Mining the Genome for Susceptibility to Diabetic Nephropathy: The Role of Large-Scale Studies and Consortia

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Summary: Approximately 30% of individuals with type 1 and type 2 diabetes develop persistent albuminuria, lose renal function, and are at increased risk for cardiovascular and other microvascular complications. Diabetes and kidney diseases rank within the top 10 causes of death in Westernized countries and cause significant morbidity. Given these observations, genetic, genomic, and proteomic investigations have been initiated to better define basic mechanisms for disease initiation and progression, to identify individuals at risk for diabetic complications, and to develop more efficacious therapies. In this review we have focused on linkage analyses of candidate genes or chromosomal regions, or coarse genome-wide scans, which have mapped either categorical (chronic kidney disease or end-stage renal disease) or quantitative kidney traits (albuminuria/proteinuria or glomerular filtration rate). Most loci identified to date have not been replicated, however, several linked chromosomal regions are concordant between independent samples, suggesting the presence of a diabetic nephropathy gene. Two genes, carnosinase (CNDP1) on 18q, and engulfment and cell motility 1 (ELMO1) on 7p14, have been identified as diabetic nephropathy susceptibility genes, but these results require authentication. The availability of patient data sets with large sample sizes, improvements in informatics, genotyping technology, and statistical methodologies should accelerate the discovery of valid diabetic nephropathy susceptibility genes.

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In the past 15 years, studies with different analytic strategies have established that diabetic nephropathy (DN) has a genetic predisposition. DN does not segregate according to Mendelian rules, but rather is a multifactorial trait that results from interplay between environmental factors and multiple genes. Given this, strategies that were used successfully to map the causal mutations for monogenic disorders could not be used for DN and other complex traits and new analytic plans were developed. Initially modest-sized study collections ranging from 100 to 200 nuclear families with affected siblings were assembled and analyzed with model-free methods in the hopes of identifying genes for DN. This was a time of uncertainty when the genome sequencing was not finished, and it was unclear which study designs and sample sizes would best facilitate the identification of genes for DN. Sample sizes generally were based on the number of subjects who could be enrolled at a single center within a 2- to 5-year time line, extrapolating successful strategies for mapping single gene disorders. Thus, these sample sizes would comprise about several hundred meiotic products (individuals) and allow a map resolution of approximately 1 megabase (1 megabase = 1 centiMorgan), assuming a single locus genome-wide. However,
neither the sample sizes nor the map resolutions were adequate for identifying DN susceptibility variants.

Subsequently, seminal studies in genetic epidemiology literature have suggested that individuals with long-standing diabetes who were discordant for nephropathy were needed to distinguish between genes responsible for diabetes and those regulating nephropathy. The rationale for this strategy was that individuals without diabetes did not have the hyperglycemic exposure necessary to develop chronic kidney disease (CKD), even if they did possess genes for DN susceptibility. Clearly, individuals who did not develop nephropathy after a number of years of exposure to chronic hyperglycemia possessed inherent (potentially genetic) mechanisms of protection; contrasting the genetic profile of diabetic individuals with and without DN would distil out the common diabetes genes, allowing identification of DN genes. The mechanisms by which persistent hyperglycemia sensitizes the kidney in individuals predisposed to develop DN is not understood completely, although tight control of hyperglycemia certainly offers renoprotection.

Concomitant with evolving approaches to gene mapping, outpatient screens for kidney disease (phenotypes) were validated. Albuminuria or proteinuria could be quantitated on a spot sample and identified individuals with early CKD initiation and progression. Simple estimating equations, based on the serum creatinine level and demographic data, allowed an estimation of the glomerular filtration rate without the need to collect urine over 24 hours. Thus, the renal community was well positioned to implement gene mapping studies with large numbers of enrolled patients with easily quantifiable kidney disease traits.

The best prospect for the successful identification of CKD susceptibility alleles would be multiple families with diabetes with multiple members with CKD and/or end-stage renal disease (ESRD). Considering the genetic load, families with more than 2 individuals on dialysis because of DN were ideal because even for a complex trait this would imply that nephropathy genes were segregating within the family. However, these types of families were difficult to locate because of early mortality from macrovascular disease. Albuminuria (microalbuminuria) may be a better predictor for cardiovascular disease than for ESRD, explaining the difficulty in identifying multiplex DN families. To relieve some of the onus of collecting families with 2 or more DN-ESRD sibling pairs, the study designs were expanded further to accommodate family members with less advanced disease. Quantitative measures of albuminuria and calculated glomerular filtration rate (glomerular filtration rate calculated based on the Modification of Diet in Renal Disease equation or the Cockcroft-Gault equation) were used as endophenotypes. Both these traits were shown to be heritable in at least some populations and are routine measures of cardiovascular and renal function in clinical settings.

The impetus to map genes for nephropathy, especially DN, was driven by the modeling of DN inheritance in several multigenerational, family data sets or by looking at concordance in phenotypes between family members. Segregation and comingling analyses are the only established methods for determining if a phenotype (clinical measure or trait) fits a particular genetic model. Two segregation analyses have suggested that a major locus controls the albumin excretion rate. In one study, proteinuria was analyzed as a continuous variable, with the conclusion that proteinuria was influenced by multiple genes with variable effects. The report by Imperatore et al in diabetic Pima families considered overt proteinuria as a discrete variable, and determined that this trait was regulated by a major gene effect. Two studies of type 2 DN partitioned the genetic and environmental influences in albumin excretion rate and estimated heritability, a measure of genetic predisposition. Both studies estimated the heritability for urinary albumin excretion to be approximately 30%. The estimates of heritability for urine albumin excretion were statistically significant, even after adjusting for potential confounding covariables such as age, sex, body weight, diabetes duration, and environment, suggesting a major genetic effect for proteinuria. Finally, a renal biopsy study in type 1 diabetic siblings showed high degrees of correlation between severity and patterns of
glomerular injury, despite the frequent lack of concordance of glycemic control between siblings. In constructing the studies for detecting genetic susceptibility to DN, consideration had to be given to several important issues. First, is DN in type 1 diabetes a different entity from that in type 2 diabetes, such that genes for type 1 and type 2 DN differ? This remains an unanswered question, but one that hopefully will be resolved by comparing the results from several large-scale gene mapping studies discussed later. However, current evidence suggests that kidney disease in type 1 and type 2 diabetic patients may result from common genes. Both type 1 and type 2 DN cluster in the same families and show a similar disease tempo with nephropathy onset approximately 10 to 15 years after diabetes onset. If hyperglycemic exposure can be considered an environmental condition that primes the glomerulus regardless of the cause, then the similar patterns of response under the influence of either type 1 or type 2 diabetes may have an explanation.

Second, how long should the diabetic relatives of probands with DN remain free of nephropathy after the initiation of diabetes to be considered truly unaffected (and contribute to a discordant relative pair)? Absent a more sensitive marker of DN progression, a lack of albuminuria (<30 mg/g albumin-to-creatinine ratio in repeated measurements) after 10 years of diabetes was considered strong evidence of resistance to DN. The figure of 10 years is not based on an arbitrary threshold but is based on epidemiologic studies that established that the peak of nephropathy initiation is within 10 to 20 years after the initiation of diabetes and the risk for nephropathy peaks approximately 25 to 30 years after disease onset. In contrast, retinopathy risk increases with diabetes duration. Intrinsic within this simple idea is a complex model—individuals can be nephropathy-free because they do not carry a specific susceptibility allele at a particular locus, or they can be nephropathy-free because they have protective alleles elsewhere in the genome that suppress the outcome of a deleterious allele, or, finally, their glomerular cells are resistant to the effects of the environmental insult, such that they can withstand hyperglycemic exposure for longer periods without consequence. The latter also would be the result of the action of modifier genes, just not genes directly controlling an obvious avenue to DN. It may not be possible to disentangle all these possibilities in a single experiment, and the genetic epidemiology study designs chosen may influence which of these outcomes are detectable. The strengths and weaknesses of the study designs pertinent to gene identification are discussed later after consideration of some of these problems.

GENETIC COMPLEXITY: A DILEMMA OF MODEST SAMPLE SIZES?

Gene mapping studies run the gamut from collection of unrelated cases and controls, to family-based studies, to cohort studies (Fig. 1, Table 1). All of these study designs have been applied to identifying genes for DN, and nested within these basic designs specific molecular (genetic) contrasts have been devised with the goal of best using the study population at hand. Several early studies attempted to identify genes for DN with small to modest sample sizes, but subsequent investigations (both linkage and association) were unable to replicate findings in other samples or attempts at replication were not reported. Few of these investigations have produced results that meet modern guidelines for statistical significance in the context of a multifactorial trait. Primed by the successes in monogenic disorders (eg, polycystic kidney disease) in which the gene effect size is large, the lack of speedy identification of DN genes led the general scientific audience to conclude that genetic complexity precluded the identification of genes for DN susceptibility. However, as reviewed later the sample sizes were modest in the majority of investigations and ascertainment criteria were not standardized between studies, nor were the chromosome locations or candidate genes that were investigated similar between the studies. Thus, direct comparison between the studies was difficult and not surprisingly the results seldom were concordant. Some of these early reports reflected either false-positives findings (type 1 error) or sample-specific signals whose effect was enhanced by the modest size of the sample. Fail-
Figure 1. Study designs used in gene mapping experiments for complex traits. Case control and trio (or TDT)-based designs test for association whereas family based designs can test for linkage or association. The first 2 designs generally are more powerful for association testing in the context of common variants that cause disease. The latter has more power for rare variants. Some rare variants are difficult to detect and only direct sequencing may be able to find these types of mutations. (A) Potential uses for the illustrated study designs: (a) candidate genes; (b) gene x gene interaction; (c) gene x environment interaction; (d) genome-wide scans. (B) Potential uses for the illustrated study designs: (a) candidate genes; (b) gene x gene interaction; (c) gene x environment interaction; (d) genome-wide scans; (e) parent-of-origin effects; (f) sex-influenced traits. (C) Potential uses for the illustrated study designs: (a) candidate genes; (b) gene x gene interaction; (c) gene x environment interaction; (d) genome-wide scans; (e) parent-of-origin effects; (f) sex-influenced traits.

ure to replicate gene mapping results in independent samples, a key requirement for proving that a gene variant causes a disease, may be the result of small sample sizes, genetic heterogeneity between samples from various studies, ascertainment bias (also leading to heterogeneity), phenocopies, unaccounted environmental correlates, and other epigenetic mechanisms. Replication studies face some unique design issues. The exact hypothesis being examined in replication studies is not the same as the null hypothesis in the original study. As an example, if in an initial scan there are 10 genes in total genome-wide that can cause nephropathy, finding sufficient evidence for linkage to any 1 of these 10 is considered a success. In a replication study, genetic evidence at a specific locus identified in the initial scan must be reproduced. If patient phenotypes in the second data set are not identical to those characteristics in the first set, gene mapping results may be at variance. This does not necessarily imply that the results of the second scan or the first scan are false, merely that more evidence is needed to advance the experiment forward.

Each method for gene mapping has its own advantages and disadvantages, and these may change with advances in technology. All methods are subject to ascertainment bias, and the
extent of the bias depends on the study design. Cohort studies that adequately represent the general population have the least bias, whereas both family-based designs and case-control designs may have significant bias. The advantage for the latter is of course the increase in power for gene finding, by recruiting subjects with extreme phenotypes. Although identifying susceptibility genes is often within the means of the study, determining population-attributable risk (the fraction of risk that would be eliminated if the risk factor was removed) is difficult with common gene mapping designs.31-35

We have summarized large-scale studies that are in the process of gene identification for DN susceptibility and perhaps also for nephroprotection (Table 2). Most studies have a population-based collection strategy, the exceptions being the Family Investigation of Nephropathy and Diabetes (FIND)36 and the Genetics of Kidneys in Diabetes (GoKinD) studies.37 The FIND study had a mixed design with approximately half the sample comprising families and the other half comprising an admixture mapping approach in Mexican Americans and African Americans. The GoKinD study focused on trios of an affected type 1 participant and their 2 parents. Although the Epidemiology of Diabetes Interventions and Complications/Diabetes Control and Complications Trial (EDIC/DCCT) has a clinical trial base, efforts were made to assess the family history of complications among participants.26 Table 2 shows that the outcomes under investigation and the study designs are fairly distinct, although each generally examines some DN trait. Further, the major focus is on type 1 and not the more common type 2 diabetes; all of the studies but FIND and The Netherlands Cooperative Study on the Adequacy of Dialysis examined type 1 diabetes. Other independent type 2 diabetes studies in specific ethnic groups also have been published in the literature (eg, the DN genome scan in African Americans,38 the DN case control single-nucleotide polymorphism [SNP] scan in the Japanese39), but the sample sizes for the majority of these investigations were modest. These cohorts can be used for replication when genes are found, but the contrasting study designs will make comparing the effect

Table 1. Study Designs for Gene Mapping Studies

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Case-Control Design</th>
<th>Retrospective/Prospective Cohorts</th>
<th>Linkage Studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenotype contrast</td>
<td>Predefined choice of disease/control status</td>
<td>Can examine multiple disease stages</td>
<td>Predefined choice of disease status</td>
</tr>
<tr>
<td>Sample size required</td>
<td>Small to modest</td>
<td>Large</td>
<td>Small to modest</td>
</tr>
<tr>
<td>Collection process</td>
<td>Can be clinic or center based</td>
<td>Population-based and requires epidemiologic principles of follow-up evaluation</td>
<td>Can be clinic or center controlled</td>
</tr>
<tr>
<td>Type of genetic hypothesis examined</td>
<td>Only association analysis with genes, environment or gene × environment</td>
<td>If data on familial relationships collected, either association or linkage can be examined</td>
<td>Either association or linkage tests can be performed</td>
</tr>
<tr>
<td>Ascertainment bias</td>
<td>Yes; can lead to biased odds ratio to estimate disease risk</td>
<td>No</td>
<td>Yes; may magnify effects of rare alleles</td>
</tr>
<tr>
<td>Study of longitudinal outcomes</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>
size of the gene quite difficult and the comparisons should be accepted with caution. Thus, the majority of the studies have some bias, even ones that represent larger populations because of the sampling scheme. Several analyses were initiated as cross-sectional studies and later expanded to cohorts. The benefit of these studies is that the data are being collected prospectively and they uniformly are using strict phenotyping protocols, which will allow for computation of attributable risk.

Recently the use of standardized criteria for recruitment and large sample sizes have allowed the emergence of unifying results across studies. This should increase confidence in future trials to identify DN genes. The prospects of success already have been realized with the identification of specific DN genes, for example, carnosinase (CNDP1)\(^40\) and engulfment and cell motility 1 (ELMO1).\(^39\) although these genes need to be validated in other populations. Similarly, linkage and/or association signals have been replicated at several chromosomal regions (e.g., 10p, 18q), suggesting that these regions harbor important susceptibility loci (discussed later).

### LINKAGE STUDIES

We have compiled a list of linkage studies for DN and all-cause nephropathy (Table 3).\(^9,11,38,41-51\) The early linkage studies considered DN as a dichotomous outcome, but the later studies have included albuminuria as a quantitative trait (Table 3). Other studies with glomerular filtration rate as an endophenotype also are forthcoming. The first

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**Table 2. Large-Scale Investigations in Diabetic Nephropathy**

<table>
<thead>
<tr>
<th>Consortium Name</th>
<th>Study Design</th>
<th>Diabetes Type</th>
<th>Outcome</th>
<th>Locale</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDIC/DCCT(^{26,69})</td>
<td>Prospective cohort linked with tight glucose control trial</td>
<td>Type 1 DM</td>
<td>Multiple complications of diabetes</td>
<td>Multicenter United States/Canada</td>
</tr>
<tr>
<td>EURODIAB(^{70,71})</td>
<td>Prospective cohort</td>
<td>Type 1 DM</td>
<td>Multiple complications of diabetes</td>
<td>Multiple European centers; United States</td>
</tr>
<tr>
<td>FIND(^36)</td>
<td>Cross-sectional family based; mapping by admixture disequilibrium</td>
<td>Predominantly type 2 DM</td>
<td>Severely affected and discordant relative pairs with DN; cases and controls with and without DN</td>
<td>Multicenter United States</td>
</tr>
<tr>
<td>FINNINDIANE(^72)</td>
<td>Prospective cohort</td>
<td>Type 1 DM</td>
<td>Follow-up evaluation of DN in 25% of adult type 1 diabetics</td>
<td>Finland</td>
</tr>
<tr>
<td>GENDIAN(^73)</td>
<td>Case control; prospective</td>
<td>Type 2 DM</td>
<td>Follow-up evaluation of DN and other complications of type 2 diabetes</td>
<td>Germany</td>
</tr>
<tr>
<td>GoKinD(^37,74,75)</td>
<td>Cross-sectional trios (family based)</td>
<td>Type 1 DM</td>
<td>Probands with CKD and parents</td>
<td>Multicenter United States</td>
</tr>
<tr>
<td>NECOSAD(^76,77)</td>
<td>Prospective cohort</td>
<td>Both</td>
<td>New ESRD case follow-up evaluation</td>
<td>The Netherlands</td>
</tr>
</tbody>
</table>

EURODIAB, European Diabetes IDDM Complications Study Group; FINNINDIANE, Finnish Diabetic Nephropathy Study; GENDIAN, Genetic and Clinical Predictors of Morbidity, Mortality, and Diabetic Nephropathy with End Stage Renal Disease in Diabetes Mellitus Type 2; NECOSAD, The Netherlands Cooperative Study on the Adequacy of Dialysis.
genome scan for DN was performed in the Pima and included 98 affected pairs, but sample sizes have increased significantly since these types of investigations were begun in the late 1990s. Among family based studies, the largest samples sizes will come from FIND and GoKinD for type 2 and type 1 DN, respectively. For example, FIND has collected approximately 1,200 families from 4 different ethnic groups, Caucasian, African American, Native American, and Mexican American, across the United States. Similarly, the GoKinD collection encompasses 71 cases, 623 controls, 272 case trios with a type 1 diabetic proband having nephropathy, and 323 control trios ascertained by a diabetic proband without renal disease. This data set is better suited to genome-wide

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Diabetes Type</th>
<th>Population</th>
<th>Study Design</th>
<th>Sample Size</th>
<th>Linkage</th>
</tr>
</thead>
<tbody>
<tr>
<td>All-cause ESRD$^41$</td>
<td>Type 2</td>
<td>African American</td>
<td>ASP</td>
<td>65 families</td>
<td>Renin-angiotensin-aldosterone*</td>
</tr>
<tr>
<td>All-cause ESRD$^42$</td>
<td>Type 2</td>
<td>African American</td>
<td>ASP</td>
<td>65 families</td>
<td>Cytokine genes*</td>
</tr>
<tr>
<td>DN$^9$</td>
<td>Type 2</td>
<td>Pima Indian</td>
<td>ASP</td>
<td>98 ASPs</td>
<td>3q, 7q, 9, 20</td>
</tr>
<tr>
<td>All-cause ESRD$^11$</td>
<td>Type 2</td>
<td>African American</td>
<td>ASP</td>
<td>142 ASPs</td>
<td>Kalikrein genes*</td>
</tr>
<tr>
<td>DN$^{10}$</td>
<td>Type 1</td>
<td>Caucasian</td>
<td>ASP</td>
<td>66 DSPs</td>
<td>3q*</td>
</tr>
<tr>
<td>All-cause ESRD$^{43,44}$</td>
<td>Type 1</td>
<td>African American</td>
<td>ASP</td>
<td>129 ASPs and</td>
<td>10p and 10q</td>
</tr>
<tr>
<td>DN$^{45}$</td>
<td>Type 2</td>
<td>Turkish</td>
<td>ASP</td>
<td>18 extended</td>
<td>18q22</td>
</tr>
<tr>
<td>Albumin-to-creatinine ratio$^{46}$</td>
<td>Multiple ethnic groups</td>
<td>Quantitative trait</td>
<td>805 families</td>
<td>19, 12q</td>
<td></td>
</tr>
<tr>
<td>DN$^{47}$</td>
<td>Type 2</td>
<td>Caucasian</td>
<td>ASP, DSP, USP</td>
<td>27 Caucasian</td>
<td>10p*</td>
</tr>
<tr>
<td>All-cause ESRD$^{38}$</td>
<td>Type 2</td>
<td>African American</td>
<td>ASP</td>
<td>166 families</td>
<td>3q, 7p, 18q</td>
</tr>
<tr>
<td>Albumin-to-creatinine ratio$^{49}$</td>
<td>Multiple ethnic groups</td>
<td>Quantitative trait analysis</td>
<td>63 extended families</td>
<td>13q33.3, 9q34.3, 4p15.32, 1q25.1, 22q, 7q, 5q</td>
<td></td>
</tr>
<tr>
<td>DN$^{50}$</td>
<td>Type 1</td>
<td>Finnish</td>
<td>DSP</td>
<td>83 DSPs</td>
<td>3q, 4p, 9q, 22p</td>
</tr>
<tr>
<td>DN and albumin-to-creatinine ratio$^{51}$</td>
<td>Both, mostly type 2</td>
<td>Multiple ethnic groups</td>
<td>ASP, DSP, USP, quantitative traits</td>
<td>378 families†</td>
<td>7q, 10p, 14q, 18q</td>
</tr>
</tbody>
</table>

ASP, concordantly affected sibling pair; DSP, discordant sibling pair; USP, concordantly unaffected sibling pair.

*Genome scan not performed.
†Does not comprise the full FIND sample.
association mapping than for linkage. Other cohorts described in Table 2, although not family based, also have the potential to discover nephropathy genes through association analyses as described later.

In total, only 5 genome scans performed for type 2 DN and 1 for type 1 DN have been reported in the literature. The definitions of DN have evolved over time, with microalbuminuric patients being considered affected in some studies but not in others. The establishment of diabetes before nephropathy initiation and the length of diabetes duration may have been variable between studies as well, especially in type 2 DN. These subtle features have not been well documented and it is possible that undetected heterogeneity has crept into the analyses as a result of differences in the clinical characteristics of recruited patients.

In comparing the genome scans, most published studies have discovered at least one unique locus (Table 3). The best evidence from the genome scans abstracted from the original articles shows little replication between studies except at 10p and 18q. Within 10p the marker D10S1435 shows evidence of linkage in at least 3 studies for type 2 DN, including FIND,40,42,43,52 and is validated further in an association study of type 1 DN.53 On chromosome 18q, the carnosinase gene has been identified after a linkage scan of 18 Turkish families multiplex for DN45 and 2 of the larger published linkage scans showed evidence in this region.38,40 Fine mapping to determine if genetic variation in carnosinase best explains the linkage signal in these 2 studies still is needed. Although chromosome 7 consistently has been reported to show linkage and association for DN, careful examination of these loci does not show overlap (Fig. 2). Evidence against linkage at specific loci has not been compared rigorously across studies, and loci that fail to meet genome-wide significance criteria in replication may show signal corroboration at more modest significance levels. One mechanism to compare genetic evidence across studies is a meta-analysis with the primary data from each study.

Figure 2. Comparison of results from four five studies of type 2 DN on chromosome 7, placed on the microsatellite map (X-axis). The approximate areas of the best regions for each study are circumscribed along the length of the chromosome. Either the log of the likelihood ratio (LOD) score or the P value are marked along the Y-axis. Therefore, the height of the bars is proportional to the significance of the linkage or association evidence. Each study is described in Table 3 and references 9, 38, 39, 49, and 51.
study, an approach that has not yet been undertaken by the kidney community but has been used with success to evaluate susceptibility alleles for type 2 diabetes and other complex genetic traits.\(^54\,56\) Meta-analyses also would enable comparisons in the phenotype definitions between studies.

Successful, systematic fine mapping of DN linkage signals to find the causative genes or variants has been reported. Janssen et al\(^40\) identified carnosinase (CNDP1) as the causative gene under the 18q linkage peak in the Turkish sample and confirmed this finding in a sample set obtained from Pima Indians. Other groups have confirmed that the trinucleotide repeat in exon 2 of the CNDP1 gene, with homozygosity of a 5-leucine repeat in the leader peptide, protects diabetes mellitus patients against nephropathy, particularly in Caucasian samples (B. I. Freedman, personal communication). Interestingly, Zschocke et al\(^57\) were unable to see any association between CNDP1 and coronary disease or survival/longevity, suggesting that this gene may be specific for renal failure susceptibility. It is unclear if a common set of genes regulates both DN initiation and progression. The distinction between initiation genes and progression genes has been difficult to address. Individuals with renal insufficiency undergo a survival bottleneck as a result of comorbid cardiovascular disease, which frequently leads to early mortality. Some investigators have speculated that death of DN patients as a result of cardiovascular disease is not random but is driven by a common gene set for kidney and cardiovascular disease. Therefore, survivors who progress to proteinuria and ESRD may retain only a fraction of DN susceptibility alleles, biasing findings toward survival alleles or less robust disease susceptibility variants. In practice, using very strict trait definitions in case-control studies may bias which genes are located as a result of this phenomenon. However, in family based studies the use of the quantitative data in all available siblings should provide some safeguards against such a trend; a linkage peak that retains a good proportion of its signal after the exclusion of microalbuminuric individuals should argue for a lack of bias. The investigation by Zschocke et al\(^57\) addressed the issue of common genes for coronary disease and survival bias in ESRD. Many more studies focusing on this gene are underway.

In addition to fine mapping efforts following up linkage signals, another study\(^58\) used the transmission disequilibrium test (TDT)\(^59,60\) to evaluate candidate type 1 DN genes in trios, a design that requires inclusion of a child affected with DN and both parents (ie, a trio). Neurphilin 1 (NRPI) is located under the 10p linkage peak identified in several genome-wide scans (discussed previously) and marginal evidence of association was reported for 2 NRPI SNPs. In the same report, the B-cell leukemia/lymphoma 2 proto-oncogene (BCL2), which is located on chromosome 18q21 near the carnosinase (CNDP1) locus, was reported to be associated with type 1 DN. The BCL2 association signal may indicate the presence of a second DN gene in the 18q region or may reflect extended linkage disequilibrium (correlation between markers near each other on the same chromosome) in this region. The linkage scans that reported the 10p and 18q signals are based predominantly on type 2 diabetes, and the investigation by Ewens et al\(^58\) and by McKnight et al\(^52\) may be the first evidence for commonality of genetic determinants between type 1 and type 2 DN, but the results, although intriguing, remain to be confirmed in larger-scale studies.

**CANDIDATE GENE STUDIES AND SYSTEMATIC GENOME-WIDE ASSOCIATION SCREENS**

Candidate gene analysis has been conducted for DN and ESRD in hundreds of genes because it is more feasible for a single investigator to assemble a modest number of cases and controls. The concern with this approach is that the genes selected are driven solely by previously known biology and therefore are limited in spectrum. We have not attempted to review the many association studies published in the literature for single genes, but have focused this review on the newer studies that systematically have examined more than 100 genes or have performed genome-wide association scans. Our rationale for this approach is that many single candidate gene reports were based on a small sample size and minimal coverage (one or only
a few markers) within or near the gene. Therefore, results are hard to interpret because most studies lack adequate power to detect an association. In recent years, standards for reporting associations have changed, and even single gene investigations need to genotype multiple SNPs in a sufficiently large sample.

Three published studies have canvassed the genome for DN variants using association or have genotyped a large number of genes at a greater depth. These articles include a genome scan using 81,315 SNPs of 87 patients with type 2 DN and 92 controls without DN in a Japanese population, scanning of 200 Irish type 1 diabetic patients with overt nephropathy and 200 type 1 diabetic patients with normal albumin excretion using 6,000 microsatellite markers, and the evaluation of 115 candidate genes using the TDT approach for type 1 DN. The Japanese association scan lead to the discovery of ELMO1, a novel gene on chromosome 7, that may be associated with DN. In the scan, 1,615 SNP loci had significant P values of less than .01 between DN and control patients. Patients and controls were ascertained at clinics and were designated according to their renal functional status. Thus, patients with DN, that is, patients with diabetic retinopathy and overt nephropathy, were individuals with urinary albumin excretion rates of 200 μg/min or greater or urinary albumin-to-creatinine ratios of 300 mg/g or more of creatinine, or patients receiving chronic renal-replacement therapy. Control subjects included patients with diabetic retinopathy but showing no evidence of renal dysfunction (ie, urinary albumin excretion rates <20 μg/min or urinary albumin-to-creatinine ratios <30 mg/g creatinine). The SNPs chosen for genotyping were selected randomly from the gene-based Japanese SNP database. Of these, the best evidence was at SNP rs741301 on 7p14. Two other nearby SNPs, rs7799004 and rs1558688, also showed good association signals in a haplotype analysis. The evidence outside of those 3 SNPs was weaker but still adequate. The association of the rs741301 SNP with DN was confirmed in a second sample of 459 patients and 242 controls. However, the haplotype around this SNP shows very weak association, probably owing to very weak linkage disequilibrium in the sample. The risk allele (G; reference allele is A) is rare in the Chinese, Japanese, and Caucasian populations, but is the more common allele in the Yoruba population. The SNP itself is intronic—in intron 18—and is not functional, and falls within a conserved haplotype block between exons 16 and 22, suggesting that the sentinel SNP may not be the causative ELMO1 variant. Because of the reduced linkage disequilibrium (lack of correlation between neighboring markers) within ELMO1, it may be difficult to replicate this report until a causal variant is identified. This hypothesis is supported by the haplotype analyses reported in this article. The G risk allele is found in haplotypes both associated and not associated with DN, suggesting that the causative variant has not yet been located. Therefore, typing only the 3 most significant SNPs from the Japanese sample, rs741301, rs7799004, and rs1558688, may or may not show evidence of association in a different sample. By comparing the haplotype blocks and examining tagging SNPs from several different populations in HAPMAP, we have estimated that in most ethnic groups it will be necessary to genotype approximately 90 to 100 SNPs to obtain full coverage of this gene, showing the need to plan replication studies carefully.

The genome scan by McKnight et al used a DNA pooling paradigm. In this study, DNA from the affected (patients) and control enrollees was pooled separately and genotyped for approximately 6,000 markers. The allele frequency profiles at each marker were compared between case and control pools, and the resulting profiles were ranked in order of the greatest to the least difference between pools. Markers on 10p (described previously) showed association with DN. Although the best evidence for association was on 10p, other loci including D6S281, D4S2937, D2S291, and D17S515, ranked next in order of priority. Participants in this study were type 1 diabetic patients and controls from Northern Ireland and the Republic of Ireland. Phenotype criteria for a case definition required an onset of persistent proteinuria (0.5 g protein/24 h) at least 10 years after the diabetes diagnosis, the presence of hypertension (blood pressure > 135/85 mm Hg and/or treatment with antihypertensive agents), and the pres-
ence of diabetic retinopathy. Control subjects had diabetes for at least 15 years, normal urinary albumin excretion, and normal blood pressures without treatment. DNA pooling, although not a commonly used disease gene mapping design, can reduce genotyping costs significantly, and when integrated into a 2-stage analytic plan it has been an efficient approach for the association of genetic variants with other disease phenotypes. With only 6,000 microsatellite markers, the screen reported by McKnight et al has a low resolution.

These 2 whole-genome association screens illustrate 2 approaches to gene mapping design. The phenotype definition in the Japanese screen was much less stringent whereas the genotyping design was more comprehensive. In contrast, the ascertainment scheme in the Irish scan was much more restrictive, but the genotyping screen was less comprehensive. Currently, there is a move to very dense genome-wide association screens with genotyping of 500,000 to 1,000,000 markers to ensure adequate coverage, and neither of these 2 investigations meet that criterion. Their efforts as landmark studies in the DN arena should be regarded as the initiation points for more detailed studies.

Finally, a study by Ewens et al, an extensive survey of 115 known and novel candidate genes, deserves mention. The study by Ewens et al examined 72 families with type 1 diabetes and nephropathy, defined as ESRD or 2 of 3 random urine albumin-to-creatinine ratios greater than 300 µg/mg collected at least 6 weeks apart, and used the TDT to test for association. This family based association study combined both linkage and association testing and controlled for population substructures that resulted in false-positive results in case-control designs from gene mapping. Modest evidence was detected for association of DN with multiple candidate genes, including aquaporin 1, B-cell leukemia/lymphoma 2 (BCL2) proto-oncogene, catalase, glutathione peroxidase 1, insulin-like growth factor (IGF) 1, laminin alpha 4, laminin gamma 1, mothers against DPP homolog (SMAD), SMAD 3, transforming growth factor beta receptors II and III, tissue inhibitor of metalloproteinase 3, and upstream transcription factor 1. However, the sample size is this study was small and the results have yet to be confirmed in other studies.

CONCLUSIONS AND FUTURE DIRECTIONS

One third of all diabetic subjects remain undiagnosed in the United States. The complications of diabetes accrue in these individuals and, as shown by Koopman et al after adjusting for age and diagnosed or undiagnosed hypertension, the association between undiagnosed diabetes and nephropathy persists (odds ratio, 2.35; 95% confidence interval, 1.38-4.01). With an unprecedented increase in the worldwide incidence of diabetes looming in the horizon, it is very important that multiple approaches to identifying individuals at risk be undertaken to reduce the burden of diabetic complications. In this review, we have shown that an array of techniques has been used by the nephrology community to map genes for DN susceptibility. Each of these techniques has specific advantages and disadvantages, but the findings require replication to be meaningful. As described earlier, replication may not be perceptible at first glance and careful secondary analysis may be necessary to reconcile apparently discrepant results.

Replication will be particularly hard if rare variants cause disease. The refocusing of gene mapping efforts from large-scale linkage studies to large-scale association studies has changed the paradigm from finding both rare and common variants to finding common variants. Discovery of the polycystic kidney disease genes (PKD1 and PKD2) was successful through the use of traditional linkage mapping and subsequent fine mapping in families. The allelic spectrum of mutations in PKD1 and PKD2 is sizeable, and it is unclear if association methods would have been able to find these genes. Therefore, the results of a single cohort (e.g., FIND, EDIC/DCCT, or GoKinD) should not be evaluated in isolation but rather put in context of the type of study design and the power of the design to find genes for specific elements of type 1 or type 2 DN. For example, the kidney traits emphasized in FIND are extreme type 2 DN phenotypes, with the majority of probands being on dialysis or having greater than 1 g
albuminuria. However, siblings of FIND probands span the range of albuminuria from no renal disease (<30 μg/mg albumin-to-creatinine ratio) to proteinuric patients (>1 g/24 h), with variable duration of diabetes. In contrast, the ascertainment strategies of EDIC/DCCT excluded subjects with overt albuminuria and nephropathy complications have developed over follow-up periods of up to 23 years. GoKinD probands included subjects with type 1 diabetes and both microalbuminuria and normoalbuminuria. The FIND design has the potential to identify both rare and common disease genes, whereas the power in EDIC/DCCT and GoKinD is geared toward common disease genes. Considering the strengths of the other cohorts, EDIC/DCCT and GoKinD have a better representation from the population with less pronounced disease, features that are conducive to the identification of initiation genes. EDIC/DCCT also has longitudinal data and may be able to distinguish initiation genes from progression genes using survival analyses–type methods.

With the advent of new SNP technology, enabling thousands of markers to be genotyped simultaneously, new DN gene mapping initiatives are in progress. FIND is in the process of a 6,000+ marker genome-wide SNP linkage scan. Admixture scans in African Americans and Mexican Americans are also on the agenda for FIND. GoKinD has been accepted for genotyping by a new public-private initiative called Genetic Association Information Network (GAIN) (http://www.fnih.org/GAIN/GAIN_home.shtml) and will undergo the genotyping for 1,000,000 SNP markers. Similarly, the EDIC/DCCT cohort also is expected to initiate a very dense genome scan in that sample. Therefore, we anticipate that a vast amount of data will be generated through these larger-scale initiatives in the future. A comparison of the variety of genetic and molecular data between studies such as FIND, GoKinD, and EDIC/DCCT is anticipated to detect genes for DN. The identification of ELMO1 and CNDP1 already has spawned investigations into the biology of these genes in an effort to translate the basic science to clinical medicine and therapeutic approaches. In conclusion, the prospects for DN genetics are very positive, and the interplay between these larger initiatives and other genome-scale projects should help to elucidate the pathobiology of DN.

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