Effect of \textit{PLCe} gene silencing on inhibiting the cancerous transformation of ulcerative colitis

KUN YANG\textsuperscript{1*}, JING YAN\textsuperscript{1*}, LAN PENG\textsuperscript{2}, YU-PEI ZOU\textsuperscript{1}, FU-QIAN HE\textsuperscript{1}, HUA-TIAN GAN\textsuperscript{1} and XIAO-LI HUANG\textsuperscript{1}

\textsuperscript{1}Center of Gerontology and Geriatrics, West China Hospital, Sichuan University, Chengdu, Sichuan 610041; \textsuperscript{2}Department of Gastroenterology, Mianyang Central Hospital, Mianyang, Sichuan 621000, P.R. China

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Abstract. The aim of the present study was to investigate the effect of phosphoinositide-specific phospholipase C\textit{e} (\textit{PLCe}) 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 \textit{pGenesil-PLCe} RNA interference vector was constructed and transfected into HEK293 cells (\textit{pGenesil-PLCe} group). HEK293 cells transfected with \textit{pGenesil} empty plasmid were set as the negative control (\textit{pGenesil-NC} group). The expression of \textit{PLCe} was observed, and molecules associated with the \textit{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-\textit{\alpha} (TNF-\textit{\alpha}) of mice in which the \textit{PLCe} gene had been silenced. Compared with the \textit{pGenesil-NC} group, the mRNA and protein levels of \textit{PLCe} were significantly decreased in the \textit{pGenesil-PLCe} group. In addition, the mRNA levels of \textit{K-ras}, \textit{NF-\kappa B}, \textit{Fas} and \textit{Bcl-2} were markedly reduced, while \textit{P53} mRNA level was notably enhanced, in the \textit{pGenesil-PLCe} group, and these changes were accompanied by similar changes in the corresponding protein levels. The serum IL-1 and TNF-\textit{\alpha} expression in the \textit{PLCe} gene-silenced mice was significantly reduced compared with that in the control mice. In conclusion, \textit{PLCe} RNA silencing can effectively inhibit the cancerous transformation of UC by regulating the colorectal cancer-related cell proliferation and cell cycle \textit{in vivo}. In addition, \textit{PLCe} RNA silencing can suppress the expression of inflammatory factors \textit{in vitro}.

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 (\textit{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, $\beta$, $\gamma$, $\delta$, $\epsilon$, $\xi$ and $\eta$; among these isoforms, \textit{PLCe} has been reported to be a key downstream effector of Ras family small GTPases (3,4).

The role of \textit{PLCe1} in tumorigenesis and inflammation has recently become a research focus. \textit{PLCe1} 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 \textit{PLCe} \textit{in vitro} and \textit{in vivo} can suppress bladder tumor proliferation (5) In \textit{PLCe}-knockout (\textit{PLCe}\textsuperscript{-/-}) 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 \textit{PLCe} plays essential roles in spontaneous intestinal tumorigenesis, with an angiogenesis-promoting effect, and inflammation (7) Furthermore, \textit{PLCe} was found to be necessary for activating cytokine production in skin cells in a wide spectrum of inflammatory reactions, and this role of \textit{PLCe} was further confirmed in \textit{PLCe} transgenic mice. \textit{PLCe} was additionally shown to be required for tumor necrosis factor-\textit{\alpha} (TNF\textit{\alpha})-induced chemokine (C-C motif) ligand 2 expression in human keratinocytes, due to its involvement in the nuclear factor \textit{\kappa B} (NF-\textit{\kappa B}) pathway. Such functions of \textit{PLCe} in inflammation are quite unique among the \textit{PLC} isozymes.

To further explore the role of \textit{PLCe} in the inflammation of UC and its conversion to malignancy, the aim of the present

Correspondence to: Dr Xiao-Li Huang, Center of Gerontology and Geriatrics, West China Hospital, Sichuan University, 37 Guoxue Lane, Tiasan Street, Chengdu, Sichuan 610041, P.R. China

E-mail: huangxiaoli_919@163.com

*Contributed equally

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study was to construct a virus vector expressing short hairpin RNA (shRNA) targeting PLCE and investigate the effect of the downregulation of PLCE 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, Tuttingen, Germany), an ultraviolet spectrophotometer (UV1601; Shimadzu Corp., Tokyo, 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 PLCE (NM016341), as well as a scramble control with the restriction sites BamHI and HindIII at the end of the oligos (PLCE shRNA sequence, 5'-GAT CCG CAA TAC TGT CAG ACG AAC TGT TCA AGA CGA CTT CGT CTG ACA GTA TGC TCT TGG TCG ACA-3'; scramble control shRNA sequence, 5'-GAT CCA CTA CCG TTG TTA TAG GTG TTC AAG AGC CAC CTA TAA CAA CGG TAG TTT TTC GTC GAC A-3'). The plasmid pGenesil-PLCE was subsequently constructed by inserting the annealed oligo into the corresponding double-digested vector. Viruses containing PLCE were generated in HEK293 cells. HEK293 cells were transfected with the empty plasmid (pGenesil-NC) or the target gene expression vector (pGenesil-PLCE) using Lipofectamine® 2000 (Life Technologies, Carlsbad, CA, USA) and incubated at 37°C with 5% CO2 for 72 h until the log phase was reached. The cells were then harvested, total RNA and protein were extracted and the expression of PLCE 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-PLCE or the negative control (pGenesil-NC).

RT-qPCR. The mRNA levels of PLCE 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 against PLCE (ab121859), K-ras (ab84573), NF-kB (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
Genesil-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
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 isofrom 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-κB, 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-γ has tumorigenic ability (21). The present results showed that PLCe 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, PLCe RNA silencing can effectively inhibit the cancerous transformation of UC by regulating the CRC-related cell proliferation and cell cycle in vivo. In addition, PLCe RNA silencing can suppress the expression of inflammatory factors in vitro.

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