Diabetic Kidney Disease (DKD) in type 2 diabetes is the leading cause of end-stage renal disease and a strong risk amplifier for cardiovascular disease and heart failure (21, 48). Efforts to reduce the burden of DKD focus on early screening for microalbuminuria to identify patients with an elevated risk of progressive renal disease (11, 14, 28). However, microalbuminuria has limitations as a test for renal impairment. First, 25–55% of type 2 diabetic patients with chronic renal insufficiency, defined as a sustained reduction in measured or estimated glomerular filtration rate (eGFR) < 60 ml-min⁻¹.1.73 m⁻², are normoalbuminuric (30, 36, 52, 57). Second, in longitudinal studies 45–55% of patients with type 2 diabetes that develop chronic kidney disease are normoalbuminuric (4, 46, 56), demonstrating that the presence of microalbuminuria is not a strong and constant predictor of progression of renal disease as was initially postulated (43, 45, 55). The limitations of microalbuminuria as a marker of early renal impairment have raised concerns regarding the use of albumin excretion as a surrogate renal endpoint in therapeutic trials (14, 22, 32). The discovery of sensitive and specific biomarkers for renal impairment would improve management and strengthen efforts to develop reno-protective drugs.

One approach for discovering noninvasive biomarkers of DKD involves profiling the urine proteome with two-dimensional gel electrophoresis and mass spectrometry to identify differentially expressed proteins (10, 39, 53). Unique patterns of urine polypeptide and protein expression have been reported in adults and adolescents with DKD (38, 40, 47). However, the unexpected complexity of the human urine proteome, estimated to contain 800 to 2,000 proteins (10, 39, 53), has complicated efforts to identify specific proteins from the mass spectrometric data. In addition, proteomic-based biomarker discovery in urine has been impeded by technical issues, including the wide dynamic range of urine protein concentration, variations in pH, and high levels of salts and urea that interfere with sample processing (1, 10). In addition, these studies used mass spectrometric techniques that provide relative, as opposed to quantitative measurements of differentially expressed proteins.

To identify candidate biomarkers of early renal impairment, we screened differentially expressed kidney mRNAs from type 2 diabetic (db/db) and nondiabetic (db/m) mice. We sought kidney mRNAs that encode secreted proteins with human orthologs. Using this strategy, we identified 36 potential biomarker candidates and measured the urinary excretion of six of these candidates for which immunoassays were available. In a pilot study, we found that that urinary protein levels of the six candidates correlated inversely with eGFR, independent of microalbuminuria, in a group of participants with type 2 diabetes. Levels of urinary endothelin-1 (ET-1), growth and differentiation factor-15 (GDF15), and interleukin-6 (IL-6) were associated with a marker of subnormal estimated glomerular filtration rate more closely than albumin and any of the six candidate markers was associated with growth factor were not increased. A composite variable of urine tensin receptor antagonists. In contrast, urinary levels of fibroblast growth factor-23 were increased in diabetic mice for differentially expressed mRNA transcripts that encode secreted proteins with human orthologs. Whether elevated urine levels of the orthologous proteins correlated with diminished glomerular filtration rate was tested in a cross-sectional study of n = 56 patients with type 2 diabetes. We identified 36 putative biomarker genes in db/db kidneys: 31 upregulated and 5 downregulated. Urinary protein levels of six selected candidates (endothelin-1, lipocalin-2, transforming growth factor-β, growth and differentiation factor-15, interleukin-6, and macrophage chemokine protein-1) were elevated in type 2 diabetic patients with subnormal glomerular filtration rate (i.e., <90 ml-min⁻¹.1.73 m⁻²), independent of microalbuminuria, age, sex, race, and use of angiotensin-converting enzyme inhibitors and angiotensin receptor antagonists. In contrast, urinary levels of fibroblast growth factor were not increased. A composite variable of urine albumin and any of the six candidate markers was associated with subnormal estimated glomerular filtration rate more closely than albumin alone. In addition, urinary endothelin-1, growth and differentiation factor-15, and interleukin-6 were associated with a marker of proximal tubule damage, N-acetyl-β-D-glucosaminidase activity. These results suggest that gene expression profiling in diabetic mouse kidney can complement existing proteomic-based approaches for renal biomarker discovery in humans.
gether, these results suggest that a renal biomarker discovery paradigm based on gene expression profiling in murine models of diabetes can complement existing proteomic-based approaches and might expedite the identification of urinary biomarkers of DKD in type 2 diabetes.

METHODS

Computational analysis of the renal transcriptome in db/db mice. We assessed our laboratory’s previously published profile of 1,016 mRNA transcripts that are differentially expressed in the kidneys of 8- and 16-wk db/db and db/m mice (41). Primary gene expression data are publicly available at the Gene Expression Omnibus http://www.ncbi.nlm.nih.gov/geo/, GEO Accession Number GSE642. In the present study, we restricted our analysis to genes that exhibited more than twofold up- or downregulation in db/db vs. db/m kidneys, identified hereafter as the “db/db gene set” (753 mRNA transcripts). To determine which genes in the db/db set encoded secreted proteins (i.e., putative biomarkers), we uploaded the EntrezGene identifiers into the Gene Ontology (3) and Ingenuity (Ingenuity Systems, www.ingenuity.com) databases to identify proteins present in the extracellular compartment. The genes encoding secreted proteins were clustered using log2-transformed mRNA levels zero-transformed to the mean expression value for each gene in 8-wk db/m mice, as described by Eisen et al. in Cluster 3.0 and Treeview (19). The EntrezGene identifiers for human orthologs of the secreted murine proteins were uploaded into the Human Kidney and Urine Proteome Project database (http://www.mapuproteome.com/urine/) to determine whether these proteins were represented by peptide fragments in the normal human proteome.

To ascertain biological functions associated with the putative biomarker proteins, the db/db gene set was assessed by enrichment analysis with the DAVID bioinformatics database (16, 26), Ingenuity Pathway Analysis (Ingenuity Systems, www.ingenuity.com), and Gene Ontology (3) platforms. The statistical significance of enrichment for biological function was calculated using a modified Fisher exact P value and a Benjamini correction for multiple testing (5) using all genes on the Affymetrix U74v2A Murine Microarray Chip as the reference group. A corrected P value <0.05 was considered significant.

Fig. 1. Putative biomarker genes identified from db/db kidneys sorted by hierarchical clustering. All mRNA expression values are normalized to 8-wk db/m kidneys (first column, db/m8). Subsequent columns are mRNA expression values from 16-wk db/m (db/m16, 1–3), 8-wk db/db (db/db8, 1–3), and 16-wk db/db (db/db16, 1–3) mice. The intensity of red and green are proportional to the magnitude of mRNA induction or repression, respectively.
Validating mRNA expression in db/db and db/m kidney by quantitative PCR. Total RNA was extracted from flash frozen (~70°C) whole db/db (n = 4) and db/m (n = 4) kidney from a new set of animals for measuring mRNA levels by quantitative PCR (qPCR), as previously described in mouse kidney (42). Five micrograms of total RNA were used for quantifying mRNA by qPCR using the ABI Prism Sequence Detection System 7000 (Applied Biosystems, Foster City, CA). Gene-specific primers were designed using Primer Express (Applied Biosystems, available on request). Relative quantification of the mRNA levels in db/db compared with db/m mice was computed using the comparative Ct method with GAPDH as the reference gene (34). A standard dilution curve was constructed to ensure the amount of input cDNA was within the linear dynamic range of detection (9). These studies were approved by the Institutional Animal Care and Use Committee at Case Western Reserve University.

Measurement of prioritized biomarker candidates in urine from patients with type 2 diabetes. Fifty-six patients with type 2 diabetes, stratified by eGFR, were recruited from the University Hospitals Case Medical Center outpatient clinics in endocrinology and nephrology. These studies were approved by the Institutional Review Board at University Hospitals Case Medical Center; all participants provided signed, informed consent. Inclusion criteria were: age 21–74 yr; diagnosis of diabetes using the revised criteria of the American Diabetes Association (2) or the use of antihyperglycemic medications; and an eGFR > 10 ml·min⁻¹·1.73 m⁻², as estimated by the Modification of Diet in Renal Disease equation (33). The exclusion criteria were a concurrent diagnosis of nondiabetic CKD, unwillingness or inability to provide informed consent, pregnancy, lactation, current substance abuse, fever, systemic and urinary-tract infections, or inflammatory disease. Healthy participants (n = 12 health professionals) without apparent renal disease and with negative urine sediments donated spot urine specimens and blood for determination of serum creatinine. Demographic and clinical data were obtained from the patient’s chart by the study coordinator.

Urine and blood collection and processing. Trained personnel obtained venous blood and spot urine specimens at the study visit. Aliquots of blood and urine were sent to the University Hospitals Case Medical Center central laboratory for measurement of serum creatinine and urine albumin (ELISA) and creatinine. For collection and processing of remaining urine, we followed a standardized protocol from the Human Kidney and Urine Proteome Project and the European Urine and Kidney Proteomics Initiative (24, 51). Briefly, deidentified random spot urine (~40 ml midstream) was collected without preservatives or protease inhibitors into sterile containers for processing. After performing conventional urine dipstick analysis (Multistix 8 SG, Bayer, Tarrytown, NY), the specimen was transferred to a 50-ml conical tube and centrifuged at 1,500 g for 10 min in a refrigerated centrifuge to pellet any cells, which were examined by microscopy. Smaller aliquots (10 ml) were centrifuged at 10,000 g to remove particulates and aliquoted for storage at ~80°C. All processed samples were frozen within 3 h of collection. Before determination of biomarker concentration by ELISA, urine pH was adjusted to 8.0 with 1 M Tris buffer (pH 8.0) to help solubilize aggregates that might form after thawing (24, 51). All biomarker assays were performed within 2 mo of urine collection after no more than one freeze-thaw cycle.

Urinary NAGase activity. NAGase is a 140-kDa brush border lysosomal enzyme that is released by proximal tubules after injury. Urine NAGase was measured using the substrate 2-methoxy-4-(2-nitrovinyl) phenyl-glucosaminide and bovine NAGase as a calibrant, according to the manufacturer’s protocols (PRR Diagnostics, London, UK). The intra-assay and interassay coefficients of variation were 8.7 and 9.6%, respectively.

Quantitative measurement of biomarker candidates in urine. Urinary ET-1, transforming growth factor-β (TGF-β), lipocalin-2 (LCN2)-neutrophil gelatinase-associated lipocalin (NGAL), GDF15, macrophage chemotactrant protein-1 (MCP-1), IL-6, and fibroblast growth factor (FGF) were measured in duplicate by ELISA using recombinant human proteins to generate a standard curve (R&D}

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**Fig. 2.** Quantitative PCR (qPCR) measurements of candidate biomarker mRNAs in db/db and db/m kidneys. We selected 17 of the 36 putative biomarkers from Fig. 1 for verification studies by qPCR in an independent set of db/db and db/m mouse kidneys (n = 4 each). The changes in renal mRNA by qPCR were concordant with the microarray measurements shown in Fig. 1. Fibroblast growth factor mRNA, a negative control, was not differentially expressed in db/db vs. db/m kidneys. edn1, Endothelin 1; edn3, endothelin 3; bmp6, bone morphogenetic protein 6; gdf15, growth differentiation factor 15; gdf5, growth differentiation factor 5; tgfβ1, transforming growth factor-β1; erd1, erythroid differentiation regulator 1; cxcl2, chemokine (C-X-C motif) ligand 2; mcp1, macrophage chemoattractant protein-1; ccl9, chemokine (C-C motif) ligand 9; il6, interleukin-6; ctf1, CCAAT transcription factor 1; scgb1a1, secretoglobin family 1A member 1; lcn2, lipocalin 2; tnfsf11, tumor necrosis factor ligand superfamily member 11; fgf, fibroblast growth factor. Values are mean ± SD of fold expression normalized to mRNA levels in 8-wk db/m control kidneys. *P < 0.05 and **P < 0.01.
Systems, Minneapolis, MN). The mean biomarker concentration was normalized for urine creatinine measured by the University Hospitals Case Medical Center central laboratory. Urine aliquots for measurement of ET-1 were extracted in acetic acid exactly as described (17). Immunoreactive TGF-β was assessed after acidification of urine to convert latent TGF-β. The intra- and interassay coefficient of variation was equal to or less than the manufacturer-reported values in normal human urine. Laboratory personnel were blinded to diabetes and nondiabetes status of all participants.

Renal fractional excretion. Twenty patients with type 2 diabetes were selected at random for determination of fractional excretion of ET-1, TGF-β, and LCN2, exactly as described by Boligano et al. (7) using serum and urine specimens obtained at the study visit.

Statistical analyses. Demographic and clinical data were grouped according to diabetes status. Means and standard deviations (SD) are presented for continuous variables and proportions for categorical variables. Continuous and categorical variables in healthy controls and participants with type 2 diabetes were compared using independent-sample t-tests and χ² statistics, respectively. Multiple linear regression with simultaneous entry of predictor variables was used to model eGFR as a function of the urine biomarkers and to adjust this relationship for possible effects of age, sex, race, and other clinical covariates. Statistical analyses were conducted in IBM SPSS 19.0.

RESULTS

Identification of differentially expressed genes in db/db kidney that encode secreted proteins. The db/db mouse is a model of type 2 diabetes with features of early DKD, including hyperfiltration, mesangial expansion, thickening of the glomerular and tubular basement membrane, inflammation, and mod-

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Fig. 3. Annotations for biological function associated with the putative diabetic kidney disease biomarkers. A: gene ontology annotations for biological function associated with 753 differentially expressed genes in the db/db gene set. Dots represent the false discovery rate-corrected P value for the annotation category (all P < 0.05). The number of genes in the db/db gene set associated with each annotation category is indicated under the dots. B: biological annotations for individual biomarker candidates, assigned in the db/db gene set in A, two-way clustered by biomarker and annotation category. A dot indicates that the biomarker belongs to the annotation category for biological function. The absence of a dot indicates that the biomarker is not associated with the biological function within the context of the db/db gene set.
controls and 56 patients with type 2 diabetes. Demographic and clinical data in 12 healthy

Table 1. Demographic and clinical data in 12 healthy controls and 56 patients with type 2 diabetes

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Type 2 Diabetes</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>eGFR, ml·min⁻¹·1.73 m⁻²</td>
<td>120 ± 9</td>
<td>79 ± 29</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Age, yr</td>
<td>48 ± 7</td>
<td>53 ± 10</td>
<td>0.037</td>
</tr>
<tr>
<td>Urine albumin/creatinine, μg/mg</td>
<td>12.3 ± 6.4</td>
<td>171.5 ± 385.0</td>
<td>0.009</td>
</tr>
<tr>
<td>CKD stage (1-5)</td>
<td>0</td>
<td>1.8 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>Urine NAckase/creatinine, U/mg</td>
<td>1.3 ± 1.7</td>
<td>5.2 ± 2.9</td>
<td>0.028</td>
</tr>
<tr>
<td>Female, %</td>
<td>33</td>
<td>53</td>
<td>0.182</td>
</tr>
<tr>
<td>African American, %</td>
<td>50</td>
<td>52</td>
<td>0.782</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>4.9 ± 0.4</td>
<td>7.7 ± 1.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Duration of diabetes, yr</td>
<td>21.6 ± 2.2</td>
<td>36 ± 8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>8</td>
<td>87</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Past/current cigarette smoking, %</td>
<td>12</td>
<td>33</td>
<td>0.032</td>
</tr>
<tr>
<td>Cardiovascular disease, %</td>
<td>0</td>
<td>86</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Chronic heart failure, %</td>
<td>0</td>
<td>8</td>
<td>0.036</td>
</tr>
<tr>
<td>Oral antihyperglycemic medication use, %</td>
<td>0</td>
<td>79</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Insulin use, %</td>
<td>0</td>
<td>50</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ACE inhibition or ARB medication use, %</td>
<td>0</td>
<td>67</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diuretic use, %</td>
<td>8</td>
<td>43</td>
<td>0.020</td>
</tr>
<tr>
<td>b-Blocker use, %</td>
<td>8</td>
<td>38</td>
<td>0.032</td>
</tr>
<tr>
<td>Statin use, %</td>
<td>21</td>
<td>63</td>
<td>0.014</td>
</tr>
</tbody>
</table>

Values are means ± SD or %. eGFR, estimated glomerular filtration rate; CKD, chronic kidney disease stage from the National Kidney Foundation Disease Outcomes Quality Initiative (http://www.kidney.org/professionals/kdoqi/guidelines_ckd4p_class_g2.htm); NAckase, N-acetyl-p-glucosaminidase; BMI, body mass index; ACE, angiotensin-converting enzyme; ARB, angiotensin receptor blocker.


eSty elevated albumin excretion (49, 50). To find biomarker candidates, we identified differentially expressed db/db renal mRNAs that encode secreted proteins, which are likely to be altered in urine. We selected these differentially expressed mRNAs from the gene expression profiles of db/m and db/db mouse kidneys (41), focusing on genes that encode secreted proteins with human orthologs. Using this approach, we identified 36 genes that encode secreted proteins (Fig. 1), of which 31 were upregulated and 5 were downregulated in db/db vs. db/m mice. None of the candidate biomarker mRNAs were significantly altered in db/m kidneys in the 8- to 16-wk transition (Fig. 1), suggesting that the observed differential expression did not reflect age-related changes in kidney gene expression.

We selected 17 of the 36 biomarker gene candidates for verification of differential mRNA expression by qPCR in an independent set of db/db and db/m mice (Fig. 2). The qPCR and microarray data were concordant (c.f., Figs. 1 and 2). ET-1, ET-3 and bone morphogenetic protein-6 mRNAs were higher in 8- and 16-wk db/db kidneys compared with db/m controls (Fig. 2). The remaining mRNAs were differentially expressed only at 16 wk. The mRNA encoding erythroid differentiation regulator-1 was lower in 16-wk db/db compared with db/m kidneys (Fig. 2), consistent with the microarray data. As a negative control, we measured mRNA for FGF, which was not differentially expressed in db/db kidneys in the microarray studies (41). The qPCR data confirmed that FGF mRNA was not differentially expressed in db/db vs. db/m kidney (Fig. 2). Importantly, none of the putative biomarker mRNAs were differentially expressed in the 16-wk compared with 8-wk db/m controls, suggesting that the changes in mRNA abundance in db/db kidney were not related to aging.

We next asked if the secreted proteins encoded by these candidate biomarker genes might be present in normal human urine. The EntrezGene identifier for each putative biomarker gene was uploaded into the Human Kidney and Urine Proteome Project database of proteins present in normal human urine. All 36 mRNAs in Fig. 1 were represented by peptide fragments identified by mass spectrometry in the Human Kidney and Urine Proteome Project database. These results sug-

Fig. 4. Distribution of candidate biomarkers in urine from controls and participants with type 2 diabetes. Spot urine collections were obtained from 12 volunteers (open boxes) with apparently normal kidney function and 56 participants (shaded boxes) with type 2 diabetes. Levels of the autocrine/paracrine first messengers were measured by ELISA and corrected for the concentration of urine creatinine. Peaks represent the 75th to 25th percentile (i.e., interquartile range), and lines indicate the 1.5 and 3 interquartile ranges. Circles above each box represent potential outliers. Two-sided P values, calculated by independent sample t-tests, are reported. ET-1, endothelin-1; Cr, creatinine; TGF, transforming growth factor; NGAL, neutrophil gelatinase-associated lipocalin; Alb, albumin.
suggest that our bioinformatic analysis of the db/db gene set appears to correctly identify kidney-derived proteins secreted in normal human urine.

We used gene set enrichment analysis (27) to associate the putative DKD biomarker mRNAs with biological functions that might be relevant to DKD. We used annotations from the Gene Ontology Consortium and Ingenuity Knowledge Database to identify biological functions associated with all 753 differentially expressed mRNAs in the db/db gene set (Fig. 3A). The 36 biomarker candidates were significantly enriched in annotations for cell death, cellular growth, cell-cell signaling, inflammation, and differentiation of epithelial cells (Fig. 3B). Less frequently, the biomarker candidates were associated with annotations for quantity of lipids and β-glucose, and for

Fig. 5. The selected urinary biomarker candidates were inversely associated with estimated glomerular filtration rate (eGFR) in a pilot study of participants with type 2 diabetes. A: albumin; B: ET-1; C: TGF-β; D: LCN2; E: GDF15; F: MCP-1; G: IL-6; H: FGF. Spot urine collections were obtained from n = 56 participants with type 2 diabetes and a range of eGFRs. GFR was estimated (Modification of Diet in Renal Disease equation) using clinical data obtained at the study visit. Urine protein levels of the biomarker candidates were measured by ELISA and normalized for creatinine. Urinary albumin normalized to creatinine was measured by ELISA in the same spot collection. The inset plots eGFR vs. urine albumin/creatinine in the range 0–600 mg/mg. The regression line (solid) and 95% confidence lines (dashed) are indicated. The Pearson’s correlation coefficient r is reported.
Table 2. MDRD eGFR regressed on the urinary biomarker values unadjusted and adjusted for age, sex, race, and urine albumin/creatinine in type 2 diabetes participants

<table>
<thead>
<tr>
<th>Urine Biomarker</th>
<th>Coefficient ± SE</th>
<th>P value</th>
<th>Adjusted for Age, Sex, Race, and Albumin/Creatinine</th>
<th>Coefficient ± SE</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ET-1</td>
<td>−6.34 ± 2.09</td>
<td>&lt;0.0001</td>
<td>−6.26 ± 2.10</td>
<td>0.0045</td>
<td></td>
</tr>
<tr>
<td>TGF-β</td>
<td>−0.17 ± 0.07</td>
<td>0.0144</td>
<td>−0.19 ± 0.06</td>
<td>0.0064</td>
<td></td>
</tr>
<tr>
<td>LCN2</td>
<td>−0.30 ± 0.23</td>
<td>&lt;0.0001</td>
<td>−0.26 ± 0.09</td>
<td>0.0056</td>
<td></td>
</tr>
<tr>
<td>GDF15</td>
<td>−1.14 ± 0.23</td>
<td>&lt;0.0001</td>
<td>−1.10 ± 0.28</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>MCP-1</td>
<td>−0.15 ± 0.05</td>
<td>0.0043</td>
<td>−0.14 ± 0.05</td>
<td>0.0003</td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>−2.79 ± 0.74</td>
<td>0.0002</td>
<td>−3.03 ± 0.72</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
</tbody>
</table>

Values are unstandardized regression coefficient ± SE. MDRD, Modification of Diet in Renal Disease; ET-1, endothelin-1; TGF-β, transforming growth factor-β; LCN2, lipocalin-2; GDF15, growth and differentiation factor-15; MCP-1, macrophage chemoattractant protein-1; IL-6, interleukin-6. Multiple linear regression was used to control for potential effects of age, sex, race, and albumin-to-creatinine ratio on the association of eGFR with urine biomarker levels. P values are two-sided.

differentiation of connective tissue cells. Although the db/db gene set was highly enriched in annotations related to oxidative stress (Fig. 3A), only one biomarker gene (TGF-β1) was assigned to this category (Fig. 3B).

Urine levels of six biomarker candidates were higher in patients with type 2 diabetes compared with controls. The small volume of urine excreted by mice precluded quantitative measurement of the putative biomarker proteins by ELISA. We, therefore, measured six biomarker candidates in the urine of patients with type 2 diabetes. We considered biomarker candidates previously reported to function in the biological processes identified as dysregulated in the db/db kidneys in Fig. 3. Biomarker candidates were considered for analysis only if ELISAs were commercially available. Using these criteria, we identified six candidates for further testing: ET-1 (EDN1), LCN2-NGAL (LCN2), TGF-β (TGF-β1), GDF15, MCP-1, and IL-6. Urine FGF was measured as a negative control.

We determined whether the six biomarker candidates were elevated in urine samples from patients with type 2 diabetes compared with nondiabetic healthy controls. Spot urine specimens were obtained from 56 patients with type 2 diabetes and 12 nondiabetic healthy controls without apparent renal disease. Demographic and clinical information is shown in Table 1. The mean age and proportion of African-Americans in healthy controls and diabetic participants were similar. The mean duration from time of initial diagnosis of type 2 diabetes was 7.0 yr. As anticipated, mean eGFR was lower, and the urine albumin-to-creatinine ratio (ACR) higher, in the diabetic participants (Table 1).

Urine ET-1, TGF-β, GDF15, MCP-1, IL-6, and albumin were significantly (P < 0.05) elevated in participants with type 2 diabetes compared with healthy controls (Fig. 4). Urine LCN2 was also elevated in type 2 diabetes, and the difference approached statistical significance. Urine FGF was not significantly changed in patients with type 2 diabetes compared with controls (Fig. 4). These results are consistent with the patterns of gene expression for these biomarkers observed in db/db kidneys (Figs. 1 and 2).

Urine levels of six biomarker candidates correlated inversely with eGFR. In participants with type 2 diabetes, urinary levels of the six biomarker candidates correlated inversely with eGFR (Fig. 5). The correlation coefficients of eGFR with ET-1, TGF-β, LCN2, GDF15, MCP-1, and IL-6 were statistically significant. The urine biomarker candidates correlated with eGFR more strongly than ACR. Urine FGF was not significantly correlated with eGFR (Fig. 5). We used multiple linear regression to adjust the association of eGFR and the biomarker candidates for age, sex, race, and ACR, which did not alter the regression coefficients (Table 2). The duration of diabetes, Hba1c, and the use of angiotensin-converting enzymes or angiotensin II type 1 receptor blockers did not affect the coefficients when examined separately in the regression model (data not shown). Taken together, these results suggest that urine levels of the putative biomarkers were independently associated with eGFR.

The renal fractional excretion of ET-1, TGF-β, and LCN2-NGAL in 20 patients with type 2 diabetes selected at random was −2.0% or lower: ET-1, 1.8 ± 2.1% (mean ± SD); TGF-β, 0.9 ± 1.4%; LCN2-NGAL, 2.1 ± 1.7%. Moreover, fractional excretion of ET-1, TGF-β, and LCN2-NGAL was not significantly correlated with their serum levels or eGFR (data not shown). Collectively, these data suggest that the amount of ET-1, TGF-β, and LCN2-NGAL filtered at the glomerulus contributed relatively little to the amount present in the urine, as previously reported in patients with chronic kidney disease (7, 23).

As shown in Fig. 3, the six selected biomarkers had gene ontology annotations for different biological functions that are relevant to DKD. We, therefore, asked whether a multivariable model with the biomarkers and urine albumin as variables would improve prediction of eGFR. We restricted our analysis in this pilot study to urine albumin with only two biomarker candidates to minimize the possibility of overfitting. Goodness of fit was assessed by R² in a multiple linear regression model. All binary combinations of the six putative biomarkers with urine albumin improved prediction of eGFR compared with regression with albumin alone (data not shown). The most robust combination of predictor variables was IL-6, GDF15, and albumin. The combination of albumin, IL-6, and GDF15 had a higher R² value than either marker alone (Table 3).

Association of urine biomarker candidates with proximal tubule damage. Recent studies suggest that damage to the proximal tubule is a determinant of early renal insufficiency in diabetes (54). A well-established noninvasive surrogate for proximal tubule damage is elevated excretion of NAGase, a 140-kDa brush border lysosomal enzyme that is released posttranslationally by proximal tubules after injury (13). Moreover, the high molecular weight of NAGase precludes glomerular filtration, making urine NAGase activity a relatively specific marker of tubular injury. We thus asked whether the urinary
level of any of the six biomarkers was associated with elevated NAGase excretion in participants with type 2 diabetes. Urine levels of ET-1, GDF15, and IL-6 were significantly positively correlated with increasing NAGase activity (Fig. 6) and remained significant after adjusting for age, sex, race, and ACR by multiple linear regression (data not shown). The association of urine TGF-β1, MCP-1, LCN2, and FGF with NAGase was not statistically significant (TGF-β1 r = 0.176, P = 0.140; MCP-1 r = 0.244, P = 0.065; LCN2 r = 0.196, P = 0.153; FGF r = 0.011, P = 0.442).

**DISCUSSION**

We sought to identify noninvasive urinary biomarkers that complement microalbuminuria in defining the degree of DKD, as assessed by eGFR. Unlike previous biomarker studies of DKD that utilized proteomic profiling in human urine (38, 40, 47), we identified candidate biomarkers from a computational analysis of a gene expression profile of the murine kidney in type 2 diabetes. In a cross-sectional study, higher urine levels of six selected candidates correlated with subnormal eGFR <90 ml·min⁻¹·1.73 m⁻², independent of age, sex, race, and ACR.

We identified candidate biomarkers of DKD by analyzing the renal gene expression profile of db/db compared with control db/m mice. Our rationale for using this comparative genomic strategy was that analogous bioinformatic analyses of gene expression profiles from murine models of cancer have identified secreted proteins that appear to be promising biomarkers for diagnosis and prognosis of human tumors (6, 35).

We evaluated differentially expressed genes in 8- and 16-wk old db/db kidneys. Eight-week-old db/db mice exhibit insulin resistance with sustained hyperglycemia, but have normal renal histology. In contrast, kidneys of 16-wk old db/db mice show mesangial expansion, basement membrane thickening, leukocyte infiltration, and modest increments in albuminuria without a measurable decline in GFR (49). Thus the phenotypic transition from 8 to 16 wk represents the murine kidney’s early phenotypic changes associated with type 2 diabetes.

We identified 36 differentially expressed genes in db/db kidney that were predicted to encode secreted proteins. None of the putative biomarker mRNAs were altered in 8- or 16-wk-old nondiabetic db/m kidneys, suggesting that differential expression of these mRNAs specifically reflects changes attributable to diabetes in db/db mice. All 36 of the putative biomarkers are represented by a peptide signature in the Human Kidney and Urine Proteome Project database of normal human urine, suggesting that our identified candidate DKD biomarker proteins are present in human urine. Several of the urinary biomarkers identified in our study (i.e., MCP-1, TGF-β, and ET-1) have been previously reported to be elevated in patients with type 2 diabetes and subnormal eGFR (25, 37), confirming the utility of our comparative genomic approach for biomarker discovery in DKD.

Elevated circulating GDF15 was reported in type 2 diabetes (12, 13), and urinary GDF15 has recently been shown to be elevated in patients with diabetic nephropathy (14). We now report the urinary GDF15 concentration as a potential biomarker for proximal tubule damage in patients with type 2 diabetes. Urinary GDF15 levels were significantly elevated in participants with type 2 diabetes (Fig. 5A), and were correlated with subnormal eGFR (Fig. 5B). Notably, the association of GDF15 with albuminuria and with subnormal eGFR remained significant after adjusting for age, sex, race, and ACR by multiple linear regression (data not shown). The association of GDF15 with urinary albumin or with subnormal eGFR was not statistically significant (r = 0.244, P = 0.065; r = 0.289, P = 0.031).

**Fig. 6.** Urinary ET-1 (A), GDF-15 (B), and IL-6 (C) correlated with a urine marker of proximal tubule damage, N-acetyl-β-D-glucosaminidase (NAGase) activity. Enzyme activity of NAGase was assessed in spot urine collections (n = 56) and correlated with ET-1, GDF-15, and IL-6 measured by ELISA in the same specimen. Biomarker values and NAGase activity are normalized for urine creatinine. The regression line (solid) and 95% confidence lines (dashed) are indicated. Pearson’s correlation coefficient r is reported.
18) and in patients with type 1 diabetes and progressive nephropathy (31), but urine GDF15 was not measured. Most of the 36 putative biomarkers identified in our experiments (Fig. 1) have not been previously examined in relation to DKD in murine models or in humans with type 2 diabetes (37) and warrant further investigation using methods for their quantitative analysis.

Urine levels of a selected set of DKD biomarkers correlated with reduced eGFR in patients with type 2 diabetes more closely than urine albumin. The correlation coefficients for ET-1, TGF-β, LCN2, GDF15, MCP-1, and IL-6 (range −0.302 to −0.478) were greater than that for urine albumin (~0.248). In a preliminary analysis using a multivariable model of eGFR, R² was significantly increased when urinary biomarker values were combined with measurements of urine albumin excretion. Because secreted (vs. filtered) urinary proteins account for ~70% of the urine proteome [for review see Decramer et al. (15) and references therein], it is likely that the observed changes in the selected set of six urinary biomarkers reflect renal secretion. Although determination of the source of the urinary biomarkers does not alter our finding of their relationship to renal disease and does not directly bear on the relevance of our results, the question of whether the biomarkers are predominantly filtered or secreted is of scientific interest. We found that the fractional excretion of ET-1, TGF-β, and LCN2-NGAL ranged from 0.9 to 2.1%, providing additional evidence that the content of these biomarkers in the urine predominantly reflects their renal secretion. Adjustment of our results by multiple linear regression suggested that age, sex, race, and ACR did not confound the relationship of eGFR to the urine biomarkers. Importantly, adjustment of the data for duration of diabetes, medication, or cardiovascular disease did not significantly reduce the regression coefficients of the biomarkers. Whether measurement of these biomarkers will prove useful in patients with type 1 diabetes remains to be explored.

Our results suggest that elevated urinary excretion of ET-1, GDF15, and IL-6 is associated with a marker of proximal tubule injury independent of ACR. Damage to the proximal tubule has emerged as a determinant of tubulo-interstitial inflammation and fibrosis in diabetic nephropathy (29, 54). Elevated excretion of NAGase, a surrogate for proximal tubule damage (13), is associated with diminished eGFR in cross-sectional (44) and longitudinal (29) studies of diabetes. We found that urine levels of ET-1, GDF15, and IL-6 are associated with NAGase. The association of NAGase with ET-1, GDF15, and IL-6 remained significant after correcting for age, sex, race, and ACR, suggesting that elevated urinary levels of these three proteins may reflect tubular injury in diabetes.

In summary, we demonstrate that a comparative genomic strategy based on gene expression profiling in the murine kidney can identify candidate biomarkers of DKD in type 2 diabetes. Additional studies of the murine kidney transcriptome, particularly in recently developed models of progressive renal insufficiency (8), may complement proteomic profiling of human urine in the search for new DKD biomarkers. Further longitudinal studies on a cohort of patients with type 2 diabetes are necessary to determine whether measurement of the urinary biomarker candidates identified herein can identify those who have normal eGFR, but are at a high risk for development of DKD.

ACKNOWLEDGMENTS

The authors thank Drs. Patrick Leahy and Martina Veigl in the Gene Expression and Genotyping Core Facility of the Case Comprehensive Cancer Center [National Institutes of Health (NIH) P30 CA43703] for help analyzing the mouse microarray data. We also thank Laura Moore, Lynn Richardson, Shahdi Malakooti, Tanya Kulow, and Erica Andrews for help recruiting participants and collecting clinical data.

GRANTS

This work was supported by the Rosenberg Foundation of the Centers for Dialysis Care, the Divisions of Nephrology and Hypertension and Endocrinology at Case Western Reserve University, and the University Hospitals Case Medical Center. This work was also supported by the Case Western Reserve University/Cleveland Clinic CTS Grant no. U11 RR024989 from the National Center for Research Resources, a component of the NIH and NIH roadmap for Medical Research.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

M.S.S., S.M.D., M.R., B.B., and D.H. designed and planned the research; M.R., B.B., D.H., and F.I.-B. performed experiments; M.S.S., M.T., S.M.D., M.R., B.B., D.H., and F.I.-B. analyzed the data; M.S.S. and F.I.-B. drafted the manuscript; M.S.S., M.T., S.M.D., M.R., B.B., D.H., and F.I.-B. critically revised and approved the final version of the manuscript.

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