

RESEARCH ARTICLE

Effects of PLCE1 Gene Silencing by RNA Interference on Cell Cycling and Apoptosis in Esophageal Carcinoma CellsLi Zhao¹, Zi-Bai Wei¹, Chang-Qing Yang¹, Jing-Jing Chen², Dan Li², Ai-Fang Ji², Liang Ma^{2*}**Abstract**

Esophageal squamous cell carcinoma (ESCC) is one of the most malignancies with a poor prognosis. The phospholipase C ϵ gene (PLCE1) encodes a novel ras-related protein effector mediating the effects of R-Ras on the actin cytoskeleton and membrane protrusion. However, molecular mechanisms pertinent to ESCC are unclear. We therefore designed PLCE1-special small interfering RNA and transfected to esophageal squamous cell (EC) 9706 cells to investigate the effects of PLCE1 gene silencing on the cell cycle and apoptosis of ESCC and indicate its important role in the development of ESCC. Esophageal cancer tissue specimens and normal esophageal mucosa were obtained and assayed by immunohistochemical staining to confirm overexpression of PLCE1 in neoplasias. Fluorescence microscopy was used to examine transfection efficiency, while the result of PLCE1 silencing was examined by reverse transcription (RT-PCR). Flow cytometry and annexin V apoptosis assays were used to assess the cell cycle and apoptosis, respectively. Expression of cyclin D1 and caspase-3 was detected by Western-blotting. The level of PLCE1 protein in esophageal cancer tissue was significantly higher than that in normal tissue. After transfection, the expression of PLCE1 mRNA in EC 9706 was significantly reduced, compared with the control group. Furthermore, flow cytometry results suggested that the PLCE1 gene silencing arrested the cell cycle in the G0/G1 phase; apoptosis was significantly higher than in the negative control group and mock group. PLCE1 gene silencing by RNAi resulted in decreased expression of cyclin D1 and increased expression of caspase-3. Our study suggests that PLCE1 may be an oncogene and play an important role in esophageal carcinogenesis through regulating proteins which control cell cycling and apoptosis.

Keywords: Esophageal neoplasms-squamous cell-gene - PLCE1 - RNA interference

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Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most aggressive cancers worldwide. Although great efforts have been made in the diagnosis and surgical treatment, the survival rate of ESCC remains poor (Chen et al., 2013). Therefore, it is necessary to study the molecular mechanisms of the development of ESCC.

PLCE1 belongs to the phospholipase family that catalyzes the hydrolysis of polyphosphoinositides such as phosphatidylinositol-4, 5-bisphosphate (PtdIns (4, 5) P2) to generate the second messengers Ins (1, 4, 5) P3 and diacylglycerol. These products initiate a cascade of intracellular responses that result in cell growth and differentiation and gene expression. Wang found that PLCE1 is correlated with the occurrence and development of ESCC (Wang et al., 2010).

In this study, we designed PLCE1-special small interfering RNA and transfected it to EC9706 cells to investigate the effects of PLCE1 gene silencing on the cell cycle and apoptosis of ESCC and indicate its important role in the development of ESCC.

Materials and Methods*Reagents*

Lipofectamine 2000 (Invitrogen, USA), PLCE1 primers and PLCE1 small interfering RNA (siRNA) and scrambled sequence siRNA from Gene Pharma (Shanghai, China), RPMI 1640 (Invitrogen, USA), primary antibody anti PLCE1 (goat anti human IgG; Santa Cruz Biotechnology, Santa Cruz, CA), anti Caspase-3 (mouse anti human IgG; Santa Cruz Biotechnology, Santa Cruz, CA), anti Cyclin D1 (mouse anti human IgG; Zhongshan Biotechnology, Beijing, China), anti β -actin (rabbit anti human IgG; Zhongshan Biotechnology, Beijing, China). Secondary antibodies, IRDye conjugated donkey anti rabbit IgG, goat anti mouse IgG, goat anti rabbit IgG were from LI-COR (LI-COR Biotechnology, Nebraska, USA). PV-9003 Polink-2 plus Polymer HRP Detection System For Goat Primary Antibody were from Zhongshan Biotechnology (Beijing, China). Western blot detection reagents were obtained from Santa Cruz Biotechnology Inc., CA, United States. RT-PCR kit (Solarbio Biotechnology, Beijing, China).

¹Department of Endoscopy, ²Department of Central laboratory, Peace Hospital Attached to Changzhi Medical College, Changzhi, China *For correspondence: maliang2004666@163.com

Cell lines and culture conditions

The human ESCC cell lines EC9706 were purchased from the Tumor Cell Bank of Chinese Academy of Sciences, and cultured in RPMI1640 and supplemented with 10% fetal bovine serum (FBS, Sigma, USA). Cells were cultured at 37°C under 5% humidified CO₂ enriched atmosphere and routinely sub-cultured every 2 d by trypsinization. Then cells were preserved with RPMI 1640 free of serum or antibiotics and prepared for transfection assays.

Liposome mediated cell transfection assays

Experiment was divided into three groups: negative control group (scrambled sequence), untreated group (mock), PLCE1-special sequence group (PLCE1 siRNA group). PLCE1-special siRNA were forward 5'-CCCUGAAUAAUUGUGAUGAUTT -3' and reverse 3'-AUCAUCACAAUUAUCAGGGTT-5'. Scrambled siRNA were 5'-UUCUCCGAACGUGUCACUUTT-3' and reverse 3'-ACGUGACACGUUCGG AGAATT-5'. Transfection was carried out using the manufacturer's instructions, using PLCE1-special siRNA or scrambled sequence siRNA at final concentrations of 30 nmol/L and 50 nmol/L and 100 nmol/L, respectively, mock group were transfected with reagent alone as control. All reproducible results were repeated for three times.

Immunohistochemistry

40 cases esophageal cancer tissue from resected specimens and matched noncancerous were obtained from Peace Hospital Attached to Changzhi Medical College between 2011 and 2013. Histological tumor typing was carried out on the basis of biopsies or resected specimens in the Department of Pathology of the same hospital. The tissues were routinely fixed in 10% natural-buffered formalin and embedded in paraffin. The sections were dewaxed using xylene and rehydrated in graded alcohols. To reduce nonspecific background staining, endogenous peroxidase activity was blocked by 3% hydrogen peroxide for 15min at room temperature. The sections were incubated overnight with primary antibody anti PLCE1 (goat anti human IgG; 1:200) at 4°C, then washed 3 times with PBS, and incubated for 20min at 37°C with secondary antibodies (IRDye conjugated donkey anti rabbit IgG). After further washing in PBS, the sections were incubated with 3, 30-diaminobenzidine dilute, lightly counterstained with hematoxylin, and observed under a photomicroscope.

RT-PCR analysis of PLCE1 mRNA levels

After being transfected with PLCE1 siRNA for 48h, cell were harvested for reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from EC9706 cells (negative control group, untreated group, PLCE1- siRNA group) using RT-PCR kit (Solarbio Biotechnology, Beijing, China). All the RNA preparations had an optical density OD₂₆₀:OD₂₈₀ ratio of 1.9-2.0. Reverse transcription was performed by using 1µg RNA in a first-strand cDNA synthesis reaction with RT-PCR kit (Solarbio Biotechnology, Beijing, China). Primers for PLCE1 were designed as follows: forward primer, 5' -TAACTGACGATGAGATGGCAAC-3'; reverse

primer, 5'-TGTTTGAAGAAGACAGCACAGG-3'. β-actin was amplified as internal control: forward primer, 5'-CATCCTCACCCCTGAAGTACCCC-3'; reverse primer, 5'-AGCCTGGATGCAACGTACATG-3'. The PCR (35 cycles) was conducted in a Mastercycler thermal cycler (Eppendorf). Each cycle included denaturation (94°C, 30 seconds), annealing (56°C, 30seconds), and extension (72°C, 30 seconds). The initial denaturation period was 3 minutes, and the final extension was 5minutes. The PCR products were electrophoresed on 1.5% agarose gels and bands were visualized by ethidium bromide staining and the results of gel electrophoresis. To quantify difference in gene expression, the relative gene expression of PLCE1 was analyzed by Quantity one 4.5.0 software. The β-actin mRNA expression was taken as a control. The PLCE1 mRNA level was evaluated by the relative intensity ratio of PLCE1 mRNA /β-actin mRNA.

Cell cycle analysis

Flow cytometric analysis was carried out as described previous (Shi et al., 2009). Cells were cultured in serum-free medium for 24h to synchronize the cells, then trypsinized and suspended in phosphate buffered saline (PBS). Single-cell suspensions were fixed using 70% ethanol overnight at 4°C, and subsequently permeabilized with PBS containing 1 mg/ml propidium iodide (Sigma), 0.1% Triton X-100 (Sigma) and 2 mg DNase-free RNase (Sigma) at room temperature. Results were analyzed using Multicycle-DNA Cell Cycle Analyzed Software.

Detection of apoptosis through fluorescein annexin V-FITC/PI double labeling

3 infected and mock-infected cells were harvested through 0.25% trypsinization, after washing with ice-cold PBS twice, cells were centrifuged at 1000 r/min for 5 min. Cells were then resuspended with four hundred µL of 1×binding buffer, then cells were stained with 5 µL of FITC-conjugated annexin V (Pharmingen) and 5 µL of PI (Pharmingen) for 15 min at 4°C in the dark. The samples were analyzed by FACS (Becton Dickinson) using Cell Quest Research Software (Becton Dickinson).

Protein extraction and Western blot

After being transfected with PLCE1 siRNA for 48h, cell were harvested for western blot analysis for detection of PLCE1. Cells were washed twice with ice-cold PBS, and collected cells were lysed in buffer (50 mmol Tris-HCl, pH 7.5, 150 mmol NaCl, 1mmol EDTA, 1% Triton X-100, 1 mmol NaF, 1 mmol activated Na₃VO₄, 0.1% sodium dodecyl sulfate and protease inhibitors. Protein concentration was measured using Bradford reagent (Sigma) and bovine serum albumin as standard, then separated by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes by electroblotting. Membranes were blocked with 5% nonfat milk in Tris buffered Saline with Tween 20 (TBST, 50 mmol Tris-HCL, PH7. 6, 150 mmol NaCl, 0.1% Tween 20) for 2h at room temperature, and subsequently incubated with primary antibody (anti-Cyclin D1, 1:200, β-actin, 1:2000, anti-Caspase-3, 1:200) in blocking buffer at 4°C overnight.

Following a wash with TBST, the membranes were incubated with IRDye conjugated secondary antibody (goat anti mouse, 1:10000, goat anti rabbit, 1:10000) for 2h at room temperature, then the membranes were washed with TBST, the protein quantity was analyzed by Odyssey Image Tool (LI-COR Inc., Nebraska, USA). The β -actin bands were taken as loading control. The target protein expression was evaluated by the relative intensity ratio of target protein/loading control.

Statistical analysis

The results are expressed as the mean \pm SEM. All data were analyzed by SPSS 18.0 statistical software (SPSS, Chicago, IL). Differences between means of two groups were tested by paired-samples T test and between multiple groups were tested by Analysis of Variance (ANOVA). Differences are significant at $P<0.05$.

Results

Immunohistochemical analysis of PLCE1

Immunohistochemical staining was used to examine the expression of PLCE1 protein in 40 cases of esophageal cancer tissues and matched noncancerous. Positive granules were mainly distributed over cytoplasm of tumor cells, the positive granules were stained brown orange (Figure 1). The level of PLCE1 protein in esophageal cancer tissue was significantly higher than that in normal tissue, with statistical significance (38.40 ± 7.47 vs 5.56 ± 0.31 , $F=24.43$, $P<0.05$).

Successful transfection of small interfering RNA

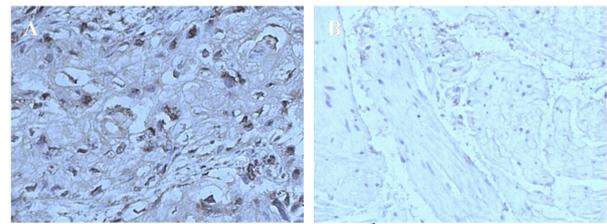
We have successfully transfected PLCE1 siRNA into EC9706 cells via lipofectamine and observed by fluorescent microscopy (Figure 2). EC9706 cells in negative control group and PLCE1 siRNA group were all took on green fluorescence, cells in mock group had no green fluorescence expression. Transfection efficiency after 6-12 hours were determined through the observation of green fluorescence; the strongest green fluorescence occurred at 48 hours. The cells that had clear structure and form took on full fluorescent protein in nucleus.

RT-PCR analysis of PLCE1 mRNA levels among different groups

Expression level of the PLCE1 mRNA 48h post-transfection of PLCE1 siRNA revealed a significant decrease in mRNA level as compared with the negative control group and mock group as shown in (Figure 3). The difference between mock group and PLCE1 siRNA group was significant (0.592 ± 0.003 vs 0.856 ± 0.002 , $T=66.454$, $P=0.000$)

Cell cycle analysis

Compared with the control group, flow cytometry results suggested that the down-regulation of PLCE1 can arrested cell cycle in G0/G1 phase and prevent cells from entering into the S phase as shown in Table 1 and Figure 4. PLCE1 gene silencing increased the percentage of G0/G1 phase and decreased the cells in S phase accordingly ($P<0.05$).



A strong expression in esophageal cancer tissues
B less expression in normal tissues

Figure 1. PLCE1 Expression in Esophageal Cancer Tissues and Normal Tissues

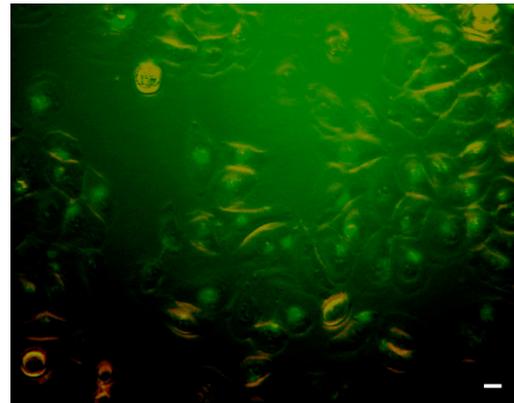


Figure 2. Effect of Transfection of PLCE1 siRNA on EC9706 Cells

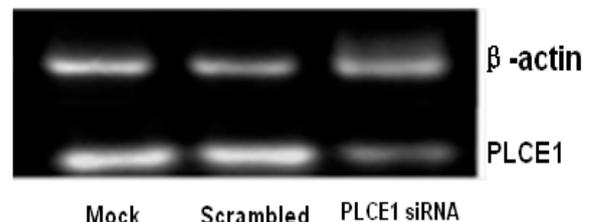


Figure 3. Small Interfering RNA-Targeting PLCE1 Was Transfected in EC9706 Cells.

PLCE1 gene silencing induces cell death by apoptosis

Apoptosis induced by PLCE1 gene silencing was further evaluated using the Annexin V-FITC/PI staining assay. The percentage of apoptosis in PLCE1-siRNA group was higher than that of the mock and the scrambled group, with statistical significance ($P<0.01$). Results showed that down-regulating PLCE1 could promote the apoptosis of EC9706 cells (Figure 5).

Western blot analysis of PLCE1 protein

The PLCE1 protein was expressed in all three groups of the EC9706 cells. The optical density value for the bands for each of the three groups was compared with that of β -actin. The expression of cell cycle related protein cyclinD1 in PLCE1-siRNA group was lower than the other two groups (Figure 6A), and the difference was significant ($P<0.05$). The expression of apoptosis related protein caspase-3 in PLCE1-siRNA group was higher than the other two groups (Figure 6B), and the difference was significant ($P<0.05$). The results showed that PLCE1 gene silencing could inhibit the expression of the cyclinD1 while promote the caspase-3.

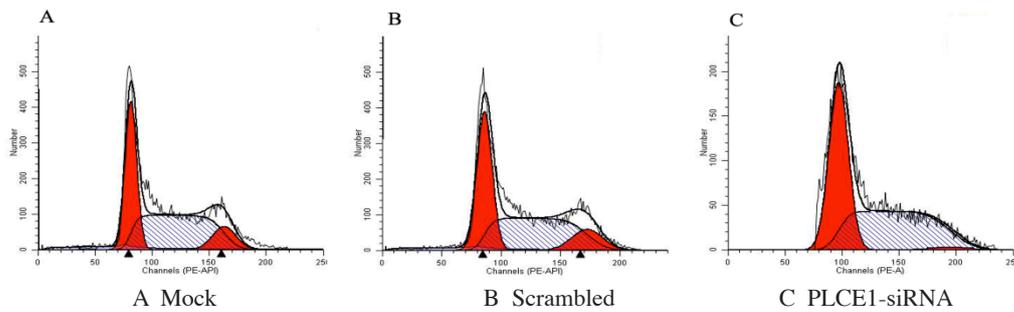


Figure 4. Cell Cycle Analysis

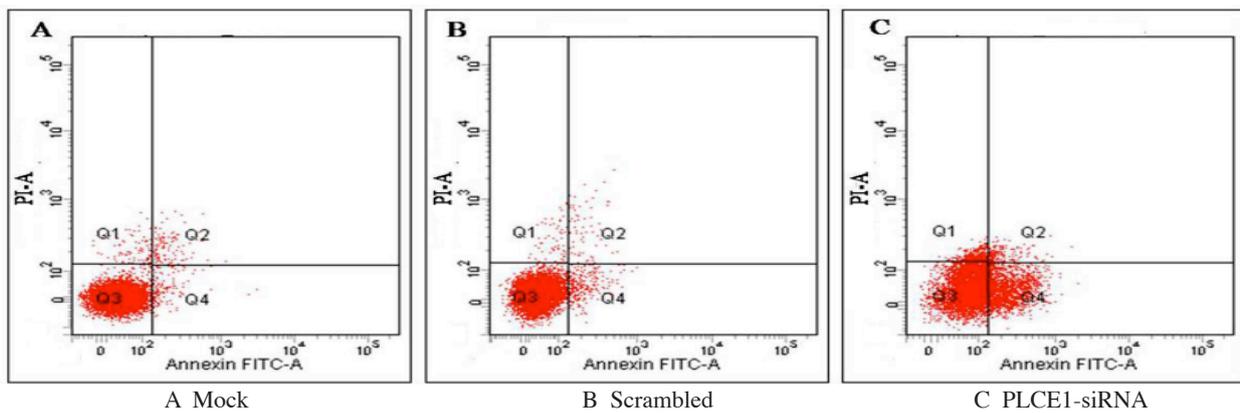
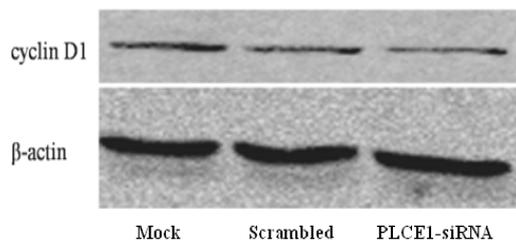
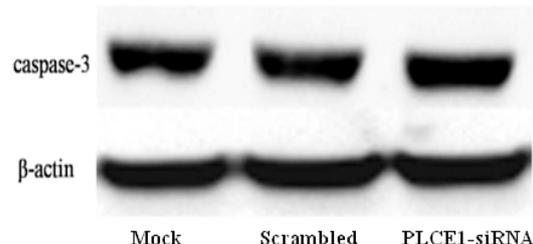


Figure 5. The PLCE1 Silencing Induced Apoptosis of EC9706



A Western blot analysis of cyclinD1



B Western blot analysis of caspase-3

Figure 6. Western Blot Analysis of CyclinD1 Protein and Caspase-3 Protein Expression in Three Groups, Normalized to β-actin Expression in EC9706 Cells

Table 1. The Cell Cycle Distribution of EC9706 after RNA interfering

Groups	G ₀ /G ₁ (%)	S(%)	G ₂ /M(%)
Mock	36.46±0.77*	51.73±0.81	11.56±0.99
Scrambled	38.87±0.32*	52.19±2.26	12.02±1.12
PLCE1-siRNA	45.65±1.39	50.81±0.61	1.90±0.17

*P<0.05 versus PLCE1-siRNA group

Discussion

Carcinogenesis is a multistage process, which involves various molecular events related to the fundamental alterations in cell physiology. It is very important to explore the molecular mechanism. In 2010, by genome-wide association study (GWAS) of 25,000 patients for the susceptibility loci to esophageal squamous cell carcinoma (ESCC) and health controls, we firstly identified PLCE1 (phospholipase Cε1, 10q23) and C20orf54 as the susceptibility loci to ESCC (Wang et al., 2010). Our previous study showed that the functional SNP in C20orf54 may modify susceptibility to esophageal squamous cell carcinoma (Ji et al., 2011). PLCE1 showed

significantly higher expression in esophageal carcinoma tissue. Some studies found that PLCE1 is correlated with the occurrence and development of gastric carcinoma (Luo et al., 2011), colorectal carcinoma (Danielsen et al., 2011), small intestine cancer (Li et al., 2009), head and neck squamous cell carcinoma (Bourguignon et al., 2006) and skin cancer (Bai et al., 2004).

PLCE1 has a Ras-associating domain at its C terminus and a CDC25-like domain at its N terminus. CDC25 domain is a key regulators in response to cell cycle checkpoints, plays a very important role in the cell's recovery from G₂/M checkpoint induced arrest. The role of PLCE1 in ESCC remains unclear. PLCE1 encodes an enzyme that produces two vital intracellular second messengers: diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃), which induce activation of protein kinase C and mobilization of Ca²⁺ from intracellular stores, respectively. Protein kinase C is an important signaling molecule in mediating tumor cell adhesion, movement, and invasion, and a PKC suppressor has displayed anti-invasion characteristics *in vitro* and *in vivo* experiments. As a special domain of PLCE1, the

CDC25-like domain could change the molecular structure of Ras family via acting on guanosine triphosphate exchange factor (GEF), and activate the Ras/MAPK signaling pathways (Cui et al., 2013) and then regulate cell apoptosis and growth, promotes cell proliferation, differentiation and even malignant change (Perrin et al., 2013). Ada-Nguema AS (Ada-Nguema et al., 2006) found that phospholipase C ϵ (PLC ϵ) is a novel R-Ras effector mediating the effects of R-Ras on the actin cytoskeleton and membrane protrusion, because R-Ras was co-precipitated with PLC ϵ and increased its activity (Martins et al., 2014). Knockdown of PLC ϵ with siRNA reduced the formation of the ruffling lamellipod in R-Ras cells. A corollary is that PLCE1 may lead to tumor formation by a chain of active reaction like regulating cell proliferation and differentiation, affecting the cell movement and cytoskeleton (Lad et al., 2006; Ou et al., 2010).

The development of research on RNA interference encourage us to further study on ESCC mechanism. Li (Li et al., 2013) found that HDAC6 siRNA can effectively downregulate the expression of HDAC6 mRNA and protein in EC9706 cells. Down-regulation of HDAC6 expression can obviously inhibit cell proliferation, arrest cell cycling in the G0/G1 phase and reduce cell migration. We designed PLCE1-special siRNA (small interfering RNA) to investigate the effects of PLCE1 (phospholipase C ϵ 1) gene silencing on the cell cycle and apoptotic of esophageal squamous carcinoma cells (ESCC), and attempt to disclose the role of PLCE1 in ESCC.

ESCC has a striking geographic distribution worldwide, with higher prevalence in some areas of China. Taihang mountain of China is one of high incidence district of esophageal cancer. Peace Hospital Attached to Changzhi Medical College is located in the taihang mountain. Clinical specimens include esophageal squamous carcinoma tissues and adjacent normal tissues were taken from our surgery department. Immunohistochemical experiment results showed that the level of PLCE1 protein in esophageal cancer tissue was significantly higher than that in normal tissue, suggesting PLCE1 is associated with development and progression of ESCC. RT-PCR result showed that the expression level of the PLCE1 mRNA 48h post-transfection of PLCE1 siRNA revealed a significant decrease in mRNA level as compared with the negative control group and mock group. Flow cytometry revealed that the down-regulation of PLCE1 can arrested cell cycle in G0/G1 phase and apoptosis was significantly higher than that of the control group. Western blot showed that the expression of apoptosis related protein caspase-3 in PLCE1-siRNA group was higher than the other two groups and the expression of cell cycle related protein cyclinD1 in PLCE1-siRNA group was lower than the other two groups, the down-regulation of PLCE1 could inhibit the expression of the cyclinD1 while promote the caspase-3. Cyclin D1 is a major regulator of the progression of cells into the proliferative stage of the cell cycle (Ferrandiz et al., 2012). Cell cycle progression from G0/G1 to the S phase requires cyclin/cyclin-dependent kinase (CDK) complexes and hyperphosphorylated retinoblastoma protein (Rb) (Takahashi et al., 2007). Our study showed that PLCE1 gene silencing by RNAi results in decreased

expression of cyclin D1 and blocked transition from G1 to S phase.

Apoptosis is an important mechanism to maintain homeostasis in mammals, and disruption of the apoptosis regulation mechanism triggers a range of diseases, such as cancer, autoimmune diseases, and developmental disorders. Caspase-3 is a member of the caspase (cysteine aspartic proteinases) family of enzymes, which are the major inducers of apoptosis. Caspase-3 is activated in the apoptotic cell both by extrinsic (death ligand) and intrinsic (mitochondrial) pathways (Huang et al., 2014). One signaling event is the introduction of granzyme B, which can activate initiator caspases, into cells targeted for apoptosis by killer T cells. This extrinsic activation then triggers the hallmark caspase cascade characteristic of the apoptotic pathway, in which caspase-3 plays a dominant role. Our experiment showed that PLCE1 gene silencing by RNAi results in increased expression of caspase-3, and then promotes the apoptosis of ESCC. Flow cytometry results have proved this.

In summary, our study revealed that PLCE1 gene is closely associated with development and progression of ESCC. PLCE1 could promote cell proliferation by up-regulating the expression of Cyclin D1, and decrease apoptosis by down-regulating the expression of caspase-3.

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