

RESEARCH ARTICLE

Silencing of PDK1 Gene Expression by RNA Interference Suppresses Growth of Esophageal Cancer

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Abstract

The current study was conducted to explore the inhibitory effects of a small interfering RNA (siRNA) on 3-phosphoinositide-dependent protein kinase 1 (PDK1) expression in esophageal cancer 9706 (EC9706) cells and the influence on their biological behavior. After transfection of a synthesized PDK1 siRNA, PDK1 mRNA and protein expression and the phosphorylation level of the downstream Akt protein were assessed using RT-PCR and Western blot analysis. Proliferation, apoptosis, cell invasion and in vivo tumor formation capacity were also investigated using MTT, flow cytometry, Transwell invasion trials, and nude mouse tumor transplantation, respectively. PDK1 siRNA effectively suppressed PDK1 mRNA and protein expression, and down-regulated the phosphorylation level of the Akt protein in the EC9706 cells ($P < 0.05$). It also inhibited cell proliferation and invasion, and promoted apoptosis; such effects were particularly obvious at 48 h and 72 h after transfection ($P < 0.05$). Growth of transplanted tumors was inhibited in nude mice, with decreased PDK1 expression in tumor tissues. PDK1 may be closely correlated with proliferation, apoptosis and invasion of esophageal cancer cells and thus may serve as an effective target for gene therapy.

Keywords: 3-phosphoinositide - dependent protein kinase 1 - RNA interference - esophageal cancer cells

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Introduction

The incidence rate of esophageal cancer (EC) is the highest in China worldwide. To develop new drugs and molecule targeted therapies has become the hotspot of study on EC nowadays. 3-phosphoinositide-dependent protein kinase 1 (PDK1) is a member of the AGC kinase family. PDK1 mediates and regulates the PI3K/Akt signal transduction pathway whose abnormality is closely correlated with the genesis and development of many malignant tumors. It is the major upstream kinase in the activation step of AGC kinases such as Akt, p70-S6K, RSK and PKC (Tamgüney et al., 2008). PDK1 was first separated and identified by Alessi DR in 1997. As an upstream kinase of Akt, it phosphorylates the Thr 308 locus of Akt to activate Akt; the activated Akt regulates its downstream pathways, which, in turn, influence carbohydrate metabolism, protein transcriptional translation, cell proliferation, cell migration and anti-apoptosis; and excessively activated Akt is closely correlated with the genesis and development, invasion and treatment tolerability of many malignant tumors such as EC (Li et al., 2007; Hildebrandt et al., 2009), bladder cancer (Knowles et al., 2009), acute myelocytic leukemia (Martelli et al., 2010), oral epithelium cancer (Watanabe et al., 2009), glioblastoma (Jiang et al., 2009), and so

on. PDK1 mediates the PI3K/Akt signal transduction pathway which is closely correlated with the genesis and development of many malignant tumors (Carnero et al., 2010). Therefore, PDK1 may be a more effective treatment target than Akt, for which PDK1 has become a new study object in molecular therapies for malignant tumors nowadays.

RNA interference (RNAi) is a phenomenon of posttranscriptional gene silencing, and has the virtues of high performance and specificity. RNAi has been extensively applied in research fields such as gene function identification and post-transcriptional control of gene expression. Because of its virtues and wide application, RNAi technique has provided a new idea for gene therapies for diseases (particularly for malignant tumors). RNAi was first discovered in threadworms by Fire A in 1998 (Fire et al., 1998). This technique promotes the formation of RNA-induced silencing complexes (RISC) through the binding of the homologous sequences in short double-stranded RNA and mRNA to induce the degradation of the target gene and inhibit the gene expression (Wilkins et al., 2005). Small interference RNA (siRNA) is a small fragment of RNA transformed by double-stranded RNA or synthesized artificially in vitro during the process of RNAi; it is comprised of 21 base pairs; and it can block gene expression specifically (Oh et al., 2009).

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Table 1. The Sequences of siRNA

Target	Sequence
PDK1 siRNA1	Sense :5'-GGUUGUUGUUGGAGAAGCATT-3' Anti-sense:5'-UGCUUCUCCAACAACAACCTT-3'
PDK1 siRNA2	Sense :5'-GGUCAGUAGUCUUGUAAUATT-3' Anti-sense:5'-UAUUACAAGACUACUGACCTT-3'
PDK1 siRNA3	Sense :5'-CGAGAACAGCACACUUAATT-3' Anti-sense:5'-UUGAAGUGUGUCUGUUCUGTT-3'
Negative control (FAM) siRNA	Sense :5'-UUCUCCGAACGUGUCACGUTT-3' Anti-sense:5'-ACGUGACACGUUCGGAGAATT-3'

In the current study, the PDK1 expression and the Akt phosphorylation level in EC9706 cells were detected after PDK1 gene silencing using siRNA. Meanwhile, cell growth, apoptosis, cell invasion and in vivo tumor formation capacity were investigated.

Materials and Methods

Cell culture and transfection

Well-differentiated human EC9706 cells were cultured with RPMI-1640 containing 10% FBS in a 5% CO₂ incubator at 37 °C. The cells in good growth were selected before transfection, and inoculated into 6-pore plates at a density of 5 × 10⁵. They were cultured with fresh serum-free medium for transfection when the coverage rate reached 50% to 70%. 10 μl siRNA (Shanghai GenePharma Co., China) the sequences are shown in (Table 1) were diluted in 82 μl serum-free medium (20 μM). 8 μl RNAi-Mate transfection reagents (Shanghai GenePharma Co., China) was added, and oscillated. The solution was placed at room temperature for the formation of siRNA/RNAi-Mate complexes. The obtained solution was added into the pores for 6-hour culture. The medium was then replaced with serum medium.

RT-PCR

PDK1 mRNA was detected using the RT-PCR technique. Total RNA was extracted from the cells after transfection using the guanidinium isothiocyanate/phenol method. The amount was then detected using ultraviolet spectrophotometry. cDNA was reversely transcribed, and synthesized according to the instructions of Quant cDNA synthesis kit (Shanghai GenePharma, China). The amplification conditions were as follows: an 95 °C initial pre-denaturation step for 2 min, followed by 35 cycles at 95 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 1 min. The final extension was extended at 72 °C for 5 min. The length of the PDK1 amplified product was 437 bp. The primers sequences were: upstream 5'-TGAAGTACCTTGCCACAT-3', and the downstream sequence was 5'-TGAAGCAGCACTGAACACG-3'. The length of the β-actin amplified product was 696 bp. The upstream primer sequence was 5'-CGGGAAATCGTGCGTGAC-3', and the downstream primer sequence was 5'-TCGCTCCAACCGACTGCT-3'. The PCR products were subjected to 1.2% agarose gel (containing 0.5 μg/ml ethidium bromide) electrophoresis. Images were obtained using an Alpha multifunctional gel imaging system, and analyzed using the Alpha View software. The relative expression of PDK1 mRNA was calculated based on the ratio of the electrophoresis band

opacity density (OD) of PDK1 to that of β-actin.

Western blot analysis

Cells were collected after trypsinization and centrifugation. They were then lysed for protein extraction (or the protein was extracted from the homogenate of the transplanted tumor tissues). The OD value at the wavelength of 562 nm in a 96-pore plate was detected using the enzyme-labeled BCA method. A standard curve was charted for protein concentration calculation. The protein was electrophoretically separated in SDS-PAGE gel (containing 10% separation gel and 5% spacer gel). The obtained sample (50 μg/well) was applied. It was electro-transferred to the PVDF membrane under a constant voltage of 100 V, and then electro-transferred under a constant voltage of 20 V for 16 min to 25 min. The membrane was sealed off in 5% skimmed milk/TBST for two hours. It was cultured with PDK1, Akt, p-Akt and β-actin antibodies (1: 50) at 4 °C overnight. Then, it was cultured with secondary antibodies (1: 2000) at room temperature for 1 h, and DAB-stained. Images were obtained using the Alpha multifunctional gel imaging system, and analyzed using the Alpha View software. The relative expression of the interest protein was calculated based on the ratio of the interest band OD to that of the β-actin band OD.

MTT assays

EC9706 cells were inoculated into a 96-pore plate. 100 μl medium was added into each well, and the density of the cells was adjusted to 4 × 10³ to 8 × 10³/well. Control and zero well were set. 20 μl MTT (5 μg/ml) solution was added at 24 h, 48 h and 72 h after transfection for 4-hour culture at 37 °C. The medium was discarded. 150 μl DMSO was added, and oscillated. The OD value at the wavelength of 492 nm was detected using an enzyme-labeled analyzer. The cell growth inhibition rate was calculated based on the formula: the inhibition rate = (1 - the OD value of the experimental group/the OD value of the control group) × 100%.

Flow cytometry

Cells were collected after transfection, and twice washed. They were resuspended with 100 μl pre-chilled 1 × binding buffer. The cell density was adjusted to 1 × 10⁶/ml. The cells were cultured with 5 μl FITC Annexin V and 5 μl PI away from light. 400 μl 1 × Binding Buffer was then added, and mixed well. The apoptotic rate was detected using flow cytometry.

Transwell invasion

The upper and lower Transwell chambers were separated with a polycarbonate membrane with 8 μm apertures. The membrane was covered with matrigel (30 μl/chamber) diluted with serum-free medium according to a 1: 8 ratio. It was then polymerized at 37 °C for 30 min. Post-transfection cells were collected, resuspended with serum-free medium, and blown into monoplast suspension (the density of the cells was 5 × 10⁵/ml). The suspension was added into the upper chamber (100 μl/chamber). 20% serum medium was added into the lower chambers (500

$\mu\text{l}/\text{chamber}$). The cells were cultured for 24 h. The upper layer matrigel and non-migrating cells were removed using a cotton bud. The membrane was stained using 0.1% crystal violet for 30 min, and PBS washed. Five visual fields were randomly selected under the microscope. The invasion inhibition rate was calculated based on the following formula: the invasion inhibition rate = $(1 - \frac{\text{number of the migrating cells in the experimental group}}{\text{that in the control group}}) \times 100\%$.

Tumor inhibition

Female BALB/c nude mice (the age was four weeks, and the weight ranged from 18 g to 21 g) were selected. EC9706 cells in logarithmic growth were collected. Each mouse was subcutaneously inoculated with 200 μl cell suspension (the density of the cells was $1 \times 10^7/\text{ml}$) into the left upper extremity for EC transplanted tumor model establishment. When the diameter of the tumor grew to 5 - 6 mm, siRNA/RNAi-Mate complexes were injected (100 μl in each mouse every three days). The control group was injected with PBS. After five times of treatment, the mice were sacrificed. The transplanted tumor was dissected, and weighted after filter paper blotting. Protein was extracted, and kept at -80°C . The PDK1 protein expression was detected using Western blot analysis.

Statistical analysis

The data were presented as means \pm standard error of means ($x \pm s$), and analyzed using the SPSS17.0 statistical software. The means were compared using one-factor analysis of variance (ANVOA), and an LSD-t test was carried out for pairwise comparisons among groups when there was an ANVOA significant difference. The size of test α was 0.05.

Results

Transfection efficiency

EC9706 cells were transfected with negative control FAM-siRNA at a final concentration of 60 nM, 80 nM and 100 nM. They were observed under the microscope at 6 h after transfection. The results showed that the 100 nM group had the highest transfection efficiency among different concentration groups (Figure 1). Therefore, siRNA at such concentration was determined in the following procedures.

PDK1 siRNA screening

Compared with the cell control group, the negative

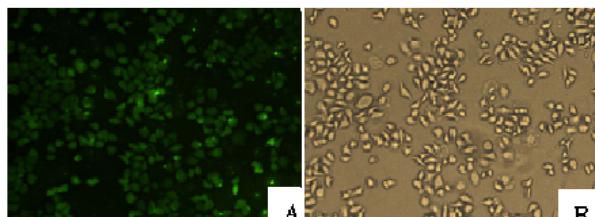


Figure 1. The Transfection Efficiency of Negative Control FAM- siRNA at the Concentration of 100 nM into EC9706 Cells. A: observation under a fluorescence microscope; and B: observation of the same visual field under a regular microscope ($\times 200$)

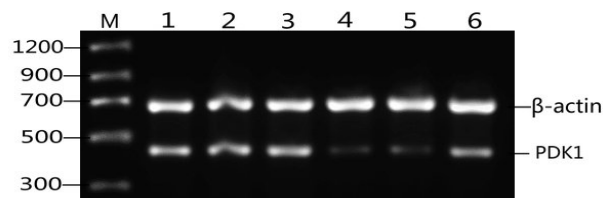


Figure 2. The Influence of PDK1 siRNA on the PDK1 mRNA Expression in EC9706 Cells. M: DNA Maker; 1: the control group; 2: the RNAi-Mate group; 3: the negative control siRNA group; 4: the PDK1 siRNA 1 group; 5: the PDK1 siRNA 2 group; and 6: the PDK1 siRNA 3 group

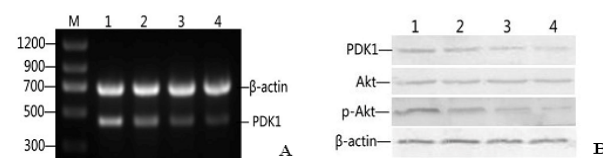


Figure 3. Downregulation of the PDK1 mRNA and Protein Expression and the Akt Phosphorylation Level by PDK1 siRNA. M: Maker; 1: non-transfection; 2: at 24 h after transfection; 3: at 48 h after transfection; 4: at 72 h after transfection; A: the PDK1 mRNA expression; and B: the PDK1, Akt and p-Akt protein expression

control siRNA group and the RNAi-Mate group did not show influences on the PDK1 mRNA expression; and no significant difference was found between the negative control siRNA group and the RNAi-Mate group ($P > 0.05$). The three PDK1 siRNA groups all had lower PDK1 mRNA expression compared with the cell control group, among which the siRNA 1 group had the highest inhibition rate ($P < 0.05$). The results are shown in Figure 2. Therefore, the PDK1 siRNA 1 sequences were determined in the following experimental procedures.

PDK1 mRNA and protein expression and Akt phosphorylation

Compared with the non-transfection (0 h) group, the PDK1 mRNA expression was decreased at 24 h, 48 h and 72 h after PDK1 siRNA 1 transfection. The inhibition rates at difference time points after transfection were $28.52 \pm 4.16\%$, $51.11 \pm 5.72\%$ and $60.75 \pm 4.07\%$, showing significant differences among them ($P < 0.05$). The PDK1 protein expression was also significantly decreased after PDK1 siRNA 1 transfection ($P < 0.05$), among which the decrease was more obvious at 48 h and 72 h, but no significant difference in the PDK1 protein expression was found between the 48 h and 72 h groups ($P > 0.05$ according to the LSD-t test). The p-Akt (the product of Akt phosphorylation) expression in the cells was noticeably decreased after the PDK1 siRNA 1 transfection, among which the most noticeable decrease was found in the 72 h group ($P < 0.05$), but the PDK1 siRNA 1 transfection did not show a significant influence on the Akt expression ($P > 0.05$). The results are shown in Figure 3.

Cell proliferation

The cell inhibition rates at 24 h, 48 h and 72 h after PDK1 siRNA transfection were obviously higher compared with the control group, the RNAi-Mate group and the negative control siRNA group ($P < 0.05$), among which the 48 h and 72 h groups showed more obvious

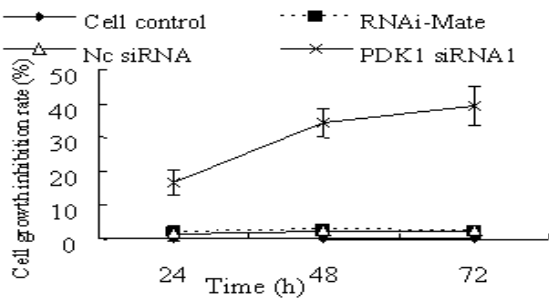


Figure 4. Comparisons of EC9706 Growth Inhibition Rates among Different Groups

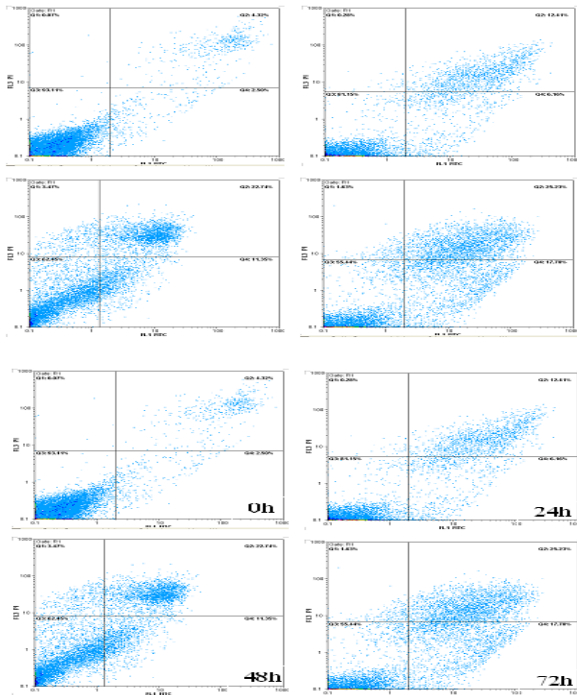


Figure 5. Comparisons of Apoptotic Rates at Different Time Points after PDK1 siRNA Transfection

inhibitory effects. No significant differences were found among the control group, the RNAi-Mate group and the negative control siRNA group ($P > 0.05$). The results are shown in Figure 4.

Apoptosis

Flow cytometry showed that the PDK1 siRNA 1 transfection increased both the early (the lower right quadrant) and late (the upper right quadrant) apoptosis compared with the 0 h group ($P < 0.05$), among which the apoptosis of the 72 h group was the most obvious (Figure 5).

Cell invasion

The Transwell invasion trial showed that the invasion inhibition rates of the three PDK1 siRNA 1 groups were $39.33 \pm 4.61\%$, $50.86 \pm 4.55\%$ and $56.47 \pm 5.12\%$; compared with the 24 h group, the 48 h and 72 groups showed more obvious inhibitory effects; but no significant difference was found between the 48 h and 72 h groups ($P > 0.05$, Figure 6).

Tumor growth and the PDK1 protein expression

The weight of the tumors in the control group was

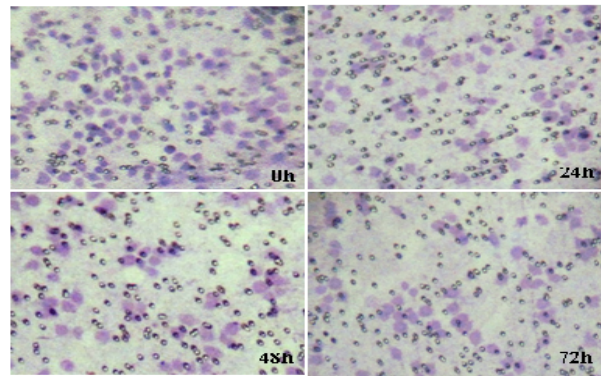


Figure 6. Comparisons of EC9706 Cell Invasion Capacities at Different Time Points after PDK1 siRNA Transfection ($\times 200$)

0.84 ± 0.08 g, which was significantly heavier than that in the PDK1 siRNA 1 group (0.51 ± 0.10 g); meanwhile, the PDK1 protein expression in the PDK1 siRNA 1 group was 32.31% lower than that in the control group ($P < 0.05$). These results were consistent to those obtained in the in vitro cell level trial.

Discussion

PDK1 is a 63 KD serine/threonine protein kinase, which contains an N-terminal kinase domain and a C-terminal pleckstrin homology domain (PH domain); the PH domain can bind with phosphatidylinositol-3, 4, 5-triphosphates (PIP3, products of P13K), which enables PDK1 to target at the cell membrane and to activate Akt (Granville et al., 2006); and the activated Akt phosphorylates a series of downstream substrates such as mTOR/p70S6K, Bad, Bax, FoxO3a and Caspase to further influence protein transcriptional translation, cell growth, cell migration and anti-apoptosis (Matheny et al., 2009). Therefore, blocking the excessively activated PI3K/Akt signal transduction pathway has become the hotspot of gene therapies for malignant tumors. Inhibition of the PDK1 expression in breast cancer MCF-7 cells (Iorns et al., 2009; Liu et al., 2009), malignant glioma U87-MG cells (Bilanges et al., 2005) and colon cancer HT-29 cells by RNA interference or micromolecular inhibitors can inhibit cell proliferation and invasion capacity, induce apoptosis, and increase the susceptibility of tumors to chemotherapeutics; and meanwhile, RNAi with PDK1 shows a better effect in increasing the susceptibility of MCF-7 cells to chemotherapeutics compared with RNAi with Akt (Liang et al., 2006). These results suggest that PDK1 might be a more effective treatment target compared with Akt.

RNAi is a phenomenon of posttranscriptional gene silencing. This technique promotes the formation of RISC through the binding of the homologous sequences in short double-stranded RNA and mRNA to induce the degradation of the target gene and to further inhibit the expression of the target gene. Small interference RNA (siRNA) is a small fragment of RNA transformed by double-stranded RNA or synthesized artificially in vitro during the process of RNAi. siRNA is comprised of 21 base pairs. As siRNA can block gene expression specifically, it has become a

potent biological means applied in tumor gene therapies. Therefore, the application of PDK1 targeted RNAi to interfere or block the excessively activated PI3K/Akt signal transduction pathway has been widely used in gene therapies for many malignant tumors. PDK1 siRNA effectively silences the PDK1 mRNA expression (Hsieh et al., 2004). PDK1 siRNA down-regulates the PDK1 expression specifically in breast cancer MDA-MB-231 cells, and inhibits the cell invasion capacity obviously (Min et al., 2009). It decreases the PDK1 mRNA and protein expression in breast cancer MCF-7, Y47D and MDA-MB-231 cells, and reduces the cell invasion capacities and tumor formation capacities in mice (Liu et al., 2009). PDK1 silencing by siRNA increases the susceptibility of MCF-7 cells to chemotherapeutics (Iorns et al., 2009; Peifer et al., 2009).

In the current study, siRNA was transfected into the EC9706 cells. The results showed that negative control siRNA transfection had no influence on the gene expression, but PDK1 siRNA 1 had the most obvious interference effect on the gene expression. Further, PDK1 siRNA obviously inhibited the PDK1 mRNA and protein expression and the phosphorylation of Akt. Moreover, PDK1 siRNA inhibited EC9706 growth and migration, promoted apoptosis, inhibited the growth of the transplanted tumors in the mice, and decreased the PDK1 protein expression in tumor tissues. These results indicate that PDK1 plays a critical role in the malignant biological behaviors of EC cells, and might serve as an effective target of gene therapies.

However, although PDK1 has attracted more and more attention in study on malignant tumors, highly efficient and susceptible PDK1 specific chemical suppressors which are applicable for in vivo use still remain to be developed. Therefore, development of such suppressors can be the focus of study in the future.

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