

Genomic Strategies for Diabetic Nephropathy

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Abstract. Insight into the molecular mechanisms that underlie the origin and progression of diabetic nephropathy remains limited in part because conventional research tools have restricted investigators to focus on single genes or isolated pathways. Microarray technologies provide opportunities for evaluating genetic factors and environmental effects at a genomic scale during the pathogenesis of diabetic nephropathy. Despite

the enormous power of the microarray technology, there are several pitfalls that need to be considered. This article discusses conceptual, practical, statistical, and logistical considerations for the use of microarrays in studies of experimental and human diabetic renal disease. New knowledge in this field will facilitate new approaches for molecular diagnosis and drug discovery.

Diabetes is the most common metabolic disorder with an estimated worldwide prevalence between 1 and 5%. One of the most severe complications of diabetes is the development of diabetic nephropathy (DNP). Diabetic renal disease is the single most common cause of ESRD in the United States, accounting for 43% of new cases (1,2). Furthermore, diabetic ESRD is associated with a poor life expectancy.

Clinical studies have identified risk factors that correlate with the development of ESRD in diabetes. The presence of microalbuminuria, hypertension, and poor glycemic control are the most important risk factors (3). It is clear, however, that poor glycemic control alone is not sufficient for the development of this complication. Long-term observational studies show that a maximum of 35% of patients develop nephropathy, irrespective of glycemic control (4). This is in contrast to retinopathy, for which the prevalence continues to rise with the duration of diabetes. Family studies showed that among patients with type 1 diabetes, when one sibling develops nephropathy, the other one has a fourfold increased risk of nephropathy compared with the sibling of a patient without nephropathy (5). These observations have clearly established the importance of genetic risk factors in the development of DNP.

Biochemical studies have described numerous important cellular pathways in the pathogenesis of DNP, including the formation of hexosamine (6), advanced glycation end products (7), and activation of protein kinase C (PKC) (8). Enhanced intracellular glucose is thought to provide the basis for the initiation of these pathways by inducing increased mitochondrial superoxide formation (9). A common downstream event

is the stimulation of matrix production via activation of TGF- β signaling (10).

General modes of intervention designed to prevent the onset or progression of complications of diabetes have been shown to slow progression of DNP. For example, the Diabetes Control and Complications Trial and the United Kingdom Prospective Diabetes Study showed the importance of strict glucose and BP control in delaying diabetic complications (4,11). Recently completed large studies showed that inhibition of the renin-angiotensin system by either angiotensin-converting enzyme (ACE) inhibitors or angiotensin receptor blockers slows the progression of DNP (12,13). However, specific molecular drug targets with the promise to prevent onset or progression of DNP in diabetics altogether remain to be discovered.

In addition, diagnostic and predictive molecular approaches for accurate assessment of the risk for DNP in individuals who receive a diagnosis of diabetes without renal manifestations are lacking. Here we discuss genomic strategies to find new diagnostic and therapeutic targets in DNP.

Genetics of Diabetic Complications in Humans

Epidemiologic studies have clearly established that only a subgroup of individuals with diabetes are at risk of nephropathy (2). To identify genetic determinants and candidate genes that confer susceptibility or progression for DNP in individuals with type 1 and type 2 diabetes, the National Institutes of Health established the ongoing Family Investigation of Nephropathy and Diabetes study consortium. The Family Investigation of Nephropathy and Diabetes is using Mapping by Admixture Linkage Disequilibrium and traditional affected and discordant sibling pair and relative pair analyses. Previous linkage analysis studies led to the mapping of several susceptibility loci for DNP on specific regions on chromosomes 3, 7, 9, 12, and 20 (14,15).

In addition, individual research groups have used association studies based on candidate gene approaches to identify genes with variants that may confer susceptibility to DNP in humans.

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Association studies involve the comparison of allele and genotype frequencies at genetic loci in individuals with the condition of interest and control subjects. Table 1 summarizes most of the proposed genetic associations based on candidate gene studies. The most studied association is between ACE insertion deletion polymorphism and DNP. Elevated pro-renin, ACE, and angiotensin II levels are observed in diabetic nephropathy (16). The blockage of the renin-angiotensin system is currently the most powerful therapy to slow the progression of diabetic renal disease (12). The ACE gene polymorphism is characterized by the insertion (I) or deletion (D) of a 256-bp segment of DNA. Each individual carries two copies of the gene; those with II genotype have the lowest level of serum ACE, and those with DD have the highest. A number of studies examined the association between the susceptibility of diabetic nephropathy and the D allele. Recent meta-analysis has suggested a weak association between the presence of D allele and the predisposition for DNP (17,18). Another line of evidence suggests that the ACE gene polymorphism may have an effect on the progression of diabetic nephropathy (19) or on the therapeutic response (20). Additional studies suggest that the e2 allele of the apolipoprotein E gene is associated with an increased risk of nephropathy (21). Aldose reductase, the first enzyme in the polyol pathway, is also thought to play an important role in the pathogenesis of DNP (22). Indeed, polymorphisms at the 5' end of the aldose reductase gene might contribute to the pathogenesis of several diabetic microangiopathic complications (23). However, existing data for most of these DNP candidate genes and susceptibility loci are not conclusive, and large studies are yet to reproduce the results of these initial observations.

New Genomic Tools and Resources

Several recently developed approaches will likely enhance the identification of associations between DNA variations and DNP considerably (24). For example, genotyping can now be performed efficiently in high-throughput format with assays based on PCR or microarray technology (25–27). These assays provide cost-effective detection of common sequence variations, single nucleotide polymorphisms (SNP), and will facilitate large-scale clinical studies to test associations between SNP and DNP. Ongoing efforts of the SNP Consortium and genome projects have generated a public SNP database. This database will provide a knowledge resource for fine mapping of chromosomal regions of interest and localizing DNP-associated SNP. Recent reports indicate that SNP are organized across the genome in so-called DNA “blocks,” transmitted for many generations without recombination. These DNA “blocks” contain distinct sets of SNP that are called “haplotype,” and with a few selected SNP each haplotype can be uniquely identified, although each block contains many more SNP. Because DNA “blocks” are characterized by a few common haplotypes, this method called haplotype mapping is expected to enhance greatly the efficiency for detecting genetic variations associated with DNP.

Comparative genomics is a sequence-based approach made possible through the availability of complete genome sequences of human, mouse, and other model organisms. Comparative genomics allows direct comparisons of genes of interest in humans and rodent models with diabetes and diabetic complications. Together, these high-throughput genetic tools and resources will greatly enhance the discovery of genetic variants in genes that may be involved in susceptibility and pathogenesis of DNP.

Table 1. Candidate gene variants in human DNP

Gene Name	Gene Symbol	Gene Variant	Chromosome	Reference
Histocompatibility antigen	HLA	DR3/4	6	51
Angiotensin-converting enzyme	ACE	D/I	17	17,20
Angiotensinogen	AGT	M235T	1	52
Angiotensin II type I receptor	AT1R	A1166C	3	53,54
Aldose reductase	ALR2	Z + 2 allele	7	22
Heparan sulphate	HSPG2	BamHI	1	55
Apolipoprotein E	APOE	e2 allele	19	56
Paraoxonase 1	PON1	T107C, Leu54Met,	7	57
Endothelial cell nitric oxide	NOS3	'a' allele	7	58
Methylenetetrahydrofolate reductase	MTHFR	C677T	1	59
Interleukin 1 β	IL1B	T105C	2	60
Atrial natriuretic peptide	NPPA	HpaII (C708T and Scal)	1	61
G-protein β 3	GPB3	C834T/Trp64Arg	12	62
Ecto-nucleotide pyrophosphatase/ phosphodiesterase	ENPP1	K121Q	6	63
Glucose transporter1	GLUT1	XbaI/HaeIII	1	64
Transforming growth factor β 1	TGFB1	Leu10Pro, Arg25Pro, Thre263Ile	19	65

In addition, the DNA microarray technology allows us to measure effects of the diabetic *milieu* on gene expression levels in kidney tissue on a genome-wide scale (26). Because the expression of genes is a major determinant of their function, this approach constitutes the field of functional genomics. Finally, computational and bioinformatics approaches that involve advanced database and data mining tools to facilitate the integration of genetic and genomic data with biologic and clinical data are being developed. This powerful systems biology approach promises data analysis across numerous studies and types of data at a global scale and will be critical for meaningful synthesis and interpretation of massive amounts of research data expected from genomic studies of DNP (24).

Functional Genomics of Diabetic Nephropathy *Current Limitations for Functional Genomics Studies of DNP in Humans*

To date, the feasibility of finding diagnostic and outcome predictor “biomarker genes” of human diseases based on gene expression profiling has been demonstrated for various malignancies, including acute leukemia (28), breast (29), and lung cancers (30). Compared with studies on tumor material, the application of microarray studies to human diabetic nephropathy poses a different challenge to the investigators (31). Highly sensitive and reproducible methods are needed to assess gene expression profiles in a limited amount of available tissue.

The standard normal *versus* diseased tissue type of comparison, which is currently the basic design principle of profiling studies, carries the limitation that gene expression profile in “normal” tissue is not well characterized. Gene expression in normal tissue is likely to be dependent on several factors, including the patient’s age, gender, and genetic background. We are currently performing gene expression profile analysis of kidney biopsy materials from living kidney donors to determine normal (healthy) gene expression profiles in separated human glomerular and tubular compartments. In this ongoing pilot project, we will analyze and estimate the influence of underlying genetic and environmental factors on renal gene expression profiles. The anticipated information will be essential for sample size calculation for future genome-wide molecular studies on human DNP.

Another major limitation for studies of diabetic renal disease at a molecular level is that current clinical practice usually does not require tissue analysis to establish the diagnosis of DNP. The diagnosis of DNP is commonly established on the basis of clinical criteria, and it is common practice to avoid kidney biopsies of patients with diabetes. Recently, this paradigm has been challenged and several reports suggest that individuals with diabetes could have diverse primary renal disease, not only DNP (32).

In cases in which kidney biopsy material is available, interpretation may be confounded by tissue heterogeneity. The proportion of glomeruli and different tubular segments presented in the biopsy material can vary significantly. Earlier studies established that manual microdissection is an effective approach that allows efficient separation of glomeruli and

tubuli in experienced hands. Laser capture microdissection technology (33,34) could present a powerful alternative to manual microdissection. It allows accurate identification and procurement of glomerular or tubular cells from tissue samples under direct microscopic visualization. Archive samples can also be analyzed with laser capture microdissection; however, a major drawback of this technique is the low amount and often inadequate quality of mRNA that is extracted from a fixed tissue.

Because renal biopsies represent a minute fraction of the diseased organ, considerable sample bias is inevitable. A representative number of glomeruli are required to obtain adequate profiles. However, in most cases, glomeruli or tubuli at different disease stages are represent in single needle biopsy material. Detailed histopathologic examination of the biopsy material, including light and electron microscopic analysis, can help to overcome these difficulties.

Another challenge is the inherent instability of mRNA. Several promising technical innovations are currently under development to overcome this problem. A new class of RNase inhibitors provided in commercial reagents (*e.g.*, RNeasy; Qiagen, Crawfordsville, IN) allows good preservation of RNA. New column-based RNA extraction methods provide high-quality RNA quickly with minimal loss during extraction. RNA quantification and quality measurements are critical steps for gene expression studies. A new technology (Lab on Chip) (35), which is based on capillary gel electrophoresis, has revolutionized this field, and as little as picogram quantities of RNA can now be accurately analyzed.

DNA array-based technology is the method of choice for comprehensive gene expression profiling. One major limitation was that earlier array technologies had required large amount (20 to 100 μg) of high-quality total RNA as a starting material. Recently, new technologies with improved RNA amplification and labeling became available (36,37). A major concern is the linearity of the amplification across different mRNA species. With highly effective amplification, even small differences in amplification efficiencies can result in significantly distorted representation of individual transcripts.

Microarrays deliver massive amounts of data on tens of thousands of genes. The result is an immense quantity of biologic information that needs to be analyzed, presented, and archived in a meaningful way. Therefore, functional genomic studies should be combined with advanced computational and statistical approaches. The gene expression data have to be analyzed in conjunction with other clinical and histopathologic variables. Despite the different variables, most of the published studies to date using microarray-based expression analysis include only limited numbers of replicates. In fact, many studies conduct the experiment only once. Considering the potential sources of assay variation, the need for sufficiently replicated studies is underscored. In addition and importantly, potential gene candidates must be assessed for relevance to disease by using parallel technologies at RNA and protein level and, more important, in functional studies.

Table 2. Renal lesions in diabetic mouse models

Mouse Strain	Defect	Diabetes Type	Hyperglycemia Onset in Males	Hyperglycemia Onset in Females	Albuminuria	ME	T1D	GBMT	VL
Ins ² Akita	Insulin2 gene	1	3–4 wk (severe)	3–4 wk (mild)	+	~20 wk	–	?	–
NOD/LtJ	Insulinitis	1	>16 wk in 40–60%	12 wk in 85–100%	+	~16–20 wk	–	+	–
Streptozotocin treatment	Beta cell death	1	~1 wk after treatment	~1 wk after treatment	+	Absent or minimal	–	?	–
Lepr ^{db}	Mutation of leptin receptor	2	6–8 wk	6–8 wk	++	~16 wk	–	+	–
Lepr ^{ob}	Absence of leptin	2	Transient 8–14 wk	Transient 8–14 wk	–	–	–	–	–
KK Cg-A ^y /J	Polygenic	2	8 wk	8 wk	++	~16 wk	–	+	–

ME, mesangial expansion; T1D, tubulointerstitial disease; GBMT, glomerular basement membrane thickening; VL, vascular lesion.

Functional Genomic Studies of Kidneys in Existing Rodent Models of Diabetes

The use of animal models of diabetes can provide an immediate and simple alternative to overcome the technical limitations of functional genomic studies of humans. Animal model systems are genetically homogeneous, and environmental factors can be easily controlled. However, the available mouse models do not represent the human disease faithfully.

There are distinct changes that one can observe during the development and progression of human diabetic nephropathy. This is characterized by an early increase in GFR, microalbuminuria, renal enlargement, glomerular hypertrophy, and widening of the glomerular basement membrane (GBM). This is followed by expansion of the mesangium, with an increase in both the cellular and the matrix components. Classically, four types of glomerular lesions are found in established human diabetic renal disease: diffuse glomerulosclerosis, nodular glomerulosclerosis, fibrin-cap lesion, and capsular-drop lesion (38). Diffuse glomerulosclerosis is the most commonly observed lesion and consists of widespread increase of eosinophilic, periodic acid-Schiff–positive matrix material in the mesangial area. The nodular lesion is the most specific histologic abnormality but is not always found in patients with DNP. As nephropathy advances to end stage, tubulointerstitial lesions such as tubular atrophy, interstitial monocellular infiltration, and fibrosis develop.

Several animal models spontaneously develop diabetes (39), manifested by a variety of clinical symptoms similar to those of human diabetes. In general, the expression and severity of metabolic, hormonal, and pathologic abnormalities vary depending on the strain, genetic background, nutrition, and age. Because of this heterogeneity, no single animal model represents all features of diabetes in humans. Similarly, diabetic animals may develop kidney disease, which resembles human diabetic nephropathy, but the renal changes are not identical. Usually, the histologic appearance of the glomerular lesions are very similar to early stages of human diabetic nephropathy; however, changes that are characteristic of later stages of DNP are absent. The explanation of this phenomenon is currently under intensive investigation. Table 2 summarizes the pathologic lesions observed in various diabetic mouse models.

From the type 1 diabetic models, the nonobese diabetic (NOD), the Akita, and the streptozotocin (STZ)-treated mice are the most frequently used. Mice develop hyperglycemia shortly (within days) after STZ treatment (40). In larger doses, STZ is nephrotoxic, making it difficult to differentiate between the direct effect of STZ and lesions caused by the diabetic milieu (41). STZ-treated mice develop a modest degree of proteinuria in conjunction with minimal mesangial matrix expansion, depending on the genetic background. The NOD mouse strain develops hypoinsulinemia secondary to autoimmune destruction of the β -cells; later, albuminuria, mild mesangial matrix expansion, and GBM thickening can be observed (42). The Akita spontaneous mutation (commonly referred to as *Mody*) is an autosomal dominant mutation in the insulin II gene (mouse homolog of preproinsulin gene). This missense mutation results in an amino acid substitution (cysteine 96 to

tyrosine). Replacement with a tyrosine causes incorrect folding of the insulin II molecule, leading to defective processing and secretion of both the insulin I and the normal insulin II proteins such that insulin release is abnormal and diabetes develops (43).

Db/db mice, which have a recessive mutation in the hypothalamic leptin receptor, develop obesity at 4 wk of age and type 2 diabetes at approximately 8 wk of age. In C57BL/6J background, the diabetes and the obesity are usually less severe than in the C57BL/KsJ background (44). Kidneys are generally enlarged in this mouse strain, and structural glomerular changes (*e.g.*, diffuse glomerulosclerosis, GBM thickening) occur without evidence of tubulointerstitial disease (40). Glomerular lesions of the KK mice are characterized by diffuse and nodular mesangial sclerosis without evidence of tubular disease (45). The lack of reliable mouse models prompted the National Institute of Diabetes and Digestive and Kidney Diseases to fund a consortium for the development and phenotyping of new diabetic mouse models that would resemble closely human DNP.

Genetic modification of the existing mouse models might improve the currently used models. Modification of the scavenger receptor system by overexpressing the receptor for advanced glycated end product (46) in endothelial cells clearly enhanced the glomerular disease in a spontaneous diabetic model system, which did not develop glomerular disease otherwise. Similarly targeted deletion of the galectin-3 gene greatly accelerated the diabetic kidney disease in STZ-treated mice (47). These observations highlight the importance of the scavenger receptor system and its ligands in the pathogenesis of DNP. Another interesting approach involved modification of the lipid signaling system via overexpression of sterol regulatory element binding proteins in the kidney (48).

Current Adaptation of Functional Genomics Studies of Kidneys in Diabetic Mice

Detailed examination and comparisons of diabetic mouse models to the human disease can identify pathways that are important in the pathogenesis and progression of diabetic renal disease. Genes and new pathways that are identified as significantly differentially expressed in mice with diabetic kidney disease can be evaluated as prognostic or diagnostic markers for development of progressive renal disease or potential drug targets.

To screen for genes that show correlation with different phenotypic outcome in diabetic mouse models, we used the cross-sectional design and performed microarray analysis on 24-wk-old STZ-treated and db/db mice with established renal pathology. In parallel with the functional genomics characterization, each individual mouse underwent a detailed renal phenotype analysis. Mice that were treated with low doses of STZ developed diabetes and moderately severe albuminuria (twice the control). In mice with C57B6/J background, the mesangial changes were mild or absent. Mice with 129SvJ genetic background developed significant glomerular changes. However, these were not significantly different from the age-matched controls (K. Sharma, K. Susztak, and E.P. Böttinger,

unpublished observations). The db/db mice became insulin resistant and developed diabetes at approximately 8 wk of age. Albuminuria was detected as early as 3 to 4 wk after the development of hyperglycemia. The glomerular histology was characterized by severe diffuse mesangial expansion, as previously reported (49).

This experimental set-up allowed us to group animals on the basis of the phenotypic outcome rather than of their underlying genotype. To identify genes with correlation to certain phenotype, we used the statistical analysis of microarray data (50). Supervised data mining algorithms were applied to find genes with the highest discriminative value for a certain phenotypic outcome (hyperglycemia, mesangial pathology) (28). This method, which is based on the k-nearest-neighborhood algorithm, first constructs a hypothetical gene expression profile that best fits the desired pattern (*e.g.*, a gene with high expression in hyperglycemic animals and low expression in animals with normal glucose values, or *vice versa*). The technique then identifies individual genes that are most similar to the hypothetical gene expression profile.

Unsupervised methods can be used to answer different questions. The main advantage of these methods is that they could uncover new, unexpected subclasses within the same diseased group. However, in general, they are less intuitive. From these methods, we used hierarchical clustering in the initial phase of the analysis. It sorted all genes (or samples) such that genes or samples with similar profiles appear near to each other. The number and size of expression patterns within a data set can be estimated quickly, although the division of the tree into actual clusters is often performed visually.

Functional genomic analysis on different diabetic mouse models provided a global view into the pathomechanisms of DNP. With advanced computational approaches, we have identified new markers that could discriminate different phenotypic appearances in mice. The discriminative value of these markers has been validated on diabetic animals that were not part of the microarray analysis. Further analysis of the marker genes based on classic biochemical approaches is currently under way in our laboratory.

Conclusions

Functional genomic approaches combined with advanced phenotypic characterization and computational algorithms are new, powerful methods. Application of these methods for diabetic nephropathy shows a great promise but also poses challenges for the investigators. Microarrays make it possible to investigate differential gene expression in normal *versus* diseased tissue, in treated *versus* nontreated tissue, and in different stages during the natural course of a disease, all on a genomic scale. Gene expression profiles may help to unlock the molecular basis of phenotype, response to treatment, and heterogeneity of disease. They may also help to define patterns of expression that will aid in diagnosis as well as define susceptibility loci that may lead to the identification of individuals at risk. Finally, as specific genes are identified and their functional roles in the development and course of disease are characterized, new targets for therapy should be identified. The

overall goal is to obtain complementary information that allows a stringent prediction concerning the diagnosis, prognosis, and differential therapy.

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