Research Article

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Serum Homocysteine, cystatin C as Biomarkers for Progression of Diabetic Nephropathy

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Abstract: Objective To investigate the clinical efficacy of serological level homocysteine (Hcy) and cystatin C (Cys-C) as biomarkers for progression of diabetic nephropathy (DN).

Methods Seventy-five patients with type 2 diabetes mellitus (DM) hospitalized in Lishui People's Hospital from January 2015 to May 2018 were included in the present study. Of the 75 cases, 28 were simple DM, 25 were early stage DN (DNe) and other 22 subjects were clinical stage DN (DNc). The serum level of Hcy and Cys-C were detected and compared among the DM, DNe and DNc groups. The efficacy of serological levels of Hcy, and Cys-C as biomarkers for diagnosis of early stage diabetic nephropathy was calculated.

Results The serological levels of Hcy were 11.53 ± 3.05 $\mu\text{mol/L}$, 15.39 ± 4.58 $\mu\text{mol/L}$ and 18.14 ± 7.03 $\mu\text{mol/L}$ for DM, DNe and DNc groups respectively ($P < 0.001$). Serum level of Cys-C, were 0.89 ± 0.23 mg/L , 1.51 ± 0.60 mg/L and 2.63 ± 0.90 mg/L respectively for DM, DNe and DNc groups respectively ($P < 0.001$). Significant positive correlation between serum Cys-C and Hcy was detected in DNe ($r_{\text{pearson}} = 0.55$, $P = 0.004$) and DNc ($r_{\text{pearson}} = 0.44$, $P = 0.04$) groups. However, there was no significant correlation of serological Cys-C and Hcy in DM group ($r_{\text{pearson}} = 0.08$, $P = 0.70$). The sensitivity and specificity in diagnosis of early stage DN were 76.0 (95%CI:54.87-90.64)%, 64.29 (54.07-81.36)% for serological Hcy and 80.0 (59.30-93.17)%, 89.29 (57.77-97.73)% for serum Cys-C respectively. The diagnostic area under the ROC curve (AUC) was 0.76 (0.63 to 0.90) and 0.84 (0.72-0.96) respectively for serum Hcy and Cys-C in detection early stage DN.

Conclusion: Serum levels of Hcy and Cys-C in diabetic nephropathy patients were elevated compared to that of

simple DM cases, making them potential biomarkers for diagnosis of early DN from DM patients.

Keywords: homocysteine; cystatin C; diabetic nephropathy; diagnosis

Introduction

Diabetes mellitus is one of the most diagnosed endocrine diseases in the clinic. According to the International Diabetes Federation (IDF), there were 415 million diabetic patients in the world in 2015, which has risen since [1]. Chinese diabetic patients account for nearly 27% of cases world-wide, of which 1.3 million died of diabetes mellitus and its complications. Diabetic nephropathy (DN) is the manifestation of diabetic microangiopathy in the kidney. More than 50% of patients with diabetes will progress to DN in 10 to 20 years, making it one of the main causes of end-stage renal disease (ESRD) [2]. Therefore, early detection and proper treatment of DN are particularly important for patients prognosis [3-5]. The serum Hcy is an intermediate metabolite of methionine and a non-paproteinogenic amino acid [6]. The main metabolic organs of Hcy are liver and kidney. Recent studies indicate that Hcy is closely related to diabetic nephropathy development [7]. Hyperhomocysteinemia is an independent risk factor for diabetic nephropathy; which can directly produce cytotoxicity, lead to oxidative stress and synergistic glycation end products, and thereafter damage vascular endothelium and induce microvascular injury. Cys-C, a cysteine protease inhibitor, is an alkaline, non-glycosylated protein. Cys-C is not affected by hepatic function and inflammation, and filters into the glomerulus freely, but is not re-absorbed or secreted. Therefore, the serum Cys-C can reflect the function of glomerular filtration and can be used as a serological marker of glomerular filtration rate [7, 8]. In the present study, we investigate the clinical efficacy of serological level Hcy, Cys-C as biomarkers for progression of DN.

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Material and methods

Patients

Seventy-five patients with type 2 DM hospitalized in Lishui People's Hospital from January 2015 to May 2018 were included in the present study. Among them, 28 cases were simple type 2 diabetes mellitus (DM), 47 cases were type 2 diabetes mellitus with diabetic nephropathy (DN). The study was approved by the Medical Ethics Committee of Lishui People's Hospital. According to Mogensen's criteria [9], the 75 subjects were further divided into three groups according to urinary albumin excretion rates (UAER) and clinical manifestations. (1) Simple diabetes mellitus group (DM group, UAER < 30mg/24h); (2) Early diabetic nephropathy group (DNe group, UAER 30-299mg/24h); (3) Clinical diabetic nephropathy group (DNc group, UAER > 300 mg/24h).

Patients inclusion criteria: (1) The DM and DN was clearly diagnosed according to the World Health Organization (WHO); (2) The patients didn't received any drugs that can affect the renal function within 30 days before recruitment; (3) All patients sign informed consent. Exclusion criteria: (1) Acute complications of diabetes mellitus such as ketoacidosis and hyperosmotic coma; (2) Primary glomerular disease; (3) Patients with malignant carcinoma; (4) Patients of acute stress state, such as stroke, acute myocardial infarction, severe infection, respiratory failure, heart failure and etc. **Ethical approval:** The research related to human use has been complied with all the relevant national regulations, institutional policies and in accordance the tenets of the Helsinki Declaration, and has been approved by the authors' institutional review board or equivalent committee.

Informed consent: Informed consent has been obtained from all individuals included in this study

Serological Hcy and Cys-C examination

All the patients fasted for more than 8 hours. 3-5 ml of cubital venous blood was collected in the morning. Cys-c and Hcy levels were tested on a Hitachi 7600-020 automatic biochemical analyzer using calibration and control reagents produced by Beijing Jiuqiang Biotechnology Co Ltd. All measurements were performed in strict accordance with the laboratory standard operating procedures.

Statistical analysis

Data was analyzed by STATA11.0 software. The difference of the DM, DNe and DNc groups were analyzed using ANOVA tests. Early diagnostic sensitivity and specificity for DN were calculated using the following equation of sensitivity = true positive/ (true positive + false negative), specificity=true negative/ (true negative + false positive). The area under the receiver operating characteristic (ROC) curve was used to evaluate the feasibility of serum Hcy and Cys-C as biomarkers for early stage DN diagnosis. The correlation between serum Hcy and Cys-C in DM, DNe and DNc groups were analyzed by Pearson's correlation test. $P < 0.05$ was considered statistically significant.

Results

Clinical character of the DM and DN

The baseline characteristics of the included cases DM, DNe and DNc are shown in **Table 1**. The age, gender and disease course were not statistical different between the groups ($P > 0.05$).

Serum level of Hcy and Cys-C

The serological levels of Hcy were $11.53 \pm 3.05 \mu\text{mol/L}$, $15.39 \pm 4.58 \mu\text{mol/L}$ and $18.14 \pm 7.03 \mu\text{mol/L}$ for DM, DNe and DNc groups respectively ($P < 0.001$). The serum levels of Cys-C were $0.89 \pm 0.23 \text{ mg/L}$, $1.51 \pm 0.60 \text{ mg/L}$ and $2.63 \pm 0.90 \text{ mg/L}$ for DM, DNe and DNc groups respectively ($P < 0.001$), **Figure 1**.

Correlation between serum Hcy and Cys-C

Significant positive correlation between serum Cys-C and Hcy was detected in DNe ($r_{\text{pearson}} = 0.55$, $P = 0.004$) and DNc ($r_{\text{pearson}} = 0.44$, $P = 0.04$) groups. However, there was no significant correlation of serological Cys-C and Hcy in DM group ($r_{\text{pearson}} = 0.08$, $P = 0.70$), **Figure 2**.

Serum Hcy and Cys-C as biomarker for early DN

The sensitivity and specificity in diagnosis of early stage DN were 76.0 (54.87-90.64)%, 64.29 (54.07-81.36)% for

Figure 1: Scatter plot of serum homocysteine and cystatin C in type 2 diabetes mellitus, early diabetic nephropathy and clinical stage diabetic nephropathy groups (a: serum homocysteine distribution; b: serum cystatin C distribution)

Figure 2: Pearson correlation between serological homocysteine and cystatin C in type 2 diabetes mellitus, early diabetic nephropathy and clinical stage diabetic nephropathy (a: homocysteine and cystatin C in type 2 diabetes mellitus group; b: homocysteine and cystatin C correlation in early diabetic nephropathy group; c: homocysteine and cystatin C correlation in clinical stage diabetic nephropathy group)

Table 1: The baseline characteristics of the included cases for type 2 diabetes mellitus and diabetic nephropathy.

Character	DM(n=28)	DNe(n=25)	DNc(n=22)	F/chi-square	P-value
Age(year)	58.6±11.3	60.3±10.6	59.8±9.8	0.18	0.84
Gender[n(%)]				0.57	0.75
Male	15(53.6)	11(44.0)	10(45.5)		
Female	13(46.4)	14(56.0)	12(54.5)		
Course(year)	10.2±5.1	9.6±4.8	11.3±5.1	0.69	0.51
UAER(mg/24h)	16.9±8.8	41.2±39.6	401.2±66.6	587.6	<0.001
Cr(μmol/L)	70.2±23.1	66.3±30.3	456.8±122.3	204.9	<0.001

serological Hcy and 80.0 (559.30-93.17)%, 89.29 (571.77-97.73)% for serum Cys-C respectively, **Table 3**. The diagnostic AUC were 0.76 (0.63 to 0.90) and 0.84 (0.72-0.96) respectively for serum Hcy and Cys-C in detection early stage DN, **Figure 3**.

Discussion

Epidemiology data shows that there are approximately 92.4 million diabetic patients China, which has thus become the largest diabetic country in the world [10].

Figure 3: ROC curve of serum homocysteine and cystatin C as biomarker in prediction diagnosis of diabetic nephropathy (a: Serum homocysteine as biomarker in diagnosis of early stage diabetic nephropathy form simple type 2 diabetes mellitus; b: Serum cystatin C as biomarker in diagnosis of early stage diabetic nephropathy form simple type 2 diabetes mellitus; c: Serum homocysteine as biomarker in diagnosis of clinical stage diabetic nephropathy form early stage diabetic nephropathy; d: Serum cystatin C as biomarker in diagnosis of clinical stage diabetic nephropathy form early stage diabetic nephropathy).

Table 2: The correlation between serological Homocysteine and cystatin C in type 2 diabetes mellitus, early stage diabetic nephropathy and clinical stage diabetic nephropathy group.

Group	R_{pearson}	95%CI	p	Equation
DM	0.08	-0.31 to 0.44	0.70	$Y = 0.005792 \cdot X + 0.8187$
DNe	0.55	0.20 to 0.78	0.004	$Y = 0.072 \cdot X + 0.4046$
DNc	0.44	0.03 to 0.73	0.04	$Y = 0.05683 \cdot X + 1.593$

Diabetic nephropathy, as a common microvascular complication of diabetes mellitus, is one of the most common causes of end-stage nephropathy [11, 12]. The clinical features of DN are proteinuria, progressive renal dysfunction, hypertension, edema and finally developed into renal failure, which was a main cause of death for DM patients. The pathogenesis of diabetic nephropathy is complex [13-16]. When proteinuria occurs, renal damage has developed to an irreversible stage. Early diagnosis

and treatment is important for the prognosis of patients with DN. In recent years, it has been reported that the occurrence of diabetic nephropathy correlated with diabetic microangiopathy [17-19]. Serum Hcy is associated with vascular disease, and may also affect the occurrence and development of diabetes. Therefore, researchers have proposed that plasma Hcy could be used as biomarker for early diagnosis of diabetic nephropathy [20].

Some researchers have pointed out that hyperhomocysteinemia is an independent risk factor for DN [21-23]. Hcy can act as an endogenous pathogenic factor due to the concentration difference between a cell and its surrounding due to active and passive transport. As a result, a large number of toxic reaction products are generated, ultimately causing a decrease of oxygen free radicals in microvascular endothelial cells. A large amount of oxygen free radicals accumulated in cells does not only lead to the destruction of cell membrane integrity, microvascular endothelial damage and endothelial

Table 3: The prediction efficacy of serum homocysteine and cystatin C as biomarker for early diabetic nephropathy and clinical stage diabetic nephropathy.

Marker	Sensitivity(95%CI)	Specificity	AUC	Likelihood ratio	Cut off
Early DN					
Hcy	76.0(54.87-90.64)%	64.29(44.07-81.36)%	0.76 (0.63 to 0.90)	2.13	>12.50
Cys-C	80.0(59.30-93.17)%	89.29(71.77-97.73)%	0.84(0.72-0.96)	7.47	>1.12
Clinical DN					
Hcy	54.6(32.21-75.61)%	80.0(59.30-93.17)%	0.63(0.46-0.80)	2.73	>19.09
Cys-C	86.4(65.09-97.09)	76.0(54.87-90.64)	0.84(0.72-0.96)	3.60	>1.75

dysfunction, but they can also change the pore size and charge selectivity of glomerular filtration membranes, increasing the intra-glomerular pressure resulting in renal injury.

In our present study, we found the serum level of Hcy in early DN patients has increased significantly, when compared with simple type 2 diabetes. This suggests that the elevated level of Hcy may also be an important manifestation of kidney injury, which may be related to the weakening of metabolic pathways that catalyze the transformation of Hcy into cysteine and alpha-butyronic acid due to impaired renal function. In addition, the decrease of renal clearance of Hcy may be another reason for the increase of serum Hcy level. However, serum Hcy level may be affected by factors such as renal function, serum folic acid, vitamin B6 and B12. Studies support a role of immune system activation in the development of hyperhomocysteinemia. Stimulation and proliferation of immune cells may lead to the production of reactive oxygen species that may oxidize antioxidants and oxidation-sensitive B-vitamins [24]. Whether using single serum levels of Hcy is suitable as a diagnosis reference for DN is currently debated. Sandhu and his colleges investigated the plasma homocysteine and insulin in diabetic nephropathy [25]. They found that no correlation between homocysteine and insulin, and homocysteine with the degree of renal failure. However, Okumura found that high plasma homocysteine concentrations are associated with plasma concentrations of thrombomodulin correlated with diabetic nephropathy. Therefore, using serum Hcy as a serological marker for evaluation early renal damage in diabetic nephropathy needs further exploration.

Cys-C is an important member of the family of cysteine protease inhibitors. It exists widely in various tissues and under normal physical conditions, Cys-C serum levels remain stable. Serum concentration is not affected by age, gender, liver function, and Cys-C can't be absorbed

or secreted by renal tubules. Therefore, Cys-C is an ideal indicator of glomerular filtration [7]. There are several advantages to using Cys-C as an indicator of glomerular filtration; Firstly, it is not bound to plasma proteins and has a low molecular weight. Secondly, it possess a positive charge and is free to pass through glomerular filtration membranes and is not secreted by renal tubules. Thirdly, it is continuously produced. Finally, the synthesis of Cys-C is unaffected by diet, exercise, muscle mass, age, sex, race or inflammation

In conclusion, serum level of Hcy and Cys-C in diabetic nephropathy patients were elevated when compared to that of simple DM cases, thus they are potential biomarkers for early detection DN in DM patients. Serum levels of Cys-C and Hcy are closely related to the stage of diabetic nephropathy. Serum Hcy and Cys-c levels can reflect the renal function of patients with diabetic nephropathy, which may become a marker of therapeutic effect of DN. A potential involvement in the pathogenesis of diabetic nephropathy remains to be explored.

The present work also had its limitations: Firstly, only 75 subjects were recruited and analyzed in the present study. This sample size is small with limited statistical power. Secondly, all the subjects were recruited from one single medical center which may lead to potential subjects selection bias. Therefore, a well-designed multicenter prospective studies with larger samples are need to further elucidate the results.

Conflict of interest: Authors state no conflict of interest

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