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PLCε1: A potential target of RNA interference therapy for gastric cancer

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ABSTRACT

Phospholipase C epsilon 1 (PLCε1) has been recently identified as a novel potential biomarker for gastric cancer because of its critical role in inflammation and tumorigenesis. Until now, there are no further reports to investigate the feasibility of gene therapy by suppressing PLCε1 expression for gastric cancer. In this study, a small interfering RNA (shRNA) targeting PLCε1 was firstly transfected into gastric cancer cells in order to silence PLCε1 expression. Both mRNA and protein expression of PLCε1 in gastric cancer cells significantly reduced by RT-PCR and Western blotting analysis. Moreover, subsequent results revealed that PLCε1 shRNA depressed the *in vitro* and *in vivo* growth of gastric cancer cells by using MTT assay and tumor xenograft experiment. Furthermore, after PLCε1 shRNA transfection, the expression of proinflammatory molecules including tumor necrosis factor-α (TNF-α), cyclooxygenase 2 (COX-2), interleukin (IL)-6 and chemokine (C–X–C motif) ligand (CXCL)-1 were unaffected, but only chemokine (C–C motif) ligand (CCL)-2 expression decreased in the gastric cancer cells. It is implied that PLCε1 may inhibit the growth of gastric cancer cells *via* CCL-2 protein mediated pathway. These results suggest that PLCε1 might be an alternative molecular target for gastric cancer gene therapy.

1. Introduction

Currently, although clinic technologies for gastric cancer has been significantly improved in recent decades, the high mortality rate of gastric cancer still threatens public health seriously worldwide. Particularly, it is urgent for Chinese to improve the prognosis of gastric cancer, because there are higher incidence and mortality in China than that in other countries [1].

Up to date, as for gastric cancer patients, the mainly curative treatment for gastric cancer is surgery. However, it is fact that nearly 91% gastric cancer patients after surgery are died of recurrences [2]. Therefore, more and more attentions have been focused on other potential treatments for gastric cancer. Gene therapy is one of the most promising therapeutic approaches. Gene therapy enables to insert a correctly functioning gene into the cells of the patient, so that the cells will regain their proper function [3]. Recently, the first gene therapy drug "Glybera" has been successfully approved by both European Commission and the United State for the treatment of lipoprotein lipase deficiency disease [4]. Similarly, there are a lot of gene therapy studies to address cancer, because most kinds of cancer mainly are caused by damaged or missing genes. However, owing to lack of a suitable molecular target, there is no an effective gene therapy for completely treating gastric cancer.

The Phosphoinositide-specific phospholipase C (PLC) represents a large family which catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate into two vital second messengers: diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (PIP2). PLC family includes six of isoforms (β, γ, δ, ε, ζ and η) [5,6]. Among them, PLCε was a newly identified member of PLC family in 2001 [7]. It is a key downstream regulator of Ras family small GTPases: Ras, Rap1, and Rap2 [7,8]. Recent studies shown that PLCε1 was considered as a risk factor that was significantly associated with gastric cancer in Chinese population by genome wide gene-expression analysis (GWAS) [9,10]. Notably, a recent work revealed that there was a different expression of PLCε1 in precancerous disease (chronic atrophic gastritis) tissues and gastric cancer tissue compared with that in normal tissues, suggesting it was a potential molecular biomarker for gastric cancer [11]. Up to date, there are few works on studying the specific role of PLCε1 as a molecular target for gastric cancer treatment.

In this study, the objective of our study is to investigate the feasibility of mediating the growth of gastric cancer cells by inhibiting the expression of PLCε1 *in vitro* and *in vivo* with shRNA interference technology.

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2. Materials and methods

2.1. Cell lines

The gastric cancer cell lines (SGC7901, AGS, MGC803) were obtained from the Chinese Academy of Sciences Cell Bank of Type Culture Collection and kept in our laboratory. The SV40-transformed immortal gastric normal epithelial cells GES-1 were preserved in our institute and maintained as recommended. All cell lines were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone), 100 units/mL penicillin and 0.1 mg/mL streptomycin. A total of 2×10^5 cells per well were cultured in 6-well plates overnight until the cultures reached approximately 90% confluence. All cells were maintained at 37 °C in a chamber with 5% CO_2.

2.2. Short hairpin RNA construction and transfection

Five shRNA candidates targeting PLCε1 (referring to GeneBank NM 016341), were prepared by method of chemical synthesis according to principle of design. They were named PLCε1 shRNA-1, PLCε1 shRNA-2, PLCε1 shRNA-3, PLCε1 shRNA-4, and PLCε1 shRNA-5, whose inhibition efficiency was 80.53%, 90.44%, 78.35%, 67.75% and 86.87%, respectively. Meanwhile, a non-silencing shRNA sequences was also designed and synthesized as a negative control. After connecting with a linear pGenesil plasmid, these shRNA reconstructed plasmids were selected for the following transfection. Both gastric cancer cells and normal epithelial cells were transfected with a mixture of either PLCε1 shRNA-2, PLCε1 shRNA-5, non-silencing shRNA, or phosphate-buffered saline buffer (PBS) and lipofectamine 2000 transfection reagent (Invitrogen, USA) at the ratio of 1:2, respectively. The transfected cells were selected using G418 (400 μg/mL). Monoclonal cells were picked after 4 weeks of exposure to selective pressure and were further identified and cultured for subsequent experimentation.

2.3. RNA extraction and reverse transcription–PCR

Total RNA from cells and tissues samples was extracted using the Trizol reagent (Invitrogen) according to the manufacturer's instruction. The extracted RNA was pretreated with RNase-free DNase, and 2 μg RNA from each sample was used for cDNA synthesis primed with random hexamers. For PCR-mediated amplification of PLCε1 cDNA, an initial amplification using PLCε1-specific primers was done with a denaturation step at 95 °C for 10 min followed by 30 denaturation cycles at 95 °C for 60 s, primer annealing at 55 °C for 30 s, and primer extension at 72 °C for 30 s. On completion of the cycling steps, a final extension at 72 °C for 5 min was carried out before the reaction was stopped and stored at 4 °C. Real-time PCR was then employed to determine the fold increase of PLCε1 mRNA. Expression data were normalized to the geometric mean of the housekeeping β-actin gene to control the variability in expression levels. Similarly, other candidate genes repeated above experiment procedures for mRNA expression analysis, including TNF-α, COX-2 and CCL-2, etc. Reverse transcription–PCR and real-time PCR primers were synthesized according to previous report [11,12].

2.4. Western blotting analysis

The transfected cells were solubilized in lysis buffer containing protease inhibitors before being subjected to sonication and centrifugation at 4 °C for 3 min. Loading buffer was added to each of the protein solutions, which were subsequently boiled for 5 min and separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis before being transferred to nitrocellulose membranes (Amersham Biosciences, Beijing, China). The membranes were blocked for 1 h with anti-PLCε1 rabbit antibody (1:250; Sigma). PLCε1 expression was detected with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2000; Amersham Pharmacia Biotech) and an enhanced chemiluminescence kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The same method was used to detect TNF-α, COX-2, IL-6, CXCL-1 and CCL-2 protein expression in cells. β-actin detected with an anti-β-actin rabbit antibody (1:1000 dilution; Sigma) was used as the loading control.

2.5. MTT assay for cell proliferation

After transfection, cell growth was evaluated using the MTT assay. A total of 2×10^4 cells per well were plated in 96-well plates that were cultured for 24, 48, 72, 96, and 120 h. Before being removed from incubation, the cells were incubated with 200 μL of 0.5 mg/mL MTT (Sigma–Aldrich, Shanghai, China) for an additional 4 h. The medium was then replaced with 200 μL dimethyl sulfoxide (Sigma–Aldrich, Shanghai, China) to resolve the crystals. The absorbance (A) at 490 nm was then measured for each well.

2.6. Tumor xenograft experiments

About 2×10^6 of transfected either PLCε1 shRNA-2, PLCε1 shRNA-5 or non-silencing shRNA MGC803 cells were harvested and resuspended in PBS buffer, respectively. Particularly, equal number of MGC803 cells without transfection treatment were treated as control group. Three groups of 4–6 week-old female BALB/c nude mice (Experimental Animal Center of Chinese Academy of Sciences, Shanghai, China) consisting of 5 mice each, were housed in a pathogen free environment. The mice were given subcutaneous injections with prepared cells and were monitored every 2 days for tumor growth. The tumor volumes (volume = $1/2ab^2$; where a is largest diameter and the b, the smallest diameter) were recorded at each point. A total of 14 days after the injection of cells, the mice were killed, and the tumor weight was recorded.

2.7. Statistical analysis

All statistical analysis were carried out using the SPSS 17.0 statistical software package. The unpaired Students' t-test was used to analyze those protein and mRNA expression in gastric cancer and normal epithelial cells, respectively. Each experiment was performed independently at least twice with similar results. $p < 0.05$ in all cases was considered statistically significant.

3. Results

3.1. Knock down of PLCε1 mRNA and protein by PLCε1 shRNA

As shown in Fig. 1A, compared with gastric normal epithelial cells GES-1, PLCε1 mRNA and protein expression were up-regulated in gastric cancer cells (MGC803, AGS and SGC7901) in phosphate-buffered saline buffer (PBS) buffer group, in agreement with previous reports [11]. After transfecting either PLCε1 shRNA-2 or PLCε1 shRNA-5, the mRNA expression of PLCε1 in both gastric cancer cells and normal epithelial cells were significantly suppressed compared with that in PBS buffer group and non-silencing group (shown in Fig. 1A). As shown in Fig. 1B, compared to non-silencing shRNA, the mRNA expression of PLCε1 gradually decreased in MGC803 accompanied with increasing treatment time after PLCε1 shRNA transfection. The PLCε1 shRNA-2 or shRNA-5 function of decreasing mRNA expression started less than

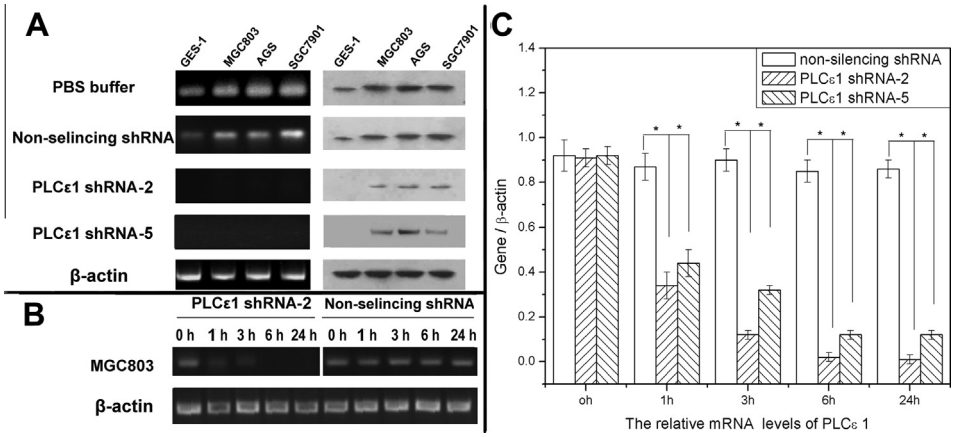

Fig. 1. (A) Expression analysis of PLCε1 protein and mRNA in gastric normal and cancer cell lines by reverse transcription–PCR and Western blotting. (B) The mRNA expression of PLCε1 in GES-1 and MGC803 after PLCε1 shRNA-2 transfection was analyzed by reverse transcription–PCR at different time intervals. (C) The mRNA expression of PLCε1 detected after PLCε1 shRNA-2 and PLCε1 shRNA-5 transfection in MGC803 at different time intervals.

1 h, and reached a peak in around 6 h, and lasted for 24 h after transfection (shown in Fig. 1C).

3.2. Inhibition of gastric cells proliferation by PLCε1 knock down

In Fig. 2, it indicated that there were no obvious changes in normal epithelial cells GES-1 in either PBS buffer, non-silencing shRNA, PLCε1 shRNA-2 or PLCε1 shRNA-5 transfection groups. However, MGC803 cells transfected with either PLCε1 shRNA-2 or PLCε1 shRNA-5 exhibited a mark growth inhibition compared with that in PBS buffer group and non-silencing group ($p < 0.05$). As shown in Fig. 2, there was particularly significant statistics difference in cell viability since 24 h. Notably, there were similar results by replacing MGC803 with either AGS or SGC7901 (date not shown).

3.3. PLCε1 knock down attenuated tumor growth in nude mice

As shown in Fig. 3A, these growth curves of xenograft tumors for 14 days showed that the both PLCε1 shRNA-2 and PLCε1 shRNA-5 exhibited a significantly smaller final tumor size than PBS buffer group and non-silencing group ($p < 0.05$). Specifically, PLCε1 shRNA-2 and shRNA-5 group showed a tumor volume of 80.02 ± 23.09 mm^3 and 112.14 ± 12.86 mm^3, respectively, compared to that of 816.24 ± 30.57 mm^3 for PBS buffer group and 759.97 ± 38.06 mm^3 for non-silencing shRNA group. Furthermore, cells that were treated with PLCε1 shRNA-2 or PLCε1 shRNA-5 exhibited lighter tumor weight than those in the PBS buffer group and non-silencing shRNA group after 14 days ($p < 0.05$) (shown in Fig. 3B), respectively.

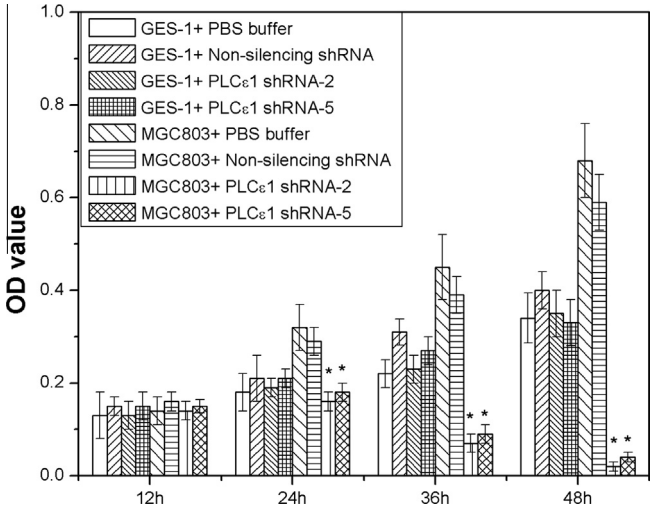

Fig. 2. Variabilities of cell proliferation in different groups over time by MTT analysis. The OD value was counted at the time points of 12 h, 24 h, 36 h and 48 h after being seeded into 96 plates. Values were expressed as the mean ± SD ($n = 5$), $p < 0.05$.

3.4. PLCε1 shRNA down-regulated proinflammatory molecules CCL-2 expression in MGC803 cells

The critical role of PLCε1 in inflammation microenvironment promoted us to further investigate the expression of proinflammatory molecules, which are implicated in tumor promotion and inflammation [13–15], involved in the PLCε1 overexpressed gastric cancer cells. The tumor necrosis factor-α (TNF-α), cyclooxygenase 2 (COX-2), interleukin (IL)-6, chemokine (C–X–C motif) ligand (CXCL)-1 and ligand (CCL)-2 protein expression levels in gastric normal and cancer cells were determined by Western blotting and real-time PCR. As shown in Fig. 3C, TNF-α, COX-2, IL-6, CXCL-1 and CCL-2 protein expression in gastric cancer cells MGC803, AGS and SGC7901 were also markedly higher than those observed in gastric normal cells GES-1. In concurrence with previous reports [16,17], IL-6 and CXCL-1 expression were detected in gastric normal microenvironment. After transfecting PLCε1 shRNA-2, only the mRNA and protein expression of CCL-2 in MGC803 cells reduced companied with the inhibition of PLCε1 compared to those in non-silencing shRNA groups ($p < 0.05$; Fig. 4A and F). On other hand, compared to non-silencing snRNA group, the level of TNF-α, COX-2, CXCL-1 and IL-6 protein in MGC803 cells were unaffected under conditions of inhibiting PLCε1 expression, both in mRNA and protein levels ($p < 0.05$, Fig. 4A–E). Similarly, with transfection of PLCε1 shRNA-5, the similar variations were found among other gastric cancer cells AGS and SGC7901 (Date not shown). These results revealed that PLCε1 shRNA reduced the mRNA and protein levels of PLCε1, but only CCL-2 protein levels correspondingly decreased in gastric cancer cells.

4. Discussion

Inflammation is emerging as an important player in carcinogenic progressions including initiation, promotion, malignant

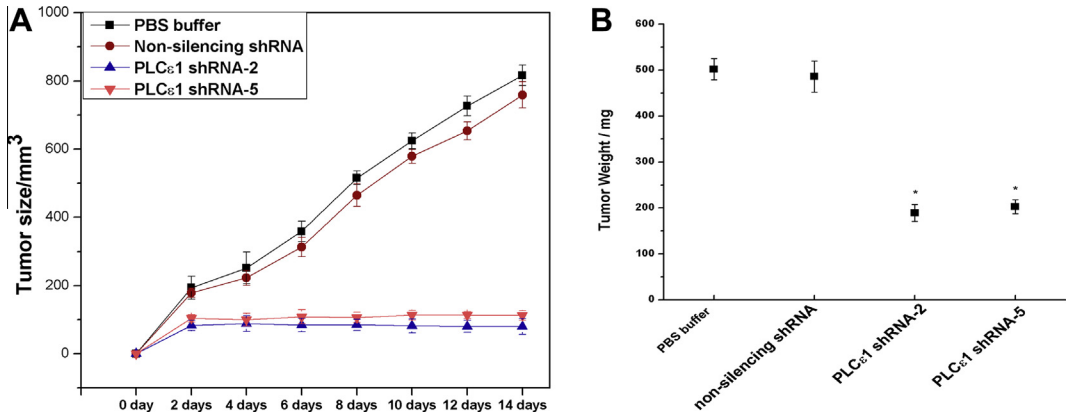

Fig. 3. (A) The size of xenograft tumors in PBS buffer group, non-silencing shRNA group, PLCε1 shRNA-2 and PLCε1 shRNA-5 group measured every 2 days, respectively. (B) The weight of tumor after 14 days. (C) Protein expression analysis of proinflammatory molecules such as TNF-α, COX-2, IL-6, CXCL-1 and CCL-2 in gastric cancer cells and normal epithelial cells by Western blotting.

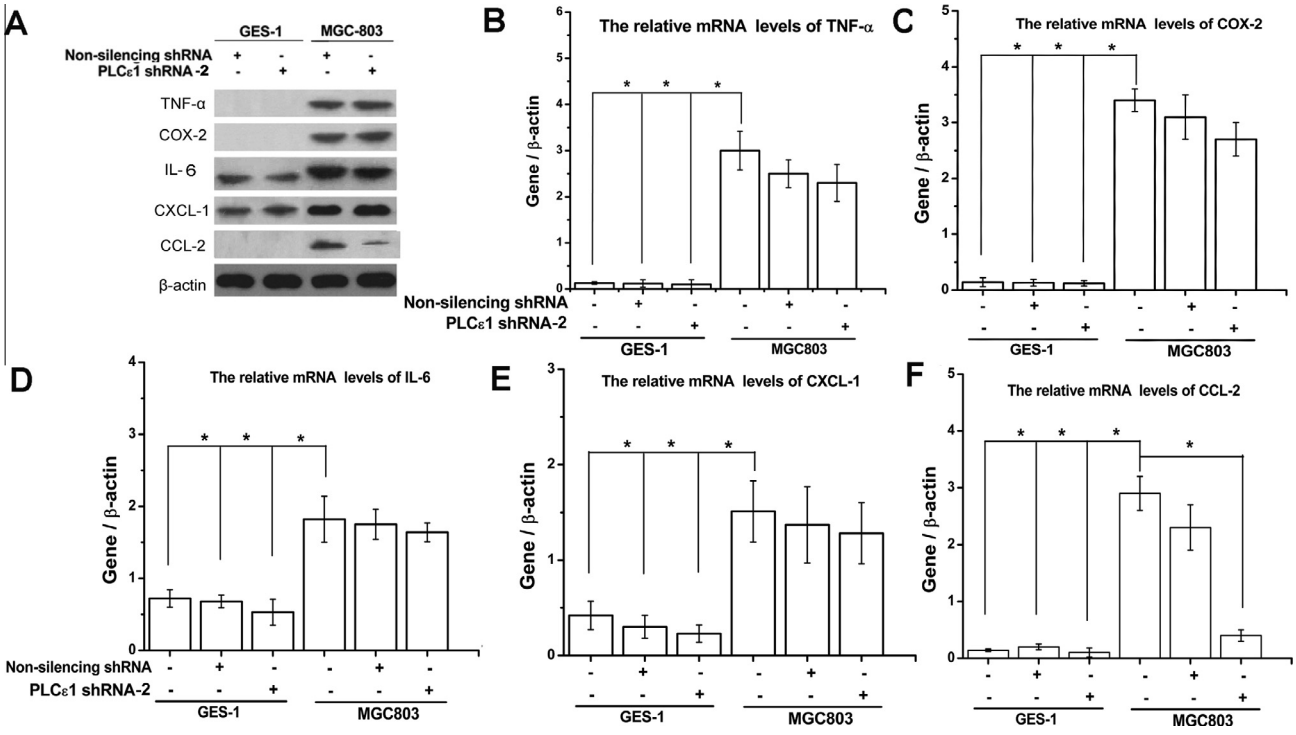

Fig. 4. Western blotting (A) and real-time PCR (B–F) analysis the changes of proinflammatory molecules in GES-1 and MGC803 after non-silencing and PLCε1 shRNA-2 transfection, respectively.

conversion, invasion, and metastasis [18]. The causal relationship between gastric tumor promotion and inflammation has been supported that the inflammation caused by *Helicobacter pylori* infection can increase the risk of gastric cancer [19]. Recently, an increasing number of reports reveal that PLCε1 is required for the activation of cytokine production in many cellular responses to inflammation reaction. PLCε$^{+/+}$ mice spontaneously developed skin inflammation by increasing production of cytokines by using the Apc$^{Min/+}$ mouse model. It also spontaneously developed skin inflammation with excessive growth of human inflammatory factors such as TNF-α, COX-2 and CXCL-1, etc. [20]. On the other hand, it demonstrated that PLCε1 played a crucial role in both inflammation and cutaneous carcinogenesis. By using transgenic mice, in which PLCε1 was regulated by gene targeting, PLCε$^{-/-}$ mice exhibited marked resistance to tumor formation and to the TPA-induce skin inflammation [21]. Subsequent studies confirmed that TPA-induced up-regulation of proinflammation molecules such as IL-6 observed in PLCε$^{+/+}$ mice was substantially attenuated in PLCε$^{-/-}$ mice [5]. IL-6 is a member of the IL family cytokines having pleiotropic functions, such as control of immune responses and inflammatory processes [22]. In this study, MTT analysis demonstrated that the cell viability of gastric cancer cells was significantly decreased after PLCε1 shRNA silence treatment, suggesting the gastric cancer cells' growth arrest could result from the suppression of PLCε1. Additional confirmation was provided by tumor xenograft experiments, clearly demonstrating PLCε1 shRNA attenuated tumor growth in xenograft mice. These above results suggested that down-regulated PLCε1 expression suppressed the aggressive phenotypes of gastric cancer cells, suggesting PLCε1 shRNA displayed a critical role in gastric cancer therapy.

In addition, many tumors express one or more proinflmmation molecules that may attract inflammatory cells, such as

macrophages, which can promote carcinogenic progression [23]. A large number of studies show that TNF-α, COX-2 and CCL-2 are over-expressed by a wide variety of cancer types in order to facilitate tumor progression [24,25]. In our study, compared to those in gastric normal epithelial cells, dramatically higher mRNA and protein expression of proinflmmation molecules including TNF-α, COX-2, IL-6, CXCL-1 and CCL-2 were also detected in gastric cancer cells (Figs. 3 and 4). This finding confirms the positive relationship between PLCε1 and inflammation reaction. Furthermore, the causal relationship between inhibition of inflammation molecules and suppression of tumor growth been supported from recent studies by using genetically engineered mice. For instance, inhibition of inflammation by pharmacologic or genetic inactivation of COX-2 reduced tumor formation and malignant progression in mice carrying the mutated Apc gene [26]. However, our Western blotting analysis showed that there were no differences on the TNF-α, COX-2 and CXCL-1 proteins expression in cancer cells before and after PLCε1 shRNA silence treatment. Particularly, only CCL-2 protein has alternation expression after non-silencing shRNA and PLCε1 shRNA transfection. This result provides an additional explanation that the arrest of gastric cancer cells growth induced by PLCε1 may *via* CCL-2 protein mediated pathway.

CCL-2 (also known as monocyte chemoattractant protein-1) is a CC chemokine that is chemotactic for monocytes, memory T cells, and natural killer cells. It is expressed by a wide variety of cancer types, and CCL-2 over-expression generally facilitates tumor progression [27,28]. Notably, CCL-2 depression expression has been shown to display an antitumor effect in some models [29,30]. In our study, we confirmed a higher mRNA and protein levels of CCL-2 existed in gastric cancer cells than that in gastric normal epithelial cells. Moreover, the circulating levels of CCL-2 correlated with PLCε1 depression in gastric cancer cells, confirmed by our Western blotting results (shown in Figs. 3 and 4). Thus, PLCε1 expression appeared to be an important determinant of gastric cancer dissemination as strongly correlated with CCL-2 protein.

5. Conclusions

In this study, a targeting PLCε1 shRNA effectively decreased gastric cancer cells' PLCε1 expression not only at mRNA level but also protein level, which could result in inhibiting the growth of gastric cancer cells *in vitro,* and hindering the formation of tumor *in vivo*. Our results suggest that PLCε1 has the potential to become a novel therapeutic target for gastric cancer. Though the intrinsic mechanism of PLCε1 action still requires for further studying, it is speculated that PLCε1 functions may be active proinflammation factors such as CCL-2 protein signaling pathway during the process of gastric cancer tumorigenesis.

Acknowledgments

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