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PLCE1 inhibits apoptosis of non-small cell lung cancer via promoting PTEN methylation

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Abstract. – OBJECTIVE: To explore the role of phospholipase C epsilon 1 (PLCE1) in regulating cell apoptosis of non-small cell lung cancer (NSCLC) and its underlying mechanism.

PATIENTS AND METHODS: The mRNA and protein levels of PLCE1 in NSCLC tissues, adjacent normal tissues and NSCLC cell lines (A549 and H1299) were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and Western blot, respectively. Methylation status in the promoter region of PTEN in NSCLC cells was accessed using the relative commercial kit. Cell apoptosis after transfection of PLCE1 siRNA in NSCLC cells was detected by flow cytometry. Protein expressions of apoptosis-related genes in NSCLC cells after altering PLCE1 expression were detected by Western blot.

RESULTS: PLCE1 was highly expressed in NSCLC tissues and cell lines than that of controls. PLCE1 knockdown promoted PTEN expression and inhibited methylation in H1299 cells. Transfection of PLCE1 siRNA in NSCLC cells remarkably induced cell apoptosis.

CONCLUSIONS: PLCE1 inhibits cell apoptosis of NSCLC by promoting PTEN methylation.

Key Words:
NSCLC, PLCE1, PTEN, Cell apoptosis.

Introduction

Lung cancer (LC) is a malignant tumor that seriously threatens human health in the world today. The morbidity and mortality of LC have been annually increased, ranking the highest in all malignancies[1,2]. Non-small cell lung cancer (NSCLC) accounts for 80%-85% of LC cases, mainly divides into squamous cell carcinoma and adenocarcinoma. Although progresses in radiotherapy, chemotherapy, and surgery have been advanced, the overall prognosis of NSCLC is poor, with the 5-year survival rate of less than 15%[3]. The incidence, evolution, and prognosis of NSCLC are related to mutations in DNA repair genes, growth factor signaling pathways, and abnormalities in cell cycle regulation[3-5]. Many oncogenes and tumor-suppressor genes are involved in the incidence and progression of NSCLC. For example, PTEN is considered as a tumor-suppressor gene that is closely related to tumorigenesis[6]. PTEN is capable of arresting cell cycle, inducing apoptosis and damaging genomic stability. However, the specific mechanism of PTEN inactivation in NSCLC still needs to be further clarified.

Phospholipase C epsilon 1 (PLCE1) is a new member of the phospholipase C family discovered in recent years. The chromosome where PLCE1 locates is found to be the susceptible site of esophageal squamous cell cancer[7]. PLCE1 exerts an important role in regulating multiple biological processes that may affect tumor development[8,9]. We first detected PLCE1 expression in NSCLC tissues and cell lines. The regulatory effect of PLCE1 on cellular functions of NSCLC was further conducted.

Patients and Methods

Reagent

Primary and secondary antibodies, PLCE1 siRNA and siRNA negative control were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); apoptosis detection kit was obtained from Sigma-Aldrich (St. Louis, MO, USA); DNA purification kit was purchased from Promega (Madison, WI, USA); methylation detection kit was purchased from Clontech (Mountain View, CA,

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USA); transfection, quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and Western blot reagents were all obtained from Invitrogen (Carlsbad, CA, USA).

Patients

32 NSCLC patients treated in The First People's Hospital of Yunnan Province from April 2015 year to September 2017 year were enrolled. Patients were pathologically diagnosed as NSCLC and did not receive preoperative anti-tumor treatments. NSCLC and adjacent normal tissues were surgically resected, followed by immediate preservation in liquid nitrogen. All patients signed the informed consent form. This investigation was approved by The First People's Hospital of Yunnan Province Ethic Committee.

Cell Culture and Transfection

293T, A549, and H1299 cells were incubated in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin and 0.1 mg/L streptomycin. Cells were incubated in a 5% CO_2 incubator at 37°C. Cell passage was performed until 85% of cell density. Culture medium was replaced every 3 days. Transfection was performed following the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

RNA Extraction and qRT-PCR

The TRIzol kit (Invitrogen, Carlsbad, CA, USA) was used to extract the total RNA, followed by reverse transcription into complementary Deoxyribose Nucleic Acid (cDNA). After the cDNA was amplified, qRT-PCR was performed to detect the expressions of related genes. Primers used in the study included: PTEN, F: 5'-ACACGACGGGAAGACAAGTT-3', R: 5'-TCCTCTGGTCCTGGTATGAAG-3'; PLCE1, F: 5'-GAGCTGCAATCGAAGTCTGG-3', R: 5'-AAGGCCTTCTGTGAGTCCTC-3'; β-actin, F: 5'-CTCCATCCTGGCCTCGCTGT-3', R: 5'-GCTGTCACCTTCACCGTTCC-3'.

Western Blot

Cells were lysed for protein extraction. The concentration of each protein sample was determined by a BCA (bicinchoninic acid) kit (Pierce, Rockford, IL, USA). The protein sample was separated by gel electrophoresis and transferred to PVDF (polyvinylidene difluoride) membranes (Roche, Basel, Switzerland). After incubation with primary and secondary antibodies, immunoreactive bands were exposed by enhanced chemiluminescence method.

Cell Apoptosis Detection

H1299 cells were digested, incubated with Annexin V and 5 mg/L propidium iodide in the dark for 15 min. Subsequently, cell apoptotic rate was detected using flow cytometry.

PTEN Methylation Detection

Cell gDNA was first extracted from NSCLC cells using DNA Purification Kit. The methylated level of PTEN was detected according to the manufacturer's recommendation. PCR products underwent agarose gel electrophoresis for observing the methylation level.

Statistical Analysis

We used Statistical Product and Service Solutions (SPSS) 19.0 software (IBM, Armonk, NY, USA) for statistical analysis. The quantitative data were represented as mean ± standard deviation ($\bar{x}\pm s$). The *t*-test was used for comparing differences between the two groups. Differences among multiple groups were compared using one-way ANOVA, followed by a post-hoc test. $p<0.05$ was considered statistically significant

Results

PLCE1 Was Highly Expressed in NSCLC

Protein expression of PLCE1 was higher in NSCLC tissues than that of adjacent normal tissues (Figure 1A). Similarly, the mRNA level of PLCE1 was also highly expressed in NSCLC tissues compared with that of adjacent normal tissues (Figure 1B). Also, both mRNA and protein levels of PLCE1 in A549 and H1299 cells were higher than those of 293T cells (Figure 1C-1E).

PLCE1 Knockdown Promoted Apoptosis of NSCLC Cells

We first constructed PLCE1 siRNA and siRNA-NC, and their transfection efficacies in H1299 cells were verified (Figure 2A). Flow cytometric results indicated that PLCE1 knockdown remarkably elevated cell apoptotic rate than that of controls (Figure 2B and 2C). Subsequently, protein expressions of apoptosis-related genes were detected by Western blot. It is found that protein expressions of cleaved caspase3 and

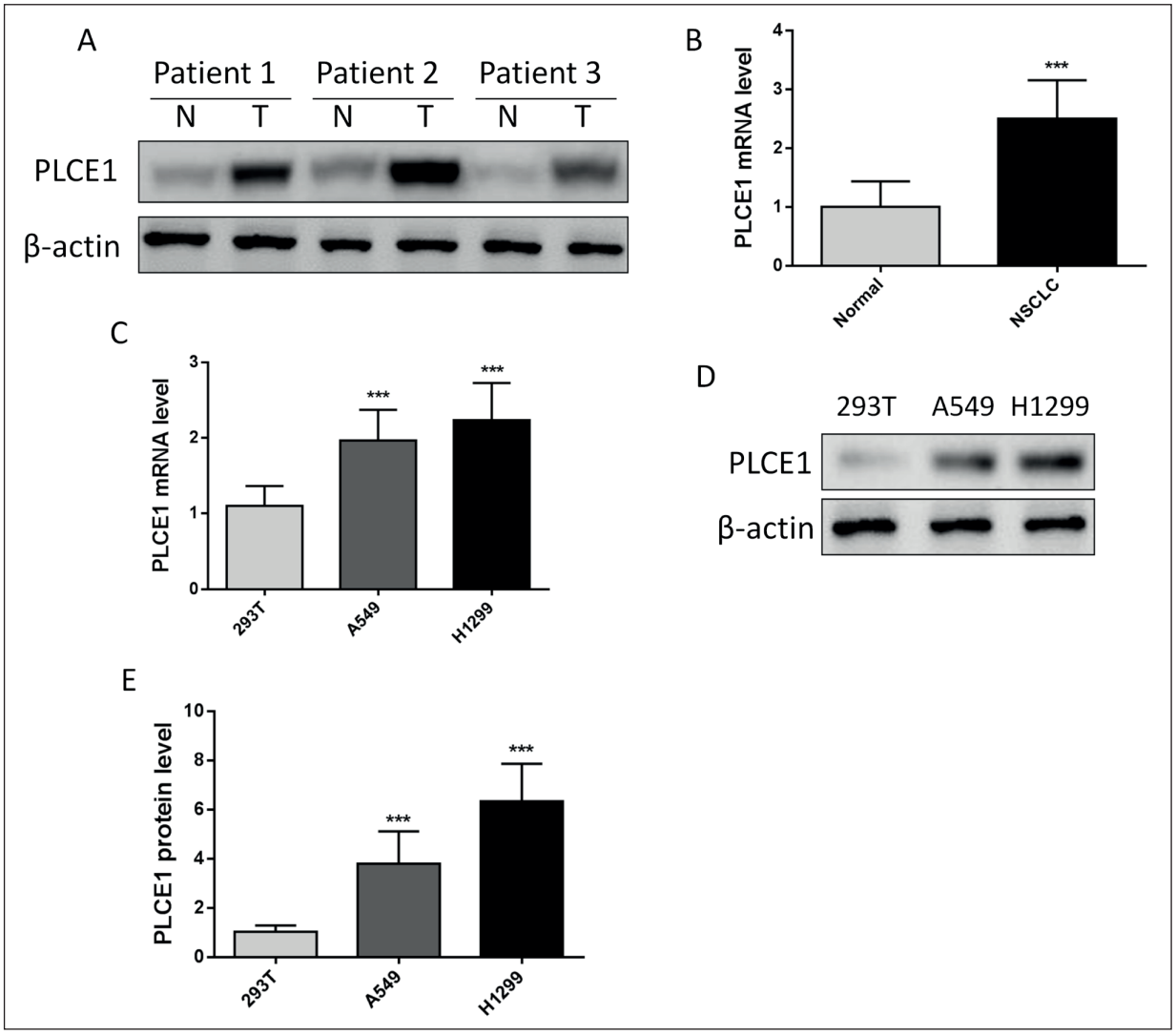

Figure 1. PLCE1 was highly expressed in NSCLC. ***A,*** Protein expression of PLCE1 in NSCLC tissues and adjacent normal tissues. ***B,*** The mRNA expression of PLCE1 in NSCLC tissues and adjacent normal tissues. ***C,*** The mRNA expression of PLCE1 in 293T, A549 and H1299 cells. ***D,*** Protein expression of PLCE1 in 293T, A549 and H1299 cells.

caspase9 were remarkably higher in H1299 cells transfected with PLCE1 si-RNA than those of controls (Figure 2D).

PLCE1 Knockdown Promoted PTEN Expression in NSCLC

Western blot was conducted to detect PTEN expression in A549 and H1299 cells. The results showed that the protein expression of PTEN is negatively regulated by PLCE1 (Figure 3A and 3B).

PLCE1 Regulated the Methylation of PTEN Promoter

QRT-PCR results showed that the percent of methylated PTEN in 293T cells was 20%, which was 50% in H1299 cells (Figure 3C). After transfection of PLCE1 si-RNA in H1299 cells, the level of methylated PTEN was markedly decreased. On the contrary, PLCE1 overexpression in 293T cells led to PTEN hypermethylation. It is concluded that PLCE1 downregulates PTEN expression *via* promoting its methylation.

Discussion

NSCLC is one of the common malignancies in China involving diverse molecular mechanisms. Studies have shown that gene mutations, loss of heterozygosity, and inactivation of tumor-sup-

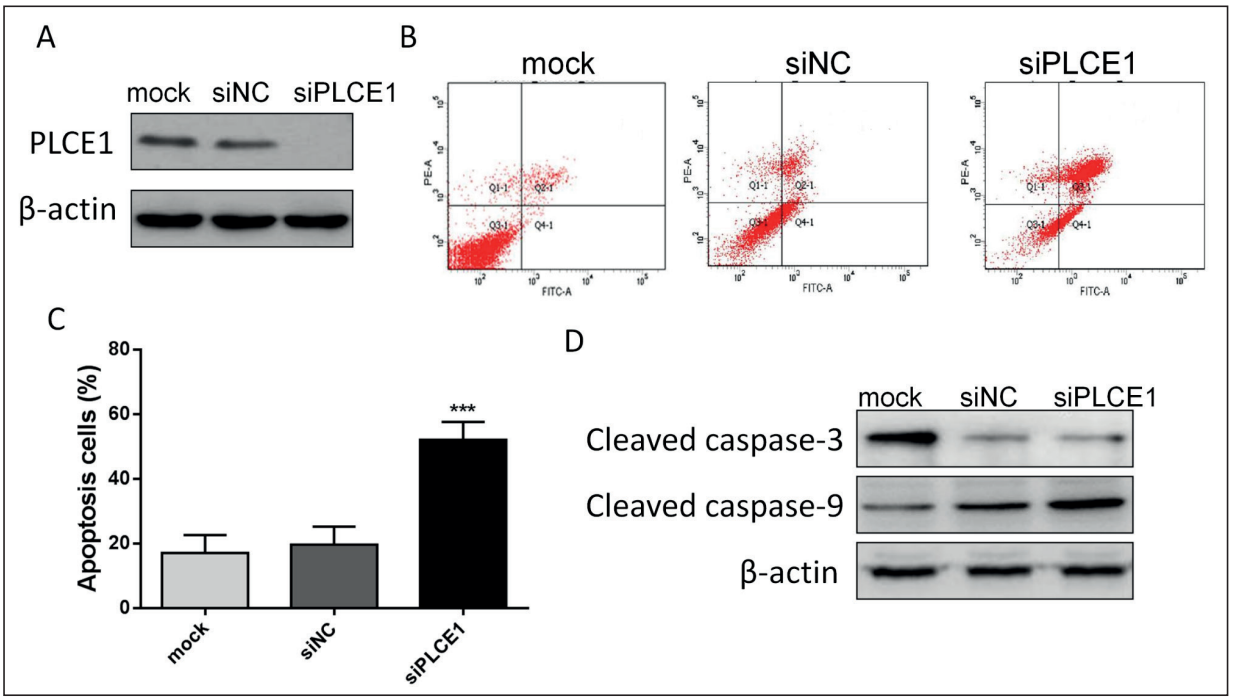

Figure 2. PLCE1 knockdown promoted apoptosis of NSCLC cells. ***A,*** Transfection efficacy of PLCE1 si-RNA in H1299 cells. ***B, C,*** Apoptotic rate of H1299 cells after transfection with PLCE1 si-RNA or si-RNA negative control. ***D,*** Protein expressions of cleaved caspase-3 and caspase-9 in H1299 cells.

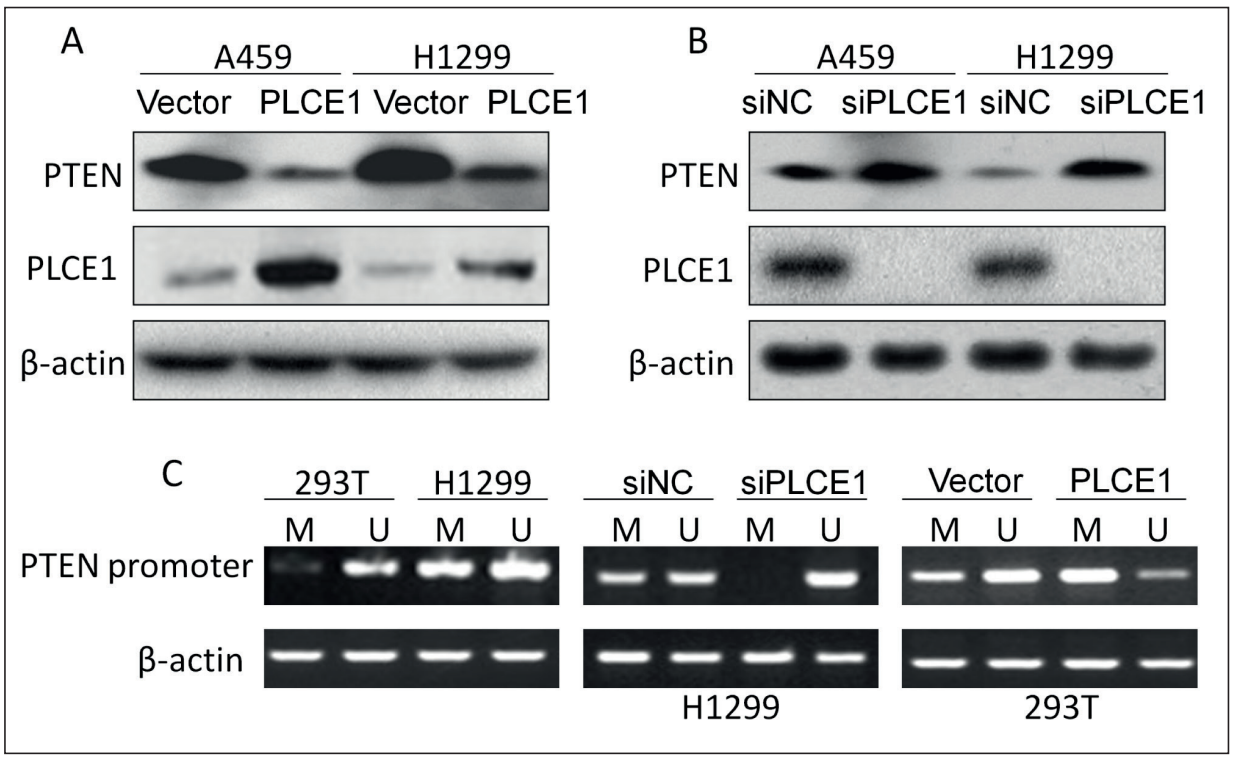

Figure 3. PLCE1 downregulated PTEN expression *via* promoting its methylation. ***A,*** PTEN expression in A549 and H1299 cells after PLCE1 overexpression. ***B,*** PTEN expression in A549 and H1299 cells after PLCE1 knockdown. ***C,*** Methylated and un-methylated PTEN levels in NSCLC cells. M: Methylated PTEN primer; U: Un-methylated PTEN primer.

pressor genes can promote the development of NSCLC[10]. PTEN served as a tumor-suppressor gene. PTEN inactivation is frequently seen in tumors, including PTEN mutation, deficiency, and low expression. The role of PTEN inactivation is of great significance in the incidence, progression, and prognosis of tumors[11]. Current researches have proved PTEN inactivation in glioblastoma, ovarian cancer, prostate cancer, thyroid cancer, and lung cancer[12]. Additionally, PTEN deletion frequently occurs in the late stage of the tumor.

Complete inactivation of PTEN is observed in the late stage of LC, where LC presents higher abilities of invasion of metastasis, indicating that PTEN deficiency may promote LC development[13]. Soria et al[14] reported that 30 (24%) of 125 patients with early-stage NSCLC are PTEN-negative, and 7 of them (35%) are methylated. Although PTEN mutations in NSCLC are rare, PTEN deficiency and low expression are commonly seen. PTEN deficiency is found in advanced prostate cancer due to its methylation. The role of PTEN in NSCLC development requires in-depth researches.

PLCE1 is a new member of the phospholipase C family discovered in recent years. Numerous biological functions could be regulated by PLCE1. So far, the role of PLCE1 in tumors is controversial. Chen et al[15] reported that PLCE1 is highly expressed in patients with esophageal cancer, which is positively correlated with tumor stage. Hu et al[16] reported that the mRNA level of PLCE1 is lowly expressed in esophageal cancer tissues. Further immunostaining analysis indicated that the staining score of PLCE1 rs2274223 GG was lower than that of AG type. Our work found that PLCE1 is highly expressed in A549 and H1299 cells, which was consistent with previous studies.

Epigenetic regulation is the frontier of life sciences and exerts an important role in gene regulation. The specific mechanism of epigenetic regulation includes DNA methylation, histone modification, and chromatin remodeling. DNA methylation is a process of methyl transfer to a specific base by s-adenosylmethionine (SAM) as a methyl donor that is catalyzed by methyltransferase (DMT)[17]. Three types of DNA methyltransferases are found in eukaryotes, namely Dnmt1, Dnmt2, Dnmt3a, and Dnmt3b. Specifically, Dnmt1 maintains methylase activity. Dnmt2 binds to a specific site on DNA. Dnmt3a and Dnmt3b can methylate CpG sites, thus participating in de novo methylation of DNA[18]. DNA methylation can shut down the activities of certain tumor-suppressor genes during the occurrence and progression of the tumor, whereas demethylation induces reactivation and upregulates expressions of oncogenes[19]. We found that the methylation status of PTEN in H1299 cells was about 50%. After PLCE1 knockdown in H1299 cells, PTEN expression was upregulated and the methylation status of PTEN promoter was downregulated, suggesting that PTEN hypermethylation is crucial in NSCLC development.

Conclusions

We showed that PLCE1 inhibits cell apoptosis of NSCLC by promoting PTEN methylation and inhibiting PTEN expression. Our research demonstrates that PLCE1 may serve as a novel therapeutic target for NSCLC treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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