Gene and protein markers of diabetic nephropathy

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Diabetic nephropathy (DN) develops in a large number of type 1 (T1D) and type 2 diabetic (T2D) patients after a variable latency period. DN absolute number is increasing because of greater longevity and the epidemic of diabetes [1]. Although this disease is progressively becoming a heavier burden for the health care system, it is as yet poorly understood in many aspects. The pathogenesis of DN is clearly multifactorial and several genes, proteins and environmental factors are likely to contribute to the onset of the disease. Several metabolic, haemodynamic and intracellular causes have been proposed to play a role in the pathogenesis of DN [2]. Acquired risks also participate in the development of kidney hyperfiltration [3]. As a primary initiator of DN, high glucose level is associated with an increased synthesis of cytokines and growth factors and with the diversion of glucose metabolism into at least three metabolic pathways, the polyol, the protein kinase C and the hexosamine pathways, as shown in Figure 1 (reviewed in [4]).

Due to the growing burden of the management of diabetes and its complications, it is important to identify DN predictors, in order to facilitate its diagnosis and treatment [5,6]. In this review, we first examine the genetic factors potentially associated with the disease, and then survey the literature on early urinary protein indicators of DN.

Genetic predisposition to DN

It is thought that specific genetic backgrounds might influence DN development. Indeed, only 30% of T1D and 40% of T2D patients will develop DN irrespective of their diabetic treatment [7], and DN often shows a familial clustering in siblings with diabetes.

The observation of a familial clustering of the disease strongly suggests that genetic factors are involved in the development of DN, whereas segregation analyses point to the existence of susceptibility genes [8,9] and have established that the onset and progression of DN are genetically determined [10,11]. The prevalence rates of DN also show marked ethnic variations. The reason for such inter-racial differences in incidence is unclear; nevertheless, genetic predisposition seems to constitute a major component in the development and/or progression of DN. Two strategies can be used to identify DN susceptibility loci: the linkage analysis (i.e. family-based studies) or the association analysis (i.e. case-control studies). Both have led to the discovery of many chromosomal and gene regions that may confer susceptibility to DN.

Linkage analysis

Genome-wide scans are valuable tools for the identification of loci that could include major genetic components of a disease. Genome-wide scans have identified several DN susceptibility loci. Two complete genome scans for DN have been performed in T2D sib pairs. In Pima Indians, four loci with multipoint LOD > 1.0 on chromosomes 3, 7, 9 and 20 were identified [12] and in African Americans, evidence for linkage was reported for chromosomes 3, 7 and 18 [13]. A partial genome-wide scan in Turkish families with type 2 DN reported linkage on chromosome 18 [14], a finding that was weakly replicated in Pima Indians (LOD = 0.3). Another genome-wide scan for type 1 DN in the Finnish population revealed a linkage to a single locus on chromosome 3q with a maximum LOD of 2.67 [15]. This locus has also been reported by others in T1D patients [16,17]. Table 1 recapitulates these studies.

Association analysis

Association studies are commonly used to test for association between traits (disease) and highly correlated single nucleotide polymorphisms (SNPs), within a candidate gene or region, selected from the literature and from the HapMap.
Aldose reductase

Aldose reductase (AKR1B1) is a cytosolic enzyme that, in the presence of NADPH, catalyzes the rate-limiting step of the polyol pathway converting glucose into sorbitol. Several mechanisms have been proposed to explain how AKR1B1 activity leads to hyperglycaemia-induced lesions in different tissues [22].

Ko et al. [23] were the first to identify seven alleles at the locus of the (AC)n dinucleotide repeat sequence upstream of AKR1B1. The most common allele contains 24 (AC) repeats and was named Z. Several studies have demonstrated a correlation between the Z-2 allele (23 repeats) and susceptibility to an increased risk of DN in both T1D and T2D [24–28]. However, other studies have reported a lack of association [29–31]. Interestingly, diabetic patients, who are homozygous for the Xbal (−) allele of the glucose transporter 1 gene and carry the Z-2 allele, present a 9-fold increased risk of DN, suggesting that this is a particularly high-risk genetic combination [32].

A second AKR1B1 polymorphism has been observed at position −106 of its promoter region. This C106T polymorphism was identified in both Caucasian and Asian subjects with T1D or T2D, and association with DN has been observed. Liu et al. [28] have suggested that the C106T polymorphism might be associated with an increased expression of AKR1B1 in the Chinese population. Neamat-Allah et al. [25] conducted a meta-analysis including seven studies and showed that the carriage of the −106 T allele was significantly associated with DN [OR = 2.2 CI95% (1.69–2.94)]. Sivenius et al. [33] and Gosek et al. [34] suggested that this polymorphism could be involved in the early development of microalbuminuria in Finnish T2D patients and is a risk factor for development of DN in T2D patients with poor glycaemic control, respectively. In the Makiishi et al. study [35], the TT genotype presented a risk for DN [OR = 4.7 CI95% (1.3–17)] in T2D Japanese subjects. All these results suggest that AKR1B1 polymorphisms play a role in DN development.

Peroxisome proliferator-activated receptor gamma 2

PPARG2 is the predominant adipose isoform of this receptor and is expressed selectively in the adipose tissue where it modulates the expression of target genes implicated in adipocyte differentiation and glucose homeostasis. PPARG2 is considered, therefore, a major candidate gene for T2D and/or obesity and, recently, for type 2 DN.

Insulin resistance may be a DN risk factor in type 2 diabetes [36] and improved insulin sensitivity could be the link between the Pro12Ala polymorphism of PPARG2 and the decreased risk of DN. Three studies have evaluated its association with type 2 DN. In the study by Herrmann et al. [37], the Pro12Ala polymorphism was associated with lower albumin excretion rates among Ala12 carriers with type 2 DN, which may indicate a protective effect of this allele. These findings were confirmed by Caramori et al. [38]. More recently, Pollex et al. [39] showed that the Ala12 allele carriers have reduced occurrence of microalbuminuria (1.5-fold reduction of the albumin/creatinine ratio). All

Angiotensin-converting enzyme

The angiotensin-converting enzyme (ACE) converts angiotensin I into the biologically active angiotensin II. Recently the DCCT/EDIC study reported a relative protective effect of the II genotype (insertion/insertion polymorphism: rs1799752) over the deletion allele (D) on the development of microalbuminuria and progression to overt proteinuria in 1300 T1D patients, followed for 17 years [10]. The II genotype is also associated with a better response to ACE inhibitors [18]. Interestingly, microalbuminuric T2D patients with the DD genotype present more severe glomerular lesions than patients with the I allele [19]. A meta-analysis including 47 studies (14727 subjects) [20] showed that subjects with the II genotype had a 22% lower risk of DN than carriers of the D allele [OR = 0.78, CI95% (0.69–0.88)]. In the Boright et al. study [10], the haplotype TIC/TIC (which corresponded to the rs1800764, rs1799752 and rs9896208 alleles) was associated with a lower risk for development of persistent microalbuminuria (P = 0.0009) and severe nephropathy (P = 0.006), whereas in the Hadjadj et al. study [21], the haplotype analysis showed that DGG (rs1799752, rs4366 and rs12449782) was associated with an increased risk for DN, compared with the ICA.

In summary, it appears that polymorphisms in the ACE gene may have a role in the progression of DN, rather than in the susceptibility to it.
Fig. 1. Pathways involved in the development of diabetic nephropathy. AGE: advanced glycation endproducts, DAG: diacyl glycerol, SLC2A1: glucose transporter 1, PPARγ: peroxisome proliferator-activated receptor gamma, ROS: reactive oxygen species and TGFβ: transforming growth factor beta.

Table 2. HUGO gene symbol and OMIM reference of the discussed genes

<table>
<thead>
<tr>
<th>Genes</th>
<th>HUGO Symbol</th>
<th>OMIM reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiotensin-converting enzyme</td>
<td>ACE</td>
<td>106180</td>
</tr>
<tr>
<td>Aldose reductase</td>
<td>AKR1B1</td>
<td>103880</td>
</tr>
<tr>
<td>Peroxisome proliferator-activated receptor gamma 2</td>
<td>PPARG2</td>
<td>601687</td>
</tr>
<tr>
<td>Glucose transporter 1</td>
<td>SLC2A1</td>
<td>138140</td>
</tr>
<tr>
<td>Carnosinase</td>
<td>CNDP1</td>
<td>609064</td>
</tr>
</tbody>
</table>

these results indicate a protective correlation between the Ala12 polymorphism and the albumin excretion rate. The mechanism underlying the protective effect of the Ala12 allele is yet unknown.

Glucose transporter 1

The glucose transporter (SLC2A1) is the major representative of the family of facilitative glucose transporters that are expressed in glomerular, mesangial, endothelial cells and podocytes. SLC2A1 is likely to be pivotal in raising intracellular glucose levels by activating pathogenic pathways [40–42].

Several works have tried to determine whether SLC2A1 might be a candidate gene conferring susceptibility to DN. These investigations have focused on testing whether the SLC2A1 Xbal SNP (rs841847) is associated with DN using a case-control study design. Although this Xbal SNP was found not to be associated with DN [43,44], heterozygosity for the Xbal (−) allele has been linked with a 1.9-fold higher susceptibility to nephropathy in Chinese T2D patients [45]. However, homozygosity for the Xbal (−) allele was also associated with an increased DN risk in other studies [32,46,47]. Inconsistent results have thus far been reported regarding the possible role of the SLC2A1 Xbal SNP in DN susceptibility. A recent meta-analysis concluded that there is, indeed, a significant association between the SLC2A1 Xbal polymorphic site and DN, but larger studies are needed [48].

Four SNPs have also been identified in SLC2A1 putative enhancer elements, i.e. enhancer-2 SNP1 (rs841847) and enhancer-2 SNP2 (rs841848), one in exon 2 (rs1385129) [47] and one in the 5’ promoter region (rs710218) [49]. The authors showed that the AA genotype of rs841847 is a ‘risk genotype’ (OR = 2.38) and that the TT genotype of rs710218 is associated with DN (P < 10−6). Moreover, they showed that patients with the AG haplotype (rs841847–rs841853) have an increased risk of DN with an OR = 2.4; also, the TT haplotype (rs710218–rs841853) is more frequent in nephropathic patients (P < 7 × 10−7) and might be associated with an enhanced glucose uptake rate via SLC2A1 in mesangial cells. Our recent findings showed that two haplotypes (composed of rs1385129–rs841847–rs841848) are associated with a 4.4- and 2.6-fold increased risk of nephropathy in the Tunisian T2D patients (to be published). In conclusion, the SLC2A1 gene seems to play an important role in the development of DN.
Carnosinase

Carnosine dipeptidase 1, a member of metallopeptidase M20 family, degrades the dipeptide carnosine. It reduces the synthesis of matrix components, fibronectin, collagen IV and TGFβ2 (transforming growth factor beta 2) in renal cell lines [50]. The carnosinase gene, CNDP1, both a functional and a positional candidate gene, resides in the 18q linkage region, as previously reported [13,14]. CNDP1 encodes for the secreted serum carnosinase. A trinucleotide repeat in exon 2, coding for a leucine repeat in the leader peptide of the carnosinase-1 precursor, has been associated with DN [50,51]. Since this polymorphism lies in the 5' coding part of the transcript, the number of trinucleotide repeats is directly related to the number of leucine residues in the leader peptide of the carnosinase precursor, i.e. five, six or seven leucines. The five-leucine allele, the shortest allelic form, is more common in subjects without nephropathy, with an OR of 2.56, and is associated with lower serum carnosinase levels [50]. This is expected to result in higher renal concentrations of the protective dipeptide carnosine. It would be interesting to evaluate whether carnosine administration or carnosinase activity inhibition could prevent the development of nephropathy in susceptible diabetic individuals.

Urinary protein markers of DN

It is currently impossible to reliably predict which, and when, diabetic patients will develop nephropathy and progress to kidney failure. Clinically, DN can be diagnosed when albumin is detected in the urine (albumin excretion 30–300 mg/day). The presence of albumin in the urine is considered predictive of the subsequent development and clinical progression of DN [52]. However, microalbuminuria could also be regarded as an indicator of an established glomerular damage. Moreover, microalbuminuria is not specific for DN, since albumin can also be detected in some other pathological conditions and the prevalence of microalbuminuria in the general population is rather high. Finally, microalbuminuria is a poor predictor of DN, since only 30–45% of microalbuminuric patients develop overt proteinuria over more than 10 years [53]. However, it is well established that regression of microalbuminuria, obtained by renin–angiotensin system blockers, is associated with protection from the development of overt DN. The conclusions of the Benedict study, where ACE inhibitors prevent development of microalbuminuria [54], and Diabiiopsies study, where ACE inhibitors reduce renal interstitial expansion [55], support this view.

If microalbuminuria is a useful indicator of developing DN and regression of renal lesions by treatment, it is far from meeting the criteria of a predictive marker. As a consequence, the identification of biological markers appearing earlier than albumin would be highly desirable. We review here some of the proteins that have recently been considered of interest to complement or even replace the dosage of albumin in the urine.

Albumin and non-immunoreactive albumin

Standard methods for measuring albuminuria are based on the immunochemical detection of serum albumin [radioimmunoassay (RIA), immunonephelometry (IN) and immunoturbidimetry (IT)]. HPLC methods can also be used to identify non-immunoreactive albumin, allowing the detection of microalbuminuria 3.9 and 2.4 years earlier than conventional measurement in T1D and T2D patients, respectively [56]. In the large Australian diabetes, obesity and lifestyle cohort, the prevalence of microalbuminuria was found to be four times higher using HPLC than by IN (20 versus 5.5%); also, 17.4% of individuals classified as normoalbuminuric with IN were reclassified as microalbuminuric by HPLC [57]. Another study showed that the mean urinary albumin concentration was 6.8 ± 4.3 mg/L by nephelometry in 998 subjects versus 17.6 ± 10.3 mg/L with HPLC [58]. Thus, conventional immunoassays may underestimate the albumin concentration because intact albumin in urine might exist in two forms, immunoreactive and non-immunoreactive [59]. However, the higher amount of albumin detected by HPLC has been attributed to contamination by proteins that co-elute with albumin [60]. Thus, although the HPLC results look promising, it is imperative to define the molecular entity that is measured. A microfluidic-based electrophoresis system has been recently used to detect both immunoreactive and non-immunoreactive albumin. This method was able to detect 3–145% more albumin (with good reproducibility: CV 3–13%) than the reference immunoassay under conditions where contamination of the albumin peak by several proteins was excluded [61]. Clearly such a system has distinct advantages over conventional methods, but needs to be evaluated in a large cohort of patients.

Type IV collagen

Type IV collagen is the major component of the glomerular extracellular matrix and the level of type IV collagen in the urine might reflect the rate of matrix turnover in diseased kidneys. Work by Watanabe showed that type IV collagen is found exclusively in the urine of diabetic patients with microalbuminuria and not in the urine of patients suffering from diseases in which albuminuria can be present [62]. These findings indicate that type IV collagen has a specificity for DN that albumin itself has not, but since urinary collagen IV is detectable only when microalbuminuria is already present [63], this marker lacks the criterion of earliness that is required for an indicator of DN onset. Nevertheless, an elevated urinary type IV collagen is one of the five criteria for early diagnosis of DN that the Japanese Diabetic Nephropathy Committee recommends to use [64].

Adiponectin

Adiponectin is an adipocyte-secreted cytokine (adipokine) of human plasma. Decreased adiponectin plasma levels are linked to obesity, insulin resistance and type 2 diabetes. Koshimura et al. found, in the urine of T2D patients with macroalbuminuria, a 30 kDa protein immunoreactive towards an anti-adiponectin monoclonal antibody [65]. By
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failure [68]. Nephrin is one of the many podocyte-specific
microalbuminuria or patients suffering from chronic renal
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filtration barrier. Podocytes and podocyte-specific proteins
can be potentially interesting urinary markers for the early
diagnosis of an alteration of the glomerulus [67]. Indeed,
urinary podocytes have been detected only in the urine of di-
abetic patients with micro- and macroalbuminuria, whereas
were absent in healthy controls, T2D patients without
Microalbuminuria or patients suffering from chronic renal
failure [68]. Nephrin is one of the many podocyte-specific
proteins that have been described in the last few years. Pätäri
et al. have looked for the presence of nephrin in urines of
T1D patients. They found immunoreactive nephrin in 30%
of normoalbuminuric diabetics, 17% of microalbuminuric,
28% of macroalbuminuric and 28% of patients who recently
became microalbuminuric [69]. Remarkably, nephrin was
totally absent in the urine of non-diabetic patients. The find-
ing that one-third of normoalbuminuric T1D patients ex-
crete nephrin is suggestive of a prognostic value of nephrin-
uria. Since Nakamura et al. did not find podocytes in the
urine of normoalbuminuric T1D patients [68], it is possible
that in the natural history of the disease, nephrin is released
as a free molecule before podocytes detach. Much atten-
tion should be paid to the possible diagnostic interest of
podocyte proteins and their fragments, since podocytes are
key elements of the kidney filtration barrier.

Advanced glycation end products
Advanced glycation end products (AGEs) are a heteroge-
 nous group of proteins and lipids to which sugar residues
are covalently bound during physio-pathological processes.
The molecular identity of the AGEs that contributes to the
development of diabetic complications is not yet
known.

Since kidneys are thought to be one of the key organs for
the clearance of AGEs, the occurrence of AGE in the urine
and its putative diagnostic interest have been studied [70].
The total AGE content in 24-h urine collections of type 2 di-
abetic patients with normoalbuminuria, microalbuminuria,
macroalbuminuria or persistent macroalbuminuria shows
statistically significant differences between normo- and mi-
icroalbuminuric patients. However, since AGE immunore-
activity has also been detected in 15% of healthy individuals
and is absent in 15% of diabetic patients with microalbum-
minuria, this parameter, in its present form, does not fulfil
the specificity/sensitivity criteria of a valuable diagnostic
test. Finally, AGE-modified proteins undergo (incomplete)
proteolytic degradation yielding LMW-AGEs (low molec-
ular weight AGES). LMW-AGEs can be monitored with
a simple fluorescence assay. In patients with T2D, serum
LMW-AGEs are 34% higher than in non-diabetic vol-
uunteers and their concentration is linked to the glomerular
filtration rate [71], indicating that this simple test may have
clinical diagnostic value.

Betaig-h3
Two recent reports point to a possible diagnostic value of
the transforming growth factor, beta-induced protein h3
(betaig-h3) urinary concentration. Betaig-h3 is an extracel-
ular matrix protein that is induced by transforming growth
factor beta, a growth factor implicated in the pathogenesis
of DN. Ha et al. [72] first showed that the betaig-h3 to cre-
atinine ratio is significantly higher in T2D patients than in
control subjects. A positive correlation was found between
urinary betaig-h3 and albumin excretion rate, and even nor-
moalbuminuric diabetic patients have a higher ratio than
controls. In another study [73], it was also observed that
the urinary level of betaig-h3 is higher in diabetic patients
(betaig-h3/creatinine ratio: 25.02 ± 8.84) than in healthy
individuals (18.67 ± 6.56). Together, these studies provide
encouraging results for a potential use of betaig-h3 in the
early detection of renal complications in T2D.

Identification of urinary markers by proteomic approaches
Proteomics refers to methods aimed at discovering and
identifying the complete set of proteins present in a given
biological sample at a given time. In 2005, Sharma et al.
compared the urinary proteome of type 1 and type 2 DN
patients (with proteinuria > 300 mg/24 h) to the urinary
proteome of healthy volunteers [74]. Using a variant of two-
dimensional gel electrophoresis (2D-DIGE), they found
that several spots in the diabetic samples were either up-
or down-regulated. This pioneer study does not, however,
reveal early markers of DN, because patients with protein-
uria were selected. Similarly, 2D-DIGE of urine samples
from T2D patients with microalbuminuria showed that four
main proteins accompany albumin: alpha-2 glycoprotein,
alpha-1 acid glycoprotein, alpha-1 microglobulin and IgG
[75]. It is not yet known whether the excretion of some
of those proteins may precede albumin in the natural his-
tory of DN. It was further reported that a set of seven
urinary proteins increased in concentration concomitantly
with increasing albuminuria, whereas four other proteins
were down-regulated [76]. Another recent report used a
proprietary proteomic method to look for polypeptide pat-
tterns specific for early DN. Eighty-eight polypeptides were
found to display significant differences between patients
with early DN and patients without. Mathematical treat-
ment of the data led to the definition of possible individual
risk factors for DN [77]. Unfortunately, the identity of the
discriminating polypeptides was not disclosed, making any
further work on them impossible. Otu et al. found a 12-peak
proteomic signature in the baseline urine of T2D patients
who subsequently developed DN [78]. The reported accu-
racy (71% sensitivity and 76% specificity) is encouraging
in view of a future diagnostic assay. Finally, in a recently
published article [79], the authors found β2 microglobulin and UbA52 as being selectively excreted in the urine of type 2 diabetics with DN.

Conclusion

Candidate gene analysis allows the study of both major and minor gene effects, but generally yields conflicting results. The observed discrepancies could be partly explained by differences in the studied populations, particularly dissimilar ethnic backgrounds and genetic heterogeneity, and by their relatively small size. The use of SNPs in association studies of complex phenotypes has attracted most of the attention. At the moment, no single gene with a large effect has been identified and only small effects of a variety of polymorphisms in a number of genes have been reported. Neither linkage analyses nor association studies performed until now support the view of major gene polymorphisms involved in DN onset, development and/or severity. The maximum LOD score was 6.1 linked to chromosome 18 [14] and the highest OR found was 9.0 for carriers of Xbal (−) and Z-2 alleles of SLC2A1 and AKR1B1 genes, respectively [32]. Hopefully, further advances in molecular biology and genetics will bring new insights into the mechanisms involved in DN development. This will permit the early identification of patients at risk of, or protected from, DN and will lead to specific preventive strategies [6,80].

As for new urinary protein markers, the ideal diagnostic protein needs to be detected earlier than albumin in the natural history of DN. Podocyte proteins, or their fragments, could harbour such candidate biomarkers, since alterations of podocytes and of the slit diaphragm are causative of albumin leakage. Proteins from the extracellular matrix could also be envisaged as DN is characterized by a deep remodelling of this structure.

Urine is a biological fluid that has only recently begun to be explored by proteomic techniques [81]. As many as 1543 different proteins have been recently identified from a pool of normal urines [82]. This unexpected diversity of the urinary proteome is both good and bad news for diagnostic purposes. On one hand, it appears that despite the urine low-protein content, the protein diversity is high and possibly not yet fully appreciated. On the other hand, more that 1500 identified urinary proteins represent a useful database for the identification of biomarkers that either would not belong to the normal protein set or would correspond to modified forms of normal proteins.

Conflict of interest statement. None declared.

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