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Research Article
Diagnostic Values of Urinary Biomarkers in Early Diagnosis of Diabetic Nephropathy

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Abstract

Background and Objective: The diagnosis of type 2 diabetic nephropathy (T2DN) patients is important to prevent the long-term damaging effects of kidney loss in patients with diabetes. The aim of this study was to explore diagnostic values of Urinary RBP levels, NGAL, ZAG and urinary transferrin in early diagnosis of diabetic nephropathy. **Materials and Methods:** The total sample size was comprised of 400 patients, 300 subjects were of type 2 diabetes mellitus and 100 subjects were considered under the control subjects without any systemic illness. According the ratio of urine and albumin urine creatinine, subjects were further divided into 3 sub-categories: normoalbuminuria, microalbuminuria and macroalbuminuria group. Human ELISA kit had been used for the evaluation of Urine urinary RBP, NGAL, Adipokine zinc-alpha-2-glycoprotein and urinary transferring. **Results:** The level of u-NCR, u-RCR, u-ZCR, u-TCR levels in the macroalbuminuria and microalbuminuria were markedly and significantly high when compared with normoalbuminuria and control subjects. So, when the stages of diabetic nephropathy are going critical, the levels of all the biomarkers also increasing in their value. urinary transferrin and RBP have excellent diagnostic accuracy with AUC = 1-0.9. **Conclusion:** This study concluded that the evaluation of urinary RBP, NGAL, ZAG and urinary transferrin could be use as novel and accurate biomarker for the early diagnosis, clinical monitoring and progression of diabetic nephropathy patients.

Key words: Diabetic nephropathy, urinary biomarkers, early diagnosis, kidney loss, clinical monitoring, microalbuminuria, urinary transferring, macroalbuminuria

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Diabetic nephropathy occurred in approximately 30-40% of the patients diagnosed with type 2 diabetes mellitus, which further increase the rate of mortality and morbidity in the population[1,2]. Microalbuminuria considered being the predictor of the progression of the diabetes mellitus[2,3]. However, they are not considered to be the very sensitive and specific biomarker in diabetes mellitus and their level believed to be on the rising side in the later stages of disease[3]. Authors concluded that the microalbuminuria was not specific for diabetes nephropathy as patients of chronic renal diseases without diabetic nephropathy have also diagnosed with microalbuminuria[2-4]. Therefore, the need of the hour is to find accurate sensitive and specific biomarker other than albuminuria to analyze the progression of the nephropathy. Recently, the studies were concentrated only on one biomarker and only on one stage of diabetic nephropathy to evaluate the diagnosis accuracy[5]. Therefore; the studies are needed on the multiple biomarkers on the various stages of diabetic nephropathy.

Retinol binding protein (RBP) belongs to the family of lipocalin and completely re-absorbed in the proximal tubules, so this could be considered as valuable biomarkers of proximal tube dysfunction[6,7].Neutrophil gelatinase associated lipocalin (NGAL) consider to be highly activated protein in renal diseases[8,9]. Recent literature showed that the urinary NGAL levels have been significantly increased in the albuminuria cases of diabetic mellitus[10,11]. Zinc alpha 2 glycoprotein (ZAG) belongs to major histo-compatability complex of class I family of proteins[12,13]. urine ZAG levels are in coordination with glucose metabolism and increases in the cases of diabetes mellitus and their levels are also associated with diabetic nephropathy stages[14]. This had been noticed that the patients with high level of urinary transferrin excretion will eventually progress to microalbuminuria very frequently[15,16]. Wu *et al.*[10] revealed that the urinary RBP and NGAL levels are associated with nephropathy in Patients with type 2 diabetes. However, this study was evaluated the levels of different urinary biomarkers at various stages of diabetic nephropathy. This study was aimed to assess the level of urinary RBP, NGAL, adipokine zinc-alpha-2-glycoprotein and urinary transferring in coordination with albuminuria for the early diagnosis of diabetic nephropathy and further to evaluate the diagnostic accuracy of these biomarkers in the different stages of diabetic nephropathy.

MATERIALS AND METHODS

The total sample size was comprised of 400 patients, out of which 300 subjects, who declared with type 2 diabetes mellitus and had been referred to Department of Endocrinology Hankou Hospital of Wuhan City, were taken as subject samples and 100 subjects taken as control healthy subjects without any systemic illness. The informed consent of the patients and Ethical Clearance had been taken from the University Ethical cum research board committee with the reference no. of HG Neph CT-2014/25. The first sample of the study was collected on 21st June 2015 and the analysis had been completed on 30th August, 2017. The total duration of the study was approximately 3 years.

Inclusion and exclusion criteria: The patients were enrolled in the study according to the following chosen criteria:

- Age was more than 18 years
- Diagnosis of diabetes had been made after the age of 30 years
- No sign and symptoms and history of any type of cardiovascular disorders like CHD, heart attack, angina etc.
- No sign and symptoms and history of nephrological disorders
- No sign and symptoms of inflammatory diseases

Patients having T2DM with >6% glycosylated hemoglobin and 7% mmoL L^{-1} fasting blood glucose level or Oral GTT 2 h post prandial level more than 11mmoL^{-1} had been taken as the inclusion criteria. The patients with longstanding history of diabetes mellitus more than 10 years and GFR of more than 65 mL min^{-1} per 1.81 m^2 were also included as study samples.

Those patients with T2DM were excluded when renal diseases attributable to other causes. Division of the Subjects: control, normoalbuminuria, microalbuminuria, macroalbuminuria according the ratio of urine albumin/urine creatinine, the subjects were further divided into 3 sub-categories:

- **Control subjects:** Without any systemic illness
- **Subjects which are having normal ratio:** Normoalbuminuria group having the ratio[11] of UAC R upto 30 mg b^{-1}
- **Subjects which are having raised ratio:** Micro albuminuria group having the ratio of UACR more[11] than 30-300 mg b^{-1}

- **Subjects which are having very high ratio:** Macroalbuminuria group having the ratio of UACR more than more[11] than 300 mg b^{-1}

Sample collection and investigation for the T2DN: The samples were collected after the fasting overnight minimum 12 h of the fasting period. Samples were stored at -90°C and analysis had been done. The collected blood was investigated for Hb1Ac analysis. Oxidase method was used for the examination of fasting blood glucose level (FBG) and Triglyceride level (TG). Alanine transaminase (ALT) and Aspartate aminotransferase were measured by the ELISA enzyme colorimetry and dynamic UV method respect. Blood uric nitrogen (BUN) and Blood Uric acid (UA) were calculated by uric acid enzymatic reaction and immune turbidimetry respectively.

Analysis of urine NGAL level by using ELISA method: ELISA human NGAL kit (Kit 314, BioPorto Diagnostics, DK-2900 Hellerup Denmark, Sweden) for the analysis of urine NGAL levels. ELISA solid phase sandwich technique had been used for the analysis of urine NGAL levels[10]. Target specific antibodies which already had coated with antibody were applied on the available microplate. After that the standard, samples and control were added into the wells. Then again 2nd reagent i.e., substrate antibody had been added. Then the enzyme-antibody target complex had been formed which further provide the readings. The intensity of the signal had been directly proportional to the concentration present in the well[10].

Analysis of urine RBP level by using ELISA method: Human Retinol binding protein (RBP) ELISA Kit (ab137991, Abcam, Cambridge, MA, USA) had been used for the analysis of urinary RBP level[11]. Retinol binding antibody precoated wells had been taken and added with samples, controls and standards as antigens. Again 2nd reagent of retinol binding protein antibody had been biotinylated in the complex followed by the buffer. Streptavidin-peroxidase complex was added and unbound conjugates were washed away with wash buffer. 3,3',5,5'- tetramethylbenzidine substrate (TMB) was then used to visualize Streptavidin-peroxidase enzymatic reaction[11].

Analysis of urine ZAG level by using ELISA method: Urinary Adipokine zinc-alpha-2-glycoprotein (ZAG) level were analyzed with commercial available ELISA kit, (RayBiotech Inc, Tebu-Bio, Le-Perray-en-Yvelines, France) as according to the manufacturer's instructions. The lowest detected value[12] for ZAG was 0.1 mg L^{-1}.

Analysis of urine transferrin by using ELISA method: The concentration of urine transferrin and transferrin to creatinine ratio were estimated on spot in the morning urinary samples. The urinary samples were centrifuged at 1000×g, for 25 min and then diving the samples into 1.5 mL aliquots and frozen at the temperature of -80°C until the analysis[13]. The urinary samples were estimated by commercially available ELISA kits from Elabscience Biotechnology Co., Ltd. Minimum and detectable dose for urinary transferrin[13] was 1.56 ng m^{-1}.

Statistical analysis: SPSS version 16.0 (Chicago, USA) had been used for the statistic analysis. For the normal distributed values and for the interquartile range or non-parametric values, mean and standard deviation of the samples had been evaluated for the distributed. One way ANOVA for the significance analysis and Kruskal wallis were used for the non-parametric values. Regression coefficient was used for the correlation of UACR and Urinary Biomarkers. The p-values of less than 0.05 considered to be the significant value.

RESULTS

Patient baseline characteristics: The demographic and clinical characteristics of 400 subjects of type 2 diabetes mellitus and control subjects with different level of albuminuria were shown in the Table 1. At the baseline, the age, gender, body mass index (BMI), body weight, triglyceride level, Insulin levels, HB1 Ac levels, C-peptide level and GFR showed non-significant different amongst the various groups. Longer duration of diabetes correlated with macroalbuminuria group as compared with normoalbuminuria and microalbuminuria with significant p-value. The levels of FBG and diastolic blood pressure (DBP) were significantly increased in T2DN groups compared with the control group (Table 1).

Differences in urinary markers with respect to albuminuria: The ratio of urine NGAL: urine creatinine (u-NCR), Urine RBP: urine creatinine (u- RCR), Urine ZAG: Urine creatinine (u-ZCR), Urine Transferrin: urine creatinine(u-TCR) had been evaluated and correlated. The values of u-NCR, u-RCR, u-ZCR and u-TCR were higher in the macroalbuminuria group with significant p-value when compared with the control, normoalbuminuria and microalbuminuria groups (Table 2).

Diagnostic accuracy of bio-markers: To evaluate the diagnostic accuracy of the biomarkers, it was evaluated the sensitivity, specificity, negative predictive value, positive predictive value and AUC (Table 3).The AUC had been taken

Table 1: Clinical characteristics of controls and T2DN patients, stratified according to albuminuria status

Parameters	Control (n = 100)	Normoalbuminuria	Microalbuminuria	Macroalbuminuria	p-value
	----------(n = 100)----------				
Age (Years)	61.2±6.7	59.5±9	67.8±11.2	64.5±5.6	0.09
Gender (M/F)	60/40	70/30	61/39	65/35	0.12
Body weight (kg)	69.2±7.7	76.4 ± 6.3	68.9±10.1	68.5±5.2	0.11
BMI (kg m^{-2})	23.45±2.1	25.63±3.1	21.11±2.9	23.43±1.2	0.18
Triglyceride level (mg dL^{-1})	178.67±21.1	198±27.4	181.2±23.2	178.3±1.9	0.08
Insulin levels	-	95.13±5.4	116.1±4.7	98.12±4.7	0.341
HB1 Ac levels (%)	-	9.54±1.2	9.89±1.1	9.12±0.9	0.223
C-peptide level (ng mL^{-1})	-	1.45±0.6	1.32±0.2	1.48±0.6	0.112
GFR Ml/min/1.73 m^2	104.2±11.2	127.3±9.8	132.3±30.1	127.8±29.1	0.234
UACR (ng mg^{-1})		17.3±6.7	71±11.2	85±18.1	<0.001
Duration (Year)		5.5±0.4	7.6±1.1	15.8±1.1	<0.001
Fasting Blood Glucose (FBG) (mmol L^{-1})	3.12±1.2	11.34±2.2	12.32±2.1	12.98±1.9	<0.001
Diastolic Blood Pressure (DBP) (mm hg^{-1})	74±4	87±3	92±5	100±5	<0.001
BUN (mmol L^{-1})	3.41±1.2	5.7±1.9	6.2±1.6	11.2±2.1	<0.001

Table 2: Differences in urinary markers with respect to albuminuria

Parameters	Control	Normoalbuminuria	Microalbuminuria	Macroalbuminuria	p-value
u-NCR (ug mg^{-1})	8.72	12.45	17.21	32.71	<0.001
u-RCR (ug mg^{-1})	9.62	14.89	20.41	40.21	<0.001
u-ZCR (ug mg^{-1})	11.32	16.89	21.89	43.21	<0.001
u-Transferrin (pg mg^{-1})	16.72	19.21	23.54	48.91	<0.001

u-NCR: Urinary nephrin creatinine ratio, u-RCR: Urinary renal clearance rate, u-ZCR: Urinary zolpidem tartrate

Table 3: Sensitivity, specificity and AUC for the 4 markers in detecting cases with macroalbuminuria

Bio-markers	Sensitivity (%)	Specificity (%)	AUC	PPV (%)	NPV (%)
RBP	90	95	0.91	96	95
NGAL	77	64	0.681	62	68
Adipokine zinc-alpha-2-glycoprotein	80	74	0.711	78	75
Urinary transferrin	95	94	0.99	99.1	98.9

RBP: Retinol binding protein, NGAL: Neutrophil gelatinase associated lipocalin, NPV: Negative predictive value, PPV: Positive predictive value, AUC: Area under curve

as the gold standard to assess the diagnostic accuracy and had been evaluated according to Albumin:Creatinine ratio (ACR). The urinary bio-markers had been further divided on the basis of AUC levels.

Biomarkers with excellent diagnostic accuracy levels between AUC = 1-0.9. Biomarkers included urinary transferrin and RBP have excellent diagnostic accuracy with AUC and RBP have excellent diagnostic accuracy with AUC value (AUC 1.0-0.9). Biomarkers with good accuracy levels between AUC = 0.8-0.7. Adipokine zinc-alpha-2-glycoprotein were found to have good diagnostic accuracy among patients with microalbuminuria with AUC value of 0.8-0.7. Biomarkers fair diagnostic accuracy levels between AUC = 0.7-0.6. Urinary NGAL biomarkers have sufficient and less diagnostic accuracy with AUC 0.7-0.6. The NGAL bio-marker revealed with 77% sensitivity, 64% specificity and area under the curve (AUC) = 0.681; RBP with 90% sensitivity, 95% specificity and AUC = 0.912; urine transferrin with 95% sensitivity, 94% specificity and AUC = 0.99 and Adipokine zinc-alpha-2-glycoprotein with 80% sensitivity, 74% specificity and area under the curve (AUC) = 0.711 (Table 3).

DISCUSSION

The results of the study showed that the urinary NGAL, RBP, ZAG and transferrin levels were significantly increased the micro and macroalbuminuria groups compared with control subjects. The results of the study marked the point that all the biomarkers NGAL, RBP, ZAG and transferrin defiantly could be novel biomarkers in the early diagnosis of T2DN.

Mahfour *et al*.[17] concluded that the serum RBP could be useful tool in diagnosis and progression of diabetic nephropathy. It was also revealed the same results as of current study and concluded that the urinary RBP levels were independently related to the risk of macroalbuminuric diabetic nephropathy which further suggested that the RBP could be used a biomarker for the clinical monitoring of diabetic nephropathy patients[7]. Galanti *et al*.[18] Revealed that the serum RBP levels are not correlated with urinary RBP levels. So, further investigations required to correlate urinary and serum RBP levels. Previous studies demonstrated that the serum NGAL levels could be good biomarker for the investigation of diabetic nephropathy[19]. De Carvalho *et al*.[20] also revealed the same results that NGAL levels are on higher

sided in T2DN cases and further increased in cases of macroalbuminuric patients[21]. It was found that urinary NGAL was elevated in type1 diabetic (T1DM) patients with or without albuminuria, demonstrating tubular damage at an early stage.[22]

The literature concluded that the levels of urinary transferrin marker was found to be increased with the presence of insulin resistance, poor glycemic control and elevated triglycerides, in addition to increased SBP[13]. The correlation between urinary excretion and these clinical markers have been tested by Cheung *et al.*[21], where the correlations were not significant except with SBP. In this study urinary transferrin and RBP have excellent diagnostic accuracy with AUC and with high sensitivity levels and needs to be further investigated with longitudinal prospective studies to evaluate the predictive power of these markers for diabetic nephropathy before any structural damage occurs. A long term follow up should also be required to know whether after the post-operative treatment, do these urinary bio markers plays any role?

CONCLUSION

Evaluation of urinary RBP, NGAL, ZAG and urinary transferrin could be use as novel and accurate biomarker for the early diagnosis, clinical monitoring and progression of diabetic nephropathy patients. Further, the study revealed that the urinary transferrin and urinary RBP had highest diagnostic accuracy with highest sensitivity and specificity levels in early diagnosis of T2DN.

SIGNIFICANCE STATEMENT

This study discovered the Urinary RBP, NGAL, ZAG and Urinary transferrin level were proved to be independently associated with the albuminuria in diabetic nephropathy cases that can be beneficial for early diagnosis, clinical monitoring and progression of diabetic nephropathy patients. This study will help the researchers to uncover the critical areas of early diagnosis of diabetic nephropathy that many researchers were not able to explore. Thus a new theory on diagnostic accuracy of urinary biomarkers may be arrived at.

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