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Original Article

Influence of protein kinase C (*PKC*) on the prognosis of diabetic nephropathy patients

Jie Yang, Jian Zhang

Second Department of Endocrinology, Tai An Central Hospital, Shandong Province China

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Abstract: Aims: To investigate the association between protein kinase C (*PKC*) and the prognosis of patients with diabetic nephropathy (DN). Methods: 92 patients with DN who had received treatments with angiotensin converting enzyme inhibitor (ACEI) or angiotensin-receptor blockade (ARB) were collected. The clinicopathologic characteristics were recorded and a 4-year follow-up with the final result of impaired renal functions (eGFR < 40 mL/min) was conducted. The expression of *PKC* was detected by immunohistochemical assay. Kaplan-Meier and Cox regression analysis were performed to estimate the effects of *PKC* on DN prognosis. Results: According to immunohistochemical analysis, there were 54 cases with positive expression of *PKC* (positive rate 58.7%). Meanwhile, during the follow-up, the urine protein, mean serum creatinine and eGFR in patients with positive *PKC* were all higher than those in negative expression group ($P < 0.05$). The expression of *PKC* was influenced by age ($P < 0.001$), course of disease ($P < 0.001$), blood pressure ($P = 0.002$), blood glucose ($P < 0.001$), HbA1c ($P = 0.002$), renal functions of patients before ($P = 0.011$) and after ($P = 0.041$) the biopsy. Besides, the Kaplan-Meier curve revealed that patients with positive *PKC* expression had shorter survival time than those with negative *PKC* expression ($P < 0.001$). Cox regression analysis indicated that HbA1c ($P = 0.009$), renal functions of patients after the biopsy ($P = 0.002$) and *PKC* ($P = 0.028$) were important factors in the prognosis of DN and they might be independent prognostic markers. Conclusion: The expression of *PKC* is relatively higher in DN patients than in healthy controls. And *PKC* may be a valuable prognostic marker for patients with DN.

Keywords: *PKC,* diabetic nephropathy, prognosis

Introduction

Diabetic nephropathy (DN), also named diabetic glomerular sclerosis pathologically, is a common capillary vessel syndrome of diabetes mellitus and it is the most common cause of end-stage renal disease (ESRD) worldwide [1, 2]. It is also a contributor for patients with diabetes to significant morbidity and mortality. With the prolongation of life and the change of lifestyle, the incidence of DN is increasing year by year in China. It is related to endothelial dysfunction and can increase the risk of long-term cardiovascular and kidney via reducing nitric oxide-dependent vasodilation and producing of pro-inflammatory factors [3]. The occurrence and development of DN are involved in many risk factors, such as genetic factors, hypertension, smoking, environmental and hemodynamic changes [4]. And genetic factors may play an important role in the risk of DN [5].

Protein kinase C (*PKC*), a group of serine/threonine kinases, is comprised by approximately 15 different isozymes [6]. The *PKC* family contains several isoforms such as classical ones (α, βI, βII, γ), novel ones (δ, ε, η, θ), and atypical ones (ξ, ι/λ). Classical and novel *PKC*s required diacylglycerol (DAG) for their activation, but atypical *PKC*s are independent of DAG and regulated in a different way [7]. There is a variety of evidence showed that altered *PKC* expression was related with tumorigenesis and drug resistance [8, 9]. However, the roles of *PKC* gene may vary in different cancer cells. In previous studies, *PKC* was confirmed to take effect on signaling events such as cardiac hypertrophy, heart failure, ischemic injury and agonist stimulation [10-12]. It was reported that *PKC* could be activated by hyperglycemia and regulated glucotoxicity in diabetic nephropathy [13, 14]. However, the influence of *PKC* in the prognosis of DN is still unclear.

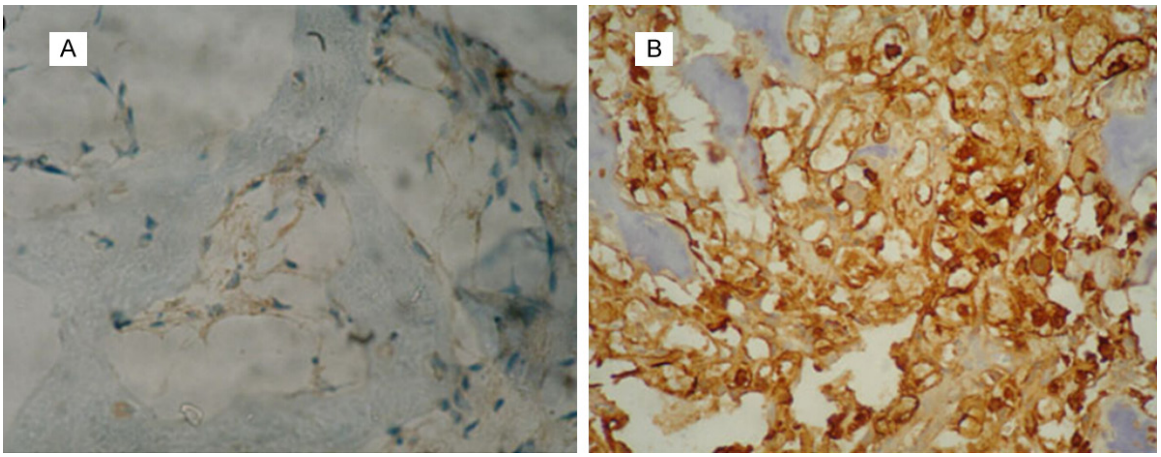

Figure 1. Immunohistochemical staining of *PKC* protein in tissue samples. The expression level of *PKC* was higher in DN patients. The original magnification was ×400. A. Showed negative expression of PKC in healthy controls; B. Showed positive expression of PKC in DN patients.

Table 1. The relationship between *PKC* and clinical characteristics in DN patients

Variable	Cases (n)	*PKC* expression		χ^2	*P*
		Negative (n = 38)	Positive (n = 54)		
Gender				3.146	0.076
Male	58	28	30		
Female	34	10	24		
Age (years)				15.898	< 0.001
≤ 45	47	10	37		
> 45	45	28	17		
Course of disease (months)				39.188	< 0.001
0~3	32	2	30		
4~10	32	12	20		
> 10	28	24	4		
Blood pressure				9.756	0.002
High	50	28	22		
Normal or low	42	10	32		
Blood glucose				28.426	< 0.001
High	45	6	39		
Normal or low	47	32	15		
HbA1c				12.614	0.002
≤ 4%	27	7	20		
5%-8%	45	16	29		
> 9%	20	15	5		
Renal functions of patients before the biopsy				6.458	0.011
Not damaged	36	9	27		
Damaged	56	29	27		
Renal functions of patients after the biopsy				4.185	0.041
Not damaged	48	15	33		
Damaged	44	23	21		

In our study, we selected patients with DN in type III as study objects according to the newest pathologic classification of DN in 2010 [15]. We aimed to investigate the association

Table 2. The comparison of clinicopathologic characteristics in DN patients with different expression of *PKC*

Clinical characteristics	Positive PKC (n = 54)	Negative *PKC* (n = 38)	*P*
24-hour urine protein (g)	4.97 ± 1.10	3.60 ± 1.10	0.039
Serum creatinine (μmol/L)	46.30 ± 10.68	16.80 ± 4.32	0.000
eGFR (mL/min)	15.60 ± 4.90	7.60 ± 2.90	< 0.01

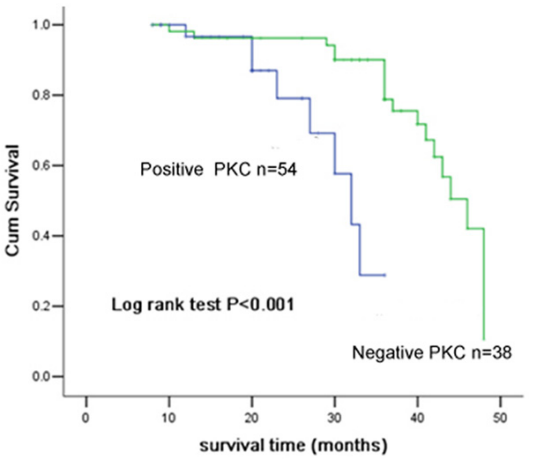

Figure 2. Kaplan-Meier survival analysis in patients with DN. The result indicated that patients with positive *PKC* expression lived shorter than those with negative *PKC* expression ($P < 0.001$).

between clinicpathological characters and *PKC* expression as well as explore the clinical value of *PKC* as a prognostic biomarker for DN patients.

Materials and methods

Patients and samples

92 patients diagnosed with DN by renal needle biopsy in Shandong Tai An Central Hospital were enrolled in the study. The inclusion and exclusion criteria for the subjects were as follows: the inclusion criteria: (1) in accordance with the newest pathologic classification of DN in 2010 [15]; (2) with normal or lightly damaged renal functions (eGFR > 45 mL/min); (3) with urine protein quantification more than 1 g in 24 hours; (4) had been received the treatments of ACEI and ARB; (5) accepted a follow-up which was over 4 years; The exclusion criteria: (1) with DN in combination with primary glomerulopathy; (2) with DN in combination with immune system diseases reducing *PKC* expression levels; (3) with C3 nephritis; (4) impaired renal functions (eGFR ≤ 45 mL/min); (5) with DN in combination with severe cardio-cerebrovascular syndromes; (6) with abnormal glucose tolerance and had a history of hypertension and coronary heart disease. Besides, 70 healthy people with matching age and sex were selected as control.

The clinicopathologic factors including age, course of disease, blood pressure, blood glucose, HbA1c and renal functions of patients before and after the biopsy were recorded in databases. All the patients were received formal drug therapy (ACEI or ARB) and fulfilled the 4-year follow-up. The changes of renal functions of patients in the follow-up were observed. The outcome (eGFR < 40 mL/min) was defined as impaired renal functions. The follow-up information of all the participants was updated every 3 months for 4 years.

Immunohistochemical analysis

The tissues from 92 patients and 70 healthy controls were fixed in 10% formaldehyde and embedded in paraffin, then were cut into 5-μm thick sections and washed for 3 times (5 min each time) with PBS. After quenching endogenous peroxidase activity with 0.3% H_2O_2 at room temperature for 30 min, the slides were subsequently washed for additional 3 times (3 min each time) with PBS. The antigens were heated in a microwave (citrate buffer, pH 6.0), then refrigerated to room temperature, washed 3 times (3 min each time) with PBS, incubated at 37°C with normal non-immunized serum solution for 10 min. Added into the primary antibody solution for overnight at 4°C, washed with PBS for 3 times (5 min each time). Secondary antibody was mixed and incubated for 30 min at 37°C, washed 3 times with PBS. Incubated with horseradish peroxidase-tagged streptavidin for 15 min and finally washed 3 times (3 min each time) with PBS. The 3, 3'-diaminobenzidine (DAB) substrate was used to develop staining color, and counterstained with hematoxylin before dehydration and mounting. Positive staining appeared in cells as yellowish-brown puncta of *PKC*. Stained tissues were scored for the proportion of positive cells out of the total number of cells. Fewer than 10% positive cells were attributed to negative while oth-

Table 3. Multivariate analysis for prognostic factors in DN via Cox regression analysis

Variables	*P*	HR	95% CI
HbA1c	0.009	0.052	0.005-0.525
Renal functions of patients after the biopsy	0.002	0.190	0.066-0.545
Positive PKC expression	0.028	3.655	1.154-11.578

ers belonged to positive. The staining was graded as either negative (-) or positive (+).

Statistical analysis

Quantitative data were shown as median ± SD. Students' t test was used to evaluate the differences between two groups. The relationship between clinicopathologic characteristics and *PCK* expression was estimated by chi-square test. The association between the overall survival and *PCK* was analyzed through Kaplan-Meier analysis. Cox regression analysis was conducted to estimate the prognostic value of *PKC* in DN. All the statistical analyses were performed using SPSS 18.0 software. It was considered to be significant difference when $P < 0.05$.

Results

Basic information of subjects

Finally, 92 patients (including 58 males and 34 females) aged from 32 to 61 with a median age of 46.49 ± 7.73 were included in our study. In the 92 subjects, 54 cases were considered as positive *PKC* expression while 38 patients belonged to negative *PKC* expression via immunohistochemical analysis. The positive rate was 58.7%. As shown in **Figure 1**, compared to the healthy control group, *PKC* expression was significantly higher in DN patients ($P < 0.05$). Among all patients, two with incomplete information in negative *PKC* expression group and one in positive *PKC* expression group were excluded in the 4-years follow-up, and finally 89 patients were taken to conduct the follow-up.

The relationship between PKC and clinicopathologic characteristics

As the positive rate of *PKC* expression was higher than negative rate of *PKC* expression in patients with DN, *PKC* was considered as a promoter in DN. To further explore the role of *PKC* in the progress of DN, we analyzed the relationship between *PKC* and clinicopathologic characteristics. The result showed that the expression of *PKC* was affected by many factors including age ($P < 0.001$), course of disease ($P < 0.001$), blood pressure ($P = 0.002$), blood glucose ($P < 0.001$), HbA1c ($P = 0.002$), renal functions of patients before ($P = 0.011$) and after ($P = 0.041$) the biopsy, which indicated that *PKC* might participate in the development of DN (**Table 1**).

Association between PKC and overall survival of patients with DN

During the follow-up, the urine protein quantities of patients in 24 hours was higher in positive *PKC* expression group than those in negative *PKC* expression group (**Table 2**, $P = 0.039$). Meanwhile, the serum creatinine increased in varying degrees while the mean of serum creatinine in patients with positive *PKC* expression was obviously higher than those with negative *PKC* expression (46.30 ± 10.68 vs. 16.80 ± 4.32, $P = 0.000$, **Table 2**). And the mean eGFR of patients with positive *PKC* expression was significantly different from those with negative *PKC* expression group (15.60 ± 4.90 vs. 7.60 ± 2.90, $P < 0.01$, **Table 2**). Kaplan-Meier analysis demonstrated that compared with the negative *PKC* expression group, the survival rate of patients who was not occur renal functions in positive *PKC* expression group was remarkably lower (Log Rank test, $P < 0.001$) (**Figure 2**). In addition, Cox regression analysis manifested that *PKC* (HR = 3.655, 95% CI = 1.154-11.578, $P = 0.028$) as well as HbA1c (HR = 0.052, 95% CI = 0.005-0.525, $P = 0.009$), and renal functions of patients after the biopsy (HR = 0.190, 95% CI = 0.066-0.545, $P = 0.009$) were influential factors in the prognosis of DN (**Table 3**).

Discussion

DN is the leading causes of chronic kidney disease and a multifactorial progressive disease involving many different cells, molecules, and factors [16-18]. DN has emerged as the leading cause of end-stage renal disease (ESRD), and more than 50% of the new ESRD cases were caused by DN every year [19, 20]. The development speed of DN is more rapidly than other

normal renal diseases. Therefore, it is meaningful to study the factors associated with the prognosis of DN for delaying the progress of the disease and improving the life quality of patients. A variety of factors are involved in the prognosis of DN such as uncontrolled blood glucose, blood pressure, cigarette smoking, anemia, nutriture and hyperlipemia [21]. Besides the genetic factors also take an vital effect on the prognosis of DN.

PKC family participated in various signal transduction pathways including cell proliferation, differentiation, cell cycle, and apoptosis. For instance, Tang et al. found the activation of *PKC* pathway might influence the expression of RGC-32 which can be a novel regulator for macrophage phagocytosis [22]. Activation of *PKC* was considered to be a promoter of cardiac fibrosis and heart failure via increasing the expression of galectin-3 in the study of Song et al. [6]. Moreover, *PKC* is a proverbial pathway of the diabetic milieu in the kidney [23]. However, the role of *PKC* in DN has not yet been fully defined.

In our study, the expression of *PKC* was detected by immunohistochemical analysis. The result demonstrated that among all the samples the positive rate of *PKC* expression was 58.7%. In addition, the mean values of serum creatinine and eGFR changed more remarkably in patients with positive *PKC* expression than those with negative *PKC* expression. These findings indicated that *PKC* might participate in DN progress. This was consistent with the previous studies [24-26]. To demonstrate the expression of *PKC* could affect the survival of DN patients Kaplan-Meier analysis was performed. The 4-year follow-up survival curve revealed that the patients with negative *PKC* expression had a longer survival time than patients with positive *PKC* expression which indicated that *PKC* could affect DN prognosis in some degree. Moreover, urine protein quantitation of patients with positive *PKC* expression in 24 hours was higher than those with negative *PKC* expression. Thus we speculated that proteinuria levels could obviously influence the risk of DN. And the reasons might be that there were too many influencing factors in DN, especially proteinuria which was related to endothelial cell disorder and chronic and low-level inflammation [27].

Up to now, although our study have demonstrated that compared with the healthy control group, the expression of *PKC* was higher in DN patients and *PKC* is a valuable prognostic biomarker, there are still other studies with different opinions about the role of *PKC* in disease. The possible reason may be that there are many members in *PKC* family and each of them may play different role in different cancer types. For example, *PKC*θ underexpression was linked to large tumor size and a poor prognosis, but *PKC*δ overexpression was linked to favorable prognostic features [28, 29].

In conclusion, the expression of *PKC* influences the quantities of urine protein and plays an important role in the prognosis of DN. However, our study has been conducted with a relative small sample size and the result may be unstable. Thus, a larger sample size is necessary in the future study. Meanwhile, in order to eliminate the interference of confounding factors and further verify the association between *PKC* and DN prognosis, specific stratified analysis of proteinuria levels needs to be carried out.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jie Yang, Second Department of Endocrinology, Tai An Central Hospital, Shandong Province, China. E-mail: doctyang@yeah.net

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