PKCζ, MMP-2 and MMP-9 expression in lung adenocarcinoma and association with a metastatic phenotype

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Abstract. The aim of the present study was to investigate protein kinase C ζ type (PKCζ), matrix metalloproteinase (MMP)-2 and MMP-9 expression in lung adenocarcinoma and to define their association with *in vitro* invasion and metastatic capacity. PKCζ, MMP-2 and MMP-9 expression was assessed by immunohistochemistry in 110 cases of lung adenocarcinoma. PKCζ small interfering (si)RNA was transfected into A549 cells, and western blotting was used to confirm PKCζ-knockdown in transfected cells and to measure MMP-2 and MMP-9 levels. A Transwell invasion assay was used to detect *in vitro* invasive capacity. The rates of positive PKCζ, MMP-2 and MMP-9 staining in lung adenocarcinoma tissues were 52.73, 55.45 and 61.82%, respectively. PKCζ expression was increased in malignant tissues compared with adjacent normal lung tissues and was associated with lymph node metastasis ($P<0.05$), although it was not associated with any other clinicopathological parameters, including sex, age, tumor size, smoking status or distant metastases (all $P>0.05$). PKCζ, MMP-2 and MMP-9 expression was markedly decreased in siPKCζ-treated A549 cells, which exhibited a significantly decreased invasive capacity in the Transwell invasion assay ($P<0.05$). In conclusion, PKCζ promoted lung adenocarcinoma invasion and metastasis, and its expression was associated with MMP-2 and MMP-9 expression. PKCζ may be a potential target for gene therapy in lung adenocarcinoma.

Introduction

Lung cancer is one of the commonest malignant carcinomas in the world, and its incidence is increasing in a number of countries. Worldwide, lung cancer is the leading cause of death from malignant tumor, accounting for ~30% of all cancer-associated mortality (1). Lung adenocarcinoma is the principal subtype of lung cancer, and metastasis is the leading cause of mortality in patients with lung adenocarcinoma.

The protein kinase C (PKC) family regulates cell growth, differentiation, metabolism and transcriptional activation. PKCs may affect the invasion and metastasis of tumor cells. PKCζ is a member of the PKC family that serves important roles in cell growth, metabolism and other associated signal transduction pathways (2,3). It has been established that PKCζ is a tumor suppressor for numerous types of human cancer (4). However, studies have additionally identified pro-oncogenic functions of PKCζ, although a complete understanding of the detailed molecular mechanisms is lacking (2-4). Additionally, it has been suggested that PKCζ may be involved in inflammatory responses to diverse stimuli *in vitro* and *in vivo* (5-8). However, PKCζ expression in lung adenocarcinoma and the possible outcomes of PKCζ signaling in the context of lung adenocarcinoma remain to be completely elucidated.

Matrix metalloproteinases (MMPs) are able to degrade the extracellular matrix and basement membrane, and serve important roles in promoting tumor invasion and metastasis (9,10). MMPs proteolytically activate or degrade a variety of non-matrix substrates, including cytokines and chemokines, exerting a regulatory function in inflammation and immunity (11). At present, the most well-established roles for MMPs are in colorectal carcinogenesis, wherein MMP-2 and MMP-9 have been implicated in colon cancer progression and metastasis (12). Studies into the role of metalloproteinases and their inhibitors in lung adenocarcinoma are limited, and the results have been varied (13,14).

Recently, studies have demonstrated that PKCs may promote the metastasis of tumor cells in breast cancer, glioma and other malignancies (15,16). PKCζ is able to activate the mitogen-activated protein kinase (MAPK) signaling pathway, which terminates with extracellular signal-regulated kinase (ERK) phosphorylation and consequent promotion of MMP-2 and MMP-9 secretion, which may facilitate invasion and metastasis (17,18). However, there have been few studies focusing on lung adenocarcinoma, and whether PKCζ may mediate the invasion and metastasis of lung adenocarcinoma by regulating MMP-2 and MMP-9 secretion remains unknown.

In the present study, PKCζ, MMP-2 and MMP-9 expression was assessed in lung adenocarcinoma and adjacent normal

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lung tissues using immunohistochemistry, and associations between their relative expression levels were analyzed. PKCζ was knocked down in the lung adenocarcinoma cell line A549, and invasive capacity, and MMP-2 and MMP-9 expression were observed, in order to examine the effects of PKCζ on invasion and metastasis in lung adenocarcinoma and to provide a novel method for the treatment of lung adenocarcinoma.

Materials and methods

Specimen collection. The present study included 110 patients with invasive lung adenocarcinoma (including all subtypes) who underwent histological diagnosis at the Second People's Hospital of Weifang (Weifang, China) between January 2012 and December 2014. Cases with preoperative therapy or a history of other known malignancies were excluded. Medical records were reviewed for clinicopathological features, including sex, age, tumor size, smoking status, lymph node metastasis, distant metastasis and pathological tumor, node, metastasis (pTNM) stage. Patients were divided into two groups by age (≤60 years and >60 years) and smoking status [smokers (>5 pack-year history) and non-smokers]. The pTNM stage was evaluated in accordance with the 7th lung cancer TNM classification and staging system (19). Adjacent normal lung tissue (taken 5 cm from the edge of the cancerous tissue) was used as the control.

Among the 110 lung adenocarcinoma patients: 59 were male and 51 were female; 66 were ≤60 years old and 44 were >60; and 44 were smokers and 66 were non-smokers. Regarding pTNM stage, 36 were stages I+II and 74 were stages III+IV. The present study was approved by the Institutional Ethics Committee of Second People's Hospital of Weifang, and written informed consent was obtained from all participants.

Reagents. Anti-PKCζ (TA312044), anti-MMP-2 (TA806846) and anti-MMP-9 (TA353338) antibodies were purchased from OriGene Technologies, Inc. (Beijing, China). Cell culture plates, Matrigel and Transwell chambers were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Lipofectamine 2000 transfection reagent was purchased from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and RIPA lysis buffer was purchased from Beyotime Institute of Biotechnology (Haimen, China). The A549 cell line was purchased from the American Type Culture Collection (Manassas, VA, USA).

Immunohistochemistry. Immunohistochemical staining was performed on 4-μm, formalin-fixed, paraffin-embedded sections. PKCζ primary antibody was diluted 1:200 and manually applied to sections. All steps were performed in accordance with the manufacturer's protocol. MMP-2 and MMP-9 were not diluted for these experiments. PBS was used as the negative control. Staining intensity and the percentage of positive cells were evaluated under a microscope (BX53; Olympus Corporation, Tokyo, Japan) in 5 high-magnification fields of vision, and 100 cells were counted in each field. The specific methods were performed according to a previous study (18).

Cell culture. The lung adenocarcinoma cell line A549 was cultured in F12K culture medium (21127-022; Invitrogen; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, Inc.) at 37˚C in 5% CO_2. Experiments were performed on cells in the logarithmic growth phase. The cells were divided into 3 groups as follows: Control group, A549 cells without any treatment; Scr/A549 group, A549 cells transiently transfected with empty plasmid; and small interfering (si)PKCζ/A549 group, A549 cells transiently transfected with the PKCζ target fragment 5'-GAGGAAGTGAGAGACATGTGT-3'. A total of 0.4 μg plasmid/siRNA were transfected into the Scr/A549 group and the (si)PKCζ/A549 group. All the vectors were synthesized by Shanghai GeneChem Co., Ltd. (Shanghai, China). Transfections were performed using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The subsequent experimentation commenced 48 h following transfection.

Western blotting. For Western blot analysis, cells or tissues were directly lysed in RIPA lysis buffer. Aliquots of 50 μg protein were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked in 5% skimmed milk for 1 h at room temperature, and then immunoblotted using the appropriate primary antibodies at 4˚C overnight and the HRP conjugated secondary antibodies at 37˚C for 2 h. They were visualized by using enhanced chemiluminescence reagents ECL (Pierce; Thermo Fisher Scientific, Inc.). Western blot data in the present study are representative from three independent experiments. The intensities of bands in western blots were quantified by densitometry analysis using AlphaImager HP (version 3.4.0; ProteinSimple, San Leandro, CA, USA) and NIH ImageJ software (version 1.44; National Institutes of Health, Bethesda, MD, USA). The following commercial antibodies were used in this study: PKCζ (TA312044; 1:1,000), MMP-2 (TA806846; 1:1,000) and MMP-9 (TA353338; 1:1,000) (all from OriGene Technologies, Inc.), β-actin (4970; 1:1,000) and HRP-linked anti-rabbit IgG antibody (7074; 1:2,000) (both from Cell Signaling Technology, Inc., Danvers, MA, USA).

Transwell invasion assay. Matrigel was added to the top chamber of a Transwell system to form the matrix layer. To this matrix was added 100 μl ($1x10^5$) Scr/A549 or siPKCζ/A549 cells; epidermal growth factor was added into the lower chamber (500 μl/well). The Transwell device was placed in an incubator (37˚C; 5% CO_2) for 24 h. Following incubation, invaded cells were fixed for 1 min in precooled methanol and Giemsa stained for 30 min at room temperature. All experiments were repeated at least three times. The number of invading cells was counted under a microscope (IX71; Olympus Corporation) in five predetermined fields, total magnification, x200, using CellSens Standard (version 1.7; Olympus Corporation).

Statistical analysis. All statistical analyses were performed using SPSS version 19.0 (IBM Corp., Armonk, NY, USA). Data are presented as the mean ± standard deviation. Statistical significance was evaluated using Student's t-test or χ^2 test. $P<0.05$ was considered to indicate a statistically significant difference.

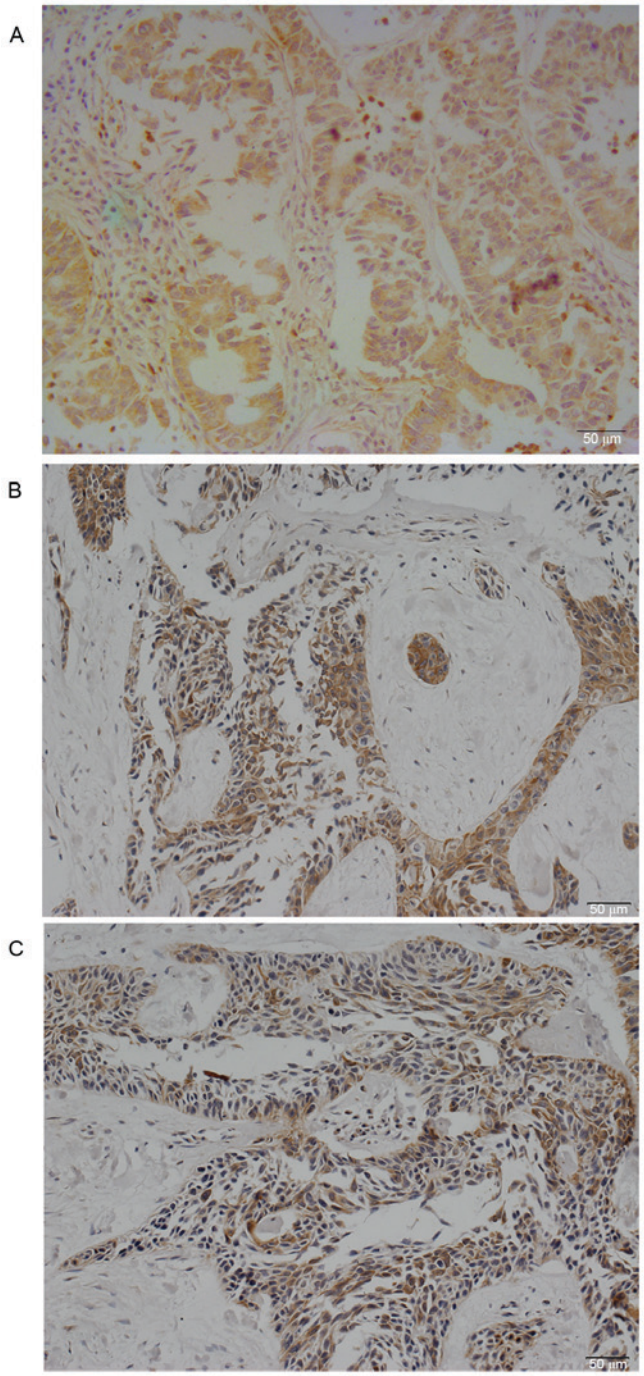

Figure 1. Expression of PKCζ, MMP-2 and MMP-9 in lung adenocarcinoma, analyzed using immunohistochemistry. (A) Positive PKCζ, (B) MMP-2 and (C) MMP-9 staining was observed in the cytoplasm of lung adenocarcinoma sections (magnification, x100). PKCζ, protein kinase C ζ type; MMP, matrix metalloproteinase.

Table I. Expression of PKCζ in lung adenocarcinoma and association with clinical pathological indices.

Clinical pathological index	Case no.	PKCζ		χ^2	P-value
		Positive	Negative		
Gender					
Male	59	32	27	0.116	0.848
Female	51	26	25		
Age, years					
≤60	66	30	36	3.501	0.08
>60	44	28	16		
Diameter of tumor, cm					
≤3	40	17	23	2.638	0.116
>3	70	41	29		
Smoker					
Yes	44	21	23	0.736	0.439
No	66	37	29		
Metastasis of LN					
Yes	39	26	13	4.710	0.045
No	71	32	39		
Distant metastasis					
Yes	42	27	15	3.642	0.077
No	68	31	37		
TNM stage					
I+II	36	16	20	1.473	0.309
III+IV	74	42	32		

LN, lymph node; TNM, tumor, node, metastasis; PKCζ, protein kinase C ζ type.

Results

Immunohistochemical findings. PKCζ was expressed in the cytoplasm of lung adenocarcinoma cells (Fig. 1A). Positive PKCζ staining was detected in 58 (52.73%) lung adenocarcinoma samples, while only 5 (4.50%) normal lung tissues exhibited weak positive staining. The difference was statistically significant (χ^2=62.479; P<0.01). The rate of positive PKCζ staining in lung adenocarcinomas with lymph node metastases (64.30%) was increased compared with non-metastatic samples (45.60%) (P=0.017). The differences among other clinicopathological parameters were not significant (Table I).

MMP-2 and MMP-9 were primarily expressed in the cytoplasm of lung adenocarcinomas (Fig. 1B and C); the rate of positive staining was 55.45 and 61.82%, respectively. PKCζ expression was associated with MMP-2 (P=0.012) and MMP-9 (P=0.006) expression in lung adenocarcinoma (Table II).

Western blot analysis results. PKCζ expression siPKCζ/A549 cells was markedly decreased compared with Scr/A549 cells, confirming that the reagent successfully disrupted the expression of the target gene (Fig. 2). In addition, MMP-2 and MMP-9 protein expression in siPKCζ/A549 cells was markedly decreased compared with Scr/A549 cells (Fig. 2).

Transwell invasion assay findings. Fewer siPKCζ/A549 cells invaded through the membrane and into the bottom chamber

Table II. Association between PKCζ, MMP-2 and MMP-9 expression in lung adenocarcinoma.

		PKCζ			
Group	Case no.	Positive	Negative	χ^2	P-value
MMP-2					
Positive	61	39	22	6.900	0.012
Negative	49	19	30		
MMP-9					
Positive	68	43	25	7.889	0.006
Negative	42	15	27		

PKCζ, protein kinase C ζ type; MMP, matrix metalloproteinase.

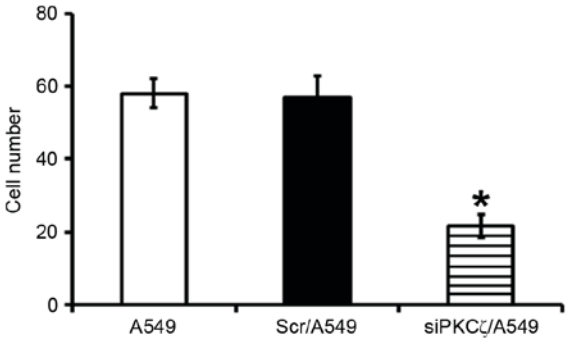

Figure 3. *In vitro* invasive ability of A549 cells with and without PKCζ. *In vitro* invasion was detected using a Transwell invasion assay, which demonstrated significantly decreased levels of invasion in the siPKCζ/A549 group compared with Scr/A549 cells. *P<0.05 vs. Scr/A549. PKCζ, protein kinase C ζ type; siRNA, small interfering RNA; Scr, scramble.

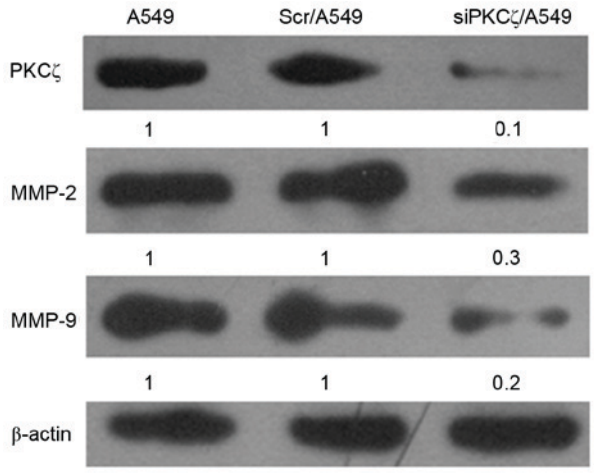

Figure 2. PKCζ siRNA-transfected A549 cells. Following PKCζ siRNA transfection, PKCζ, MMP-2 and MMP-9 were immunoprecipitated and proteins were analyzed using western blotting. PKCζ, MMP-2 and MMP-9 expression was decreased in the siPKCζ/A549 group. PKCζ, protein kinase C ζ type; MMP, matrix metalloproteinase; siRNA, small interfering RNA; Scr, scramble.

compared with Scr/A549 cells (P<0.05), suggesting that PKCζ downregulation was able to decreased the invasive ability of lung adenocarcinoma cells (Fig. 3).

Discussion

In the present study, it was observed that positive PKCζ expression in lung adenocarcinoma was associated with lymph node metastasis, and MMP-2 and MMP-9 expression. It was additionally observed that MMP-2 and MMP-9 expression was decreased in A549 cells following PKCζ knockdown by siRNA, which weakened the invasive ability of the cells *in vitro*.

PKCs are lipid-dependent serine/threonine protein kinases present in mammalian cells that serve important roles in growth, metabolism, proliferation and cytoskeletal remodeling. PKCs are also important intracellular signaling molecules that have been demonstrated to act as oncogenes and tumor suppressors, depending on the cellular context and upon which protein adaptors interact with which PKC isoforms (20-23). PKC isozymes comprise three classes: Conventional (cPKC, α, β and γ), novel (nPKC, δ, ε, η and θ) and atypical (aPKC, ζ and ι). Different PKC isotypes are known to serve distinct regulatory roles. PKCζ is an important subtype of atypical PKCs that is involved in numerous signal transduction pathways.

PKC family proteins have been intensively studied due to their association with cancer. Previous studies have demonstrated that PKCζ may promote tumor cell chemotaxis in glioma, liver cancer and breast cancer, thus promoting cancer cell invasion and metastasis (16,24). When PKCζ is activated, it may phosphorylate Lim domain kinase 1 and cofilin, promoting F-actin depolymerization and polymerization, respectively, which affects the cytoskeleton structure and inhibits cancer cell chemotaxis and migration. Moreover, PKCζ is able to activate integrin-β1, which enhances adhesion between cells, activates the MAPK pathway, and promotes vascular endothelial growth factor (VEGF) expression and angiogenesis, which may consequently promote tumor invasion and metastasis (17,18).

Ma *et al* (25) observed that PKCζ is involved in lung cancer cell adhesion and chemotaxis, and thus may affect the invasion and metastasis of lung cancer. In the present study, it was demonstrated that the rate of positive PKCζ staining in patient-derived lung adenocarcinoma paraffin sections assayed by immunohistochemistry was significantly increased compared with adjacent tissues, and that PKCζ expression was associated with lymph node metastasis. This result also suggested that PKCζ affected the invasion and metastasis of lung adenocarcinoma. *In vitro* Transwell invasion experiments using A549 lung adenocarcinoma cells further confirmed that reducing PKCζ inhibited the invasive capacity of tumor cells. Therefore, the results of the present study demonstrated that PKCζ was able to promote the invasion and metastasis of lung adenocarcinoma through *in vitro* and *in vivo* methods.

MMPs are a family of Zn^{2+}-dependent endopeptidases that are able to degrade the extracellular matrix and basement membrane, and serve an important role in physiological and pathological processes. They have been regarded as critical factors that promote tumor cell invasion. MMP-2 and MMP-9 are the most important enzymes for type IV collagen degradation, and serve important roles in tumor

angiogenesis, invasion and metastasis (26,27). The mechanism underlying this effect involves the increase of VEGF secretion from tumor cells induced by MMP-2 and MMP-9, promoting invasion and metastasis, which is dependent on MAPK activation and ERK phosphorylation. In addition, MMP-9 expression is known to cause emphysema in chronic obstructive pulmonary disorder and angiogenesis/metastasis in lung cancer (28).

Studies have demonstrated that MMP-2 and MMP-9 expression in non-small cell lung cancer is significantly increased compared with normal tissue adjacent to the cancer, and that their expression levels are associated with pathological grading and staging, invasion and metastasis (20,29). PKCζ was able to activate MAPK and the MAPK signaling pathway, and promote VEGF overexpression, angiogenesis, tumor invasion and metastasis (17). In the present study, it was observed that PKCζ expression was associated with the expression of MMP-2 and MMP-9 in lung adenocarcinoma, using immunohistochemical detection. By decreasing the expression of PKCζ in A549 cells, the invasiveness of siPKCζ/A549 cells decreased significantly; decreased PKCζ expression coincided with reduced secretion of MMP-2 and MMP-9. The above results suggested that the PKCζ may promote lung cancer invasion and metastasis by affecting MMP-2 and MMP-9 secretion in lung adenocarcinoma cells.

In conclusion, PKCζ expression was associated with the invasion and metastasis of lung adenocarcinoma, making PKCζ a potential target for gene therapy in lung cancer and providing a theoretical basis for enhancing the survival rate of patients with lung adenocarcinoma. PKCζ, MMP-2 and MMP-9 synergistically promoted lung cancer invasion and metastasis, although the specific mechanism remains unclear and requires further research.

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