A New Locus for Familial FSGS on Chromosome 2P

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ABSTRACT

FSGS is a clinicopathologic entity characterized by nephrotic syndrome and progression to ESRD. Although the pathogenesis is unknown, the podocyte seems to play a central role in this disorder. Here, we present six kindreds with hereditary FSGS that did not associate with mutations in known causal genes, and we report a new locus for the disease on chromosome 2p15 in one kindred. We performed genome-wide linkage analysis and refined the linkage area with microsatellite markers and haplotype analysis to define the minimal candidate region. Genome-wide linkage analysis yielded a maximum two-point logarithm of odds (LOD) score of 3.6 for the six families on chromosome 2p. One family contributed the largest proportion of the additive score (LOD 2.02) at this locus. Multipoint parametric LOD score calculation in this family yielded a significant LOD score of 3.1 at markers D2S393 and D2S337, and fine mapping of this region with microsatellite markers defined a minimal candidate region of 0.9 Mb with observed recombinations at markers D2S2332 and RS1919481. We excluded the remaining five families from linkage to this region by haplotype analysis. These data support a new gene locus for familial FSGS on chromosome 2p15. Identification of the mutated gene at this locus may provide further insight into the disease mechanisms of FSGS.

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FSGS is a clinicopathologic entity that is characterized by the nephrotic syndrome and is often steroid resistant. Progression to end-stage kidney disease (ESKD) is frequent. Histologically, the lesion is characterized by focal glomerulosclerosis or tuft collapse, segmental hyalinosis, occasionally IgM staining on immunofluorescence, and effacement of foot processes on electron microscopy.[1] Its incidence is estimated at seven per million.[2] It is responsible for 2 to 20% of all cases of ESKD in the United States, and it is second only to urogenital and kidney malformation as a cause of ESKD in children.[2–5] In addition, it is the most common glomerular cause of ESKD.[3] The incidence of FSGS seems to be increasing. Kitiyakara *et al.*[5] reported an 11-fold increase among dialysis patients who were older than 21 years. In every age group, the incidence is higher in black than in white patients with a striking difference in the age distribution pattern in black patients; the highest incidence occurred in the 40- to 49-year age group.[2,5,6]

FSGS can be broadly classified into primary (idiopathic), secondary, syndromic, and familial. Fa-

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Table 1. Clinical characteristics of six large kindreds with familial FSGS

Study No.	Race	No. Affected	Age at Onset (years; median [range])	Proteinuria (g/d; median [range])	Age at ESKD (years; median [range])	Maximum Attainable LOD Score[a]	Maximum LOD Score (Mean [SE])[a]
6505	White	9	28 (20 to 37)	2.15 (0.30 to 4.50)	44.0 (28.0 to 50.0)	2.26	0.76 (0.017)
6509	Black	6	29 (25 to 32)	NA	31.0 (23.0 to 59.0)	0.89	0.44 (0.013)
6510	White	8	NA	6.50 (0.30 to 10.00)	40.0 (36.0 to 51.0)	1.47	0.43 (0.015)
6524	White	9	25 (16 to 29)	1.00 (1.00 to 3.00)	26.5 (17.0 to 56.0)	2.00	0.66 (0.016)
6543	White	13	19 (2 to 27)	3.00 (1.00 to 14.80)	33.0 (27.0 to 47.0)	3.85	1.58 (0.029)
6590	White	8	NA	NA	50.5 (48.0 to 66.0)	3.00	0.87 (0.021)

NA, not available.
[a]Statistical power to detect linkage assuming a rare dominant genetic model and a microsatellite marker with four alleles and a recombination fraction of 0.05.

milial cases are increasingly being recognized. The primary defect in FSGS seems to be in the filtration barrier of the glomerulus. This barrier is made up of the fenestrated endothelium, the glomerular basement membrane, and podocytes that have a slit diaphragm between their interdigitating foot processes. Disruption of the filtration barrier results in loss of permselectivity and albuminuria. The pathogenesis of FSGS has yet to be fully explained. Early theories included T lymphocyte dysfunction and dysregulation of cytokines and growth factors.[7,8] Recent results from genetic studies reveal mutations in genes that encode slit diaphragm and podocyte cytoskeletal proteins. There is now strong evidence to suggest that FSGS is essentially a disease of the podocyte. The first major breakthrough was the cloning of nephrin (*NPHS1*) as a cause of congenital nephrotic syndrome of the Finnish type.[9] Subsequently, five additional genes have been identified, including podocin (*NPHS2*); actinin-α4 (*ACTN4/FSGS1*); transient receptor potential cation channel, type 6 (*TRPC6/FSGS2*); CD2-associated protein (*CD2AP/FSGS3*); and phospholipase c, ε1 (*PLCE1/NPHS3*) as causes for FSGS and hereditary nephrotic syndromes.[10–14]

The discovery of these genes has allowed us to begin to unravel signaling events and cytostructural abnormalities in the podocyte and the slit diaphragm. Further studies of familial cases will help in understanding the fundamental pathways for the ultrastructural and functional changes seen in FSGS and the subsequent development of rational approaches to therapy. In addition, because the pathologic findings in the more common sporadic FSGS are similar to those of inherited FSGS, it is possible that common mechanisms are responsible for disease evolution in both familial and sporadic cases. Despite the advance in knowledge regarding inherited FSGS, there remains a subset of families who have hereditary FSGS but do not carry known disease-causing mutations.

We present a comprehensive genetic study of six kindreds with familial FSGS, which includes the sequencing of known FSGS genes and genes for other hereditary nephrotic syndromes, genome-wide linkage analysis, fine-mapping, and haplotype analysis. There is no evidence for linkage or mutations in previously identified genes in these families. Genome-wide linkage analysis identified three regions of linkage. Concentrated analysis of one very large family supported strong linkage to one of these regions, and fine-mapping narrowed the linkage region to a 0.9-Mb interval on chromosome 2p15.

RESULTS

Clinical Data and Power Studies

Six families met the inclusion criteria for this study. All kindreds had male-to-male transmission and affected members in at least three generations consistent with an autosomal dominant inheritance pattern. Basic demographics and clinical data are shown in Table 1. The pedigree of one of the largest families studied is shown in Figure 1 (family 6543), and the remaining pedigrees are shown in Supplemental Figure 1S. Family 6543 is

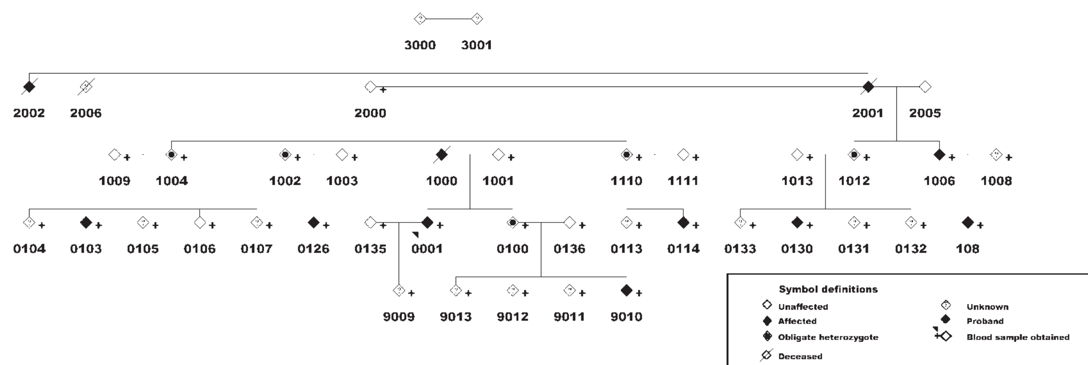

Figure 1. Autosomal dominant inheritance demonstrated in family 6543. This is a 65-member family from Central Europe dating back five generations. The median age of onset of disease in this family is 19 years. Individuals are coded as diamonds to protect anonymity.

Table 2. Clinical characteristics of affected individuals in family 6543

Individual No.	Age at Diagnosis (years)	Gender	Urinary Protein (g/24 h)	Renal Biopsy Findings	Age at ESKD (years)	Transplant/ Recurrence	Extrarenal Findings
1	6	F	NA	FSGS	33	Y/N	None
103	23	M	13	FSGS	28	Y/?Y	Ocular defect[a]
108	21	F	14	NA	27	NA/NA	Cardiomyopathy
114	14	F	NA	NA	NA	NA/NA	None
126	17	F	10	NA	NA	NA/NA	None
130[b]	27	M	1	NA	NA	NA	None
1000	NA	M	3	NA	47	NA/NA	MR
1006	21	M	3	FSGS	33	Y/Y[c]	Mild MR
2001	NA	F	5	NA	61	NA/NA	Malignancy
2002	NA	M	NA	NA	NA	NA/NA	None
9010	2	F	1	NA	NA	NA/NA	None

NA, not applicable; MR, mental retardation.
[a]Congenital grouped pigmentation of the pigment epithelium.
[b]Individual 130 was the only member of the family who received treatment. He was steroid-responsive and at last examination was in stable remission with normal renal function.
[c]Recurrence was biopsy-proven.

a 65-member kindred from Central Europe dating back at least five generations. The disease in this kindred is characterized by onset in the first two decades of life and rapid progression to ESKD by the third decade. Three affected individuals underwent kidney biopsy, and all had FSGS histology. Three affected individuals received kidney transplants; of these, two developed proteinuria after transplantation, and one has histologically confirmed FSGS recurrence in the graft. One affected individual responded to steroid therapy and is in stable remission with normal renal function. Extrarenal manifestations in this kindred include mental retardation (two affected individuals) and congenital grouped retinal pigmentation (one affected person). The clinical characteristics of the affected individuals in family 6543 are summarized in Table 2.

We evaluated the statistical power to detect linkage in the full set of families and in each family individually. This analysis revealed that the six pedigrees are capable of generating a mean (SE) maximum logarithm of odds (LOD) score of 4.000 (0.051) assuming a rare dominant genetic model and a microsatellite marker with four alleles and a recombination fraction of 0.05. This represents power of 72% to detect a LOD score of 3.0 and 90% to detect a LOD score of 2.0. The power analysis for the individual families is shown in Table 1. Family 6543, the largest family, has the highest power to detect linkage.

Mutation Analysis in Known FSGS/Nephrotic Syndrome Genes

No mutations were found in *NPHS1*, *NPHS2*, *ACTN4*, *TRPC6*, and *PLCE1* in any of the six families *via* direct exon sequencing.

GWLA of Six Kindreds with Familial FSGS

Two-point parametric and multipoint parametric LOD scores of >3 were obtained on chromosomes 2p, 2q, and 5p for the six families (Figure 2). By-family LOD score analyses were carried out for each of these regions and revealed that only family 6543 had a suggestive LOD score in the chromosome 2p region. The remaining five families did not have suggestive LOD scores on chromosome 2p, 2q, and 5p peaks (Figure 2S). We therefore

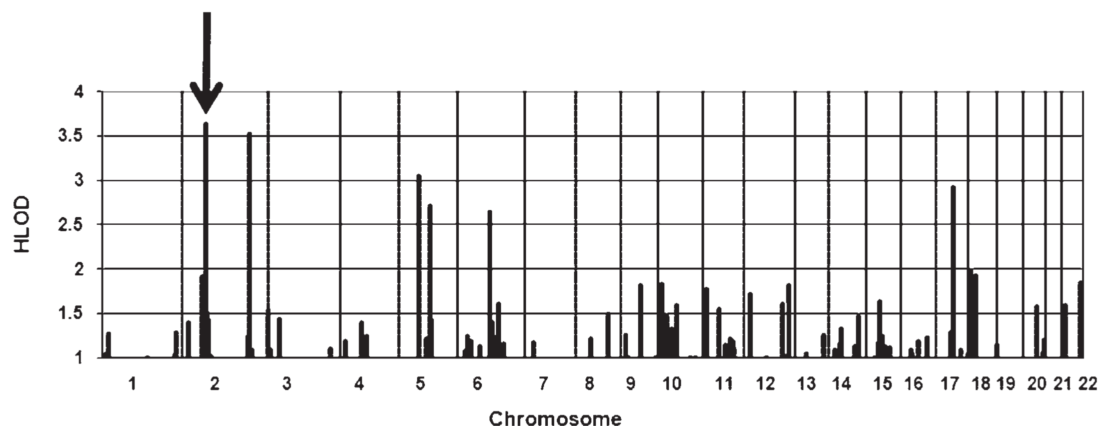

Figure 2. GWLA in six kindreds with familial FSGS showing peak heterogenous LOD (HLOD) score on chromosome 2p. Genome-wide linkage scan using the Illumina Infinum II HumanLinkage-12 genotyping beadchip assay yields a peak two-point parametric LOD score of 3.6 on chromosome 2p (arrow). Chromosome numbers are shown on the *x* axis and HLOD score on the *y* axis.

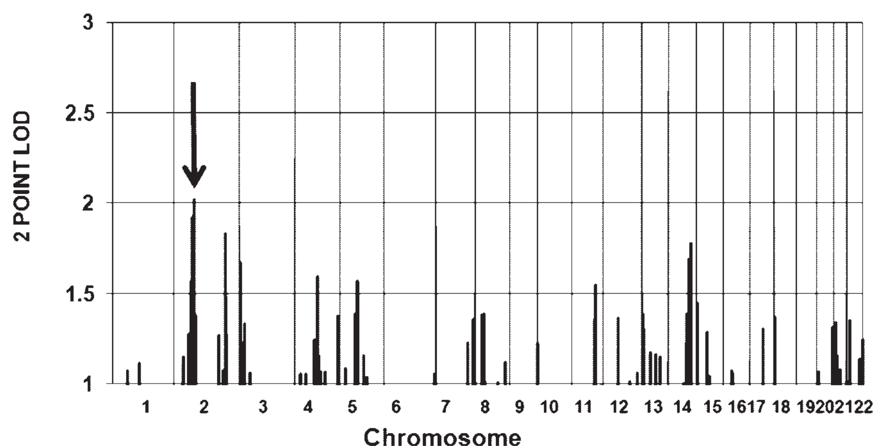

Figure 3. GWLA in family 6543 yielded a LOD score of 2.0 on chromosome 2p. Individual family analysis of the GWLA data yields a maximum two-point parametric LOD score of 2.02 in family 6543 (arrow); this represents the highest LOD score across the entire genome for this family.

carried out further analysis on the chromosome 2p locus in family 6543.

GWLA of Family 6543

We obtained a two-point parametric LOD score of 2.02 for family 6543 in the chromosome 2p region (Figure 3); a genome-wide multipoint parametric LOD score of 2.29 was obtained in the same region. This represents the highest LOD score obtained for family 6543 across the entire genome. Other regions with LOD scores >1.5 in family 6543 (2q, 3p, 4p, 5p, 11p, and 14q) were excluded by single-nucleotide polymorphism (SNP) haplotype analysis. Of the remaining five families, none attained LOD scores of >2 at this locus (Supplemental Figure 3S).

Fine-Mapping of the Chromosome 2p Region

To refine this region further, we genotyped microsatellite markers at the chromosome 2p locus for all six families. The two-point affecteds-only heterogeneity LOD score for all families was 2.60 using a recombination fraction of 0.05. As in the SNP linkage analysis, family 6543 contributed the majority of the evidence for linkage in this area with a LOD score of 2.40 at a recombination fraction of 0.05 (Table 3).

Given the high LOD scores for family 6543 and seeming heterogeneity in the other families at this region, we carefully examined the evidence for linkage to establish the peak linkage region and to define a minimum candidate region (MCR) critical interval using recombination events. Pairwise affecteds-only analysis yielded a LOD score of 2.7 ($\theta = 0$) at marker D2S337 in family 6543 (Table 4). Supporting evidence for linkage was obtained at marker D2S393 (LOD score of 2.4). The maximum multipoint LOD score of 3.1 was obtained using markers D2S393, D2S2160, and D2S337 in family 6543 (Figure 4). Haplotype analysis assuming a dominant model revealed clear evidence that a single disease-carrying chromosome segregated with affected individuals in family 6543 (Figure 5A). The recombination events observed among affected individuals established a minimal MCR of 0.9 Mb between markers D2S2332 and RS1919481, effectively narrowing the region to chromosome 2p15 (Figure 5, Supplemental Figure 4S). The LOD scores for families 6510 and 6524 support this localization (Table 3); however, the LOD scores are NS and haplotype analysis is inconclusive. These families may be useful for mutation analysis once the causative gene and mutation are identified in family 6543.

DISCUSSION

In this study we report six new cases of familial FSGS from the United States and Central Europe and define a new gene locus on chromosome 2p15 in one of these kindreds. The families in this series illustrate distinct heterogeneity in disease phenotype as indicated by wide variability in the age of onset of disease and progression to ESKD. The mode of inheritance in all families seems to be autosomal dominant, because they have at least three affected generations and both male and female individuals are affected. The demonstration of variability in the genotype is in accordance with the divergence of the clinical phenotype in these families and thus suggests that there are different disease loci and different genetic causes of FSGS in these kindreds. It is interesting to note that in family 6543, two

Table 3. Affecteds-only two-point LOD scores for all families at the chromosome 2p locus

Physical Distance (bp)	Markers	All Families	Family No.					
			6543	6505	6509	6510	6524	6590
58746029	D2S2734	0.000	−1.200	−0.240	−0.110	−0.940	−2.080	0.560
60462217	D2S357	0.019	0.700	−0.860	−0.460	−2.140	−1.170	−1.180
60707437	D2S2160	0.029	0.530	−1.680	0.000	0.129	−0.820	−0.870
61204074	D2S2332	1.420	0.990	−1.240	−0.460	1.320	0.490	−1.050
61581582	D2S337	2.600	2.400	−1.700	−0.460	0.350	0.530	−0.020
62968808	D2S2320	0.160	0.500	−0.070	−0.460	−0.800	0.050	−2.840

All scores are at $\theta = 0.05$.

Table 4. Affecteds-only two-point LOD scores for family 6543

Location (cM)	Physical Distance Start (bp)	Marker	Recombination Fraction (θ)							Z_{max}
			0.00	0.05	0.10	0.15	0.20	0.30	0.40	
68.80	44053930	D2S2298	−8.20	−2.00	−1.10	−0.60	−0.30	−0.02	0.04	0.04
71.90	46177320	D2S2182	−0.70	−0.10	0.36	0.58	0.67	0.64	0.40	0.67
78.40	54620134	D2S2153	−0.10	1.00	1.30	1.40	1.30	1.10	0.60	1.40
80.50	57215303	D2S378	−0.40	0.70	1.10	1.20	1.20	0.90	0.50	1.20
81.70	58746029	D2S2734	−2.30	−1.20	−0.80	−0.50	−0.40	−0.20	−0.10	−0.10
82.30	59423514	D2S393	2.40	2.20	2.00	1.80	1.50	1.10	0.60	2.40
83.98	61581582	D2S337	2.70	2.40	2.20	1.90	1.70	1.20	0.60	2.70
84.68	62968808	D2S2320	−4.00	0.54	0.88	0.96	0.93	0.71	0.37	0.96
87.70	65859547	D2S2293	−1.80	0.30	0.50	0.50	0.50	0.40	0.20	0.50
91.76	68809195	D2S358	−2.20	−0.00	0.20	0.20	0.20	0.20	0.10	0.20
97.20	72291413	D2S2112	−7.70	−1.80	−0.80	−0.30	−0.00	0.20	0.20	0.20
100.30	75742036	D2S2114	−4.40	−0.80	−0.10	0.20	0.30	0.40	0.30	0.40
108.18	85198750	D2S2161	−8.30	−3.30	−2.20	−1.50	−1.00	−0.40	−0.10	−0.10

The deCODE genetic map was used to estimate the genetic distance (http://www.decode.com/services/microsatellite-genotyping-genome-wide-scans.php).

affected individuals developed recurrence of FSGS in their renal allograft; this may suggest that the gene defect in this family may not necessarily be localized to the kidney or the podocyte alone. Further evidence for this is the presence of prominent neurologic and ocular findings in some of the affected individuals. An alternative explanation for the finding of disease recurrence would be similar to disease recurrence after transplantation in individuals with nephrin mutations caused by the introduction of a neo-antigen and subsequent development of anti-nephrin antibodies.[15,16]

The chromosome 2p region was initially narrowed to an MCR of 4.2 Mb and later to a 0.9-Mb interval. Genes that may have a role in the maintenance of the structural and functional integrity of the filtration barrier, those that are involved in cellular signaling, and cytokines are potential candidates. This includes *AHSA2*, an activator of heat shock protein. Heat-shock proteins are families of proteins that act as molecular chaperones that promote stabilization, repair, and disposal of denatured protein.[17,18] They are also known to assist in the assembly, sorting, and targeting of native protein to specific cellular domains. In addition, several members of the heat-shock protein family have been reported to be upregulated in

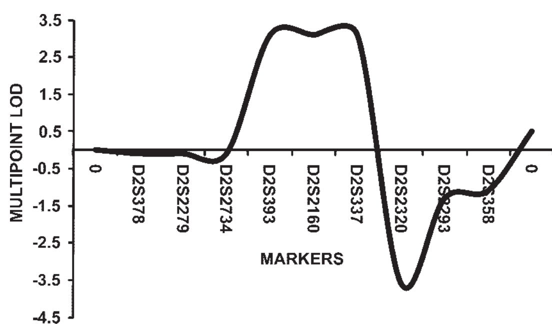

Figure 4. Multipoint parametric analysis using informative microsatellites confirms linkage to the chromosome 2p region. A maximum LOD score of 3.1 is obtained at markers D2S393 and D2S337. The flanking markers are D2S2734 and D2S2320, defining an interval of 4.2 Mb.

murine podocytes after glucocorticoid stimulation, and they have also been shown to regulate the morphologic and actin cytoskeleton response of podocytes in an *in vitro* model of podocyte injury.[19,20] The chaperonin containing TCP1 subunit 4 delta (*CCT4*) is also in this region. These genes assist the folding of protein after ATP hydrolysis, and they are known to play a role in *in vitro* folding of actin and tubulin.[21] It is therefore plausible that mutations in this gene may affect the cytoarchitecture of the glomerular slit diaphragm. We sequenced the coding regions of these two genes and did not find any disease-causing mutations. There are also many genes encoding for transmembrane proteins within the chromosome 2p region; mutation of genes encoding for these proteins may affect signaling at the slit diaphragm.

The kindred that mapped to chromosome 2p also displays significant variability in the phenotype of affected individuals, for example, steroid responsiveness and recurrence of disease in renal allografts. Identifying the gene mutated in this family may not only improve our understanding of the events that lead to the development of FSGS in both familial and sporadic cases but also provide further insight into mechanisms of disease recurrence and also possible therapeutic targets. In conclusion, we have defined a new locus for familial FSGS on chromosome 2p; future studies will include identification of the mutated gene and functional studies to verify and elucidate the mechanism of pathogenesis.

CONCISE METHODS

Ascertainment and Evaluation

Institutional review board approval was obtained from Duke University Medical Center (Durham, NC). Families were identified from responses to advertisements placed by the International Collaborative Group on Familial FSGS, advertisements in nephrology journals, published cases, and the personnel in the Division of Nephrology at Duke University Medical Center. Clinical material on these families

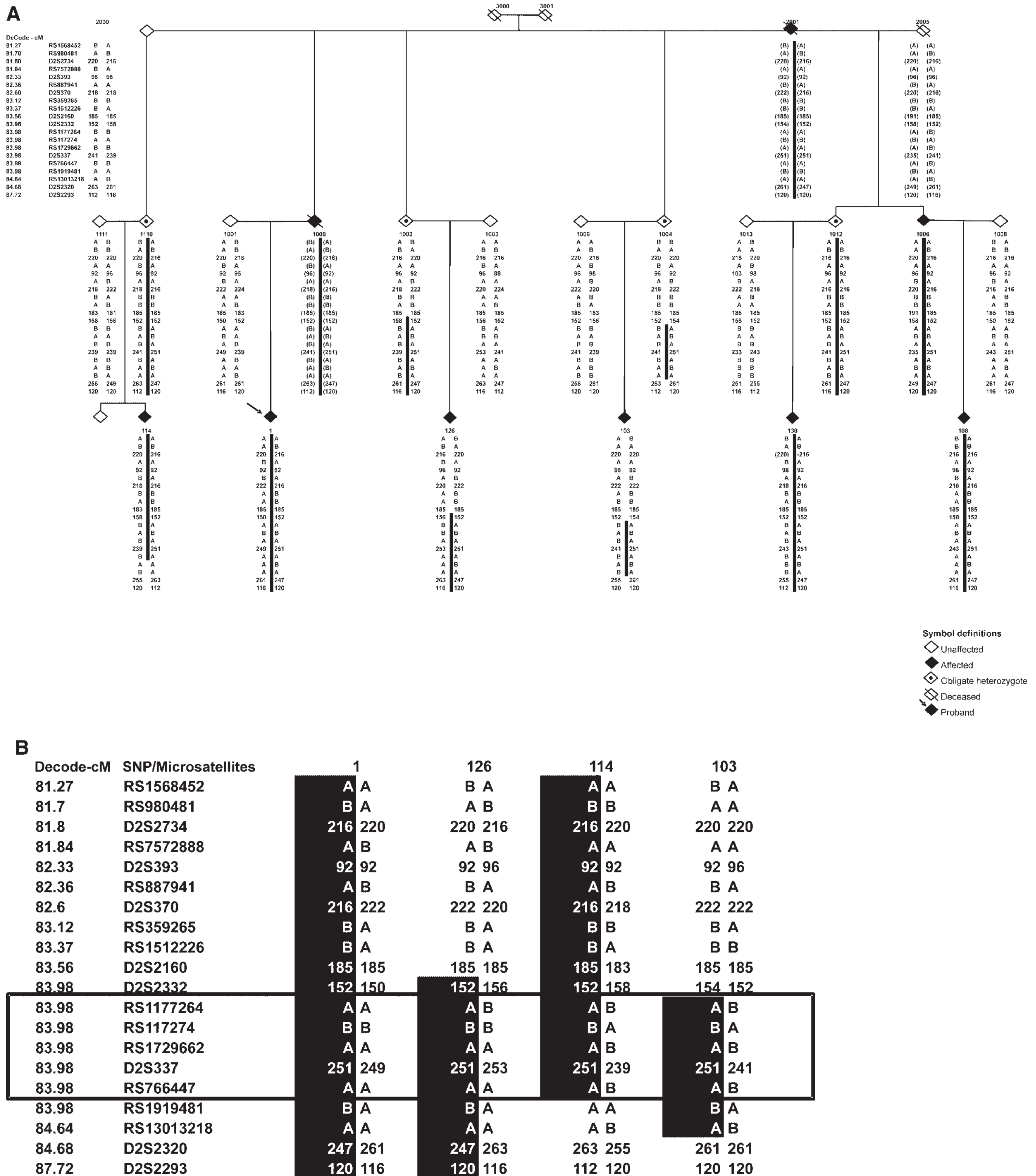

Figure 5. Haplotype analysis in family 6543 showing the minimal candidate region (MCR). (A) Haplotype analysis narrowed the minimal candidate region in family 6543 to a 0.9-Mb interval. Simplified pedigree that shows fine-mapping of the chromosome 2p region with informative microsatellites and SNPs yields the disease-causing haplotype (black rectangles). Recombination events in affected individuals in the family delimit the chromosome 2p region to an interval flanked by markers D2S2332 (defined by affected individual 103) and RS1919481 (defined by affected individual 114). The proband (solid arrow) carries the ancestral haplotype. Alleles or SNPs in parentheses are inferred because of missing genotype information. (B) Haplotypes of individuals defining the MCR in the chromosome 2p locus; individual 1 is the proband and has the ancestral haplotype. The centromeric flanking marker D2S332 is defined by affected individual 103, and telomeric flanking marker RS1919481 is defined by affected individual 114.

was compiled by clinical personnel from the Center for Human Genetics in conjunction with their primary physicians.

Inclusion criteria and determination of affection status are as previously reported.[22] Briefly, inclusion in this analysis required at least one individual with biopsy-proven FSGS and a second family member with FSGS and/or ESKD. Clinical evaluation of these kindreds included a full family history, physical examination, urinalysis with qualitative or quantitative proteinuria, and serum creatinine assay when appropriate. Renal pathology reports and slides were reviewed when available for affected individuals.

Diagnostic Criteria

Individuals were classified as follows:

Affected: When they required dialysis, had undergone renal transplantation, had 2+ to 4+ proteinuria by qualitative urinalysis or ≥500 mg/24 h on quantitative urinalysis, or had a renal biopsy demonstrating FSGS without evidence of other systemic diseases known to cause FSGS or chronic renal failure.

Probably affected: When they had trace to 1+ proteinuria on qualitative urinalysis. These individuals were categorized as unknown in the linkage analysis.

Unaffected: Individuals who had no detectable proteinuria on qualitative urinalysis and unrelated married-in spouses.

Power Analysis

Power analysis for the families who met inclusion criteria was performed using SIMLINK[23] to determine overall and family-specific statistical power using a rare dominant affected-only model with disease allele frequency of 0.001 and markers with four alleles with frequencies of 0.4, 0.3, 0.2, and 0.1. A total of 1000 replicates were simulated. Mean maximum LOD scores and SEs were calculated at a recombination fraction of 0.05.

DNA Isolation, Sequencing, and Genotyping

Genomic DNA was isolated from peripheral blood through the Center for Human Genetics, DNA Banking Core, Duke University Medical Center, using PureGene. The six families were screened for mutations in *NPHS1*, *NPHS2*, *ACTN4*, *TRPC6*, and *PLCE1* by exon sequencing. Exons for each gene were amplified using the PCR with primers that were designed using the Primer 3 design software (http://frodo.wi.mit.edu/primer3/) from known genomic sequence (http://genome.ucsc.edu/) using standard PCR protocols. Targeted sequence included all exons and a minimum of 25 to 50 bp of the intronic sequence surrounding each exon. PCR products were purified using the QIAquick PCR purification kit (Qiagen, Frederick, MD). Sequencing reactions were performed using BigDye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster, CA) and purified using Edge Biosystems Gel Filtration Columns (Edge Biosystems, Gaithersburg, MD). Sequencing was carried out using the ABI 3730 DNA Analyzer (Applied Biosystems) and analyzed using Sequencher DNA sequencing analysis software (Gene Codes Corp., Ann Arbor, MI).

A genome-wide linkage scan was performed using the Illumina Infinum II HumanLinkage-12 genotyping beadchip assay (Illumina Inc., San Diego, CA). This assay contained >6000 SNPs with an average genetic distance of 0.58 cM and call rate of >99%. Genotyping was performed on 120 of the most informative individuals from the six kindreds. Individuals 1 and 2 from CEPH family 1330 and replicate samples were also genotyped as quality controls. Microsatellite markers were identified in the chromosome 2p region. Primers were designed for the microsatellites. The forward primers were prelabeled with fluorescent dye, and a standard PCR reaction was carried out. Genotyping was performed on ABI 3730 Genetic analyzer (Applied Biosystems) at the Center for Human Genetics Duke University sequencing core.

Linkage and Haplotype Analysis

We performed a multistage analysis beginning with the genome-wide SNP linkage scan and followed by microsatellite fine-mapping. Markers and samples that did not meet quality control benchmarks for genotyping efficiency were removed. Genotypes were independently verified by three individuals who were blinded to the pedigree information.

Two-point LOD scores were calculated for all 6000 informative SNPs, and multipoint LOD scores were calculated for all regions of the genome with a two-point LOD score of >2 using the Vitesse statistical program.[24] A LOD score of ≥3.0 is considered significant evidence for linkage, and ≤−2.0 is significant evidence for exclusion of linkage to the region. Values between these are inconclusive and additional data are needed before a conclusion can be reached. For two-point LOD scores ≥3.0, a 1-LOD-unit-down support interval was calculated as an approximation to a 95% confidence interval.[25] A rare dominant model was assumed. A conservative low-penetrance affecteds-only analysis was performed to ensure that results obtained were not due to asymptomatic individuals who were nonpenetrant carriers of the FSGS gene. Map distances for the marker loci were obtained from published data. For further confirmation or exclusion of a region of linkage, microsatellites were genotyped at 5-cM intervals and the two-point and multipoint LOD scores were calculated as described already. Haplotype analysis was carried out as described previously to identify critical recombination events by visual inspection and was confirmed by using SIMWALK software.[26] Under a rare dominant model with reduced penetrance, all affected individuals in a family will share at least one haplotype in common. Thus, a candidate interval was excluded when two affected individuals within the pedigree inherited different haplotypes.

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DISCLOSURES

None.

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