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Effect of *PLCε* gene silencing on inhibiting the cancerous transformation of ulcerative colitis

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Abstract. The aim of the present study was to investigate the effect of phosphoinositide-specific phospholipase Cε (*PLCε*) gene silencing on the inhibition of cancer development in ulcerative colitis (UC) and to explore the pathogenesis and carcinogenic mechanism of UC, in order to facilitate the establishment of novel strategies for the treatment of UC, prevent the cancerous transformation of UC and discern the association between inflammation and cancer. A pGenesil-*PLCε* RNA interference vector was constructed and transfected into HEK293 cells (pGenesil-*PLCε* group). HEK293 cells transfected with pGenesil empty plasmid were set as the negative control (pGenesil-NC group). The expression of *PLCε* was observed, and molecules associated with the *PLC* signaling pathway were detected using a reverse transcription-quantitative polymerase chain reaction and western blotting. ELISA was used to determine the expression of serum interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) of mice in which the *PLCε* gene had been silenced. Compared with the pGenesil-NC group, the mRNA and protein levels of PLCε were significantly decreased in the pGenesil-*PLCε* group. In addition, the mRNA levels of *K-ras*, *NF-κB*, *Fas* and *Bcl-2* were markedly reduced, while *P53* mRNA level was notably enhanced, in the pGenesil-*PLCε* group, and these changes were accompanied by similar changes in the corresponding protein levels. The serum IL-1 and TNF-α expression in the *PLCε* gene-silenced mice was significantly reduced compared with that in the control mice. In conclusion, *PLCε* RNA silencing can effectively inhibit the cancerous transformation of UC by regulating the colorectal cancer-related cell proliferation and cell cycle *in vivo*. In addition, *PLCε* RNA silencing can suppress the expression of inflammatory factors *in vitro*.

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Introduction

Patients with ulcerative colitis (UC) have a 2.4-fold-increased overall colorectal cancer (CRC) risk. Irritable bowel syndrome (IBD)-related CRC accounts for 1-2% of all CRC cases in the general population, and CRC accounts for 15% of all mortality in patients with IBD (1). It has been generally accepted that the risk of developing CRC is associated with the extent of inflammation in the colon, as well as the duration of disease (2). Phosphoinositide-specific phospholipase C (*PLC*) represents a large gene family characterized by the ability to catalyze the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) into two vital secondary messengers: Diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). The family is composed of six isoforms, β, γ, δ, ε, ζ and η; among these isoforms, *PLCε* has been reported to be a key downstream effecter of Ras family small GTPases (3,4).

The role of *PLCε1* in tumorigenesis and inflammation has recently become a research focus. *PLCε1* expression has been found to be positively associated with human cancer, such as bladder and skin cancer, as well as the severity of the inflammation. Furthermore, the downregulation of *PLCε in vitro* and *in vivo* can suppress bladder tumor proliferation (5) In *PLCε*-knockout (*PLCε*$^{-/-}$) mice, a substantial resistance to tumor formation and to 12-*O*-tetradecanoylphorbol-13-acetate-induced skin inflammation has been observed (6). Another study, which used an adenomatous polyposis coli mouse model, has demonstrated that *PLCε* plays essential roles in spontaneous intestinal tumorigenesis, with an angiogenesis-promoting effect, and inflammation (7) Furthermore, *PLCε* was found to be necessary for activating cytokine production in skin cells in a wide spectrum of inflammatory reactions, and this role of *PLCε* was further confirmed in *PLCε* transgenic mice. *PLCε* was additionally shown to be required for tumor necrosis factor-α (TNFα)-induced chemokine (C-C motif) ligand 2 expression in human keratinocytes, due to its involvement in the nuclear factor κB (NF-κB) pathway. Such functions of *PLCε* in inflammation are quite unique among the *PLC* isozymes.

To further explore the role of *PLCε* in the inflammation of UC and its conversion to malignancy, the aim of the present

study was to construct a virus vector expressing short hairpin RNA (shRNA) targeting *PLCε* and investigate the effect of the downregulation of *PLCε* on the extent of inflammation and tumorigenesis.

Materials and methods

Equipment and reagents. Glycine and sodium dodecyl sulfate (SDS) were purchased from Sigma (St. Louis, MO, USA). All restriction enzymes and ligases were obtained from New England Biolabs (Ipswich, MA, USA). Plasmid Miniprep kits and transfection kits were purchased from Tiangen (Beijing, China). All other materials were domestic or imported analytical reagents. The equipment used in this study included a high-speed, refrigerated centrifuge (Universal 16R; Hettich, Tuttlingen, Germany), an ultraviolet spectrophotometer (UVl601; Shimadzu Corp., Kyoto, Japan), a GeneGenius gel image analysis system (Synoptics Ltd., Cambridge, UK), a microelectrophoresis and transfer system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Animal model. Swiss Webster mice (weighing 25-30 g, 8 weeks old) were obtained from the Beijing Laboratory Animal Research Center (Beijing, China). The mice were fed a standard diet and treated with appropriate medicine or sacrificed when in pain or distress. This animal protocol was approved by the local Ethics Committee (8). The animal model of colitis was established by the *ad libitum* feeding of the mice with 5% dextran sulfate sodium (DSS; ICN Pharmaceuticals, Inc., Costa Mesa, CA, USA), as described by Cooper *et al* (6). Briefly, the mice were exposed to four cycles of DSS with a basic cycle composed of 7 days of DSS followed by 14 days of tap water. Animals were sacrificed by an overdose of sodium phenobarbital at the end of the four cycles, i.e. 84 days. Once the mice had been sacrificed, the entire bowel was sampled and the diagnosis of colitis was confirmed by at least two experienced pathologists.

Cells and vector construction. The viruses were prepared using HEK293 cells derived from a human embryonic kidney, obtained from the Cell Resource Center (Institute of Basic Medical Sciences; Beijing, China). We designed an shRNA targeting the 3'-untranslated region of *PLCε* (NM016341), as well as a scramble control with the restriction sites *Bam*HI and *Hin*dIII at the end of the oligos (PLCε shRNA sequence, 5'-GAT CCG CAA TAC TGT CAG ACG AAC TGT TCA AGA CGA CTT CGT CTG ACA GTA TGT GCT TTT TTG TCG ACA-3'; scramble control shRNA sequence, 5'-GAT CCA CTA CCG TTG TTA TAG GTG TTC AAG ACG CAC CTA TAA CAA CGG TAG TTT TTT GTC GAC A-3'). The plasmid pGenesil-*PLCε* was subsequently constructed by inserting the annealed oligo into the corresponding double-digested vector. Viruses containing *PLCε* were generated in HEK293 cells. HEK293 cells were transfected with the empty plasmid (pGenesil-NC) or the target gene expression vector (pGenesil-*PLCε*) using Lipofectamine® 2000 (Life Technologies, Carlsbad, CA, USA) and incubated at 37˚C with 5% CO_2 for 72 h until the log phase was reached. The cells were then harvested, total RNA and protein were extracted and the expression of *PLCε* was determined using a reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting for the protein. The titer of the standard virus was determined using the copy number of serially diluted plasmid DNA. Mice were divided into two groups (n=5 per group), which were treated with either the pGenesil-PLCε or the negative control (pGenesil-NC).

RT-qPCR. The mRNA levels of *PLCε* and other candidate genes were quantified using the TaqMan® Real-Time PCR Detection kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. The total RNA of sampled cells was extracted, and the expression level of target RNA and U6 (correction standard) was quantified via RT-qPCR using an ABI Prism® 7000 Sequence Detection system (Applied Biosystems). The ratio of the target RNA to U6 was then calculated. Primer sequences were synthesized by Takara Biotechnology Co., Ltd. (Dalian, China). The qPCR reaction was performed according to the manufacturer's protocol: 50˚C for 2 min and 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min (Applied Biosystems).

Western blot analysis. Fresh tissue samples were ground to powder in liquid nitrogen and then lysed in sampling buffer [62.5 mmol/l TrisHCl (pH 6.8), 2% SDS, 10% glycerol and 5% 2-mercaptoethanol]. The Bradford assay (Bio-Rad Laboratories, Inc.) was used to determine the total protein concentration. Briefly, protein samples were loaded onto 10% SDS polyacrylamide gels, separated by electrophoresis and then transferred onto polyvinylidene fluoride membranes (GE Healthcare Life Sciences, Little Chalfont, UK). The membrane was incubated with rabbit polyclonal againt PLCε (ab121859), K-ras (ab84573), NF-κB (ab16502), Fas (ab15285), β-actin (ab59381) rabbit monoclonal Bcl-2 (ab32124) and mouse monoclonal P53 (ab1101) primary antibodies (all 1:5,000; Abcam, Cambridge, UK) overnight at 4˚C. Next, the membranes were incubated with corresponding horseradish peroxidase-conjugated goat (ab6721) or mouse (ab6789) anti-rabbit IgG (1:1,000; Abcam) for 2 h at 37˚C according to the manufacturer's instructions. β-actin (ab59381; Abcam) was used as the loading control.

ELISA. Samples were analyzed in a blinded manner. The serum levels of the cytokines IL-1 and TNF-α were determined using commercially available ELISA kits (Mybiosource LLC, San Diego, CA, USA), and the assays were performed according to the manufacturer's instruction.

Statistical analysis. All statistical analyses were carried out using the SPSS 17.0 software package (SPSS, Inc., Chicago, IL, USA). Analysis of variance or the Student's t-test was used to analyze the data from the RT-qPCR, western blotting and ELISA. Each experiment was performed independently in triplicate, and data are presented the mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Transfection efficiency of recombinant expression vector. HEK293 cells were transfected with the empty plasmid

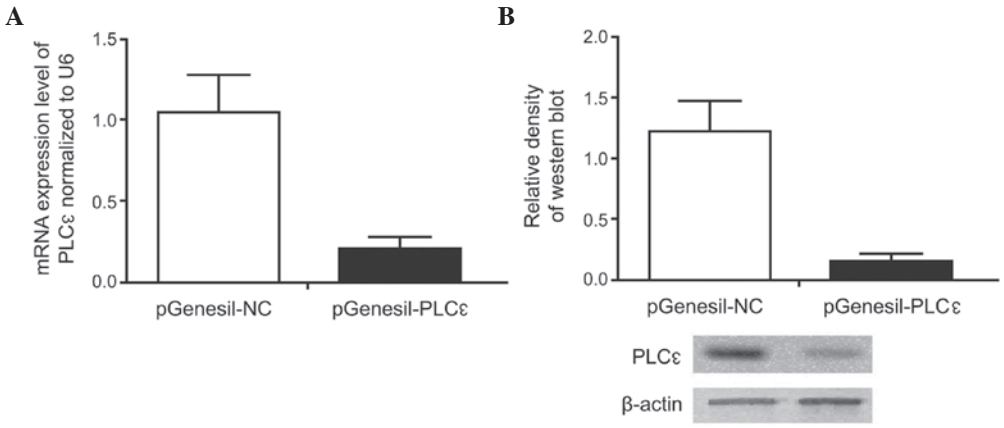

Figure 1. Vectors containing *PLCε*-targeting shRNA or a scrambled shRNA were constructed and transfected into a colon cancer cell line to assess the silencing effect of the shRNA. (A) The mRNA level of *PLCε* in the cells transfected with pGenesil-*PLCε* was significantly lower than that in cells transfected with control vector. (B) Lower panel: The protein level of *PLCε* in cells transfected with pGenesil-*PLCε* was significantly lower than that in cells transfected with control vector. Upper chart: Densitometric analysis of the western blotting results. *PLCε*, phosphoinositide-specific phospholipase Cε; shRNA, short hairpin RNA.

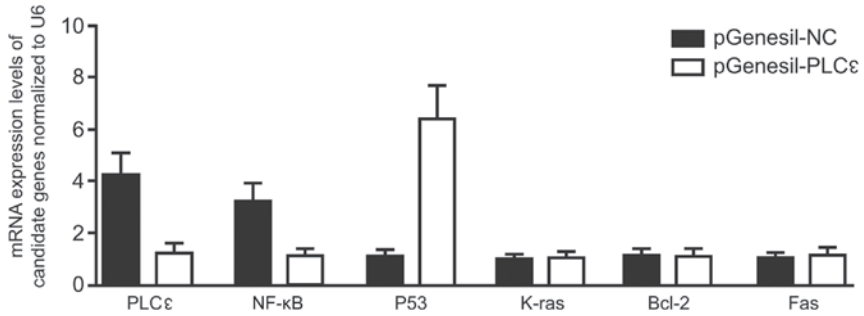

Figure 2. Comparison of the mRNA levels of *PLCε* and other candidate genes that have been reported to be functionally associated with *PLCε* between the pGenesil-PLCε-treated and pGenesil-NC-treated mice with dextran sulfate sodium-induced colitis. The *PLCε* and *NF-κB* mRNA levels were markedly lower in the pGenesil-PLCε-treated group than those in the control group, while the mRNA level of *P53* was markedly higher in the pGenesil-PLCε-treated group than that in control group. PLCε, phosphoinositide-specific phospholipase Cε; NF-κB, nuclear factor κB; Bcl-2, B-cell lymphoma-2.

(pGenesil-NC) or the target gene expression vector (pGenesil-*PLCε*), and the expression of *PLCε* was determined using an RT-qPCR. In addition, western blotting was used to determine the *PLCε* protein expression. The results showed that, compared with the pGenesil-NC group, the mRNA and protein levels of *PLCε* were significantly decreased in the pGenesil-*PLCε* group (Fig. 1), suggesting that the recombinant expression vector pGenesil-*PLCε* effectively inhibited the expression of *PLCε*.

Comparison of the mRNA levels of PLCε-related genes. The mRNA levels of *K-ras*, *NF-κB*, *Bcl-2* and *P53* were also detected using RT-qPCR, and the results demonstrated that the introduction of pGenesil-*PLCε* significantly reduced the mRNA levels of *K-ras*, *NF-κB*, *Fas* and *Bcl-2*, while notably enhancing the mRNA level of *P53* (Fig. 2). This indicated that *PLCε* RNA silencing could inhibit the expression of *K-ras*, *NF-κB*, *Fas* and *Bcl-2* and promote the expression of *P53*.

Comparison of protein levels of PLCε-related genes. Western blot analysis showed that, compared with the control group, the protein levels of K-ras, NF-κB, Fas and Bcl-2 in the pGenesil-*PLCε*-treated group were significantly reduced, while the level of P53 protein was substantially increased (Fig. 3). These data suggested that the downregulation of *PLCε* expression was associated with the inhibition of tumor-related proteins and enhanced P53 protein expression.

Changes in the levels of serum IL-1 and TNF-α in PLCε gene-silenced mice. The *PLCε* gene-silenced mouse model was generated, and changes in the levels of serum IL-1 and TNF-α in the mice were detected using ELISA. The serum IL-1 and TNF-α levels in the *PLCε* gene-silenced mice were significantly reduced compared with those in the control mice (Fig. 4).

Discussion

UC is a common clinical disease that severely threatens human health and is associated with an annually increasing incidence in Germany (9). In addition to the progress in medical technology, numerous drugs have been developed and used for the treatment of UC, achieving a continually improving prognosis. In the absence of early treatment, however, UC can undergo a transformation into colon cancer (10). A previous study showed that, for patients with colon cancer, the 5-year survival rate was 65%, which increased to 90% for patients in the early stages of the disease. Patients whose cancers had metastasized

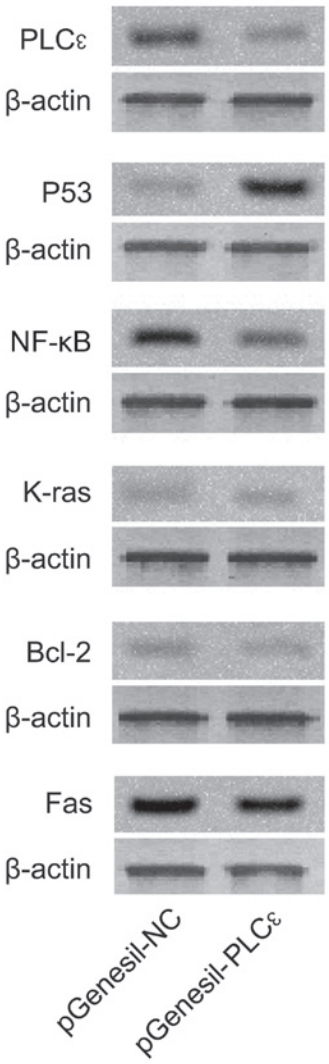

Figure 3. Comparison of the protein levels of *PLCε* and candidate genes that have been reported to be functionally associated with *PLCε* between the pGenesil-*PLCε*-treated and pGenesil-NC-treated mice with dextran sulfate sodium-induced colitis. *PLCε* and NF-κB protein levels were markedly lower in the pGenesil-*PLCε*-treated group than those in the control group, while the protein level of P53 was markedly higher in the pGenesil-*PLCε* treated group than that in control group. *PLCε*, phosphoinositide-specific phospholipase Cε; NF-κB, nuclear factor κB; Bcl-2, B-cell lymphoma-2.

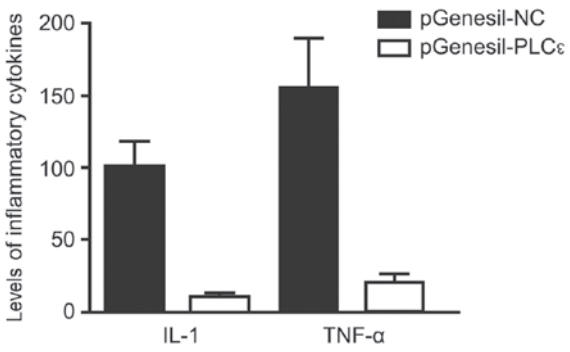

Figure 4. Concentrations of IL-1 and TNF-α were found to be significantly lower in the pGenesil-PLCε-treated group compared with those in the control group. PLCε, phosphoinositide-specific phospholipase Cε; IL-1, interleukin-1; TNF-α, tumor necrosis factor-α.

had a 5-year survival rate of <10%; therefore, the early detection of the cancerous transformation of UC is crucial (11).

It is well known that the cancerous transformation of UC is associated with hereditary and environmental factors, and the pathogenic process involves a complex regulatory network comprising multiple genes, steps and stages, which leads to cell proliferation disorders, apoptosis inhibition and diffusion through different signal transduction pathways. Finally, normal cells undergo a series of carcinogenic changes and metastasis. Overall, the cancerous transformation of UC is closely associated with the expression imbalance of multiple genes and proteins (12).

PLC is a key enzyme in the phosphoinositide signaling pathway and can be activated by a number of molecules, including hormones, neurotransmitters and growth factors, catalyzing the hydrolysis of PIP2 into DAG and IP3, which activate protein kinase C (PKC) and induce intracellular calcium release, respectively, thereby triggering a downstream cascade reaction (13). PLCε, an isoform of PLC, is an effector molecule of Ras protein that is regulated by GTP-dependent Ras. *PLCε* can affect inflammation and tumorigenesis (14,15) and is an important signal transducer. It has been suggested that the concentration of *PLCε* is significantly increased in cancer cells, indicating a link between *PLCε* and cancer. *In vivo* and *in vitro* studies have found that the overexpression of *PLCε* promotes cell transformation and increases the invasion of cancer cells (16). By contrast, blocking the expression of *PLCε* reverses the phenotypic characteristics of malignant cells and reduces the invasion of cancer cells (17).

In the present study, a *PLCε* expression inhibition system was constructed through shRNA technology, and the effect of *PLCε* gene silencing on the inflammation and cancerous transformation of UC was investigated. The data showed that, compared with the pGenesil-NC group, the mRNA and protein levels of *PLCε* were significantly decreased in the pGenesil-*PLCε* group, suggesting that the recombinant expression vector pGenesil-*PLCε* effectively inhibited the expression of *PLCε*. A previous study demonstrated that certain isoforms of PLC, depending on their phospholipase activity, could promote cell mitosis by transmitting mitogenic signals (18). Consistently, it is possible that PLCε upregulates the PKC signal transduction pathway through its phospholipase activity, acting as a mitosis promoting factor. On the other hand, PLCε may inhibit mitosis caused by the abnormal expression of *PLCε* and Ras. It has also been suggested that PLC is associated with the Ras/raf/mitogen-activated protein kinase kinase/mitogen-activated protein kinase pathway through its activation of PKC, enhancing cell proliferation via its SH3 domain (19). The present findings showed that the mRNA levels of *K-ras*, *NF-κB*, *Fas* and *Bcl-2* were significantly reduced in the presence of pGenesil-PLCε; however, there was a marked increase in the level of *P53* mRNA, which indicated that *PLCε* RNA silencing could inhibit the expression of *K-ras*, *NF-B*, *Fas* and *Bcl-2*, while enhancing the expression of *P53*. The present data indicated that the suppression of tumor-related proteins could promote the expression of P53 protein, effectively preventing the development of cancer.

Previous *in vitro* studies have found that *PLC-γ1* promotes cell transformation and tumorigenesis, and the overexpression of *PLC-γ1* in mouse fibroblasts induced malignant

transformation. In addition, tumorigenesis has been shown to occur following the implantation of *PLC-γ1* into nude mice (20). These data indicate that *PLC-γ1* has tumorigenic ability (21). The present results showed that *PLCε* RNA silencing effectively suppressed the expression of IL-1 and TNF-α *in vivo*, contributing to the anti-tumor and anti-inflammatory effects; however, its specific mechanisms, including acting elements and binding proteins, remain to be further studied.

In conclusion, *PLCε* RNA silencing can effectively inhibit the cancerous transformation of UC by regulating the CRC-related cell proliferation and cell cycle *in vivo*. In addition, *PLCε* RNA silencing can suppress the expression of inflammatory factors *in vitro*.

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