Linkage Analysis of Candidate Loci for End-Stage Renal Disease due to Diabetic Nephropathy

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Abstract. Diabetic nephropathy (DN), a major cause of ESRD, is undoubtedly multifactorial and is caused by environmental and genetic factors. To identify a genetic basis for DN susceptibility, we are collecting multiplex DN families in the Caucasian (CA) and African-American (AA) populations for whole genome scanning and candidate gene analysis. A candidate gene search of diabetic sibs discordantly affected, concordantly affected and concordantly unaffected for DN was performed with microsatellite markers in genomic regions harboring nephropathy susceptibility loci. Regions examined were at human chromosome 10p,10q (orthologous to the rat renal susceptibility Rf-1 locus), and at NPHS1 (nephrin), CD2AP, Wilms tumor (WT1), and NPHS2 (podocin) loci. Linkage analyses were conducted using model-free methods (SIBPAL, S.A.G.E.) for AA, CA, and the combined sample. Allele frequencies and the identity by descent sharing were estimated separately for AA and CA, and race was included as a covariate in the final linkage analysis. To date, we have collected 212 sib pairs from 46 CA and 50 AA families. The average age of diabetes onset was 46.8 yr versus 36.2 yr for CA and 39.5 yr versus 40.2 yr for AA, in males versus females respectively. Genotyping data were available for 106 sib pairs (43 CA, 63 AA) from 27 CA (44% male probands) and 38 AA families (43% male probands). Average AA and CA sibship size was 2.73. Singlepoint and multipoint linkage analyses indicate that marker D10S1654 on chromosome 10p is potentially linked to DN (CA only multipoint P = 4 × 10⁻⁵). Interestingly, the majority of the linkage evidence derives from the CA sib pairs. We are now adding sib pairs and increasing marker density on chromosome 10. We have excluded linkage with candidate regions for nephrin, CD2AP, WT1, and podocin in this sample. In conjunction with previous reports, our data support evidence for a DN susceptibility locus on chromosome 10.

ESRD: a Common Complex Disease With a Genetic Basis

ESRD is a multifactorial disease with increasing worldwide incidence, due in part to the aging population (1). Progression of diabetic nephropathy (DN) to ESRD can be effectively slowed by aggressively treating hyperglycemia and hypertension, using either angiotensin converting enzyme inhibitors or angiotensin receptor blockers. Although modifiable risk factors have been identified, epidemiologic data, primarily from Caucasian and Pima Indian populations, indicate that DN is genetically determined, with multiple genes regulating the phenotype (reviewed in reference 2–5). Several lines of evidence suggest that ESRD pathogenesis is mediated by genes. First, there is significant evidence of familial aggregation for ESRD (6–10). Second, animal models also suggest that ESRD pathogenesis is mediated by genes (11–14). Lastly, environmental factors alone cannot adequately explain the excessive clustering of ESRD in families.

Complex segregation of families in which DN was clustered, performed independently by two groups of investigators, demonstrated a major genetic effect on susceptibility to DN. Imperatore et al. (15) defined nephropathy as a urine protein to creatinine ratio ≥500 mg/g in 715 nuclear, Pima Indian families, and rejected models for no major gene effect and no transmission of a major effect (P = 0.00001; P = 0.003). In contrast, they were unable to reject Mendelian transmission models (P = 0.85), although the specific mode of inheritance could not be determined. Fogarty et al. (16) examined segregation of a quantitative trait, urine albumin to creatinine ratio, in 96 large multigenational pedigrees ascertained for type 2 diabetes and found that a multifactorial mode of inheritance best fit the ratio of albumin to creatinine levels. Together, these segregation analyses support the hypothesis of a major genetic effect on susceptibility to DN.

Strategies that have identified the genes responsible for monogenic renal diseases such as polycystic kidney disease (17–20) and Alport’s syndrome (21,22) are being applied to more common causes of progressive renal disease, such as DN, hypertensive nephrosclerosis (HN), and glomerulonephritis.
(GN). Classical linkage analysis of extended families has met with moderate success in mapping genes for familial forms of focal segmental glomerulosclerosis (FSGS) and IgA nephropathy (IgAN). Familial FSGS has been linked to markers on chromosome 19q13 (23,24), 11q21–q22 (25), and 1q25–31 (26). Mutations in the gene encoding α-actinin-4, an actin filament cross-linking protein, were identified in three families with an autosomal dominant form of FSGS linked to the 19q13 locus (27), identifying yet another molecular basis for a common histologic phenotype. Gharavi et al. (28) used genome-wide linkage analysis of 30 multiplex IgAN kindreds and identified a locus for IgAN on 6q22–23, with an autosomal dominant model of transmission with incomplete penetrance. The investigators obtained a lod score of 5.6, and observed that 60% of kindreds were linked to markers on chromosome 6.

Collection of extended family data are problematic for the most common causes of nephropathy, such as DN and HN, because the age of disease in probands is later in life and relatives are often deceased or unavailable for collection. Furthermore, basic assumptions of traditional linkage analysis for Mendelian traits often cannot be applied to multifactorial diseases. The nature of complex diseases precludes the use of specific genetic models in the linkage analysis, because the mode of inheritance is often unknown. Locus heterogeneity, as observed for FSGS above, as well as allelic (mutation) heterogeneity, further constrains the usefulness of traditional linkage methods. New linkage methods have been developed that use nuclear family structures, such as affected and discordant sibling pairs, which make no assumptions about the mode of inheritance of the disease (for reviews see references 29 and 30). These novel, model-free methods have formed the basis of genetic investigations in common forms of nephropathy. In aggregate, these data suggest that genes mediating ESRD pathogenesis are complex in etiology.

Candidate Loci for Diabetic Nephropathy

In an effort to reduce genetic heterogeneity, our group has limited recruitment to families with DN. Association analyses of candidate DN pathogenesis genes using case-control designs are common, but results often cannot be replicated. Only two linkage analyses of families in which DN is clustered have been reported. Moczulski et al. (31) performed a linkage study using 66 type 1 diabetic Caucasian sib pairs who were discordant for DN. Chromosomal regions containing genes for ACE, angiotensinogen, and angiotensin II type 1 receptor (AT1) were examined. The investigators observed significant evidence of linkage at microsatellites near AT1. Follow-up analyses demonstrated that DN was linked with a major nephropathy susceptibility locus in a 20-cM region, which included AT1 ($P = 7.7 \times 10^{-7}$). Imperatore et al. (32) undertook a more comprehensive genome-wide survey in 98 diabetic sibling pairs concordant for diabetes and nephropathy to identify DN susceptibility loci among the Pima Indians. They observed suggestive evidence for linkage on chromosomes 3, 7, 9 and 20.

Although our goal is to identify genes that regulate DN, several epidemiologic studies indicate that inherited susceptibility to progressive renal failure is independent of the etiology of ESRD (8,33), suggesting that genes or loci linked to non-diabetic causes of kidney disease should be assessed as candidate pathogenesis genes for DN. Whole genome linkage analysis in the fawn-hooded rat, a model of hypertension and nephrosclerosis, identified two renal failure susceptibility genes, Rf-1 and Rf-2 (11). The region of human homology for Rf-1 was identified as the distal portion of chromosome 10q. Yu et al. (34) investigated if markers on human chromosome 10 were linked with chronic renal failure in 129 African-American nondiabetic sibling pairs concordant for ESRD. The investigators observed weak evidence for linkage with markers on 10p, but not in the Rf-1 region.

In vitro, animal and human data suggest that podocyte dysregulation can initiate and/or perpetuate progressive glomerular scarring (35). Both transgenic animal models and familial glomerulosclerosis have been linked to mutations in two novel and two previously known genes, which encode podocyte proteins that comprise components of the slit diaphragm. Although mesangial expansion is the classic lesion of DN, recent work suggests that podocyte injury occurs early in the course of DN and filtration barrier dysfunction is a hallmark of the disease. NPHS1 (nephrin) and NPHS2 (podocin) were identified by positional cloning in families with congenital nephrotic syndrome of the Finnish type (36), and families with steroid-resistant nephrotic syndrome (37), respectively. Nephrin, an Ig superfamily member, is thought to be a major transmembrane component of the slit diaphragm (38–40). Podocin, which is similar to stomatin family scaffold molecules, has a single hairpin-like structure with both the N-terminal and C-terminal domains in the cytosol (37). ACTN4, which encodes the actin cross-linking protein α-actinin-4 (41), was identified as a candidate gene on the 19q13 locus mapped from three families with FSGS. CD2 associated protein (CD2AP) was originally identified as an adapter protein that interacts with the cytoplasmic domain of CD2, a T cell adhesion protein but subsequently was found to cause progressive glomerulosclerosis in mice lacking CD2AP (42).

Materials and Methods

Sib Pair Study Design

In our ongoing project we are collecting sibling pairs who are concordant for diabetes and renal disease (affected sib pairs, ASP), based upon the rationale that genes predisposing to ESRD are more likely to segregate in families with more than one affected sibling. To distinguish between diabetes susceptibility loci and DN loci, we are also collecting sibling pairs who are concordant for diabetes and discordant for DN (discordant sib pairs, DSP). In diseases such as DN where the recurrence risk for diabetes among siblings is high, DSP may provide as much information as ASP (43). Our selection criteria for ASP and DSP with type 2 diabetes and DN, and the strategy for data collection are described in a previously published methods paper (44). Briefly, the clinical characteristics (phenotype) of index cases from dialysis clinics and family members (predominantly sibs) are ascertained with a questionnaire, from medical record review, and from measurement of proteinuria, serum creatinine and HgbA1C. The general classification scheme for sib pairs with varying degrees of albumin excretion is described in Figure 1. Diabetes is defined by
Candidate Gene and Linkage Analyses

Database

HgbA1C ≥ 7.0 mg/dl, fasting serum glucose ≥ 126 mg/dl, random glucose ≥ 200 mg/dl, or prevalent treatment with insulin or oral hypoglycemic agents. DN is defined by diabetes duration ≥ 10 yr, urine protein ≥ 500 mg/g creatinine, and background or proliferative retinopathy is defined by ophthalmology records, or history of laser surgery. Index cases and affected sibs must meet diabetes and DN criteria. Discordant sibs must have diabetes ≥ 10 yr, but no proteinuria (< 30 µg albumin/g creatinine).

Results

Data Collection

To date, we have obtained information on 1577 type 2 diabetic index cases (see Figure 2 and Table 1). Comparison of family history records from subjects with all causes of ESRD (n = 2804) versus DN only (n = 1577) revealed several trends (Table 1). First, there is a significant excess of females with a family history among all-cause ESRD and DN (P = 8.5 × 10^{-9}), although this gender difference is great in DN (P = 3.4 × 10^{-13}). Second, there is no difference in the percentage of African Americans (AA) and Caucasians (CA) with and without a family history for all cause ESRD versus DN (with family history P = 0.968, without family history P = 0.091). We also compared the DN probands enrolled in the genetic portion of the study (n = 229) to those who participated in the initial questionnaire (n = 964), but did not meet study criteria (Table 2). There were no significant differences in the average ages of the two groups. Comparing index cases with a living diabetic sib versus the unenrolled population, we did observe an increase in the percentage of AA compared with CA (P = 0.023). The source of this difference is likely to be the decrease in the percentage of male CA with living diabetic sibs.

Candidate Gene and Linkage Analyses

A candidate gene search of diabetic sibs discordantly affected, concordantly affected, and concordantly unaffected for DN was performed with microsatellite markers in regions suspected to harbor nephropathy susceptibility loci. Regions examined were at chromosome 10p and 10q, orthologous to the rat renal susceptibility Rf-1 locus, NPHS1 (nephrin, 19q), Wilm’s tumor (WT1), CD2AP, and podocin (NPHS2) loci. Linkage analyses were conducted using model-free methods (SIBPAL2, S.A.G.E.) for AA, CA, and the combined sample. Allele frequencies and the identity by descent sharing were estimated separately for AA and CA. Race was included as a covariate in the final linkage analysis.

The protocol has been approved by Institutional Review Boards at MetroHealth Medical Center and University Hospitals of Cleveland.

Figure 1. Classification of sib pairs for linkage analysis on the basis of albumin excretion. Model-free linkage analysis used three types of sib pairs; concordantly affected, concordantly unaffected, and discordant. Unaffected sibs were long-standing diabetics (diabetes mellitus duration > 10 yr) who did not demonstrate evidence of incipient nephropathy. In contrast, affected sibs were individuals who progressed to overt nephropathy.

Table 1. Summary of the study population.

| NPHS1 (nephrin, 19q), Wilm’s tumor (WT1), CD2AP, and podocin (NPHS2) loci. Linkage analyses were conducted using model-free methods (SIBPAL2, S.A.G.E.) for AA, CA, and the combined sample. Allele frequencies and the identity by descent sharing were estimated separately for AA and CA. Race was included as a covariate in the final linkage analysis.

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Figure 2. Recruitment update for the Cleveland ESRD population (updated from 44).
families contain 212 sibs pairs, of which 59 and 72 meet ASP and DSP criteria, respectively. The remaining sibs pairs (n\text/H11005/81) fail to meet phenotype requirements, due primarily to sibs with microalbuminuria or diabetes duration <10 yr. Sixty additional families are under evaluation.

We also compared the age-at-diabetes-onset in probands and their diabetic sibs (Table 3). When analyzed as a group, ages of diabetes onset are similar in sibs and probands. After stratification of the sibs by phenotype, DSP sibs are significantly older than the probands at the onset of diabetes, despite having diabetes duration as long as the proband.

Candidate Gene Analysis

Genotyping data are available for 106 sib pairs (43 CA, 63 AA) from 25 CA (44% male probands) and 37 AA families (43% male probands). Singlepoint and multipoint linkage analyses indicate that markers on chromosome 10p demonstrate suggestive evidence for linkage with a putative nephropathy susceptibility locus (Figure 3, \( P \text/H11005/0.03 \) for multipoint analysis) (45). Interestingly, the majority of the linkage evidence derives from the CA sib pairs. A second peak, with weaker evidence for linkage, is identified at the Rf-1 locus on 10q D10S1230). In addition, we have excluded linkage with candidate regions for CD2AP, WT1, podocin, nephrin, and ACTN4 (Table 4).

Discussion

The linkage results demonstrate strong evidence for a DN susceptibility locus on chromosome 10p in CA families. Yu \textit{et al.} (34) also showed linkage of two adjacent markers on 10p, D10S1435 and D10S249, to ESRD in sibling pairs with non-diabetic causes of ESRD (\( P \text/H11005/0.035 \) pairwise, \( P \text/H11005/0.082 \) multipoint for D10S1435; \( P \text/H11005/0.074 \) pairwise, \( P \text/H11005/0.063 \) multipoint for D10S249). In contrast to Yu \textit{et al.} (34), we also observed a second smaller linkage peak at the Rf-1 locus. Our results indicate the existence of one DN susceptibility locus of strong effect and a second locus of weak/moderate effect on chromosome 10, consistent with emerging evidence from genetic linkage studies performed in other populations (46,47).

We have previously demonstrated that the sibling recurrence risk and sibling recurrence risk ratio are greater in CA versus

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<th>Table 1. Comparison of the family history in all ESRD \textit{versus} diabetic nephropathy only</th>
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<td>Family history</td>
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<td>ESRD</td>
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<td>African American (%)</td>
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<th>Table 2. DN probands enrolled in the study \textit{versus} those not enrolled\textit{a}</th>
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<td>Index Cases with Living Diabetic Sib</td>
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<td>Age (yr)</td>
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\textit{a} All diabetic index cases (n = 1,577) were not included in these estimates. AA, African American; CA, caucasian.

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<th>Table 3. Comparison of DM onset and duration in probands \textit{versus} sibs\textit{a}</th>
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\textit{a} ASP, affected sib pairs; DSP, discordant sib pairs.
The results of our linkage analyses confirm that CA sib pairs are a powerful tool to map susceptibility genes for DN. We anticipate that fine mapping of chromosome 10p and 10q in a CA subpopulation that includes additional sibs may yield specific candidate genes, which regulate susceptibility for the development of DN and progression to ESRD.

**Acknowledgments**

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**References**