Advances in early biomarkers of diabetic nephropathy

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Summary

Diabetic nephropathy is the main cause of chronic kidney disease, and represents the most common and serious complication of diabetes. The exact pathogenesis is complex and not elucidated. Several factors and mechanisms contribute to the development and outcome of diabetic nephropathy. An early diagnosis and intervention may slow down disease progression. A variety of biological markers associated with diabetic nephropathy were found in recent years, which was important for predicting the occurrence and development of the disease. Therefore, this article provides an overview of early biomarkers that are associated with diabetic nephropathy.

Keywords: Diabetes Mellitus. Diabetic Nephropathies. Biomarkers.

Introduction

Diabetes mellitus (DM) is an endocrine and metabolic disease that has serious impact on human health. The morbidity and mortality of DM have risen continually at an alarming rate in recent years, and the population with diabetes mellitus is predicted to be about 439 million worldwide by 2030.1 The complications of DM include diabetic retinopathy, diabetic cardiovascular diseases and diabetic nephropathy (DN), which is the most common and serious complication of DM. DN has become the leading cause of chronic kidney failure, starting with normoalbuminuria, microalbuminuria, macroalbuminuria and ultimately leading to end stage renal disease (ESRD).2 For a long time, proteinuria has been considered the gold standard for evaluation and monitoring of renal function. However, renal function declines in about one-third of the patients before the occurrence of proteinuria,3 which makes it inadequate to detect proteinuria alone to monitor the incidence and progression of DN. Therefore, we need to look for laboratory biomarkers that are earlier than microalbuminuria or those appearing at the same time. This review focuses on the early biomarkers associated with the pathogenesis and pathology of DN and changes in renal function.

Biomarkers associated with DN pathogenesis

A large number of prospective studies confirm that hyperglycemia is the most important risk factor for DN.4,5 Hyperglycemia promotes mitochondrial electron transport chain to generate excessive reactive oxygen species (ROS) through formation of the advanced glycation end products (AGEs) and activation of the polyol pathway, hexosamine pathway, protein kinase C (PKC) and angiotensin II. Then, the ROS initiate or enhance the oxidative stress and eventually cause the inflammatory response and formation of fibrosis.6,7 In addition, lipid metabolism abnormality, renin-angiotensin-aldosterone system (RAAS) activation, systemic and glomerular hypertension, insulin signaling impairment, increased growth factors and pro-inflammatory cytokines, and intracellular signaling pathway activation also play a role in the occurrence and progression of DN.6,8

Biomarkers of oxidative stress

The occurrence and progression of DN is closely related with oxidative stress. Excessive ROS, which are induced by hyperglycemia, are involved in oxidative stress causing direct oxidation and damage of deoxyribonucleic acid (DNA), proteins and lipids.8,9

Biomarkers of DNA injury

8-hydroxy-2′-deoxyguanine (8-OHdG) is a sensitive biomarker of DNA damage to assess oxidative stress in the human body. In 1994, Ha et al.10 found that the 8-OHdG levels were significantly higher in cortex and nipples of diabetic mice induced by streptozotocin than in control mice, and they decreased after insulin treatment, which
suggested that DN might be associated with oxidative stress and the formation of 8-OHdG. The following study by Hinokio et al. showed that urinary 8-OHdG excretion in patients suffering from type 2 diabetes mellitus complicated by nephropathy was higher than in patients without complications or in healthy control subjects. Moreover, there was a correlation between urinary 8-OHdG level and glycosylated hemoglobin (HbA1c). In this report, 8-OHdG was speculated to be a useful biomarker associated with complications secondary to DM. Zhao et al. measured the serum concentration of 8-OHdG using enzyme-linked immunosorbent assay (ELISA) and drew a similar conclusion. However, Serdar et al. demonstrated that there was no difference in urinary 8-OHdG levels between the groups with and without diabetic nephropathy on liquid chromatography-mass spectrometry, suggesting that 8-OHdG in urine was not a sensitive biomarker regarding albumin to creatinine ratio (UACR) for distinguishing DN patients from DM patients. Different biological fluids and methods might contribute to the lack of consistency in these studies, so that the predictive value of 8-OHdG in the early stages of DN needs further research to be determined.

**Biomarkers of protein and lipid injury**

Biomarkers associated with protein injury comprise pentosidine, 2,4-dinitrophenylhydrazine (DNPH) and advanced oxidation protein product (AOPP). F2-isoprostaglandin and 4-hydroxy-nonenal (HNE) are related to lipid injury. Calabrese et al. found that both urinary and serum levels of pentosidine, DNPH, F2-isoprostaglandin and HNE of DN patients were higher than those of control subjects. Tabak et al. showed that the level of AOPP in type 2 diabetes mellitus patients with complications such as DN and diabetic retinopathy was significantly higher than in patients without complications. These two studies have confirmed that oxidative stress damage is involved in the development of diabetic nephropathy.

**Biomarkers of glutathione antioxidant system and lipid peroxidation**

A growing number of studies reported that DM and its complications were closely related to oxidative stress, so we supposed that the biomarkers related to antioxidant defense system and lipid peroxidation (LPO) induced by free radicals may be potential biomarkers of kidney damage in diabetic patients. Glutathione s-transferase (GST), a kind of enzyme involved in cell detoxification, promotes inactivation and excretion of toxins by combining toxic drophobic compounds with glutathione.

Experimental data from a study by Jiang et al. showed that the expression level of GST in diabetic rats induced by streptozotocin was remarkably higher than in control rats, suggesting that hyperglycemia may be the major cause for elevated GST. Eight weeks after treatment with resveratrol, the GST expression decreased and several indicators suggesting the occurrence of DN such as urinary protein excretion, creatinine, cellular apoptosis and renal hypertrophy were all improved, leading researchers to suppose that resveratrol likely played a role in renal protection by lowering the expression level of GST. In agreement with GST, animal experiments on LPO have yielded the same results. In addition, genetic investigation also found that knockout of GST coding genes can lead to decreased GST levels and increased malondialdehyde (MDA) levels, an important biomarker of LPO, demonstrating that GST has an effect against oxidative stress.

Human research was consistent with the experimental studies above. Compared with healthy subjects, increased activity of GST and increased level of MDA were found in type 2 diabetes mellitus patients. These results suggested that oxidative stress was involved in the occurrence of DM and GST was likely to play an important role in antioxidation. In the study about GST and DN, Noce et al. reported that GST activity in type 2 diabetes mellitus patients with and without nephropathy were both significantly higher than that of control subjects, appearing to be closely related with the stages of DN and indicating that GST was likely to be a potential biomarker in early stage DN.

**Biomarkers of inflammation**

Inflammatory response could be activated by biochemical, metabolic or hemodynamic disorders when a large number of white blood cells gather in the kidney. Then, pro-inflammatory cytokines and a variety of chemokines secreted by leukocytes may guide the latter into the kidney directly. Thus, a new cycle of inflammatory response is induced. The inflammatory cytokines and chemokines involved were hypothesized as potential biomarkers of DN. Liu et al. detected urinary levels of 27 kinds of inflammation-related factors of type 2 diabetes mellitus patients by multiplex-27 bead immunoassay. They found that the levels of proinflammatory cytokines such as interleukin-8 (IL-8), tumor necrosis factor (TNF-α) and chemokines such as monocyte chemoattractant protein-1 (MCP-1), interferon-inducible protein-10 (IP-10) in patients with microalbuminuria were all significantly higher than those of patients with normoalbuminuria and...
the control subjects. Besides, the levels of MCP-1 and IP-10 were positively correlated with proteinuria and HbA1c, while negatively correlated with the estimated glomerular filtration rate (eGFR). These outcomes suggest that urinary inflammation-related factors may contribute to the diagnosis in early stages of DN.

In addition, some studies have shown that serum interleukin-18 (IL-18) level was elevated in DN patients and associated with HbA1c or UACR, thus being speculated as a potential biomarker of diabetic nephropathy. On the other hand, the value of interleukin-6 (IL-6) in early diagnosis of diabetic nephropathy remains to be further confirmed. A number of studies have found that serum IL-6 levels of patients with normoalbuminuria or microalbuminuria were higher than those of control subjects and showed a positive correlation with UACR. However, some other studies have found that serum IL-6 level was elevated in patients with macroalbuminuria alone, and its early diagnosis value was not as good as that of urinary albumin excretion.

Some studies demonstrated that an increase in both urinary and serum levels of TNF-α in patients with nephropathy secondary to DM was found compared to those with normoalbuminuria and control subjects. Besides, levels of TNF-α in urine and serum were both significantly associated with urinary albumin excretion. These results revealed that TNF-α might be an early biomarker of kidney damage in diabetic patients. Soluble CD40 ligand (sCD40L) is a transmembrane protein of the tumor necrosis factor superfamly and regulates inflammatory response by binding with CD40. A study by El-Asrar et al. showed that serum sCD40L level in type 1 diabetes mellitus patients with microangiopathy such as diabetic nephropathy, retinopathy or neuropathy was significantly higher than that of patients without complications and healthy control subjects, and diabetic patients without any of these complications presented higher sCD40L concentration as compared to healthy subjects. The researchers also found that serum sCD40L was significantly associated with the severity of kidney damage and the level of glycemic control.

In addition to the biomarkers cited above, glycosyl hydrolase family of 18 members, including chitotriosidase (CHIT1) and cartilage glycoprotein 40 (YKL-40), commonly activated by macrophages cells and neutrophils, were also involved in the inflammatory response. Several studies showed that both CHIT1 activity and YKL-40 level of type 2 diabetes mellitus patients in all subgroups were higher than that of control subjects. CHIT1 activity and YKL-40 level increased gradually along with the stages of DN according to UACR, which was correlated with activity of CHIT1 and level of YKL-40 even after adjustment for clinical parameters, suggesting that they were both associated with kidney damage of DN patients. However, because of the higher sensitivity and specificity, CHIT1 activity was better in the diagnosis of persistent microalbuminuria compared with serum level of YKL-40.

**Biomarkers of RAAS activation**

Renin-angiotensin-aldosterone system (RAAS) plays an important role in regulating blood pressure by producing aldosterone in human body. Angiotensinogen, produced by liver, was reported in patients with chronic glomerulonephritis in a previous study. The following study found that urinary angiotensinogen excretion of type 2 diabetes mellitus patients with microalbuminuria and macroalbuminuria were both significantly increased compared to control subjects, as well as to normoalbuminuric patients, suggesting that angiotensinogen appeared prior to the establishment of albuminuria. Also, angiotensinogen level shows a strong association with urinary albumin excretion, which is an indicator of the severity of kidney damage in diabetic patients. Angiotensinogen may be a promising biomarker in the early stages of DN due to its high sensitivity and specificity in diagnostic analysis of diabetic nephropathy.

These biomarkers were summarized in Figure 1.

**Biomarkers associated with DN pathology**

**Biomarkers of damage of glomerular filtration membrane**

Under normal circumstances, podocyte and foot process, glomerular basement membrane and capillary endothelial cells constitute the glomerular filtration barrier. The damage of this filtration barrier can affect the glomerular filtration function. Markers such as podocytes, basement membrane and endothelial cell damage may have potential to indicate kidney damage in DN patients.

**Biomarkers of podocytes injury**

Studies have shown that a decline in the number of podocytes and disappearance of foot processes often occur in the early stages of DN due to apoptosis or shedding of podocytes. Therefore, urinary podocytes and their specific protein products may be regarded as potential biomarkers of podocyte injury. Currently, the studies focused on the podocyte-specific protein products because it was difficult to detect urinary podocytes directly. One study by Wang et al. showed that urinary mRNA levels of podocin, synaptopodin and nephrin in DN patients were extremely higher than those found in control subjects.
by real-time quantitative PCR. These results were also proved by renal biopsy. Also, synaptopodin level was positively correlated with urinary albumin excretion and serum creatinine concentration while negatively correlated with GFR. Patients, however, were not divided into different subgroups according to their average level of urinary protein. The validation of these podocyte-specific protein products in early stages of DN was not confirmed in this study.\textsuperscript{41} Further research performed by Hara et al. revealed that urinary synaptopodin level of type 2 diabetes mellitus patients complicated by nephropathy was higher when compared to control subjects, even before the occurrence of proteinuria and associated with the level of urinary albumin and HbA\textsubscript{1c}, indicating that synaptopodin was a biomarker with high sensitivity to podocyte injury in diabetic patients.\textsuperscript{42} Another report by Jim et al. revealed that nephrin level in urine was elevated in all DN patients and 54\% of normoalbuminuric subjects. In addition, urinary level of nephrin showed a strong association with UACR so that it might be a useful biomarker for nephropathic patients in preclinical stage.\textsuperscript{43}

**Biomarkers of basement membrane injury**

Type IV collagen is the main component of the glomerular basement membrane and extracellular matrix, and does not pass through glomerular filtration barrier under normal circumstances. Therefore, type IV collagen could be used as a biomarker of basement membrane injury. The study found that urinary type IV collagen levels were higher before microalbuminuria and associated with urinary albumin and serum creatinine, suggesting that urinary type IV collagen may be a promising biomarker for early diagnosis of DN.\textsuperscript{44}

**Biomarkers of endothelial cells injury**

Endothelial cells injury can directly affect the permeability of the glomerular filtration membrane. Generally, von Willebrand factor (vWF) is mostly synthesized by endothelial cells. Plasma vWF levels increase when endothelial cells are stimulated or damaged. Jensen\textsuperscript{45} first discovered that plasma levels of vWF are higher in type 1 diabetes mellitus patients, indicating that there is endothelial cell dysfunction in diabetic patients. Subsequently, a number of studies have shown that plasma vWF levels in patients with DN are significantly higher than those in patients without kidney disease and control subjects, indicating that plasma vWF may contribute to the early diagnosis of diabetic nephropathy.\textsuperscript{46-48}

Hyperglycemia does aggravate vascular endothelial injury by up-regulating the expression of adhesion molecules by endothelial cells.\textsuperscript{49} The study about type 2 diabetes mellitus patients from Malaysia discovered that plasma levels of intercellular adhesion molecule-1 (ICAM-1) are elevated in DN patients.\textsuperscript{50}

Vascular endothelial growth factor (VEGF) can affect the filtration of large molecular weight proteins through
glomerular filtration barrier by promoting endothelial cell proliferation and increasing vascular permeability. Researchers have found that plasma and urinary levels of VEGF in DN patients were both elevated. Especially in type 2 diabetes mellitus subjects, urinary VEGF level was higher in normoalbuminuric patients than in control subjects and gradually increased along with the DN stages. These findings suggested that VEGF may be an effective biomarker for early diagnosis in DN patients.

Biomarkers of mesangial expansion and fibrosis
Fibrosis is one of the pathological features of diabetic complications caused by extracellular matrix alterations and mesangial expansion. Hyperglycemia up-regulates the expression of transforming growth factor-β1 (TGF-β1), which is considered to be the most crucial cytokine in glomerulosclerosis and tubulointerstitial fibrosis. Data by Xie showed that serum TGF-β1 level of patients with microalbuminuria was significantly higher than that of patients with normoalbuminuria and control subjects. Interestingly, urinary levels of TGF-β1 are already elevated in normoalbuminuria subjects and gradually increase along with DN progression, so that TGF-β1 was considered a sensitive biomarker in the early phase of diabetic nephropathy.

Pigment epithelial-derived factor (PEDF) is a member of the serine protease superfamily and is involved in the formation of extracellular matrix and vascular endothelial growth factor. PEDF levels were found to be decreased in the kidney of diabetic mice, suggesting that it may have a protective effect in diabetic microvascular lesions. Researchers also found that urinary PEDF levels in DN patients are significantly higher than in control patients, indicating that PEDF is probably an effective biomarker of DN.

These biomarkers were summarized in Table 1.

Biomarkers associated with renal function changes
The level of proteinuria in the early stages of DN can tell us whether there is glomerular damage or not and the extent of the damage. Investigation of proteinuria continues to be the gold standard for diagnosis and staging of DN. In addition, albumin, transferrin (TRF), ceruloplasmin (CER) and immunoglobulin G (IgG) in urine can also reflect functional changes in glomerular filtration. Narita et al. found that urinary levels of TRF, CER and IgG in normoalbuminuric patients were significantly higher than those in control subjects and they strongly correlated with each other, indicating that TRF, CER and IgG may be more sensitive makers for changes in filtration function than albuminuria in the early stages of DN.

Biomarkers of renal tubular dysfunction
Tubulointerstitial injury plays an important role in DN development process and even prior to glomerular injury. In addition, about one third of the patients with diabetes mellitus have decreased renal function prior to proteinuria. Therefore, we should pay more attention to the biomarkers of tubulointerstitial injury, which can contribute to the early diagnosis and treatment of DN patients.

- a1-Microglobulin, retinol-binding protein 4 (RBP4) and other low molecular weight proteins can freely pass through the glomerular filtration membrane and then be reabsorbed in the tubules. These were early biomarkers of tubular injury because of their increase in urine after renal tubular damage. Researchers found that urinary levels of a1-microglobulin and RBP4 in patients with normoalbuminuria were significantly higher than those in control subjects and were both associated with the levels of HbA₁c, so that detection of two biomarkers may be helpful for early diagnosis of diabetic nephropathy.

- Some other biomarkers of tubular injury, such as neutrophil gelatinase-associated lipocalin (NGAL), N-acetyl-β-D-glucosidase (NAG), kidney injury molecule-1 (KIM-1) and heart-type fatty acid binding protein (H-FABP) applied only to predict acute kidney injury.

A recent study discovered that urinary levels of NGAL, NAG, KIM-1, H-FABP of patients with normoalbuminuria were significantly higher than those of control subjects and increased gradually along with the DN stages. In addition, they all significantly correlated with urinary albumin levels, indicating that they might be early biomarkers for DN diagnosis.

These biomarkers were summarized in Table 2.

Conclusion
In recent years, there has been an important achievement regarding the finding of associated biomarkers in all aspects of diabetic nephropathy. These research findings contribute greatly to our understanding of disease mechanisms. So far, there is no biomarker that can replace proteinuria. We believe that advances in research methods based on genomics, proteomics and metabolomics will provide much more convenience in future. However, we should also take all the questions into our consideration, such as the fact that there is no universally accepted standard for subject inclusion and staging, not all researches made adjustment for significant parameters and few studies have discussed the effectiveness of multi-biomarker detection.
TABLE 1  Summary of biomarkers associated with DN pathology.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Mechanism</th>
<th>Sample</th>
<th>Method</th>
<th>Study object</th>
<th>Level in DN</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Podocin</td>
<td>Podocytes injury</td>
<td>Urine</td>
<td>RT-QPCR</td>
<td>21 DN patients by biopsy</td>
<td>9 —</td>
<td>Increased [42]</td>
</tr>
<tr>
<td>Synaptopodin</td>
<td>Podocytes injury</td>
<td>Urine</td>
<td>RT-QPCR</td>
<td>21 DN patients by biopsy</td>
<td>9 —</td>
<td>Increased [42]</td>
</tr>
<tr>
<td>Nephrin</td>
<td>Podocytes injury</td>
<td>Urine</td>
<td>RT-QPCR</td>
<td>21 DN patients by biopsy</td>
<td>9 —</td>
<td>Increased [42]</td>
</tr>
<tr>
<td>Type IV collagen</td>
<td>Basement membrane injury</td>
<td>Urine</td>
<td>ELISA</td>
<td>698 DM (264 normo/169 micro/181 macro/84 renal failure)</td>
<td>191 —</td>
<td>Increased [45]</td>
</tr>
<tr>
<td>vWF</td>
<td>Endothelial cells injury</td>
<td>Plasma</td>
<td>ELISA</td>
<td>109 (66 normo/26 micro/17 macro)</td>
<td>— 31 nondiabetic</td>
<td>Increased [47]</td>
</tr>
<tr>
<td>VEGF</td>
<td>Endothelial cells injury</td>
<td>Plasma</td>
<td>ELISA</td>
<td>387 T1DM (188 normo/199 DN)</td>
<td>— —</td>
<td>Increased [52]</td>
</tr>
</tbody>
</table>

DN: diabetic nephropathy; T2DM: type 2 diabetes mellitus; Ref: reference; RT-QPCR: real-time quantitative polymerase chain reaction; ELISA: enzyme-linked immunoassay assay; normo: normoalbuminuria; micro: microalbuminuria; macro: macroalbuminuria; DM: diabetes mellitus; vWF: von Willebrand factor; VEGF: vascular endothelial growth factor; T1DM: type 1 diabetes mellitus; TGF-β1: transforming growth factor-β1; PEDF: pigment epithelial-derived factor. Control 1: healthy subjects; control 2: not healthy subjects. #: increased prior to albuminuria.

TABLE 2  Summary of biomarkers associated with renal function changes.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Mechanism</th>
<th>Sample</th>
<th>Method</th>
<th>Study object</th>
<th>Level in DN</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRF/CER/IgG</td>
<td>Glomerular dysfunction</td>
<td>Urine</td>
<td>IRMA</td>
<td>61 (61 normo)</td>
<td>17 —</td>
<td>Increased [59]</td>
</tr>
<tr>
<td>a1-microglobulin</td>
<td>Renal tubular dysfunction</td>
<td>Urine</td>
<td>Latex immunoassay</td>
<td>587 (375 normo/181 micro/31 macro)</td>
<td>— —</td>
<td>Increased [62]</td>
</tr>
<tr>
<td>RBP</td>
<td>Renal tubular dysfunction</td>
<td>Urine</td>
<td>ELISA</td>
<td>59 T1DM (48 normo/11 micro)</td>
<td>40 —</td>
<td>Increased [63]</td>
</tr>
<tr>
<td>NGAL/NAG/</td>
<td>Renal tubular dysfunction</td>
<td>Urine</td>
<td>ELISA</td>
<td>94 DM (41 normo/41 micro/12 macro)</td>
<td>— 45 nondiabetic</td>
<td>Increased* [66]</td>
</tr>
</tbody>
</table>


REFERENCES


