Proteomic Identification of Urinary Biomarkers of Diabetic Nephropathy

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OBJECTIVE — Diabetic nephropathy is a serious complication of both type 1 and type 2 diabetes, and, unless arrested, leads to end-stage renal disease. Current diagnosis consists of urine assays of microalbuminuria, which have inadequate specificity and sensitivity.

RESEARCH DESIGN AND METHODS — We used proteomic analyses to identify novel biomarkers of nephropathy in urine from type 2 diabetic patients with demonstrated normo-, micro-, or macroalbuminuria. Samples were analyzed by fluorescence two-dimensional (2-D) differential in-gel electrophoresis (DIGE), and protein identification was performed by liquid chromatography-tandem mass spectrometry.

RESULTS — 2-D DIGE analysis of the urinary proteome in diabetes with nephropathy identified 195 protein spots representing 62 unique proteins. These proteins belonged to several functional groups, i.e., cell development, cell organization, defense response, metabolism, and signal transduction. Comparisons between control and diabetic subjects with different stages of renal dysfunction revealed the differential expression of several proteins. Spot volume quantification identified 7 proteins that were progressively upregulated with increasing albuminuria and 4 proteins that exhibited progressive downregulation. The majority of these potential candidate biomarkers were glycoproteins.

CONCLUSIONS — These data demonstrate the ability of proteomic analyses to reveal potential biomarkers for diabetic nephropathy in urine, an important step forward in advancing accurate diagnosis and our understanding of disease mechanisms.

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In the U.S. alone, diabetes currently affects 18.2 million people or 6.3% of the population. As the general population ages and as specific at-risk populations increase, these numbers are set to increase dramatically. Diabetes is the leading cause of kidney failure, accounting for 44% of new cases in 2002, and ~60,000 patients with end-stage renal disease (ESRD) die yearly in the U.S. (1). The prevalence of type 2 diabetes in particular is projected to double within the next 15 years, and the incidence of ESRD is expected to continue to increase. However, traditional risk factors do not adequately explain disease risk, and predictors of diabetic nephropathy in biological fluids, such as urine, that can substitute for invasive biopsy have yet to be characterized and validated.

A limited set of novel risk factors for overt nephropathy was examined in the Pittsburgh Epidemiology of Diabetes Complications (EDC) cohort of individuals with childhood-onset type 1 diabetes. In addition to the urinary albumin excretion rate, the differential presence of immune complexes and lipoprotein subclasses provided additional information for the assessment of overt nephropathy risk (2). Plasma prekallikrein activity was also correlated positively and independently with albumin excretion rate in 636 type 1 diabetic patients in the microalbuminuric subgroup of the Diabetes Control and Complications Trial/Epidemiology and Diabetes Intervention and Complications Study cohort. Furthermore, plasma prekallikrein was categorically elevated within the subgroups of these patients with microalbuminuria, suggesting that plasma prekallikrein could be a marker for progressive nephropathy (3).

Advances in proteomic methods of protein separation, identification, and quantification enable comprehensive surveys of low- and high-abundance proteins in various biological fluids. These technologies are now being applied to diabetic nephropathy (4). Thongboonkerd et al. (5) analyzed kidney proteins in a murine type 1 diabetes model and found differential expression of components of the elastin-elastase system. Capillary electrophoresis-mass spectrometry analyses of urine from type 1 (6) and type 2 (7,8) diabetic patients with and without micro- and macroalbuminuria revealed characteristic polypeptide patterns and identified a limited number of differentially abundant peptides derived from high-abundance urine proteins such as albumin. Recently, protein expression profiles of urine were compared between diabetic patients with nephropathy and control subjects using two-dimensional (2-D) gel electrophoresis and matrix-assisted laser desorption ionization-time of flight mass spectrometry (9). This study was focused primarily on a restricted set of targeted proteins and changes in global protein profiles were not evaluated, although sev-

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Abbreviations: A1AT, α_1-antitrypsin; AMBP, α_1-microglobulin/bikunin precursor; DIGE, differential in-gel electrophoresis; ESRD, end-stage renal disease; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectroscopy; RBP, retinol-binding protein; VDBP, vitamin D–binding protein.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

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Table 1—*Demographics of type 2 diabetic patients without albuminuria (group 1), with microalbuminuria (group 2), or with macroalbuminuria (group 3), and control subjects*

Sex (*n*)	Age (years)	Duration of diabetes (years)	BMI (kg/m^2)
Control subjects			
M (4)	48.50 ± 4.73		22.71 ± 3.64
F (5)	56.80 ± 8.98		25.82 ± 1.72
Study group 1			
M (5)	51.80 ± 5.17	6.20 ± 3.96	23.88 ± 3.35
F (5)	56.20 ± 11.08	11.75 ± 9.03	22.55 ± 2.64
Study group 2			
M (6)	48.00 ± 5.74	5.60 ± 5.73	27.75 ± 5.40
F (7)	54.00 ± 5.54	5.86 ± 4.02	24.94 ± 4.89
Study group 3			
M (5)	50.00 ± 16.19	9.75 ± 10.87	26.65 ± 4.69
F (5)	48.20 ± 8.50	15.60 ± 1.52	22.37 ± 5.20

Data are means ± SEM.

eral protein spots appeared to be upregulated in the patients with diabetic nephropathy. In our study, we have applied proteomics technologies for an unbiased examination of urine to detect novel biomarkers and potential molecular networks that could play a critical role in diabetic nephropathy. Specifically, we used fluorescence 2-D differential in-gel electrophoresis (DIGE), a sensitive gel-based separation and quantification approach, combined with liquid chromatography (LC)-tandem mass spectrometry (MS/MS), for in-depth proteome coverage and to explore global changes in the urinary proteome in diabetic nephropathy, and report the identification of distinct proteomic signatures characteristic of disease state.

RESEARCH DESIGN AND METHODS

— Urine samples were collected from a total of 33 subjects with type 2 diabetes and from 9 healthy control subjects. In the diabetic group, 10 subjects had normoalbuminuria (urinary albumin <20 mg/g creatinine; group 1), 13 had microalbuminuria (20–200 mg/g creatinine; group 2), and 10 had macroalbuminuria (>200 mg/g creatinine; group 3). The subjects in group 3 exhibited creatinine clearance rates ≤50% of those of control subjects, and blood urea levels >60 mg/dl and serum creatinine levels >1.5 mg/dl. Control subjects (4 women and 5 men) were 45–69 years of age with BMIs between 19.67 and 27.64 kg/m^2. They had normal renal function and blood pressure, no proteinuria, normal urinary sediment, and no significant clinical events during the past 6 months, and none of them were taking medication. The clinical characteristics of control subjects and the patients in study groups 1, 2, and 3 are shown in Table 1. Informed consent was obtained from the subjects following the institutional review board guidelines for human subjects (Nizam's Institute of Medical Sciences, Hyderabad, India).

All subjects had provided second voided clean-catch urine samples in the early morning after an overnight fast in a 5- to 10-ml volume in 15-ml conical tubes. These specimens were frozen and stored at −20°C until being shipped to Oregon Health and Science University for further analysis. Urine samples were centrifuged at 10,000*g* for 20 min at 4°C to remove cellular debris and nuclei. The supernatants were transferred to 15-ml Ultrafree 5K membrane concentrators (Millipore, Billerica, MA) and spun at 7,000*g* to reduce the volumes to ~1 ml. Precipitates formed during the concentration step were removed by centrifugation at 7,000*g* for 15 min.

Immunodepletion of abundant serum proteins in human urine

Urine samples were depleted of six major proteins (albumin, IgG, IgA, antitrypsin, transferrin, and haptoglobin) using the Agilent multiple affinity system (Agilent Technologies, Palo Alto, CA). Urine (1 ml) equilibrated in Agilent buffer A was processed using an Agilent immunoaffinity column (4.6 × 100-mm) attached to a Waters high-performance liquid chromatography system. Appropriate fractions were collected, buffer-exchanged with 10 mmol/l Tris, pH 8.4, and concentrated using 5,000 molecular weight cutoff filters. Protein concentration was determined using the Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA).

Fluorescence 2-D DIGE

A total of 15 2-D-DIGE gels were run, each representing a pair of samples from control and diabetes subjects (5 each for diabetes without albuminuria, diabetes with microalbuminuria, and diabetes with macroalbuminuria). Urine proteins (50 μg) were labeled with CyDye DIGE Fluor minimal dye (Cy3 for control, Cy5 for diabetes, or Cy2 for reference [diabetes + control]; Amersham Biosciences, Piscataway, NJ) at a concentration of 100–400 pmol/l dye/50 μg protein, and all three labeled samples were multiplexed and resolved in one gel. Labeled proteins were purified by acetone precipitation and dissolved in isoelectric focusing buffer and rehydrated on to a 13-cm IPG strip (pH 4–7) for 12 h at room temperature. The IPG strip was then equilibrated with dithiothreitol equilibration buffer and iodoacetamide equilibration buffer for 15 min sequentially. Second-dimension 8–16% SDS-polyacrylamide gel electrophoresis was conducted at 80–90 V for 18 h.

Image analysis

Gels were scanned in a Typhoon 9400 scanner (Amersham Biosciences) using appropriate lasers and filters with photomultiplier tube voltage set between 550 and 600. Images in different channels (control and diabetes) were overlaid using pseudo-colors, and differences were visualized using ImageQuant software (Amersham Biosciences). 2-D gel image analysis to identify differentially abundant protein spots was performed using Phoretix 2D Evolution software (version 2005; Nonlinear USA, Durham, NC). A fixed area was selected from every gel, and a cross-stain analysis protocol was performed. Background subtraction was done using the "mode of nonspot" method, and images were wrapped to maximize spot matching. A ratiometric normalization algorithm was applied to account for potential concentration differences in protein labeling. Normalized protein spots in the Cy3 and Cy5 channels were compared with the internal standard (Cy2) to generate a ratio of rela-

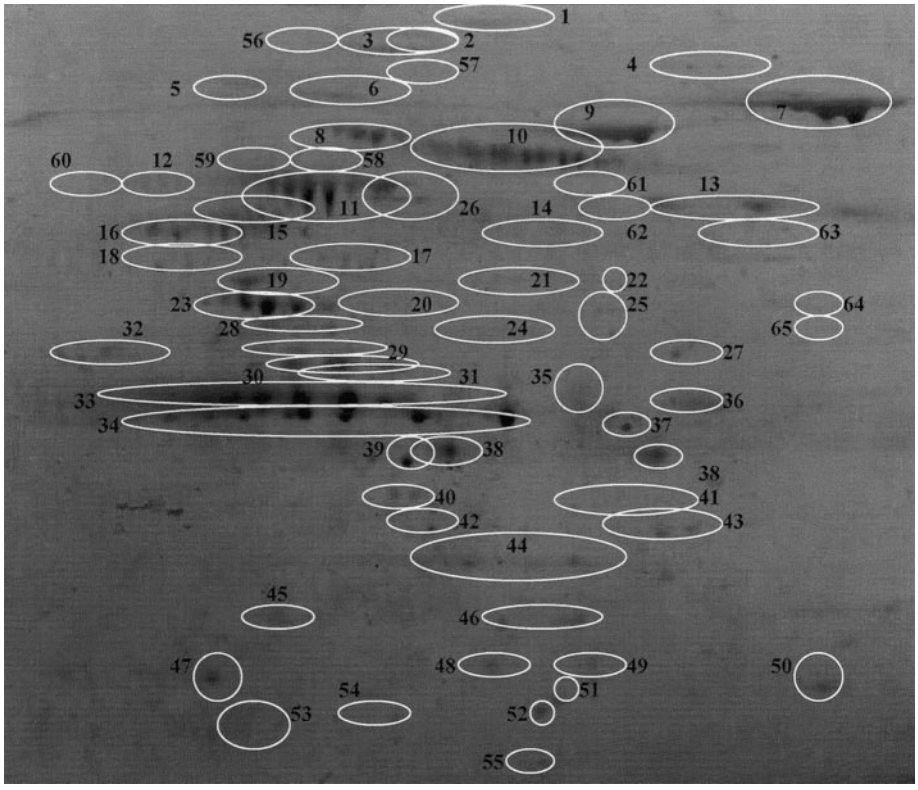

Figure 1—*Urinary proteomic profile of type 2 diabetic patients with macroalbuminuria (group 3) and protein identification. Urine protein (1 mg) was subjected to 2-D DIGE and stained with Coomassie Blue R-250. Individual spots were cut from the gel, destained, and digested in gel with trypsin for 24 h at 37°C. Protein identification was obtained by LC-MS/MS (Table 2).*

tive amount. The statistical significance of differences in the intensity of protein spots was determined by *t* tests on the averaged gels for each group. Protein spots with a relative ratio >1.5 and a *t* test value of <0.05 were considered to be significantly differentially abundant.

Preparative 2-D gel electrophoresis and protein identification

Urine protein (1 mg) was subjected to 2-D DIGE and stained with Coomassie Blue R-250. Individual spots were cut from the gel, destained, and digested in-gel with trypsin for 24 h at 37°C. Tryptic peptides were extracted with 0.1% trifluoroacetic acid, purified using Zip-Tip$_{C18}$ pipette tips from Millipore, and analyzed on a Waters hybrid quadrupole time-of-flight mass spectrometer (Q-Tof-2) connected to a Waters CapLC. An MS/MS-MS survey method was used to acquire MS/MS-MS spectra. Masses of 400–1,500 Da were scanned for MS survey, and masses of 50–1900 Da were scanned for MS/MS. Data analysis was performed using the ProteinLynx Global Server (version 2.1; Waters) and by de novo sequencing using a PEAKS algorithm combined with the OpenSea alignment algorithm (version 1.3.1) (10). Peptides consisting of five or more amino acids were used and matched to either a nonredundant human IPI or the Swiss-Prot database to identify the corresponding proteins. Proteins with two or more peptides by both ProteinLynx (significance score >10.6) and OpenSea (significance score >100) scoring algorithms (6) were chosen.

RESULTS

Urinary proteome profiles in diabetic nephropathy (macroalbuminuria)

To improve the detection of low-abundance proteins, we removed the six most abundant serum proteins (albumin, IgG, IgA, transferrin, haptoglobin, and antitrypsin). Because of the high levels of urinary protein in diabetic nephropathy, preparative 2-D gels were prepared from group 3 (macroalbuminuria) to identify proteins expressed in the urinary proteome. As shown in Fig. 1, 65 areas from the 2-D gels representing single or multiple spots potentially corresponding to the same protein were picked for identification. Analysis of MS/MS spectra by ProteinLynx, de novo sequencing algorithms, and a search of a nonredundant human IPI database identified 195 peptide MS/MS spectra that corresponded to 62 unique proteins (Table 2).

Functional annotation of the urinary proteome

We classified identified proteins by function with DAVID (Database for Annotation, Visualization and Integrated Discovery; National Institute of Allergy and Infections, National Institutes of Health) and sorted by Gene Ontology (GO) Consortium terms (Table 2). Further sorting for relation to metabolism was also performed on the basis of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway terms and the Bioinformatic Harvester Web site using SOURCE, STRING, and TrEMBL. The subgroups assigned were cell development, cell organization, defense response, metabolism, and signal transduction.

2-D DIGE analysis of differential expression of urinary proteins in diabetic nephropathy

To enhance sensitivity, reproducibility, and detection over a wide dynamic range, we used a multiplex proteomic analysis approach to label proteins with fluorescent cyanine dyes (Cy3, Cy5, and Cy2; control, diabetes, and reference, respectively), and each pair of diabetic and control samples was resolved in the same gel. Five individual gels representing each group were analyzed to identify differentially abundant proteins. Spot quantification and statistical analysis (Phoretix 2D Evolution software; Nonlinear) of gels containing individual study group samples identified a total of 195 spots from all conditions. After background subtraction and ratiometric normalization, matched spots across all the gels were used for statistical analysis. By using the criteria of differential abundance >1.5-fold change and $P < 0.05$, 65 spots were classified as significantly differentially abundant.

Differential expression by relative abundance from the gel spots identified individual proteins whose levels differed between groups 3 and 1 and group 3 and control subjects (Tables 3 and 4) by this 2-D DIGE approach. To evaluate proteins exhibiting significant differences in abundance in different stages of diabetic nephropathy (normoalbuminuria versus microalbuminuria versus macroalbuminuria), we examined specific differentially abundant spots that were present in all

Table 2—*Urine proteins identified by LC-MS/MS in type 2 diabetic patients with macroalbuminuria*

Swiss-Prot/ TrEMBL Accession No.	Protein name	Peptide count	Spot No.	MW (kDa)	pI	Function
P01009	α_1-Antitrypsin	286	5, 6, 8, 11, 12, 14–21, 23–25, 27–29, 31–37, 39, 40, 42–45, 47, 51–56, 59	46,707	5.52	Defense response
P02787	Serotransferrin precursor	60	7, 13, 14, 18, 23, 36, 52	77,000	7.19	Transport
P00450	Ceruloplasmin precursor	56	2, 3, 4, 6, 17, 24, 28, 57	122,128	5.64	Transport
P02790	Hemopexin	42	8, 10, 14, 19, 28, 29, 58	51,643	6.96	Transport
P02760	AMBP protein	38	12, 38, 30–34, 40, 42, 64, 65	38,974	6.21	Metabolism
P08603	Complement factor H	37	1, 2	139,034	6.64	Defense response
Q56G89	Serum albumin	37	8, 9, 10, 17	71,658	6.79	Transport
P01011	α_1-Antichymotrypsin precursor	35	11, 12, 28, 29, 54, 60	47,651	4.6	Defense response
P01008	Antithrombin-III	35	11, 12, 15, 17, 19, 20, 29, 53, 54, 61, 62	52,658	6.41	Defense response
P01024	Complement component C3	34	9, 19, 21, 22, 28, 45	187,046	6.32	Defense response
P02647	ApoA-1	31	25, 38, 39	30,759	5.72	Metabolism
P25311	α_2-Glycoprotein 1, zinc	27	19, 20, 23, 24, 28	34,223	5.97	Defense response
P01857	Ig γ-1 chain C region	27	13–16, 24, 28, 45	51,628	8.1	Defense response
P01042	Kininogen precursor	27	11, 12, 15, 53, 59, 60	47,853	6.64	Signal transduction
Q5JP68	B-factor, properdin	26	29, 30, 31, 35, 36	68,829	6.48	Metabolism
P04217	α_{1B}-Glycoprotein	23	8, 17, 21, 58	54,239	5.82	FNA
P06727	ApoA-IV	22	17, 27, 40	45,343	5.37	Metabolism
P00737	Haptoglobin precursor	22	19, 20, 21, 24, 44	31,362	8.65	Transport
P10909	Clusterin	21	28, 29, 32	52,461	6.19	Signal transduction
P02749	β_2-Glycoprotein 1 precursor	20	13, 14, 19, 20, 21, 22, 62	38,273	8.42	Metabolism
P00751	Complement factor B	17	24, 29, 30, 36	85,479	7.06	Defense response
P05156	Complement factor I	17	14, 24, 28	65,677	7.87	Defense response
P10643	Complement component C7	16	4	93,457	6.43	Defense response
Q8IZY7	Hepatocellular carcinoma associated protein TB6	16	5, 6	83,232	5.69	Transport
Q6LDQ3	Sulfated glycoprotein 2	16	28, 29	57,796	6.62	Signal transduction
P01019	Angiotensinogen	13	11, 17, 27, 36, 62	53,121	6.22	Signal transduction
P02750	Leucine-rich α_2-glycoprotein	13	18, 63	38,154	6.88	FNA
P02766	Transthyretin precursor	11	45, 46, 47, 48	15,877	5.71	Metabolism
P02765	α_2-HS-glycoprotein precursor	10	15, 16	39,300	5.64	Metabolism
P41222	Prostaglandin H_2 D-isomerase precursor	10	38, 39, 41	21,015	8.15	Metabolism
P06702	Calgranulin B	9	17, 41, 48	13,234	6.04	Defense response
P02748	Complement component 9 precursor	9	8, 32	63,133	5.53	Defense response
P01876	Ig α-1 chain C region	8	13, 14, 58, 59	53,163	6.06	Defense response
Q14624	Inter-α-trypsin inhibitor heavy chain h4 precursor	8	3, 6, 56	101,179	6.57	Metabolism
P02753	Plasma retinol-binding protein precursor	8	40	22,995	5.95	Signal transduction
Q12805	Epidermal growth factor–containing fibulin-like extracellular matrix protein	7	20	54,517	4.99	Signal transduction
IPI00061246	Hypothetical protein	7	34, 36, 37, 38, 44	52,553	7.9	FNA
IPI00178198	Predicted immunoglobulin	7	38, 43, 49, 50	23,530	7.6	FNA
P01834	Ig κ chain C region	7	38, 39	15,170	8.65	Defense response
P01884	β_2-microglobulin	6	50, 52, 55	13,706	6.45	Defense response
Q14118	Dystroglycan precursor	6	18, 19, 20	97,520	8.9	Metabolism
IPI00181119	Hypothetical protein	6	12, 61	70,309	7.9	FNA
IPI00186312	Hypothetical protein	6	22	37,651	6.4	FNA
P02774	VDBP	5	26	52,964	5.2	Transport
Q15668	Epididymal secretory protein E1	4	40	16,559	8.04	Defense response
IPI00151992	Hypothetical protein FLJ31320	4	9	67,970	8.49	FNA
P51884	Lumican precursor	4	11	38,405	6.54	Cell Development
P01861	Ig γ-4 chain C region	4	13	35,941	8.2	Defense response
P08697	α_2-Antiplasmin precursor	3	11, 16	54,531	6.2	Defense response
P02652	ApoA-II precursor	3	53	11,168	6.82	Metabolism
P02675	Fibrinogen β chain precursor	3	25	55,892	8.66	Metabolism
IPI00168010	Hypothetical protein FLJ35322	3	4, 61, 62	18,511	6.2	FNA
P05090	ApoD	2	34	21,262	5.11	Metabolism
P08185	Corticosteroid-binding globulin precursor	2	12	45,112	5.93	Transport
Q5JXG3	Novel protein	2	18	94,331	5.47	FNA
P04004	Vitronectin precursor	2	12	54,306	4.6	Defense response
P02679	Fibrinogen γ chain	1	18	51,479	5.51	Metabolism
Q9H647	Hypothetical protein FLJ22612	1	34	58,511	4.62	FNA
P01859	Ig γ-2 chain C region	1	14	46,032	7.88	Defense response
P24592	Insulin-like growth factor binding protein-6	1	35	25,306	8.26	Cell organization
P19823	Inter-α-trypsin inhibitor heavy chain H2	1	54	106,596	6.4	Metabolism
P15144	Membrane alanine aminopeptidase precursor	1	6	109,312	5.36	Cell development

Identified proteins are listed with their SwissProt or TrEMBL accession numbers. Molecular weight (MW) and pI were calculated using the CalPI/MW tool available on the SwissProt Web site. FNA, function not assigned.

Table 3—*Urine proteins differentially expressed in type 2 diabetic patients with macroalbuminuria (group 3) compared with type 2 diabetes without albuminuria (group 1) detected by 2-D DIGE*

Swiss-Prot accession no.	Protein name	-Fold change
P04217	α_{1B}-Glycoprotein	6.95
P25311	α_2-Glycoprotein 1, zinc	5.86
P02774	VDBP	4.84
P02765	α_2-HS-glycoprotein	4.71
P06702	Calgranulin B	3.87
P01009	α_1-Antitrypsin	2.89
P02790	Hemopexin	2.39
P02753	Plasma RBP	−1.52
P02760	AMBP protein	−1.61
P02647	ApoA-1	−3.20
P02766	Transthyretin	−4.32

three groups by spot volume quantification (Phoretix software) (Fig. 2). The relative values across three subgroups of albuminuria (groups 1, 2, and 3) representing normo-, micro-, and macroalbuminuria associated with diabetes are shown in Table 5. Whereas 11 proteins were progressively increased from normoalbuminuria to overt nephropathy, 4 proteins were progressively decreased with increasing disease severity.

Upregulated urinary proteins in diabetic nephropathy

Seven proteins were upregulated >1.5-fold ($P < 0.05$) in diabetic nephropathy with macroalbuminuria when compared with diabetes without albuminuria. Normalized volumes of these upregulated proteins were also progressively increased across three categories of diabetic patients with normo-, micro-, and macroalbuminuria, indicating their positive association with disease progression (Table 5). α_{1B}-Glycoprotein showed the greatest overall increase (7.0-fold), followed by zinc-α_2-glycoprotein (5.9-fold), α_2-HS-glycoprotein (4.7-fold), vitamin D–binding protein (VDBP) (4.8-fold), calgranulin B (3.9-fold), α_1-antitrypsin (A1AT) (2.9-fold), and hemopexin (2.4-fold). When compared with control subjects, VDBP exhibited the greatest increase (11.1-fold), followed by zinc-α_2-glycoprotein (6.0-fold), α_2-HS-glycoprotein precursor (2.3-fold), and A1AT (2.2-fold). A preliminary analysis of urine VDBP levels using immunoassays verified a significant difference between healthy subjects (0.025 ± 0.002 μg/μl, $n = 5$) and macroalbuminuric patients (75 ± 10 μg/μl, $n = 5$) (data not shown).

Downregulated urinary proteins in diabetic nephropathy

Four proteins were downregulated <1.5-fold (P value < 0.05) in diabetic nephropathy with macroalbuminuria compared with diabetes without albuminuria. Transthyretin was highly downregulated (4.3-fold), followed by apolipoprotein (apo) A-I (3.2-fold), α_1-microglobulin/bikunin precursor (AMBP) (1.61-fold), and plasma retinol-binding protein (1.52-fold). The reduction in the normalized volumes of plasma retinol-binding protein and AMBP across the control and three diabetic groups was consistent with worsening diabetic renal disease (Table 5). Compared with control subjects, AMBP was the least abundant in macroalbuminuria versus control (5.06-fold), followed by plasma retinol-binding protein (2.56-fold) and apoA-I (2.53-fold).

CONCLUSIONS — To date, 2-D gel electrophoresis–based methods have been most successful in identifying individual disease-specific protein biomarkers. 2-D gel electrophoresis and protein identification using LC-MS/MS allowed us to identify 62 unique proteins expressed in urine (Table 2); ~65 distinct urinary protein spots associated with these 62 individual gene products unique to diabetic nephropathy were mapped. Further subgrouping of their annotated functions revealed the presence of transport proteins, proteases and protein inhibitors, glycoproteins, growth factors, extracellular proteins, and complement factors and immunoglobulins.

With the multiple proteomic comparisons used in this study, we identified distinct sets of proteins that were differentially abundant in individuals with diabetic nephropathy compared with those without diabetic nephropathy or control subjects. Seven urine proteins exhibited high relative abundance in diabetic nephropathy (macroalbuminuria) compared with diabetes without albuminuria,

Table 4—*Urine proteins differentially expressed in type 2 diabetic patients with macroalbuminuria (group 3) compared with control subjects detected by 2-D DIGE.*

Swiss-Prot accession no.	Protein name	-Fold change
P02774	VDBP	11.10
P25311	α_2-Glycoprotein 1, zinc	5.97
P02748	Complement component 9 precursor	5.74
P01011	α_1-Antichymotrypsin precursor	5.14
P00738	Haptoglobin-2 precursor	4.56
P02765	α_2-HS-glycoprotein precursor	2.32
P00751	Complement factor B	2.27
P01009	α_1-Antitrypsin	2.17
P02787	Serotransferrin precursor	2.11
P10643	Complement component C7	2.05
P02652	ApoA-II precursor	2.00
P43652	Afamin precursor	1.82
P02647	ApoA-1	−2.53
P02753	Plasma RBP	−2.56
P02760	AMBP protein	−5.06
P41222	Prostaglandin H_2 D-isomerase	−6.02
P00450	Ceruloplasmin precursor	−6.37

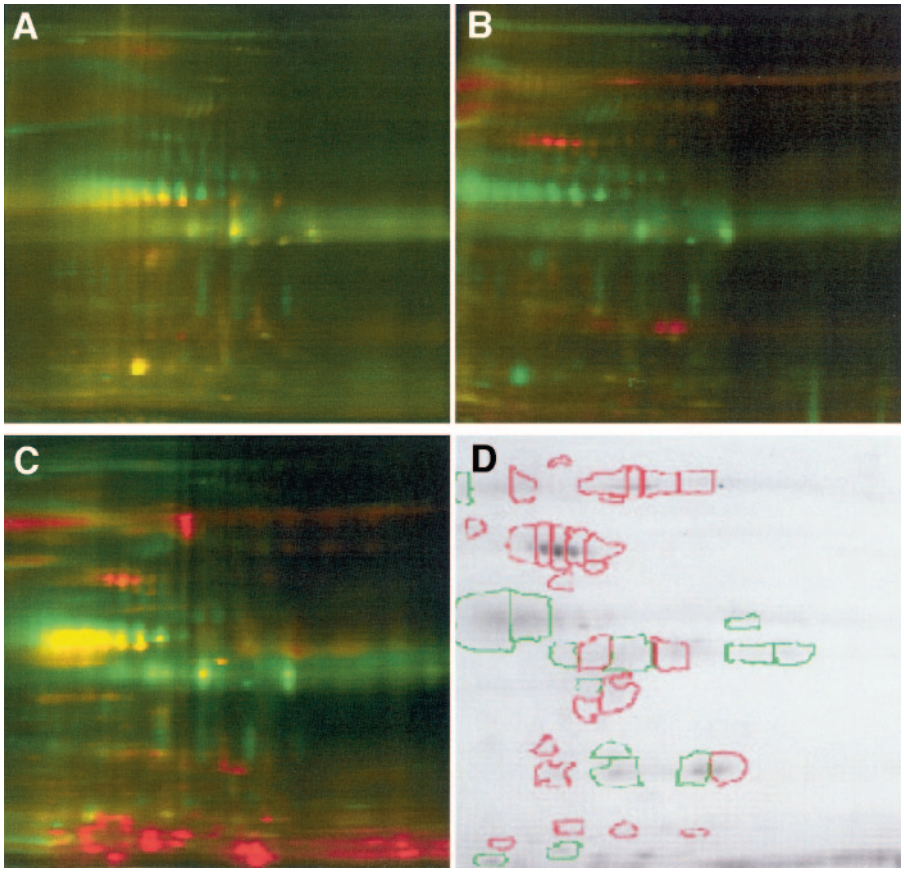

Figure 2—*Proteomic analysis of diabetic urine using 2-D-DIGE. 2-D DIGE images of* (A) *control (green) versus diabetes with no albuminuria (red),* (B) *control (green) versus diabetes with microalbuminuria (red), and* (C) *control (green) versus diabetes with macroalbuminuria (red).* D*: Difference map generated by Phoretix Evolution comparing diabetes with macroalbuminuria and without albuminuria. Spots outlined in red were consistently >1.5-fold upregulated* ($P < 0.05$)*, whereas spots outlined in green were consistently >1.5-fold downregulated* ($P < 0.05$) *in diabetes with macroalbuminuria compared to diabetes without albuminuria.*

and their differential abundance was consistent across groups of nephropathy. Four urine proteins were of low relative abundance in macroalbuminuria compared with diabetes without albuminuria, and the differential abundance of two of them was consistent across three groups of nephropathy. It is likely that further studies of these potential markers will facilitate better understanding of the mechanism of diabetic nephropathy and provide new avenues for therapy.

Upregulated urinary proteins in diabetic nephropathy

α_{1B}-Glycoprotein contains five Ig-like V-type (immunoglobulin-like) domains and is homologous to the immunoglobulin supergene family, suggesting its possible role in the autoimmunity involved in nephropathy. Zinc-α_2-glycoprotein was the second most abundant urinary protein in diabetic nephropathy. This protein stimulates lipid degradation in adipocytes and may also bind polyunsaturated fatty acids. An earlier study of type 2 diabetes described zinc-α_2-glycoprotein and three other proteins, α_1-acid glycoprotein, α_1-microglobulin, and IgG, as specific markers for diabetic nephropathy (9). α_2-HS-glycoprotein precursor (fetuin A) is an inflammation-related calcium-regulatory glycoprotein that acts as a systemic calcification inhibitor. Both chronic inflammation and uremia may contribute to exhausting fetuin A release in the late stages of kidney disease. Deficiencies of calcification inhibitors such as fetuin A are relevant to uncontrolled vascular calcification and may offer potential for future therapeutic approaches (11). Hemopexin (β_{1B}-glycoprotein) binds heme and transports it to the liver for breakdown and iron recovery, after which the free hemopexin returns to the circulation. Hemopexin levels have been described to be higher in type 2 diabetes, its relationship with C-reactive protein is lost in both type 1 and type 2 diabetes, and its levels are independently determined by triacylglycerol and the diabetic state (12). The hemopexin molecule can potentially act as a toxic protease, leading in the rat to proteinuria and glomerular alterations (13). Although there were no direct references to its possible effects in humans with diabetes or nephropathy, because diabetes is an inflammatory condition associated with iron abnormalities, it can be postulated that hemopexin is altered in diabetes.

VDBP is a multifunctional protein found in body fluids and on the surface of many cell types. In plasma, it carries vitamin D sterols and prevents polymerization of actin by binding its monomers. VDBP also associates with membrane-bound immunoglobulin on the surface of B lymphocytes and with IgG Fc receptors on the membranes of T lymphocytes, suggesting its possible role in the immunopathogenesis and progression of the disease. Furthermore, an association of VDBP with type 1 and type 2 diabetes, glucose intolerance, hypoinsulinemia/hyperinsulinemia, and/or insulin resistance and pregnancy associated-diabetes was reported in Dogrib Indians (14), American Anglos and Hispanics (15), Pima Indians (16), Japanese (17,18), Russians (19), Polynesian Island populations (20), Alsacian French (21,22), Bantu (23), and Finnish populations (24). VDBP was also found in the vitreous in diabetic macular edema, along with pigment epithelium-derived factor, apoA-4, apoA-1, trip-11, and plasma retinol-binding protein (RBP). These chemical mediators in the posterior vitreous may play a role in the pathogenesis of diabetic macular edema (25).

Calgranulin B is expressed by macrophages in inflamed tissues and is an inhibitor of protein kinases. Differences in the isoforms and abundance of several urine proteins, including calgranulin B, inter-α-trypsin inhibitor, prothrombin fragment 1, and CD59, were known to be associated with stone formation (26). There have been no previous reports on their association with diabetes or other renal diseases. A1AT is an inhibitor of serine proteases, and its primary target is elastase, but it also has a moderate affinity for plasmin and thrombin. The serum levels of A1AT and a α_1-acid glycoprotein, as well as their glycosylated protein fractions, were reported to be significantly greater in sera from patients with diabetic

Table 5—*Urine proteins exhibiting progressive changes in abundance with degree of albuminuria*

Swiss-Prot/TrEMBL accession no.	Protein name	Normalized volume		
		Group 1 vs. C	Group 2 vs. C	Group 3 vs. C
P01009	α_1-Antitrypsin	0.58	0.65	0.78
P02774	VDBP	0.37	1.84	4.23
P00450	Leucine-rich α_2-glycoprotein	0.39	1.04	1.10
P02790	Hemopexin	0.18	0.66	1.02
P25311	α_2-Glycoprotein 1, zinc	0.39	2.11	2.48
P06727	ApoA-IV	0.31	0.39	0.53
P02787	Serotransferrin precursor	0.15	0.50	0.87
P00751	Complement factor B	0.28	0.30	0.47
P04217	α_{1B}-Glycoprotein	0.55	1.07	1.30
P02765	α_2-HS-glycoprotein precursor	0.64	1.39	2.09
P06702	Calgranulin B	0.38	0.59	2.26
P00450	Ceruloplasmin precursor	0.33	0.32	0.23
Q8IZY7	Hepatocellular carcinoma-associated protein TB6	1.23	0.62	0.56
P02760	AMBP protein	0.53	0.25	0.20
P02753	Plasma RBP precursor	0.51	0.42	0.37

Normalized volumes sequentially expressed in type 2 diabetic patients compared with control subjects (C) as determined by 2-D DIGE.

nephropathy compared with healthy adults. Marked linear deposition of these proteins in the glomerular or dermal vascular walls was also observed in the same patients (27), linking them to diabetic nephropathy. Whereas the association between A1AT deficiency and glomerulonephritis has been reported only sporadically, A1AT deficiency was more frequently linked to lung emphysema with or without hepatic cirrhosis (28).

Downregulated urinary proteins in diabetic nephropathy

Transthyretin (prealbumin) is a thyroid hormone-binding protein, which transports a small part of thyroxine from the bloodstream to the brain. About 40% of plasma transthyretin circulates in a tight complex with plasma RBP. Transthyretin was reported as a better and suitable marker for nutrition assessment in patients with chronic renal failure (29). Elevated plasma RBP in insulin-resistant humans with obesity and type-2 diabetes was known to induce hepatic expression of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase and impairs insulin signaling in muscle (30). It is possible that low RBP-4 in diabetic nephropathy increases insulin sensitivity and causes spontaneous hypoglycemia, because low RBP-4 is potentially hypoglycemic.

AMBP contains both α_1-microglobulin and inter-α-trypsin inhibitor light chain (bikunin). Bikunin is an important anti-inflammatory substance to modulate inflammatory events. Whether decreases in these protein levels affect immunocompetence in diabetic renal disease remains unstudied. The other component of AMBP protein, α_1-microglobulin, in urine was directly related to progressive albuminuria in Chinese, Malays, and Asian Indians with type 2 diabetes (31). This is in contrast to the decreasing levels of this protein observed in our study. Urinary α_1-microglobulin indicates proximal tubular dysfunction and could be a useful biomarker for the early detection of nephropathy in diabetic subjects in addition to albuminuria, which indicates glomerular dysfunction.

ApoA-I, acting as a cofactor for the lecithin cholesterol acyltransferase, participates in the reverse transport of cholesterol from tissues to the liver for excretion. ApoA1, as well as apoB/A1, was included among several nontraditional cardiovascular risk factors in the progression to pediatric metabolic syndrome (32), but there have been no reported references to its association with diabetes or nephropathy.

In summary, this study differs from previous proteomics studies of urinary proteins in diabetic nephropathy in several aspects. Earlier analyses have been focused on kidney-derived proteins in a particular mouse model of type 1 diabetes (4) or have described peptide patterns in human type 1 and type 2 diabetic urine, in which the identity of a only small fraction of the source proteins was identified, and which were, in those cases, high-abundance proteins (6–8). One other recent study assessed a limited number of candidate biomarkers by 2-D gel electrophoresis and MS and found a small number of proteins that were upregulated in diabetic urine (9). In the current study, on the other hand, we used a more robust 2-D DIGE approach coupled with LC-MS/MS to produce a comprehensive list of proteins in human urine and identified a significant number that exhibited either increased or decreased abundance in the presence of diabetic nephropathy, including several whose levels correlated with increasing degree of albuminuria.

Seven potential upregulated urinary biomarkers for diabetic nephropathy found in this study were α_{1B}-glycoprotein, zinc-α_2-glycoprotein, α_2-HS-glycoprotein, VDBP, calgranulin B, A1AT, and hemopexin. The detection of a significant number of glycoproteins in diabetic urine could potentially be representative of the hyperglycosylated state of these proteins in the serum proteome. This is the first report of an elevated transport protein (VDBP) and two defense-response proteins (A1AT and calgranulin B) in the urine in diabetic nephropathy.

A limitation of the current study, as well as of the other studies of the human urinary proteome in diabetic nephropathy cited above, is that the candidate biomarkers and peptide patterns described could be indicative of nephropathy per se, rather than being uniquely associated with diabetic nephropathy. Distinguishing between these alternatives would require large-scale analyses in light of the wide spectrum of nondiabetic causes of nephropathy (33–37). However, because

these biomarkers have been derived from a well-characterized patient population and would be used in a similarly characterized population, this issue does not detract from the potential utility or possible mechanistic significance of the candidate proteins.

The identification of novel protein biomarkers of diabetic nephropathy represents an important step forward in advancing our understanding of the physiologic perturbances that lead to ESRD. Further studies of these proteins may elucidate the mechanisms and pathways that underlie the pathogenesis of diabetic nephropathy, thus facilitating the design of effective therapeutic agents. Characterization and identification of the differentially abundant proteins seen in this study can also provide the basis for development of robust, noninvasive, multianalyte assessment of diabetic nephropathy. We acknowledge that our findings should be considered preliminary until validated in a larger cohort and that to be clinically significant, the utility of these markers in practice must be better than that of currently available tests. However, to reverse the alarming trend of the steady rise in the rate of ESRD in the U.S., innovative treatment strategies based on the reliable identification of diabetic patients at high risk for nephropathy must be developed.

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