D1 decreased in the transfection group compared with the blank group. After addition of taxol, the expression of cyclin D1 in the three groups significantly decreased, with the most obvious decrease observed in the transfection + taxol group (Figure 3).

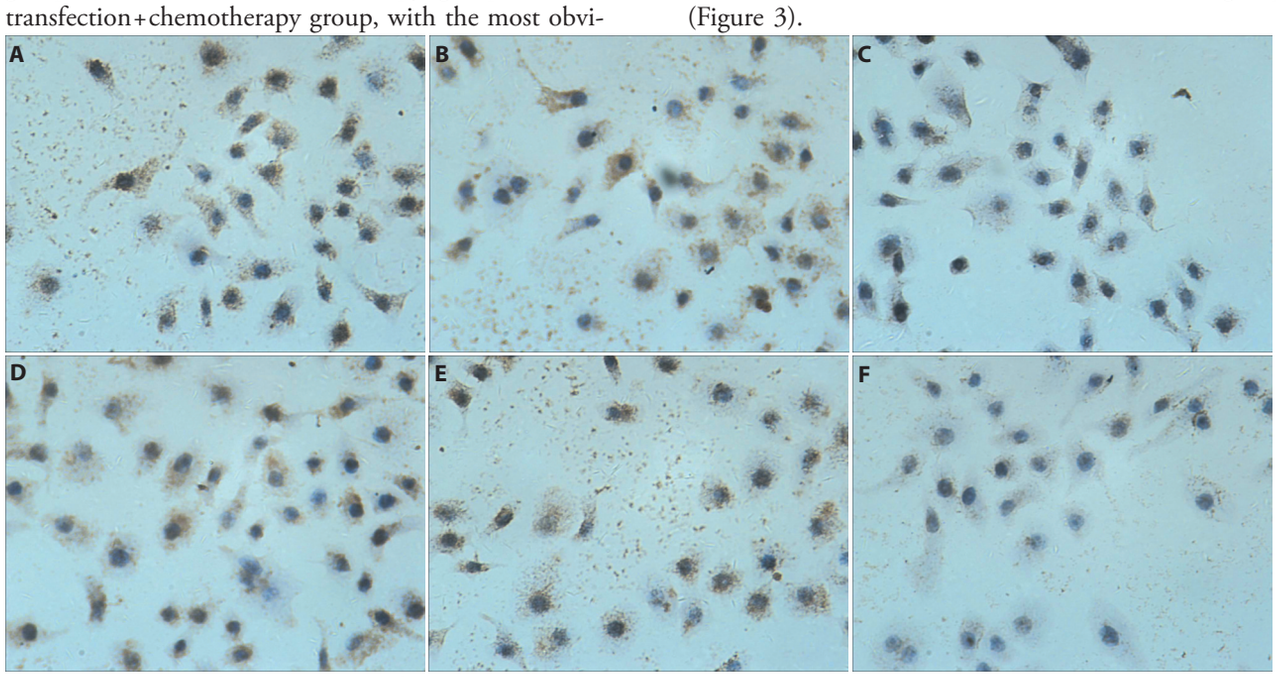

Figure 1 Expression of K-Ras in the transferted cells

A: blank group; B: no-carrier group; C: transfection group; D: taxol chemotherapy group; E: no-carrier + taxol chemotherapy group; F: transfection+taxol chemotherapy group. K-Ras: K-RAS gene; MAPK1: mitogen-activated protein kinases1.

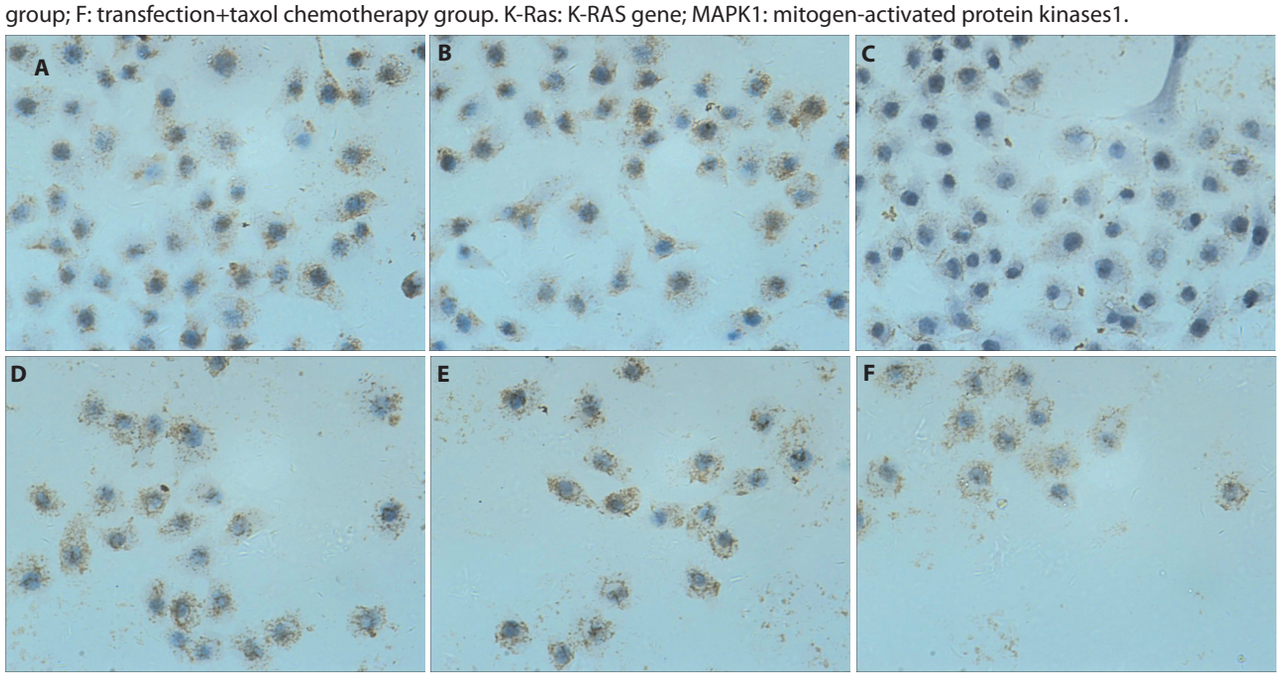

Figure 2 Expression of MAPK1 in the transfected cells

A: blank group; B: no-carrier group; C: transfection group; D: taxol chemotherapy group; E: no-carrier + taxol chemotherapy group; F: transfection+taxol chemotherapy group. MAPK1: mitogen-activated protein kinases1.

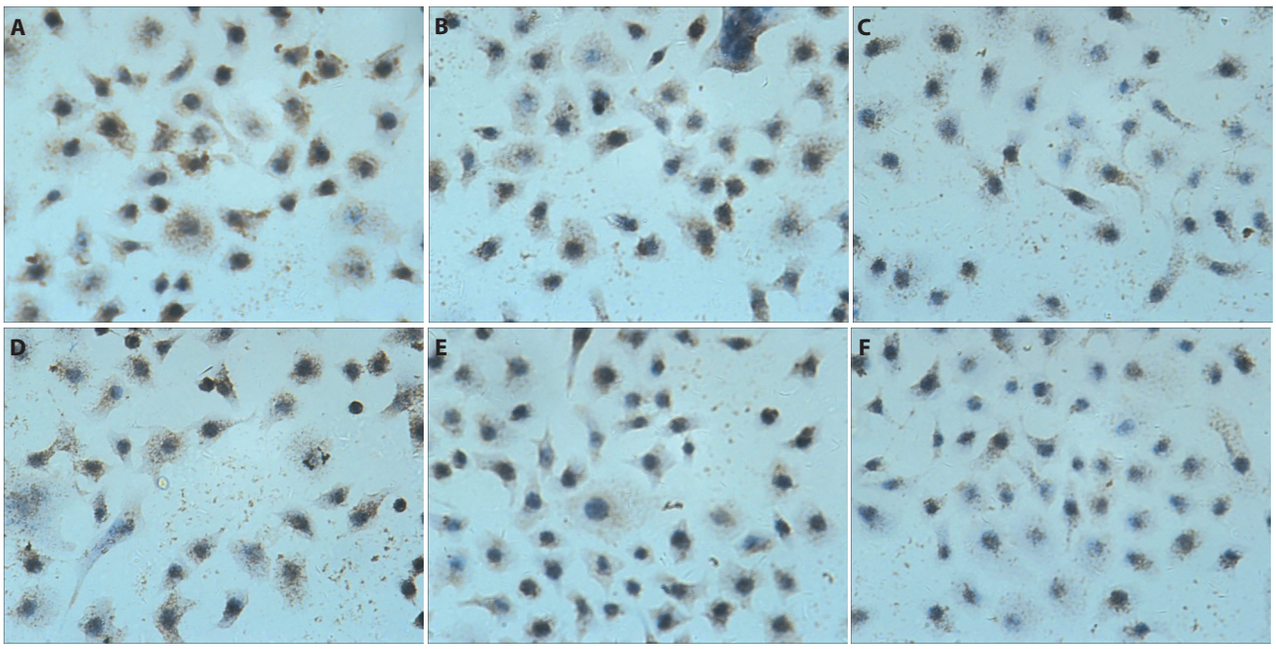

Figure 3 Expression of cyclin D1 in the transfected cells
A: blank group; B: no-carrier group; C: transfection group; D: taxol chemotherapy group; E: no-carrier + taxol chemotherapy group; F: transfection+taxol chemotherapy group.

Effects of siRNA transfection on MAPK1 and cyclin D1 expression by RT-PCR analysis

The expression of MAPK1 in the three groups showed a tendency to decrease, with no significant difference between the no-carrier group and the blank group, and with a significant difference between the transfection group and the blank group (P< 0.05) (Table 1).

Table 1 Expression of MAPK in the transfected cells

	Blank (Control)	No-carrier	Transfection (SiRas)	P value
Actin	17.629 74	17.7359	17.763 04	
MAPK1	18.4774	18.6382	19.051 86	<0.05
$^{\Delta\Delta}$Ct	0	0.054 627	0.441 153	
Fold	1	0.962 843	0.441 153	

Notes: MAPK1: mitogen-activated protein kinases1; SiRas: small interference Ras.

The expression of cyclin D1 protein in the three groups showed a tendency to decrease, with no significant difference between the no-carrier group and the blank group, and with a significant difference between the transfection group and the blank group (P<0.05) (Tables 2, Figure 4).

Table 2 Expression of cyclin D1 in the transfected cells

	Blank (Control)	No-carier	Transfection(SiRas)	P value
Actin	17.629 74	17.7359	17.763 04	
CyclinD1	20.035 51	20.177 91	20.746 19	<0.05
$^{\Delta\Delta}$Ct	0	0.036 236	0.577 373	
Fold	1	0.975 196	0.670 183	

Note: SiRas: small interference Ras.

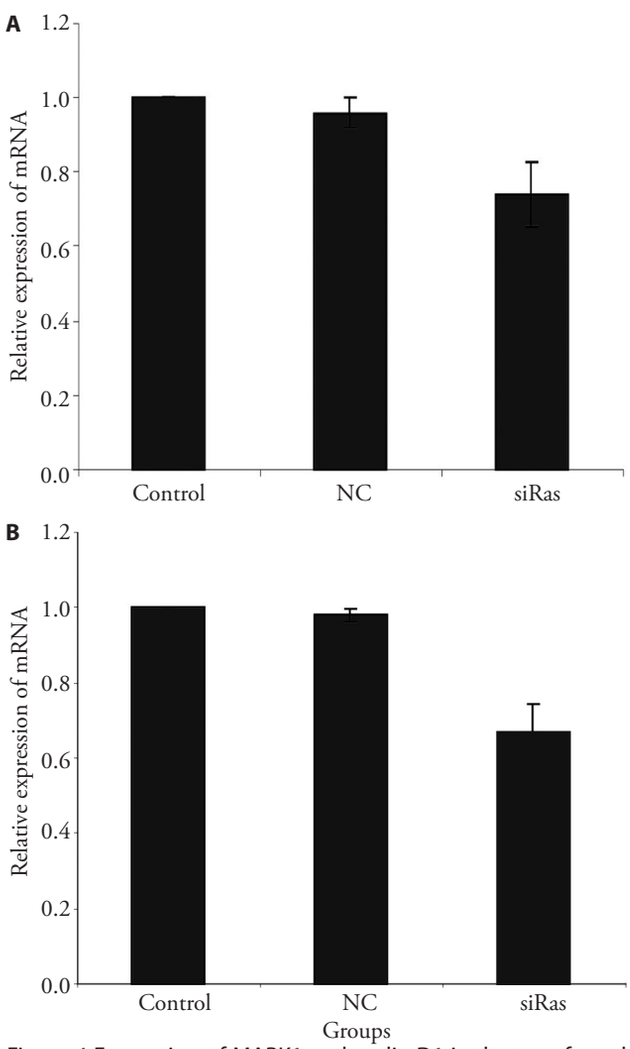

Figure 4 Expression of MAPK1 and cyclin D1 in the transfected cells
A:MAPK1; B: Cyclin D1. MAPK1: mitogen-activated protein kinases1; NC: no-carier; SiRas: small interference Ras.

Effects of siRNA transfection on MAPK1 and cyclin D1 expression

Western blots show the significant inhibition of MAPK1 and cyclin D1 expression in the siRas experimental groups (Figure 5).

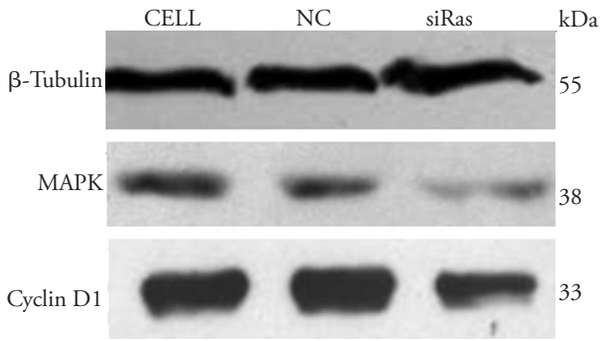

Figure 5 Expression of MAPK1 and cyclin D1 proteins in the cells transfected with K-Ras siRNA
MAPK1: mitogen-activated protein kinases1.

Figure 5 shows that 72 h after transfection of EC9706 cells, MAPK and cyclin D1 decreased in all groups, and their expression was significantly inhibited in the transfection group as compared with the blank group.

Cell nuclei were identified by DAPI (4',6-diamidino-2-phenylindole), staining (blue fluorescence). In addition, the apoptotic cells emitted green fluorescence. Therefore, apoptosis was determined according to the ratio between blue and green fluorescence. Apoptosis significantly increased after Ras siRNA transfection or addition of taxol compared with the blank group and the no-carrier group (Figure 6).

DISCUSSION

Mutation of the Ras gene is the most common oncogene abnormality, and it occurs in about 30% of human tumors. The Ras gene family includes the three members, H-ras, K-ras and N-ras. Among them, mutations in the K-ras gene, which is located on chromosome 12 with a length of about 45 000 base pairs, are most common. Abnormal activation of the Ras gene leads to the production of constitutively active p21ras protein, which activates signaling molecules downstream of MAPK to induce unlimited proliferation and tumor production.[13] The high activity of Ras is often accompanied by excessive phosphorylation and amplification of ERK (Extracellular signal regulated kinase), which promotes malignant transformation. Ras activates the MAPKKK (mitogen-activated protein kinase kinase kinase)-MAPKK (mitogen-activated protein kinase kinase)-MAPK signal transduction cascade[14] to induce cell proliferation via cyclin D1, the activity of which is closely related to other cancer genes. In some cell lines, cyclin D1 shows a weak cancer-inducing action. Although it has no transforming action itself, it can cooperate with the activated Ras gene to transform rat fibroblasts.[15] In the present study, siRNA interference was used to silence the K-ras gene in the EC9706 esophageal cancer cell line and the expression of the key signal proteins, MAPK1 and cyclin D1, downstream of Ras was investigated. The results indicated that siRNA silencing of K-Ras in EC9706 cells led to decreased expression of MAPK1 and cyclin D1. RT-PCR analysis indicated that the expression of MAPK1 and cyclin D1 was lower in the transfection groups than in the blank group (P<0.05). Western blot analysis showed that 72 h after transfection of EC9706 cells, the expression of MAPK1 and cyclin D1 proteins decreased in all groups, with significant inhibition being observed in the transfection group as compared

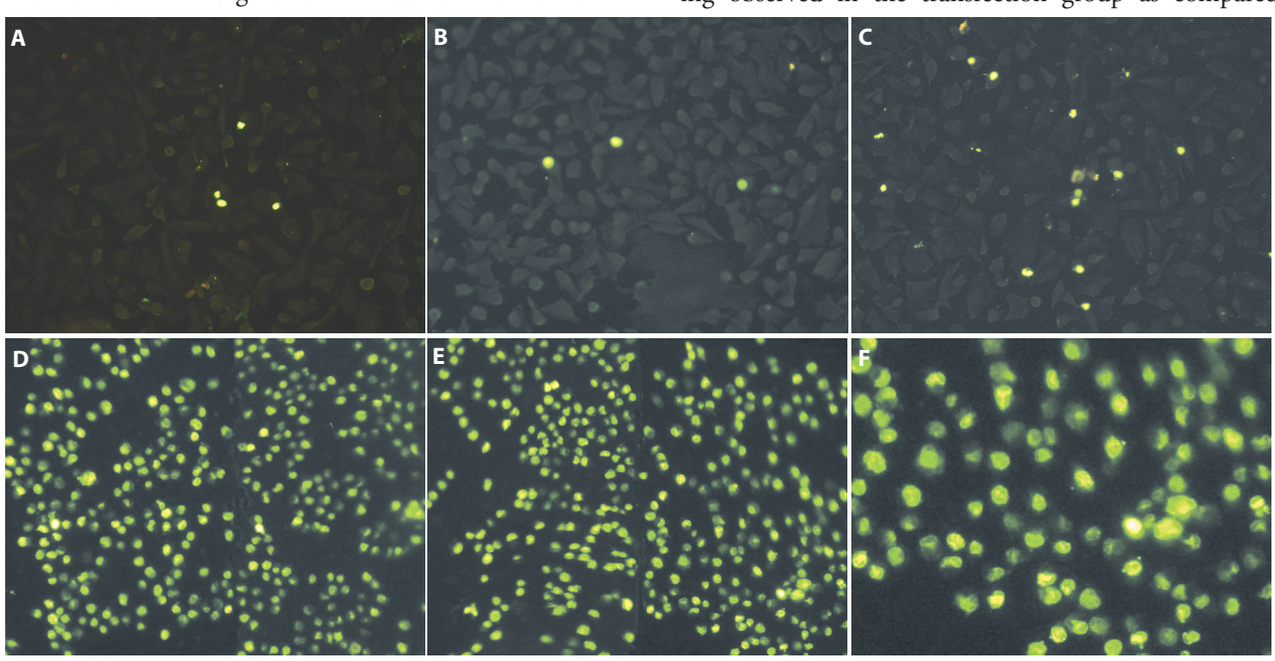

Figure 6 Morphological changes in the transfected cells (TUNEL assay)
A: blank group; B: no-carrier group; C: transfection group; D: taxol chemotherapy group; E: no-carrier + taxol chemotherapy group; F: transfection+taxol chemotherapy group. TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling.

with the blank group. Apoptosis significantly increased in the transfection group or after addition of taxol, as compared with the blank group and the no-carrier group, and the degree of apoptosis was most severe in the taxol plus transfection group.

The above results indicate that siRNA silencing of K-Ras in vitro blocks the Ras signal transduction pathway in the EC9706 esophageal carcinoma cell line and significantly increases apoptosis. The degree of apoptosis was more severe in the transfection plus taxol chemotherapy group. Ras knockdown therefore increased cellular sensitivity to the chemotherapeutic agent, taxol. Ras knockdown also down-regulated the expression of the downstream genes, MAPK1 and cyclin D1, thus inhibiting the growth, proliferation and metabolism of esophageal cancer cells.

REFERENCES

1 **Tanaka T**, Ishiguro H, Kuwabara Y, et al. Vascular endothelial growth factor C in esophageal cancer correlates with lymph node metastasis and poor patient prognosis. Shi Yan Yu Lin Chuang Ai Zheng Za Zhi 2010; 29(1): 83.

2 **Wang XJ**, Zheng YL, Fan QX. Activation expressions of Ras pathway and cyclin D1 in tissues of esophageal squamous cancer. Di San Jun Yi Da Xue Xue Bao 2011; 33 (24): 2626-2628.

3 **Yoo YA**, Na AR, Lee MS, Yoon S, Kim JS, Yoo YD. RASSF1A suppresses oncogenic H-Ras-induce dc-Jun N-terminal kinase activation. Int J Oncol 2006; 29(6): 1541-1547.

4 **Song MS**, Chang JS, Song SJ, Yang TH, Lee H, Lim DS. The centrosomal protein RAS association domain family protein 1A (RASSF1A)-binding protein 1 regulates mitotic progression by recruiting RASSF1A to spindle poles. J Biol Chem 2005; 280(11): 3920-3927.

5 **Pijnenborg JM**, Dam-de Veen GC, Kisters N, et al. RASSF1A methylation and K-ras and B-rafmutations and recurrent endometrial cancer. Ann Oncol 2007; 18(2): 491-497.

6 **Chen YH**, Lan ZP, Hui QY, Li BL, Yang YL. Expressions of RASSF1A and Cyclin D1 in lesion tissues of gastric mucosa and their relationship. Zhong Hua Xiao Hua Za Zhi 2008; 16(9): 2604-2609.

7 **Schimanski CC**, Zimmermann T, Schmidtmann I, et al. K-ras mutation status correlates with the expression of VEGFR1, VEGFR2, and PDGFR alpha in colorectal cancer. Int J Colorectal Dis 2010; 25(2): 181-186.

8 **Lo PH**, Xie D, Chan KC, et al. Reduced expression of RASSF1A in esophageal and naso-pharyngeal carcinomas significantly correlates with tumor stage. Cancer Lett 2007; 257(2): 199-205.

9 **Zhang TW**, Wang SF, Cong DG, et al. Suppression of RASSF1A gene on human esophageal carcinoma cells: experiments *in vitro* and *in vivo*. Zhong Hua Yi Xue Za Zhi 2007; 87(17): 1214-1216.

10 **Cong DG**, Wang SF, Zhang TW. MRNA expression of RASSF1A in esophageal squamous cell carci-noma and clinical significance. Zhong Hua Yi Xue Za Zhi 2006; 86 (23): 1624-1627.

11 **Pu HW**, Chen X. Expressions of hMLH1 and RASSF1A in the tissue of esophageal cancer and relationship with prognosis of the patient. Shi Jie Hua Ren Xiao Hua Za Zhi 2011; 19(13): 1347-1352.

12 **Li J**, Zhao ZG. Expression of ras protein in the tissues of esophageal cancer and precancerous lesion of esophageal cancer. Zhong Guo Zhong Liu Lin Chuang Yu Kang Fu 2009; 6(1): 11-13.

13 **Margarit SM**, Sondermann H, Hall BE, et al. Structural evidence for feedback activation by Ras. GTP of the Ras-specific nucleotide exchange factor SOS. Cell 2003; 112(5): 685-695.

14 **Bar-Sagi D**, Hall A. Ras and Rho GTPases: a family reunion. Cell 2000; 103(2): 227-238.

15 **Lovec H**, Sewing A, Lucibello FC, et al. Oncogenic activity of cyclin D1 revealed through cooperation with Ha-ras: link between cell cycle control and malignant transformation. Oncogene 1994; 9(1): 323-326.