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Original Article

Significance of joint detection of urinary proteins with low molecular weight as prognostic indicators for early diagnosis of diabetic nephropathy

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Abstract: Diabetic nephropathy (DN) is a main cause of end-stage renal disease. Early diagnosis and intervention can greatly increase the prognosis. This study was aimed at investigating the clinical significance of urinary excretion of proteins with low weight including microalbuminuria (MA), α_1-microprotein (A1M), β_2-microglobulin (β_2-MG) and retinol binding protein (RBP) as indicators for early diagnosis of DN. A total of 196 DN patients were further classified into 4 groups (non-albuminuria, normoalbuminuria, microalbuminuria and macroalbuminuria) and 80 healthy subjects served as a control group. The level of urinary A1M, MA β_2-MG and RBP was simultaneously evaluated. Both ACR and eGFR were measured for all subjects. The urinary level of A1M, MA and β_2-MG showed a statistically significant increase in all diabetic patients compared to the healthy group. Urinary A1M, MA β_2-MG and RBP were positively correlated with ACR levels in all patients and the four proteins strongly correlated with each other. Combined detection of urinary MA, A1M, β_2-MG and RBP should be examined reliable for the early diagnosis of DN.

Keywords: Diabetic nephropathy, microalbuminuria, α_1-microprotein, β_2-microglobulin, retinol binding protein

Introduction

The prevalence of type 2 diabetes has been globally increasing in obesity and an aging population in recent years. Diabetic nephropathy (DN), which is defined as decline in renal function in a diabetes patient without urinary tract infection or any other renal damage, has become a common complication of diabetes and also a frequent cause of end-stage renal disease both in China and worldwide [1, 2]. Statistics have revealed that approximately 25~40% of patients with diabetes eventually progress to DN. Thus, early prediction of prognosis at the time of initial DN is necessary to optimize therapy in order to delay the progression of diabetes. Some indicators have been identified as predictors of monitoring renal function including serum creatinine (Cr), severe proteinuria and glomerular filtration rate (GFR).

Several biomarkers for tubular damage recently have been proposed as useful diagnostic indicators of primary glomerular damage [3, 4]. Proteins with low molecular weight are filtered freely and reabsorbed lately at the glomerulus by proximal renal tubular cells, some of which have been applied as biomarkers of glomerulus and tubular damage in different renal diseases. Microalbuminuria (MA) is the earliest clinical indicator of microvascular diseases as well as renal disease and the most common used in detection of DN in calculation of urinary albumin excretion rate (UAE) [5, 6]. However, urinary MA is prone to be affected by numerous factor including arterial blood pressure, aldosterone and some foods, moreover, its inter-individual variation is as high as 40% [7]. Similarly, urinary excretion of α_1-microprotein (A1M), β_2-microglobulin (β_2-MG) and retinol binding protein (RBP) are filtered freely and reabsorbed lately at the glomerulus by proximal renal tubular cells, which are not normally detected in healthy people. However, a minimal degree of proximal tubular damage leads to an increase of proteins with low molecular weight in urine [8, 9]. One or

two of these indicators has been extensively applied in predicting the occurrence of renal tubulointerstitial injury, evolution of end-stage renal diseases in idiopathic membranous nephropathy. Standard assays for detection of joint urinary markers are not widely available in laboratories in northeast of China [10].

In the present study, a total of 196 DN patients and 80 healthy subjects were enrolled, and the level of MA, A1M, β_2-MG and RBP in the urine was examined, aiming to evaluate kidney injury at the early stages of diabetes.

Materials and methods

Study participates

A total of 196 patients were enrolled from the people's hospital of Jilin in China from January 2012 to December 2014 and included 98 males and 98 females with mean age of 53.2 ± 8.1 years as well as the mean duration of disease, 9.3 ± 3.1 years. 80 healthy subjects who received routine physical and laboratory examination were also enrolled as control groups including 39 males and 41 females with a median age of 49.3 ± 8.1 years. Renal tumors, trauma, infections and inflammatory diseases were excluded in these patients, besides, liver disease, renal disease other than diabetic nephropathy and pregnancy were also considered as exclusion criteria. In the meanwhile, ECG was performed for the exclusion of cardiovascular diseases.

Human experimental protocols to collect the clinical samples were approved by ethical committees of people's hospital of Jilin (formal ethical approval number: Protocol Number 2012-01-16), and written informed consent was gained from all participants in the study.

Calculation of eGFR and ACR

All the patients with type 2 diabetes and healthy volunteers received a vegetarian diet for longer than 3 days and there was no strenuous exercise during the study period. The urine was gathered in the morning and centrifuged. The supernatant was collected and subjected to urine biochemical analysis (Beckman AU-680, USA) for qualitative urinary protein. At the same time, the volume of 24-h urine was collected and 3 ml of urine was processed for determination of albumin/creatinine ratio (ACR). Estimated glomerular filtration rate (eGFR) was calculated using the Modification of Diet in Renal Disease (MDRD) formula [11].

Detection of the urinary MA, A1M, β_2-MG and RBP

Immune turbidimetric method was utilized to detect the urinary MA, A1M, β_2-MG and RBP with an automatic biochemical analyzer (AU-2700; OLYMPUS) according to manufacturer's instructions (R & D, USA).

The intra-batch CV was < 9% and the inter-batch CV was < 15% in detection of MA, A1M, β_2-MG and RBP respectively. The cutoff values were set at 30 mg/L, 20 mg/L, 0.3 mg/L, 1.5 mg/L for urinary MA, A1M, β_2-MG and RBP.

Statistical analysis

Statistical analysis was performed with SPSS version 19.0 using the mean. Standard deviation and quantitative data were compared with the *t* test. Qualitative and quantitative data were compared with chi-square test and *t* test. Analysis of variance (one-way ANOVA) was used to compare differences among all groups. The relationship among urinary four biomarkers was determined using the Spearman correlation analysis in addition to the linear regression assay. A value of $P < 0.05$ or $P = 0.05$ was regarded as statistically significant.

Results

Characteristics of all groups

One hundred and ninety-six diabetic patients were enrolled in our study, and divided into four groups group I non-albuminuria (n = 30), group II normoalbuminuria (n = 74), group III microalbuminuria (n = 60), group IV macroalbuminuria (n = 32) according to the presence of ACR in addition to eGFR. Additionally, 30 cases in group I were found to be negative for qualitative urinary protein, moreover, further diagnosed by renal pathological examination (data not shown). No patient was excluded from all groups for any reason.

Demographic data were comparable between groups while duration of the disease was significantly longer in four groups compared to control group. On the contrary, eGFR showed a statistical significant decrease in group IV compared to group III and group III compared to

Table 1. Demographic characteristics of all subjects

Variable	Controls (n = 80)	Group I (n = 30)	Group II (n = 74)	Group III (n = 60)	Group IV (n = 32)	*P* value
Age	49.2 ± 3.1	51.2 ± 4.2	53.2 ± 5.1	52.2 ± 7.2	54.2 ± 8.1	0.941
Gender M/F	39/41	16/14	36/38	31/29	15/17	0.624
Disease duration	-	5.2 ± 0.44	8.3 ± 1.62	10.7 ± 6.54	14.4 ± 11.5	0.034*
ACR (mg/mmol)	-	≤ 3	≤ 3	3-30	> 30	
eGFR (ml/min)	112 ± 24.3	110 ± 23.5	94 ± 18.5	70 ± 16.4	56 ± 13.4	0.001*

M = male, F = female, eGFR = estimated glomerular filtration rate, ACR = albumin/creatinine ratio, *= significant.

Table 2. Comparison of levels of A1M, MA, β_2-MG and RBP in different groups (x ± s) (mg/L)

Variable	Controls (n = 80)	Group I (n = 30)	Group II (n = 74)	Group III (n = 60)	Group IV (n = 32)	*P* value
A1M	7.37 ± 1.46	7.46 ± 1.67	19.74 ± 5.74	147.4 ± 51.9	245.7 ± 71.9	0.005*
MA	7.82 ± 1.92	7.92 ± 2.62	21.34 ± 13.5	39.78 ± 10.43	79.7 ± 12.41	0.002*
β_2-MG	0.72 ± 0.34	0.82 ± 0.44	3.23 ± 1.62	16.72 ± 6.54	34.72 ± 11.55	0.001*
RBP	1.15 ± 0.13	3.13 ± 1.14	3.15 ± 1.52	6.73 ± 1.52	13.13 ± 5.56	0.001*

*= significant. A1M: α_1-microprotein; MA: mcroalbuminuria; β_2-MG: β_2-microglobulin; RBP: retinol binding protein.

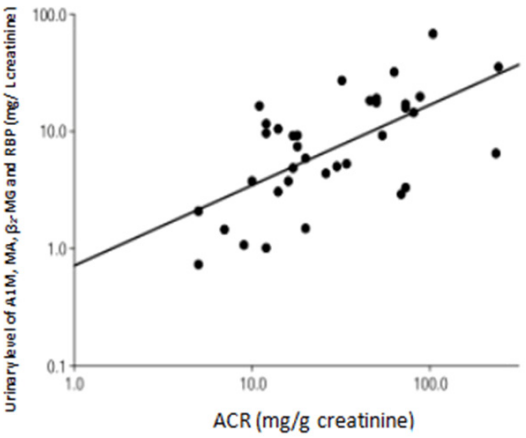

Figure 1. Correlation between urinary A1M, MA, β_2-MG and RBP and albumin/creatinine ratio in diabetic patients (n = 196). Logarithm-transformed urinary A1M, MA, β_2-MG and RBP levels positively correlate with Logarithm-transformed albumin/creatinine ratio (ACR) levels (r = 0.617; P < 0.0001).

group II and the all groups compared to the control group except group I. No marked difference was found in clinical information of these patients among all groups ($P > 0.05$). Preliminary demographic data of the 196 patients and control group were listed in **Table 1**.

Laboratory findings and association of albuminuria with four parameters

Of laboratory findings, the urinary level of A1M, MA and β_2-MG showed a statistical significant increase in group IV compared to group III and group III compared to group II and group II compared to the group I and the three groups compared to the control group ($P < 0.01$). The urinary level of RBP in four groups was significantly greater than that of the control group ($P < 0.01$). However, statistical analysis showed no difference in the level of urinary RBP between group I with non-albuminuria and group II ($P > 0.05$). The four indicators in all groups are presented in **Table 2**.

Mean levels of urinary A1M, MA, β_2-MG and RBP were positively correlated with ACR levels in all patients (r = 0.617; $P < 0.0001$) (**Figure 1**). The urinary A1M, MA, β_2-MG and RBP strongly correlated with each other (data not shown).

Discussion

A nationwide type 2 diabetes surveillance study in 14 provinces and municipalities in China between 2007 and 2008 showed that the prevalence of diabetes had increased to 9.7%, approximately 60% of adults aged 20 have not yet been diagnosed with obscure symptom, only were confirmed in physical examination [12]. The WHO has identified diabetes or DN as a major health problem in Asia, especially in China as well as India. The mortality of DM patients with DN is 30 times higher than that of DM patients with DN. Once DN is present, the glomerular filtration and the structure in addition to function of renal tubules are liable to be

damaged, hence, resulting in albuminuria. This is the clinical stage of DN and it is difficult to treat or block the progression of DN. Thus, early diagnosis and prevention of diabetes or DN have become a high priority among health policies [13, 14].

In previous studies, GFR was considered as the best indicator of renal excretory function, which was used as gold standard primarily to evaluate the glomerular filtration, however, the developing methods for determining GFR have a nature of time-consuming, requirement of experienced stuff, complex procedures, which are limited in clinical practice, along with risk for radiation induced injury [15]. Our study demonstrated that eGFR was significantly lower in diabetic patients than that of the healthy control. However, in those without any clinical evidence of tubular dysfunction, the only eGFR cannot reflect the activity of the disease in addition to the residual functional capacity of the kidney. Although blood urea nitrogen (Bun) and Serum muscle (Scr) have been applied to assess kidney function, both are influenced by several factors and vary among individuals, besides, changes in Bun and Scr are only observed in patients with severe kidney dysfunction. Thus, these indicators are not in a position to reflect the kidney disease at an early stage. In addition, kidney biopsy is a gold standard for the diagnosis of DN, but it is an invasive tool and has risks [16].

Comparison among all the studied groups as regard urinary A1M, MA, β_2-MG and RBP showed a statistically significant increase in all groups than the healthy control. Traditionally, MA was the first of renal involvement in predicting overt nephropathy among low weight proteins. Regardless of the fact that MA remains an essential indicator of DN for monitoring renal damage progression, a number of evidence demonstrate that only thirty percent of patients with MA progress to overt NP after following-up for ten years [17]. In addition, there was a statement that a sizable variety of patients with MA can revert from nomoalbuminuria to normoalbuminuria, suggesting a reduction in glomerular filtration rate without progressing from normo-to MA [18]. These documents illustrate that MA is more a diagnostic marker than an indicator to predict DN [19].

Increase in urinary β_2-MG excretion is proved to be elevated in DN patients with reduced GFR and tubular injury. However, its sensitivity for early diagnosis is limited because of poor stability at acidic pH. An alternative tool of evaluating renal tubular function is stable A1M or RBP. Normally, urinary excretion of A1M and RBP is extremely low in the healthy population. There is an evidence that urinary A1M or/and RBP excretion markedly increases with the degree of type 2 diabetes, suggesting A1M or/and RBP may be complementary to MA, both have been served as a sensitive indicator for proximal tubule injury. In addition, urinary RBP increases only in those with concomitant microvascular lesions in studies on DN of patients with type 2 DM. Thus, RBP may serve as an indicator to predict the concomitant microvascular lesions or macrovascular lesions in DM patients [20]. Some studies have shown that the RBP of different forms in the blood increases when the blood flow in kidney reduces, i.e., RBP increases markedly when GFR reduces, and thus RBP may be used to evaluate the kidney injury at an early stage.

In the present study, our results showed DN was associated with elevated urinary excretion of A1M, MA, β_2-MG and RBP values compared to a healthy control. This increase in four indicators was parallel to the severity of renal dysfunction among four groups regarding albuminuria. These results suggested that increased urinary MA, A1M, β_2-MG and RBP had a diagnostic value for DN, which were in agreement with other findings [21]. In addition, the urinary level of RBP in nonalbuminuric DN has increased and was significantly higher than those in healthy subjects. This suggested that urinary excretion of RBP was invariably elevated in the absence of any clinical evidence of kidney damage. The result was in agreement with other reporters that stated that changes in urinary RBP occurred prior to microalbuminuria [11].

To our knowledge, this study is the first to reveal a significant correlation between urinary proteins with low molecular weight and DN in northeast of China. Those, who live in the regions, like to eat high-salt, high-fat food, are liable to obesity without physical inactivity. All factors become risk-diabetic. Therefore, a noninvasive diagnosis of type 2 diabetes is useful assay for the management of DN patients,

prognosis, and therapy in addition to nephroprotective strategies.

We speculate that measurement of urinary MA, A1M, β_2-MG and RBP may serve as earlier indicators for the initial diagnosis of DN at the initial stage and their sensitivity are higher than that of urea and Scr. Furthermore, collection of urine samples is obtained noninvasively and relatively easily. Screening of urinary MA, A1M, β_2-MG and RBP is also helpful for the early therapy of DN. Studies on the excretion of MA, A1M, β_2-MG and RBP have indicated that these may be indicators of renal impairment. This is consistent with the findings of Nakayama A [22].

Our study has several limitations. The study was not population-based, and the numbers of DN patients included in the study were not sufficient to determine the predictive powers. Further large scale validation is needed in order to elucidate their values as multiple prognostic biomarkers for DN.

In conclusion, measurement of urinary MA, A1M, β_2-DM and RBP is sensitive in the diagnosis of these lesions when DN patients develop renal tubular or glomerular injury at the early stage. Combined detection of four proteins with urinary low weight is beneficial for the early diagnosis of DN and helps to identify the severity of kidney injury.

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Disclosure of conflict of interest

None.

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