Urine Proteome Specific for Eye Damage Can Predict Kidney Damage in Patients With Type 2 Diabetes: A Case-Control and a 5.3-Year Prospective Cohort Study

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OBJECTIVE

The predictive value of microalbuminuria (MAU) for kidney damage is limited in type 2 diabetes (T2D). We studied whether a urine proteome specific for sight-threatening proliferative diabetic retinopathy (PDR) is an indicator to predict chronic renal insufficiency (CRI) in patients with T2D.

RESEARCH DESIGN AND METHODS

A shotgun urine proteomic analysis was performed in patients with MAU and PDR (case subjects) and in patients with MAU and a duration of T2D for >10 years but without any degree of retinopathy (control subjects). In the cohort study, 210 patients with T2D with an estimated glomerular filtration rate (eGFR) ≥80 mL/min/1.73 m^2 were followed for a median of 5.3 years. Urine proteins specific for PDR were used for predicting CRI (eGFR <60 mL/min/1.73 m^2).

RESULTS

The top two urine proteins with the highest difference in ratio of case subjects to control subjects were haptoglobin (8.7 times; $P < 0.0001$) and α-2-macroglobulin (5.7 times; $P < 0.0001$). In the cohort study, patients with baseline urinary haptoglobin ≥20 ng/min (haptoglobinuria) had a higher incidence of CRI than those without (hazard ratio [95% CI] 3.27 [1.41–7.58]; $P = 0.006$). The overall CRI rate was 3.2% for patients without haptoglobinuria or MAU, 9.5% for those with MAU, and 13.3% for those with haptoglobinuria. The highest rate for CRI (22.4%) was in patients with both MAU and haptoglobinuria ($P < 0.001$).

CONCLUSIONS

Urine haptoglobin, which is specific for PDR, is a novel biomarker and complement to urine albumin for predicting kidney damage in patients with T2D.

The major microvascular complications of diabetes—diabetic kidney disease (DKD) and diabetic retinopathy (DR)—are the most common causes of end-stage renal disease (ESRD) and blindness worldwide. Although, early detection of DR is simple through digital fundus retinography (1,2), diagnosis of the early stages of DKD is difficult. Because diabetes is a common chronic condition, coincidence with other nondiabetic chronic kidney disease (CKD) is relatively frequent. Kidney biopsy is required but not practical for the precise diagnosis of DKD (3). At present, clinically

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detectable DKD begins with the development of microalbuminuria (MAU) (4). However, the predictive value of MAU is now questioned. First, a large proportion of patients with DKD and MAU can revert to normal albuminuria (NAU) (4,5). Second, only a minority of patients with MAU progress to proteinuria (6,7). Third, advanced DKD develops in one-third of patients with type 1 diabetes (T1D) soon after the onset of MAU, not proteinuria (8).

As early as 1992, the presence of DR suggested that DKD is the cause of MAU in patients with type 2 diabetes (T2D) (9). In 2007, the National Kidney Foundation recommended that in most patients with T2D, CKD be attributed to DKD if MAU is co-present with diagnosed DR. Other causes of CKD are considered in certain circumstances, such as the absence of DR and low or rapidly decreasing glomerular filtration rates (GFRs) in patients with MAU (10). In a 15-year follow-up prospective study, a positive relationship was found between retinopathy, especially the severe form DR, and overt nephropathy (10). Therefore, the presence of DR is considered a vital clinical biomarker for the diagnosis of DKD.

We hypothesized that sight-threatening proliferative DR (PDR) is a strong indicator for DKD. Quantitative values of proteins in the urine of patients with PDR, in contrast to patients with diabetes but without retinopathy, may contribute to diabetes-induced kidney damage. To address this hypothesis, we analyzed urine specimens by shotgun proteomic analysis in patients with both T2D and MAU (Fig. 1*A*) obtained from our sight-threatening PDR case-control study (1,11). The proteins with comparatively greater value in patients with T2D, MAU, and PDR (case subjects) than in patients with T2D and MAU but without any degree of retinopathy (NDR) (control subjects) were further verified by ELISA. Finally, we verified whether this protein was correlated with renal function decline in participants with diabetes from a 5.3-year follow-up cohort study. This study demonstrates that urinary haptoglobin, which is specific for eye damage, is a putative clinical biomarker for predicting kidney damage related to diabetes.

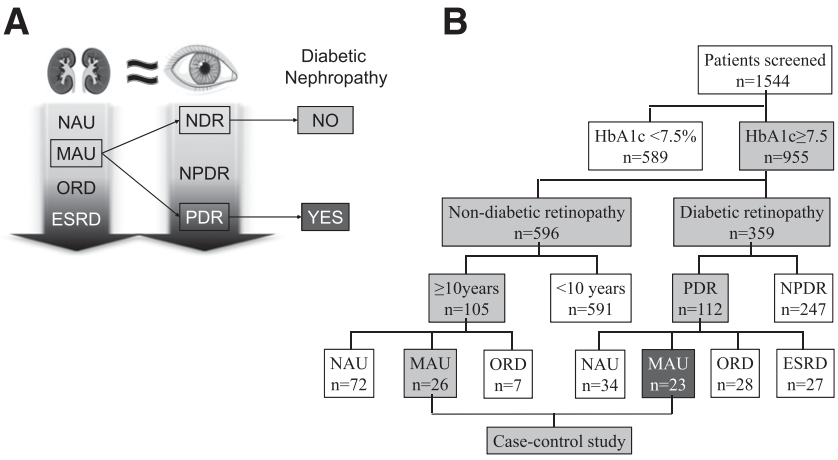

Figure 1—Design and inclusion flowchart of the case-control study. *A*: Hypothesis: In patients with MAU, sight-threatening PDR is a strong indicator for DKD. *B*: Inclusion and exclusion flowchart of the case-control study based on the hypothesis. NPDR, nonproliferative DR; ORD, overt renal disease.

RESEARCH DESIGN AND METHODS

Study Population

Case-Control Study

This extreme eye phenotype case-control study was performed to find a urine protein specific for eye damage that can be used to predict kidney damage. Patients with T2D, MAU, and PDR were suspected of having kidney damage, whereas patients with T2D, MAU, and NDR were suspected of having no kidney damage (Fig. 1*A*). We chose patients with glycated hemoglobin A_{1c} (HbA_{1c}) $\geq$7.5% (58 mmol/mol) for eye phenotype screening. To match clinical parameters between case and control subjects, patients with sight-threatening PDR or those with a duration of diabetes of $\geq$10 years but with NDR (diabetes duration was not considered) were eligible for further urinary albumin testing. Patients with MAU and with eligible extreme eye phenotypes (NDR or PDR) were assigned for the case-control study (Fig. 1*B*).

Cohort Study

The cohort study was designed to verify whether elevated urinary protein, which was increased in the PDR (case) group in the case-control study could be used to predict renal function decline in a T2D cohort. Participants were from our inpatient follow-up cohort. Patients with T2D who had an estimated GFR (eGFR) $\geq$80 mL/min/1.73 m^2, were free of clinical or laboratory evidence of other causes of renal disease, and had finished clinical examination monitoring at least three times during the follow-up period were eligible. The kidney function end point was chronic renal insufficiency (CRI) (eGFR $<$60 mL/min/1.73 m^2) (12). These studies were approved by the Medical Ethics Committee of Beijing Tongren Hospital, Capital Medical University, and all participants provided written informed consent.

Biochemical Measurements

Biochemical parameters were measured with a Beckman-UniCel Dxc 800 biochemistry analyzer (Beckman Coulter, Carlsbad, CA), including fasting plasma glucose, postprandial plasma glucose, total cholesterol, triglycerides, LDL and HDL cholesterol, serum creatinine, alanine aminotransferase, and aspartate aminotransferase. HbA_{1c} was measured by high-performance liquid chromatography (VARIANT II; Bio-Rad, Hercules, CA).

Eye Examination

The presence of DR was diagnosed by using digital retinal photographs (two eyes $\times$ two fields) taken with a TRC-NW7SF non mydriatic camera at 45° (Topcon, Tokyo, Japan). These photographs were subsequently examined independently by two qualified retinal photography graders while following quality assurance protocols. A research ophthalmologist performed a confirmatory grading of DR based on international clinical DR and diabetic macular edema disease severity scales (13).

Kidney Function and End Points

We performed dynamic imaging with 99mTc diethylene-triamine pentaacetic acid to obtain the GFR. We calculated the eGFR according to the modified MDRD equation for the Chinese population (14). The kidney function end points were determined as CRI (eGFR $<$60 mL/min/1.73 m^2), which corresponds to the proposed National Kidney Foundation

Kidney Dialysis Outcomes Quality Initiative guidelines for defining CKD stages 3–5 (12). The definition of CRI has been widely used since 2003 (15,16).

Automated 2D-Nano-LC-ESI-MS/MS Analysis of Urine Proteomics

Urine samples were kept at −80°C until shotgun proteomic analysis. The urinary proteins of all samples were isolated and digested into the peptide mixture. The peptide fragments fingerprint was detected by 2D-nano-LC-ESI-MS/MS (two-dimensional nano–liquid chromatography electrospray ionization tandem mass spectrometry). All spectrums were identified by using SEQUEST against the Swiss-Prot human database. Trans-Proteomic Pipeline software was then used to identify proteins by their biological information (Supplementary Data). The proteomic measurements of proteins are presented in relative concentrations [(indexed protein)/(total proteins in the urine)].

Urine Albumin Quantitation

Urinary albumin was determined by chemiluminescence immunoassay with an IMMULITE 2000 system (Diagnostics Products Corporation, Los Angeles, CA). According to the protocol proposed by the American Diabetes Association (17), a urinary albumin excretion rate (UAER) $<$20 μg/min was defined as NAU. MAU was defined as levels of UAER ranging from 20–200 μg/min. Macroalbuminuria was defined as UAER $\geq$200 μg/min. The 8-h urine sample was collected carefully from 10:00 P.M. to 6:00 A.M. for testing. The following conditions were excluded: exercise within 24 h, infection, fever, congestive heart failure, pyuria, and hematuria.

Urine Haptoglobin and α-2-Macroglobulin Quantitation

The top two proteins (haptoglobin and α-2-macroglobulin) found increased in the urine from the PDR (case) group were further analyzed by ELISA (CUSABIO, Wuhan, China), according to the manufacturer's instructions. The concentration of haptoglobin in the samples was calculated by comparing the optical density value to the standard curve. All measurements were made in duplicate.

Statistical Analysis

Data are presented as mean ± SD or median (25th, 75th interval). Student *t* test, Mann-Whitney *U* test, or Fisher exact test was used as appropriate. Time-to-event analyses were performed using Kaplan-Meier plots and log-rank testing. To determine whether an independent association existed between urine haptoglobin and the CRI end point, we performed logistic regression analysis. To compare the ability of urine haptoglobin and albumin measurements to detect the presence or absence of the CRI end point, we calculated receiver operating characteristic (ROC) curves and compared the areas beneath them. All *P* values were two-sided. The statistical analyses were performed using SPSS version 17.0 software (IBM Corporation, Chicago, IL).

RESULTS

Case-Control Study

Characteristics and Prevalence of Kidney Disease

Of the 1,544 consecutive patients with T2D screened, 955 had an HbA_{1c} $\geq$7.5% (58 mmol/mol). According to the screening criteria of the case-control study, 112 patients had PDR and 105 patients had NDR with a diabetes duration $\geq$10 years. The number of patients with NAU, MAU, overt renal disease, and ESRD were 72, 26, 7, and 0 versus 34, 23, 28, and 27 for the NDR versus PDR groups, respectively. The trend to overt renal disease and ESRD in patients with PDR was significantly higher than in patients with NDR (χ^2 = 25.05; $P < 0.0001$) (Fig. 1*B*).

Patients with MAU and eye extreme phenotypes (26 NDR, 23 PDR) were assigned as case subjects (T2D with MAU and PDR) and control subjects (T2D with MAU and NDR) (Fig. 1*B*). To match clinical parameters of the case-control study, patients with a diabetes duration of $\geq$10 years (HbA_{1c} $\geq$7.5% [58 mmol/mol]) but NDR were eligible as control subjects, whereas those with sight-threatening PDR (not considering diabetes duration) were eligible as case subjects. Therefore, patients with and without retinopathy were matched in the duration of diabetes. No differences between case and control subjects were observed regarding sex, age, BMI, blood pressure, serum creatinine, total protein, albumin, liver enzymes, lipid profiles, fasting blood glucose, and HbA_{1c} (Table 1). The PDR group had a higher (but not statistically different) prevalence of hypertension than the NDR group (62 of 110 vs. 67 of 100, respectively; χ^2 = 2.50; P = 0.114).

PDR and Kidney Damage

Levels of GFR and eGFR were 73.2 ± 17.6 mL and 77.9 ± 21.2 mL/min/1.73 m^2 in case subjects, which was lower than in control subjects (87.1 ± 21.5 mL and 93.6 ± 24.2 mL/min/1.73 m^2; P = 0.030 and 0.022, respectively) (Fig. 2*A*). According to the inclusion criteria, all subjects must have MAU. The average UAER was 77.7 ± 13.0 and 70.9 ± 10.3 mg/24 h in case and control subjects, respectively (P = 0.767) (Fig. 2*B*). Therefore, patients with PDR had a decreased kidney function, although urine albumin levels were compatible between the groups.

Urine Proteome

One hundred eighty-six proteins were identified in urine samples from patients in both groups by 2D-nano–LC-ESI-MS/MS proteomic analysis at a single unique peptide threshold ($P \geq 0.9$). Distinct chromatographic differences were observed between case and control subjects. We detected 89 proteins (Supplementary Table 1) in urine samples from both groups (50 in the case group [Supplementary Table 2] and 47 in the control group [Supplementary Table 3]).

The extreme eye DR proteome was functionally annotated using gene ontology annotations from DAVID and BioHarvester informatics resources. The majority of specific urine proteins for case subjects have immune response functions (20%), including humoral immune response, regulation of humoral immune response, complement activation, regulation of complement activation, and immune system process. Other cellular functions were fibrinolysis (5%) and multicellular organismal process (5%) (Supplementary Fig. 1). Pathway analysis was performed using the Kyoto Encyclopedia of Genes and Genomes Pathway database. The results demonstrated that complement and coagulation cascades are relevant to the PDR proteome (Supplementary Fig. 2).

The proteomic measurements of proteins are presented in relative concentrations according to housekeeping proteins. Of the 89 common proteins, albumin was 1.8 times more in the case group than in the control group. Among the common proteins, levels of 21 proteins were greater than albumin in the case group, and haptoglobin and α-2-macroglobulin were the top two proteins with the highest difference in ratio of the case group

Table 1—Baseline demographics in the case-control and cohort studies

	Case-control study		Cohort study		
	Control	Case	All	UHER (<20 ng/min)	UHER (≥20 ng/min)
n	26	23	210	110	100
Sex					
Male	16	9	109	59	50
Female	10	14	101	51	50
Age (years)	60.9 ± 10.8	61.0 ± 10.2	59.6 ± 10.0	58.7 ± 10.5	60.5 ± 10.7
Diabetes duration (years)	13.7 ± 4.5	14.5 ± 8.5	11.1 ± 7.2	9.7 ± 6.4	12.5 ± 7.8**
BMI (kg/m^2)	25.9 ± 3.6	26.0 ± 3.5	25.2 ± 3.3	25.1 ± 3.2	25.4 ± 3.3
SBP (mmHg)	138.3 ± 17.1	143.7 ± 19.9	131.8 ± 16.8	128.8 ± 16.5	135.2 ± 16.4**
DBP (mmHg)	83.1 ± 7.4	83.7 ± 10.5	79.0 ± 10.3	78.1 ± 9.4	80.0 ± 11.4
BUN (mmol/L)	5.1 ± 1.8	5.4 ± 1.9	5.2 ± 6.8	4.5 ± 1.5	4.6 ± 1.3
Cr (μmol/L)	76.0 ± 18.1	83.0 ± 20.1	67.1 ± 15.0	66.5 ± 15.6	67.7 ± 14.1
UA (μmol/L)	324 ± 55	299 ± 57	303 ± 86	309 ± 79	297 ± 82
TP (g/L)	65.1 ± 4.4	65.9 ± 5.7	63.6 ± 7.3	63.8 ± 5.3	64.0 ± 6.3
ALB (g/L)	37.3 ± 3.1	35.9 ± 3.5	37.5 ± 5.4	37.8 ± 3.1	36.8 ± 5.1
TBIL (μmol/L)	14.9 ± 5.1	13.4 ± 4.9	14.0 ± 4.8	13.3 ± 3.8	13.4 ± 3.8
DBIL (μmol/L)	2.8 ± 1.5	2.6 ± 0.9	3.1 ± 1.3	3.2 ± 1.2	2.9 ± 1.1
ALT (IU/L)	18 (16, 25)	18 (15, 22)	18 (14,24)	18 (14, 24)	18 (14, 24)
AST (IU/L)	21 (17, 26)	20 (17, 23)	21 (17, 28)	22 (17, 28)	21 (17, 25)
TG (mmol/L)	1.6 (1.2, 2.3)	1.6 (0.9, 1.9)	1.5 (1.0, 2.3)	1.4 (0.9, 1.9)	1.7 (1.2, 2.5)**
TC (mmol/L)	4.5 ± 0.8	4.6 ± 1.0	4.8 ± 1.1	4.7 ± 1.0	4.9 ± 1.2
LDL-C (mmol/L)	3.0 ± 0.7	2.9 ± 0.7	3.1 ± 1.2	3.0 ± 0.8	3.1 ± 1.0
HDL-C (mmol/L)	1.0 ± 0.3	1.1 ± 0.2	1.5 ± 1.5	1.2 ± 0.3	1.1 ± 0.3
FBG (mmol/L)	7.5 ± 2.2	7.1 ± 2.8	7.9 ± 2.8	7.4 ± 2.5	8.4 ± 3.0*
HbA_{1c}					
%	8.7 ± 1.6	8.8 ± 1.3	8.4 ± 2.2	8.0 ± 2.0	8.9 ± 2.3*
mmol/mol	72 ± 13	73 ± 11	68 ± 18	64 ± 16	74 ± 19

Data are mean ± SD or median (25th, 75th interval) unless otherwise indicated. Statistical analyses were by Student *t* test or Mann-Whitney *U* test. ALB, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; Cr, creatinine; DBIL, direct bilirubin; DBP, diastolic blood pressure; FBG, fasting blood glucose; HDL-C, HDL cholesterol; IU, international unit; LDL-C, LDL cholesterol; SBP, systolic blood pressure; TBIL, total bilirubin; TC, total cholesterol; TG, triglyceride; TP, total protein; UA, uric acid. *$P < 0.05$ and ** $P < 0.01$, case vs. control subjects or UHER ≥20 ng/min vs. UHER <20 ng/min group.

compared with the control group (8.7 and 5.7 times, respectively, both $P < 0.0001$) (Supplementary Table 1).

Using ELISA, we verified the top two proteins with the highest difference in ratio of case to control subjects by 2D-nano-LC-ESI-MS/MS analysis. A significant elevation of urine haptoglobin (Fig. 2*C*) and α-2-macroglobulin (Fig. 2*D*) in case subjects was confirmed ($P = 0.009$ vs. 0.027 in control subjects). To adjust for the imbalance of urine albumin between groups, we calculated the ratio of the two specific urine proteins to albumin. The ratio of haptoglobin to albumin (Fig. 2*E*) and α-2-macroglobulin to albumin (Fig. 2*F*) also were elevated in case subjects versus control subjects ($P = 0.011$ vs. 0.035, respectively).

Cohort Study

Characteristics of the Cohort

We conducted a cohort study to verify the value of a urine biomarker of PDR for predicting kidney damage. Two hundred ten patients with T2D were eligible according to the inclusion criteria. In the first visit, urine was obtained for testing haptoglobin identified by urine proteomic analysis in the case-control study. The median follow-up time was 5.3 years. Characteristics of the patients participating in the cohort study are summarized in Table 1.

At baseline, levels of urine haptoglobin, calculated as the urinary haptoglobin excretion rate (UHER), were moderately correlated with urine albumin (Pearson $r = 0.54$ [95% CI 0.44–0.63]; $P < 0.0001$). According to the baseline levels of urine haptoglobin, participants were categorized into two groups: UHER ≥20 ng/min ($n = 100$) and UHER <20 ng/min ($n = 110$). At baseline, there was no significant changes between groups for sex, age, BMI, diastolic blood pressure, serum creatinine, total protein, albumin, and liver enzymes. Duration of T2D was longer and systolic blood pressure, total cholesterol, fasting blood glucose, and HbA_{1c} were higher in the UHER ≥20 ng/min group (Table 1). Kidney function, calculated as eGFR, was compatible between the groups (99.4 ± 2.2 vs. 104.4 ± 2.2 mL/min/1.73 m^2, respectively). Cohort participants were categorized into two groups: UAER ≥20 μg/min ($n = 73$) and UAER <20 μg/min ($n = 137$). Because patients with T2D and an eGFR ≥80 mL/min/1.73 m^2 were eligible for this study at baseline, this explains why eGFR was compatible between the groups in terms of UHER ≥20 ng/min and UHER <20 ng/min at baseline (101.4 ± 2.6 vs. 102.3 ± 1.9 mL/min/1.73 m^2, respectively).

Urine Biomarkers of PDR for Predicting Kidney Damage

Survival time of follow-up was calculated from the first clinical examination to the occurrence of the end point (CRI) or the end of the study for patients who

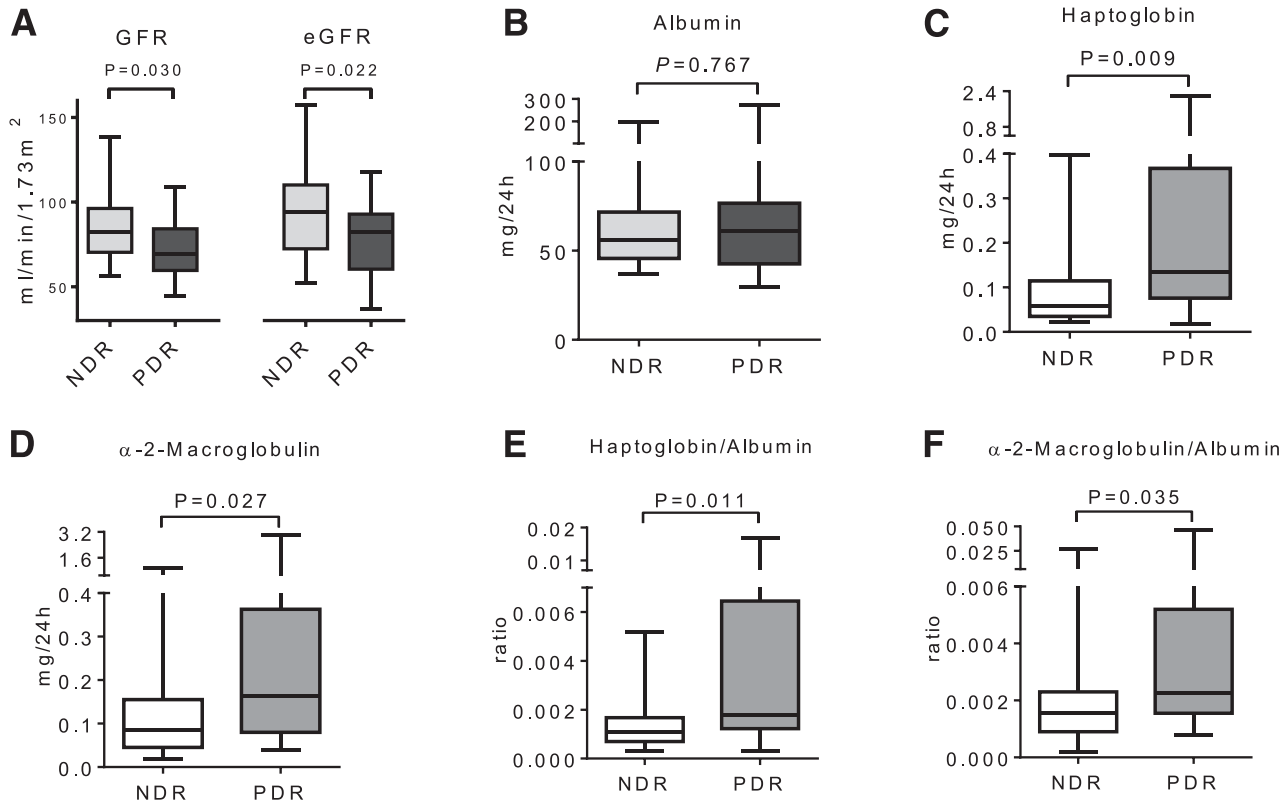

Figure 2—Kidney function and quantitative analysis of urine proteins in the case-control study. *A*: Kidney function as indicated by GFR and calculated eGFR. *B–F*: Quantitative analysis of urine albumin, urine haptoglobin, urine α-2-macroglobulin, urine haptoglobin corrected with albumin, and urine α-2-macroglobulin corrected with albumin. Data are median (25th, 75th interval) and range. Significance values were calculated by Mann-Whitney *U* test.

did not progress to CRI. The number of end point events was 18 of 100 and 5 of 110 in UHER ≥20 ng/min and UHER <20 ng/min groups, respectively, during the follow-up period (χ^2 = 9.72; *P* = 0.002). Survival analysis indicated that patients with UHER ≥20 ng/min had a significantly higher percentage of progression to CRI (log-rank test *P* = 0.006). The unadjusted hazard ratio (95% CI) for incident CRI among patients with UHER ≥20 ng/min was 3.27 (1.41–7.58) (Fig. 3*A*).

The predictive value of urine albumin for kidney damage was also evident. The number of end point events was 16 of 73 and 7 of 137 in UAER ≥20 μg/min group and UAER <20 μg/min group, respectively, during the follow-up period (χ^2 = 13.80, *P* = 0.002). Survival analysis indicated a significant difference of progression to CRI between groups (log-rank test *P* < 0.001). The unadjusted hazard ratio (95% CI) for incident CRI among patients with UAER ≥20 μg/min was 4.86 (2.02–11.69) (Fig. 3*B*).

The overall CRI rate for patients with neither haptoglobinuria (defined as UHER ≥20 ng/min) nor albuminuria (defined as UAER ≥20 μg/min) was 3.2%; for those with albuminuria, it was 9.5%; and for those with haptoglobinuria, it was 13.3%. The highest rates for CRI (22.4%) were in patients with both albuminuria and haptoglobinuria (Fisher exact test *P* < 0.001) (Fig. 3*C*). These findings indicate that both UHER and UAER have identical meaning for predicting kidney function decline, and urine haptoglobin, which is specific for PDR, is a complement to urine albumin for predicting kidney damage in patients with T2D.

To determine whether an independent association exists between urine haptoglobin and CRI, we performed a logistic regression analysis. With an adjustment for albuminuria (UAER), sex, age, BMI, systolic blood pressure, eGFR at baseline, HbA_{1c}, and duration of diabetes, urine haptoglobin (UHER) was significantly associated as an independent risk factor for CRI (*P* = 0.032) (Supplementary Table 4). The area under the ROC curve was 76.1% (95% CI 64.0–88.1) for UAER, 76.8% (95% CI 62.5–84.8) for UHER, and 78.5% (95% CI 68.3–88.8) for UHER and UAER combined (Fig. 3*D* and Supplementary Table 5).

CONCLUSIONS

Persistent albuminuria in the range of 20–200 μg/min (MAU) has been shown to be the earliest stage of diabetic nephropathy in T1D and a marker for the development of nephropathy in T2D. However, not all people with diabetes, kidney disease, and reduced eGFR have albuminuria (18). Many reports have demonstrated that nearly 10% of patients already had impaired renal function before MAU was observed, which suggests that patients progress to renal function decline earlier than the onset of MAU. As previously suggested, MAU may not be as sensitive and specific as a predictor of DKD. The positive predictive value of MAU as a marker of risk for DKD is 43%, and the negative predictive value is 77%. Other markers of risk for DKD are needed for optimal clinical management (19).

The current literature suggests that the presence of one preexisting microvascular complication (retinopathy or nephropathy)

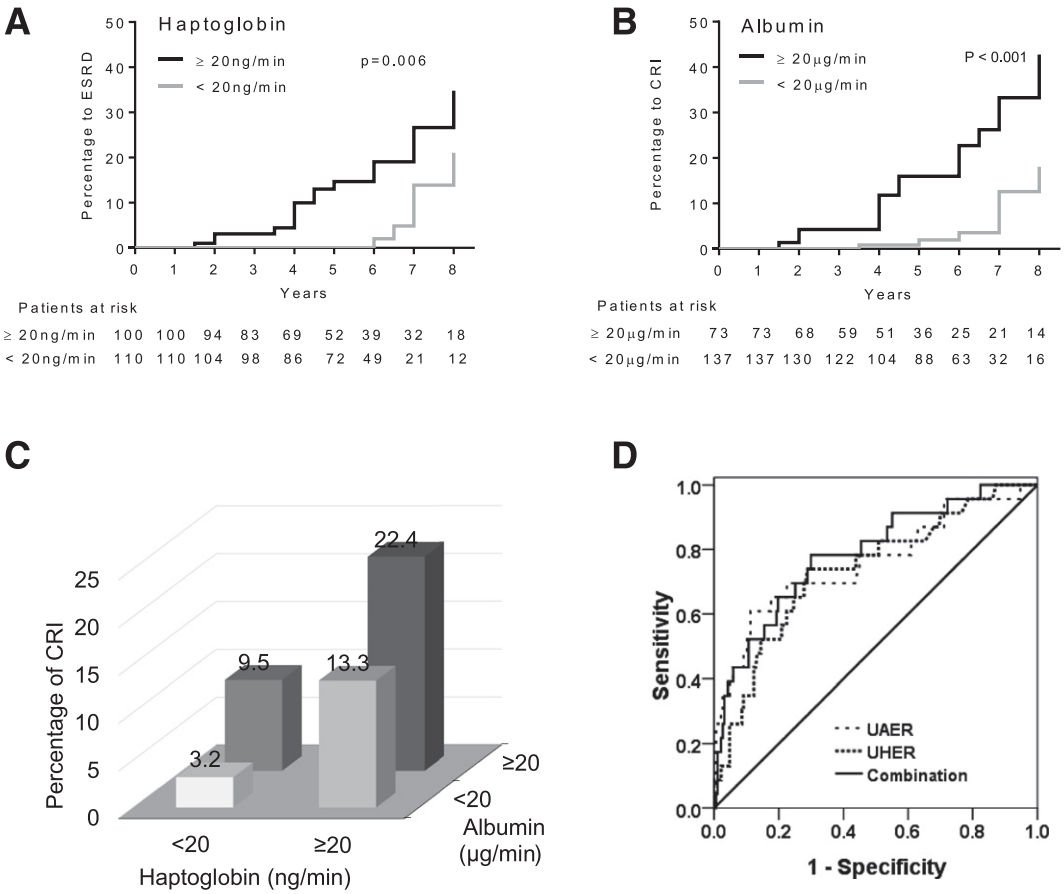

Figure 3—Urine proteins and kidney function decline in the cohort study. *A*: Kaplan-Meier curves of CRI in 210 patients with T2D according to urine haptoglobin levels ≥20 ng/min vs. <20 ng/min. *B*: Urine albumin levels ≥20 μg/min vs. <20 μg/min. Log-rank test was used for comparing both groups. *C*: Percentage of CRI in subgroups divided according to urine levels of albumin and haptoglobin. *D*: ROC curves for UAER, UHER, and UAER and UHER combined.

may contribute to the development of another (20). Retinal venular geometry can independently predict incident renal dysfunction and may be a useful tool to identify individuals at high risk for renal disease early in the course of T1D (21). El-Asrar et al. (22) studied the prevalence of diabetic nephropathy and found a link with an increasing severity of DR. Their analyses indicated that patients with T1D and DR were 13.39 times more likely to have DKD than those without DR. Almost no study about the relationship between nephropathy and retinopathy has been reported in patients with T2D. In the current study, we analyzed urine proteome in patients with sight-threatening PDR and selected the PDR-specific urine protein for predicting renal function decline in patients with T2D. We found that urine proteome specific for eye damage could predict kidney damage in a 5.3-year prospective cohort of T2D.

Biofluids such as blood and urine have been analyzed by various proteomics techniques. However, detection of low-abundance proteins and the identification of the statistically significant proteins are still positioned as bottlenecks for proteomics analysis. In a previous study, most findings were from two-dimensional gel electrophoresis or capillary electrophoresis mass spectrometry proteomic approaches to clarify the mechanisms of T1D and its complications (5). The current study focused on the discovery of potential biomarkers of PDR by using an automated 2D-nano-LC-ESI-MS/MS method. Through the shotgun proteomic analysis, a number of clinically relevant proteins as the indicators of PDR were found in patients with T2D. To our knowledge, this report is the first regarding the urine proteome of DR.

Oxidative stress plays an important role in the development of diabetic vascular complications, including nephropathy and retinopathy. Haptoglobin and α-2-macroglobulin, the two most closely related proteins, were elevated in patients with PDR. Haptoglobin is the major hemoglobin-binding protein in the plasma of most vertebrates and all mammals. The haptoglobin-hemoglobin complex is eliminated from the circulation, leading to protection against heme-driven oxidative stress (23). The haptoglobin polymorphism contributes to the prevalence and clinical evolution of many inflammatory diseases, including dyslipidemia (24), T2D (25), atherosclerosis (26), and autoimmune disorders (27). These effects may be a result of a phenotype-dependent modulation of oxidative stress. Haptoglobin appears to be a reliable biomarker for early diagnosis of acute allograft rejection after kidney transplantation. Urine haptoglobin is upsecreted in patients with clear cell renal carcinoma identified by mass spectrometry (28). Bhensdadia et al. (29) used urine of patients with T2D and NAU to identify candidate markers for loss of renal function and found that haptoglobin was the best predictor of early renal functional decline. The antioxidant protection provided by haptoglobin is genotype dependent. Many studies have shown that

individuals with diabetes and a haptoglobin genotype (Hp2-2) are more likely to develop nephropathy, retinopathy, and cardiovascular disease (30).

Complement activation occurs in the kidney during the progression of a broad range of renal diseases and could contribute to the inflammatory environment in which fibrosis occurs (31). A recent report indicated that local activation of the complement system mediates renal injury in diabetic nephropathy, wherein complement C3 reactivity in tubules leads to broader renal activation of the complement system, which is sustained by suppression of complement regulators, and contributes to renal inflammation, impaired function, and fibrosis (32). In the current study, pathway analysis shows that the C3-mediated complement cascade may be relevant to PDR and DKD.

Some potential limitations to our study exist. First, we only verified the top two urine proteins (haptoglobin and α-2-macroglobulin) with the highest difference in the ratio of case to control subjects. These proteins were present in urine samples from both groups. Actually, 50 proteins were present only in the urine from the PDR (case) group. One-half of these proteins have immune response functions (Supplementary Table 2). In the cohort study, we only chose haptoglobin to predict renal function decline. The reason was mainly because no proper kit was commercially available for testing these proteins in urine samples. Second, the prospective cohort study was conducted in only one academic hospital. A large-scale multicenter prospective cohort study should be conducted in the future. Nonetheless, with a cohort of 210 patients followed for 5.3 years, we found urine haptoglobin to be a strong biomarker for predicting kidney damage in patients with T2D. Third, as with any observational study, we cannot rule out the possibility of unmeasured confounding, such as haptoglobin genotype (30), eGFR, HbA_{1c}, blood pressure, and renin-angiotensin system blocking treatment–based differences, although with an adjustment for urine albuminuria, sex, age, BMI, systolic blood pressure, eGFR at baseline, HbA_{1c}, and duration of diabetes, urine haptoglobin was an independent risk factor for CRI.

In conclusion, this study provided preliminary data on how urine haptoglobin may be used as a biomarker for PDR as well as for DKD. Although the predictive value of urine albumin and haptoglobin for kidney damage have been found, patients with both MAU and haptoglobinuria show higher rates for progressing to CRI than those with MAU alone, and those with neither haptoglobinuria nor MAU have a very low rate for progressing to CRI. Therefore, urine haptoglobin that is specific for PDR serves as a novel biomarker and a complement to urine albumin for predicting kidney damage in patients with T2D. We speculate that the elevation of urine haptoglobin is involved in the pathological changes associated with kidney function decline in patients with T2D.

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