DOWNREGULATION OF STK33 AND ITS EFFECTS ON THE PROLIFERATION, INVASION, AND APOPTOSIS OF ESOPHAGEAL CANCER CELLS BY REGULATING THE S6K1/RPS6/BAD SIGNALING PATHWAY

Shoujun Hu[1], Yannan Han[1], Jing Zhou[1], Chao Zhou[2], Zhongtao Sun[3*]
[1]Department of Thoracic Surgery, Fuyang People's Hospital of Anhui Province, Fuyang 236000, PR China - [2]Shanghai Chest Hospital, Shanghai 200030, PR China - [3]Fuyang People's Hospital of Anhui Province, Fuyang 236000, PR China

ABSTRACT

Objective*: To analyze the effect of downregulation of stk33 on the proliferation, invasion, and apoptosis of esophageal cancer cells by regulating the S6K1/RPS6/BAD signaling pathway.*

Methods: *Cancer tissues and normal tissues adjacent to the cancer were collected from 33 patients with esophageal squamous cell carcinoma who underwent surgical treatment in our hospital. The expression levels of si-stk33 in the esophageal squamous cell carcinoma and normal tissues adjacent to the cancer were detected using real-time quantitative PCR. The Eca109 cells were cultured. The si-stk33 plasmid was transfected into the cells and then cultured for 4 h to obtain the si-stk33 group. Control group cells were set, with 5 duplicate groups for each group. The expression levels of si-stk33 in the si-stk33 group and the control group were determined using real-time quantitative PCR and western blotting. The cell proliferation of each group was measured using the MTT method. The changes in the cell invasion ability and migration ability of the two groups were detected with a Transwell test and the changes in the cell cycle and apoptosis in each group with flow cytometry. The expression levels of p-S6K1, p-RPS6, and p-BAD in the two groups were determined using western blotting.*

Results: *Compared with normal tissues adjacent to the cancer, the expression level of si-stk33 in esophageal squamous cell carcinoma was significantly higher (P<0.01). Compared with the control group, in the si-stk33 group cells, the expression level of si-stk33, the cell migration and invasion ability, the S-phase and G2-phase cells, and the expression levels of p-S6K1, p-RPS6, and p-BAD were significantly reduced, and the cells greatly increased at the G1 phase (P<0.01). There was no significant difference in OD value between the two groups at 24 h (P>0.05). From 48 h, the OD value of cells in the si-stk33 group was significantly reduced compared with that of the control group (P<0.01).*

Conclusions: *Downregulating the expression of stk33 can limit the proliferation, invasion, and metastasis of esophageal cancer cells, block the cell cycle at the G1 phase, and induce apoptosis by inhibiting the S6K1/RPS6/BAD signaling pathway.*

Keywords: *stk3, esophageal cancer, cell proliferation, cell invasion, cell apoptosis, mechanism.*

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Introduction

Esophageal cancer is one of the most common malignant tumors in the digestive system. According to statistics, more than 300,000 people die from esophageal cancer each year worldwide, over 150,000 of which are in China. China ranks first globally for mortality and incidence of esophageal cancer. Esophageal squamous cell carcinoma accounts for approximately 90% of esophageal cancer in China and the patient survival rate is poor[(1)]. With the continuous advances in social technology and the rapid development of medical technology, surgical technologies and comprehensive treatments such as surgery, chemotherapy, and radiotherapy have undergone important developments. Despite this, the five-year survival rate of patients has not improved greatly-only by approximately 14%~22%. Apart from having a serious impact on patients' health and quality of life, the prevalence of this cancer also

gives rise to a huge burden on the social economy[2]. Serine/threonine kinase 33 (stk33), a member of the serine/threonine kinase protein family, is a human serine/threonine kinase gene. Serine and threonine in proteins can be phosphorylated through the action of this kinase, thereby regulating the proliferation, differentiation, and apoptosis of cells[3].

Stk33 has potential anti-cancer properties, and in-depth research into its role in the occurrence and development of esophageal cancer will help develop new clinical treatment methods for esophageal cancer[4]. According to related reports, stk33 is clearly expressed in lung cancer, breast cancer, liver cancer, and other tumor cells, but the specific mechanism of its effect is still unclear and further discussions are necessary[5]. In this study, we first detected the expression of stk33 in esophageal squamous cell carcinoma and then used siRNA interference technology to silence the expression of stk33 in esophageal cancer cell lines to downregulate it. We detected the effect of stk33 on the biological behavior of the proliferation, invasion, and apoptosis of esophageal squamous carcinoma cells. We also tried to determine whether stk33 can impact the ability of cell proliferation, invasion, and apoptosis and its influence by regulating ribosomal S6 kinase 1/ribosomal protein S6/death factor bad (S6K1/RPS6/BAD) signaling pathway.

Materials and methods

Experimental materials

Cancer tissues and normal tissues adjacent to the cancer were collected from 33 patients with esophageal squamous cell carcinoma who underwent surgical treatment in our hospital. They came from esophageal squamous carcinoma cell line Eca109 (provided by the Cell Bank of the Chinese Academy of Sciences).

Experimental reagents and instruments

Fetal bovine serum (Shanghai Yubo Biotechnology Co., Ltd.); phosphate buffer (Shanghai Yuanye Biotechnology Co., Ltd.); trypsin (Shanghai First Biochemical Pharmaceutical Co., Ltd., batch number: 31022018, specification: 2.5 U); penicillin-streptomycin solution double antibody [Juneng Century Information Technology (Suzhou) Co., Ltd., specification: 100 ml]; MTT kit (Shanghai Zeye Biotechnology Co., Ltd.); DMEM medium (Heilongjiang Jiufeng Bioengineering Co., Ltd.); protease inhibitor [Thermo Fisher Scientific (China) Co., Ltd.]; carbon dioxide incubator (Shanghai Danding International Trade Co., Ltd., model: MCO-20AIC); electronic balance (Shanghai Shunyu Hengping Scientific Instrument Co., Ltd., model: AE523); pure water distiller (Shanghai Precision Instrument Co., Ltd., model: SZ-93); low-temperature high-speed centrifuge (Shanghai Luxiangyi Centrifuge Instrument Co., Ltd., model: TGL-18M); high-pressure steam sterilizer (Jinan Bo Xin Biotechnology Co., Ltd., model: BKQP-L); constant temperature water bath (Changzhou Jintan Youlian Instrument Research Institute, model: HH-600); Flow cytometry (Beckman Coulter Co., Ltd., model: CytoFLEX); real-time quantitative fluorescence PCR detector (Jinan Guangyao Medical Equipment Co., Ltd., model: CFX384Touch); inverted biological microscope (Jingtong Life Science Instrument Co., Ltd., model: VMB1900A).

Cell culture

An Eca109 cell cryopreservation tube was removed from the liquid nitrogen tank and thawed in a water bath. The cell suspension in the cryotube was aspirated into a 10 ml centrifuge tube equipped with an incubator and centrifuged at 1500 r/min for 5 min, and the supernatant was removed. We added 1 ml of the medium to the centrifuge tube to resuspend the cells. We inoculated the cell suspension into a 25 cm^2 culture flask, and the flask was gently shaken to distribute the cells evenly. It was then placed in a 5% CO_2 incubator at 37°C. The medium was replaced when the cells adhered to the wall.

When the cell density in the culture flask reached approximately 85%, the cells were passaged. The primary culture medium was removed from the flask, and then the cells were washed 3 times before 3 ml of phosphate buffer was added. The cells were digested for 3 min with 1 ml of trypsin, and then gently pipetted for suspension. The cell suspension was taken and then 5 ml of the solution containing 10% fetal bovine serum and 1% penicillin-streptomycin double-antibody solution was added. This was incubated in a 5% CO_2 incubator at 37°C.

Main cell information such as cell passage, type, and freeze date was recorded in a cryopreservation tube. The tube was put into a cell gradient cryopreservation box in a fixed order for linear cooling, and then moved into a liquid nitrogen tank.

Transfection

Eca109 cells with good growth in the logarithmic growth phase were digested and centrifuged. The

supernatant was removed and they were resuspended in a DMEM medium containing 10% fetal bovine serum. The cells measured prior to being seeded in a 6-well plate. They were placed in a 5% CO_2 incubator at 37°C for 24 h.

The si-stk33 plasmid was transfected into the cells, and then the medium was replaced with a serum-free medium and cultured for 4 h to obtain the si-stk33 group. The control group cells were set in the same period. Each group had 5 duplicate groups.

Observation indexes

Real-time quantitative PCR was used to determine the expression levels of si-stk33 in esophageal squamous cell carcinoma and in normal tissues adjacent to the cancer. The expression levels in the si-stk33 group and the control group were determined using real-time quantitative PCR and western blotting. The cell proliferation of each group was measured using the MTT method. Cells at the logarithmic growth phase were digested, centrifuged, and made into a cell suspension, which was inoculated into a 96-well plate at 1000 cells/well. Then, 10 μL/well MTT solution was added to each well. The cells groups were cultured for 24 h, 48 h, 72 h, 96 h, and 120 h, respectively, and then incubated continuously for 2 h. The absorbance (OD value) at 470 nm was measured using a multifunctional microplate reader.

Cell invasion

A Transwell invasion test was used to determine the changes in the cell invasion ability of the two groups. After culturing the transfected cells in each group for 48 h, the adherent cells were digested with trypsin and washed 3 times with phosphate buffer to adjust the cell concentration to 5×105 cells/ml. 200 μL of the cell suspension of each group was pipetted to the upper chamber of the Transwell chamber and 600 μL of RPMI 1640 culture solution was added to the lower chamber and incubated in a 5% CO_2 incubator at 37°C for 24 h. We took the small chamber out and removed the residual liquid in the upper chamber. We added 75% ethanol to the lower chamber, fixed for 30 min at room temperature. The residual liquid was removed, and the cells attached to the microporous membrane of the upper chamber were gently wiped with a clean cotton swab. It was observed under a microscope and recorded.

Cell migration

The changes in the cell migration ability of the two groups were determined using a Transwell migration experiment. We took 60 μL of Matrigel and medium dilution, and covered the basement membrane of the upper chamber. It was then stored in an incubator at 37°C overnight, and then the Matrigel was dried. The solidified Matrigel was transferred into 40 μL of RPMI 1640 culture solution on the second day, standing in a 5% CO_2 incubator at 37oC for 30 min, and the residual liquid that remained after Matrigel hydration was removed. The remaining steps were the same as in the cell invasion experiment. The changes in the cell cycle and apoptosis of each group were detected using flow cytometry. Cells in the si-stk33 group and the control group were seeded in 6-well plates. We added 2 ml of complete medium to each well, and then placed the well plates in a cell incubator. When the cell density reached 80% or more, the cells were reserved. The cells were digested with trypsin and centrifuged. The centrifuged cells were fixed with 70% ethanol at 4°C for 2 h after the supernatant was removed. The fixed cells were collected and recentrifuged, and then the supernatant was removed. We slowly added 500 μL of staining buffer to different samples, and then the cells were resuspended with a pipette tip. Subsequently, we mixed the cells well with 25 μL of propidium iodide staining solution, and then added 10 μL of RnaseA, which was incubated at 37°C for 30 min in the dark. Adenocarcinoma was stored in an ice bath in a dark bath for flow detection.

The expression levels of the p-S6K1, p-RPS6, and p-BAD of the cells in the two groups were determined using western blotting.

Statistical analysis

All data were analyzed using SPSS18.0 software. The measurement data were compared using an independent sample t-test. The expression levels of si-stk33 in the esophageal squamous cell carcinoma and the normal tissues adjacent to the cancer were determined using real-time quantitative PCR. The expression levels of si-stk33 in the si-stk33 group and in the control group were determined with real-time quantitative PCR and western blotting. The cell proliferation of each group was measured with the MTT method. The changes in the cell invasion and migration ability of the two groups were detected using a Transwell test. The changes in the cell cycle and apoptosis in each group were determined using flow cytometry. The expression levels of p-S6K1, p-RPS6, and p-BAD of the cells in the two groups were determined using western blotting. The results are statistically significant when $P<0.05$.

Results

Expression levels of si-stk33 in esophageal squamous cell carcinoma and in normal tissues

Compared with the normal tissues adjacent to the cancer, the expression level of si-stk33 in esophageal squamous cell carcinoma was significantly increased (P<0.01). See Table 1.

Group	n (n)	si-stk33
Esophageal squamous cell carcinoma	33	11.64±2.57
Normal tissue adjacent to cancer	33	4.18±2.39
t		12.211
P		<0.001

Table 1: Comparison of expression levels of si-stk33 in esophageal squamous cell carcinoma and normal tissues ($\bar{x}\pm s$).

Expression levels of si-stk33 in cells in the si-stk33 group and the control group

Compared with the control group, the expression level of the si-stk33 of cells in the si-stk33 group was significantly reduced (P<0.01). See Figure 1 and Table 2.

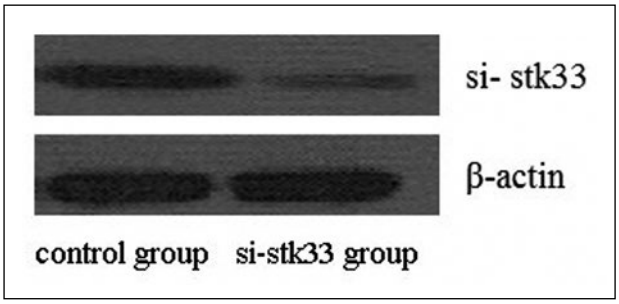

Figure 1: Expression levels of si-stk33 of cells in the si-stk33 group and in the control group.

Group	si-stk33
Control group	1.00±0.03
si-stk33 group	0.36±0.09
t	15.085
P	<0.001

Table 2: Comparison of expression levels of si-stk33 of cells in the si-stk33 group and the control group ($\bar{x}\pm s$).

Comparison of cell proliferation between the two groups

There was no significant difference in the OD value between the two groups at 24 h (P>0.05). From 48 h, compared with the control group, the OD value of the cells in the si-stk33 group was significantly reduced (P<0.01). See Table 3.

Group	Cell proliferation (OD value)				
	24 h	48 h	72 h	96 h	120 h
Control group	0.39±0.02	0.57±0.06	0.81±0.04	1.01±0.07	1.03±0.06
si-stk33 group	0.37±0.03	0.42±0.03	0.61±0.04	0.73±0.05	0.74±0.04
t	1.240	5.000	7.906	7.278	8.993
P	0.250	0.001	<0.001	<0.001	<0.001

Table 3: Comparison of cell proliferation between the two groups ($\bar{x}\pm s$).

Changes in the cell invasion ability and migration ability of the two groups

Compared with the control group, the cell migration ability and invasion ability of the si-stk33 group were significantly reduced (P<0.05). See Figure 2 and Table 4.

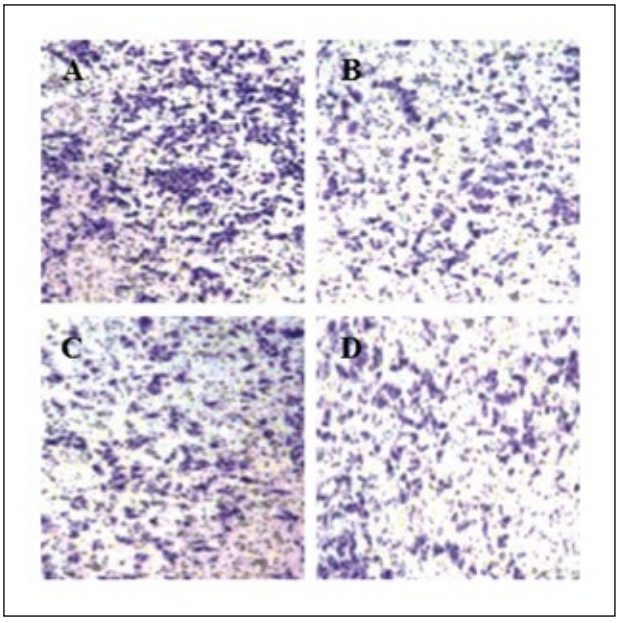

Figure 2: Changes in cell invasion ability and migration ability of the two groups.
Cell invasion ability Figure A-B: Figure A: control group; Figure B: si-stk33 group. Cell migration ability Figure C-D: Figure C: control group; Figure D: si-stk33 group.

Group	Cell migration ability	Cell invasion ability
Control group	372.55±43.83	246.18±48.73
si-stk33 group	184.67±33.89	111.33±64.71
t	7.583	3.722
P	<0.001	0.006

Table 4: Comparison of changes in cell invasion and migration ability of the two groups ($\bar{x}\pm s$).

Comparison of apoptosis between the two groups

Compared with the control group, the apoptosis rate of the si-stk33 group increased significantly (P<0.05). See Table 5.

Group	Apoptosis rate (%)
Control group	3.69±0.71
si-stk33 group	12.57±1.44
t	12.368
P	<0.001

Table 5: Comparison of apoptosis between the two groups ($\bar{x}\pm s$).

Cell cycle changes of the two groups

Compared with the control group, the G1 phase cells in the si-stk33 group increased significantly, while the S phase and G2 phase cells decreased significantly (P<0.05 or P<0.01). See Table 6.

Group	Cell cycle (%)		
	G1 phase	S phase	G2 phase
Control group	62.27±6.73	22.47±2.63	15.52±3.42
si-stk33 group	75.46±9.62	16.73±3.35	7.22±2.41
t	2.512	3.014	4.436
P	0.036	0.017	0.002

Table 6: Cell cycle changes of the two groups ($\bar{x}\pm s$).

Comparison of p-S6K1, p-RPS6, and p-BAD expression levels of cells in the two groups

Compared with the control group, the expression levels of p-S6K1, p-RPS6, and p-BAD in cells in the si-stk33 group were significantly reduced (P<0.05). See Figure 3.

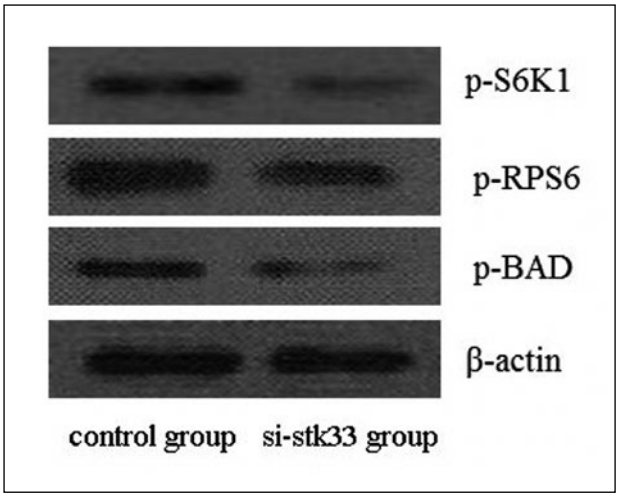

Figure 3: Cp-S6K1, p-RPS6, and p-BAD expression levels of cells in the two groups.

Discussion

Esophageal cancer is one of the six most typical tumors in clinic, and China is the country with the highest incidence of esophageal cancer. Squamous cell carcinoma cancer is the main type of esophageal cancer in China and poses a serious threat to human health. According to related reports, the occurrence and development of esophageal cancer is a complex process involving the accumulation and interaction of multiple factors and gene variants, and is closely related not only to lifestyle habits, environmental factors, and human papillomavirus infections, but also to genetic factors[6]. Esophageal cancer has an insidious onset, often without obvious manifestations in the early stage, and is often in the middle or late stages when it is diagnosed.

Due to strong invasion and metastasis abilities, the tumor is mainly characterized through direct infiltration, lymphatic metastasis, and bloody metastasis. Surgical treatment is the primary therapy for esophageal cancer, with the aim of removing the intact lesion and dissecting relevant lymph nodes, supplemented by chemotherapy or radiotherapy if necessary[7]. Although the treatment options are diverse, the five-year survival rate is relatively low: only 14% to 22%. Therefore, early diagnosis and early treatment are necessary to obtain a good prognosis. The causes of many diseases are closely related to the abnormal expression of genes, and many plasmid transfection techniques are involved in the process of gene therapy. Plasmid transfection includes DEAE-dextran, calcium carbonate co-precipitation transfection, electroporation, liposomes, non-liposome, and other methods, which can transfect the target plasmid into eukaryotic cells to play a relevant role[8]. In this study, liposome transfection was mainly used to transfect si-stk33 into the esophageal cancer cell line Eca109 to explore the biological role and related mechanisms of stk33 in esophageal cancer cells.

Stk33, a new type of serine/threonine protein kinase, usually located in the cytoplasm, is weakly expressed in the nucleus, and has the characteristics of soluble proteins. It can phosphorylate serine and threonine residues on proteins and plays an important role in regulating major cellular processes such as signal transduction pathways, DNA replication, cell differentiation, cell proliferation, apoptosis, and tumor occurrence and development[9]. Some research has shown that stk33 is expressed in the heart, brain, and spinal cord, and that it is also an important medium for the proliferation and invasion of small cell lung cancer, esophageal cancer, and other cells[10]. Wang et al.[11] found that stk33 helps the migration and invasion of human large cell lung cancer cells and that controlling the stk33 gene can

inhibit the migration and invasion of these cell lines, which are results similar to those of this study.

Cell proliferation, a key biological feature, is an important basis for biological occurrence, development, and inheritance. Cell proliferation can produce new cells via cell division. Mitosis is one of the most common forms of division in the animal kingdom, and it is also the main mode of eukaryotic cell proliferation. Eukaryotic cells are cyclical and have a cell cycle during mitosis. The complete cell cycle includes the interphase and the division phase. The interphase includes the G1 phase, S phase, and G2 phase. RNA replication and related protein syntheses occur at the G1 phase and G2 phase, and DNA replication occurs at the S phase, so if the cells are blocked at the G1 phase, it plays a crucial role in blocking the cell cycle[12]. Apoptosis is a basic biological phenomenon of cells, which involves the activation, expression, and regulation of a series of genes, and plays a significant role in the evolution of organisms, the stability of the internal environment, and the development of multiple systems. In addition, cell invasion and metastasis are important causes of tumor treatment failure[13]. This study found that the downregulation of stk33 expression can significantly increase the cell viability of esophageal cancer, induce apoptosis, and significantly inhibit the invasion and migration of esophageal cancer.

S6K1 is a downstream effector molecule of mTOR, which can participate in the regulation of biophysiological processes including gene transcription, protein synthesis, and cell proliferation through the phosphorylation of various substrates. It is reported that S6K1 plays an important role in the proliferation and invasion of tumor cells[14]. RPS6 is a component of the small ribosomal subunit S6, which can undergo a series of phosphorylation reactions, thereby playing an important role in promoting glucose metabolism and protein synthesis[15].

As an apoptosis gene, BAD is a member of the Bcl-2 family and plays a key role in cell apoptosis. Some studies have found that the BAD gene can be combined with viral receptors to regulate cell apoptosis through a tyrosine kinase signaling pathway[16]. Studies have confirmed that inhibiting the expression of stk33 can control the expression of S6K1 due to the certain positive correlation between their expressions, thereby promoting apoptosis and further exerting effects[17]. The results of this study found that the S6K1/RPS6/BAD signaling pathway is involved in the development of esophageal cancer cells, and downregulation of stk33 expression can significantly inhibit the activity of the S6K1/RPS6/BAD signaling pathway.

In summary, the downregulation of stk33 expression can inhibit the proliferation, invasion, and metastasis of esophageal cancer cells by inhibiting the S6K1/RPS6/BAD signaling pathway, blocking the cell cycle in the G1 phase and inducing apoptosis.

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Corresponding Author:
Zhongtao Sun
Email: ndgdf6@163.com
(China)