Am. J. Hum. Genet. 53:1256–1261, 1993

Mapping of a Gene for Familial Juvenile Nephronophthisis: Refining the Map and Defining Flanking Markers on Chromosome 2

Friedhelm Hildebrandt,* Iva Singh-Sawhney,* Birgit Schnieders,* Linda Centofante,* Heymut Omran,* Andreas Pohlmann,* Cornelius Schmaltz,* Horst Wedekind,* Christian Schubotz,* Corinne Antignac,† James L. Weber,‡ Matthias Brandis,* and Members of the APN Study Group§

*University Children's Hospital, Freiburg University, Freiburg; †INSERM U192, Hôpital Necker-Enfants Malades, Paris; ‡Marshfield Medical Foundation, Marshfield, WI; and §Members of the Study Group of the Arbeitsgemeinschaft für Pädiatrische Nephrologie (APN)[1]

Summary

Familial juvenile nephronophthisis (NPH) is an autosomal recessive kidney disease that leads to end-stage renal failure in adolescence and is associated with the formation of cysts at the cortico-medullary junction of the kidneys. NPH is responsible for about 15% of end-stage renal disease in children, as shown by Kleinknecht and Habib. NPH in combination with autosomal recessive retinitis pigmentosa is known as the Senior-Løken syndrome (SLS) and exhibits renal pathology that is identical to NPH. We had excluded 40% of the human genome from linkage with a disease locus for NPH or SLS when Antignac et al. first demonstrated linkage for an NPH locus on chromosome 2. We present confirmation of linkage of an NPH locus to microsatellite markers on chromosome 2 in nine families with NPH. By linkage analysis with marker AFM262xb5 at locus D2S176, a maximum lod score of 5.05 at a $\theta_{max} = .03$ was obtained. In a large NPH family that yielded at D2S176 a maximum lod score of 2.66 at $\theta_{max} = .0$, markers AFM172xc3 and AFM016yc5, representing loci D2S135 and D2S110, respectively, were identified as flanking markers, thereby defining the interval for an NPH locus to a region of approximately 15 cM. Furthermore, the cytogenetic assignment of the NPH region was specified to 2p12-(2q13 or adjacent bands) by calculation of linkage between these flanking markers and markers with known unique cytogenetic assignment. The refined map may serve as a genetic framework for additional genetic and physical mapping of the region.

Introduction

Familial juvenile nephronophthisis (NPH) (Smith and Graham 1945; Fanconi et al. 1951) is an autosomal recessive renal disease of unknown etiology. This disease, which is also known as recessive medullary cystic disease of the kidney, is characterized by the development of cysts at the cortico-medullary junction of the kidneys (Waldherr et al. 1982). Cyst formation leads to end-stage renal failure at around age 13 years, following the initial symptoms of polyuria, polydipsia due to renal salt loss, development of anemia, and growth retardation. NPH is the commonest genetic cause for end-stage renal disease (ESRD) in childhood, accounting for up to 15% of ESRD in children in Europe (Kleinknecht and Habib 1992). Associations of NPH with extrarenal organ involvement have been described, comprising liver fibrosis (Boichis et al. 1973), cone-shaped epiphyses of bone, cerebellar ataxia (Mainzer et al. 1970), and autosomal recessive retinitis pigmentosa (RP) (Løken et al. 1961; Senior et al. 1961). NPH is associated with RP in about 16% of cases. This association is known as the Senior-Løken syndrome (SLS). Renal histology is characteristic but not specific for the dis-

[1] O. Amon (Hamburg), J. Brodehl (Hannover), B. Brouhard (Cleveland), J. Dippel (Frankfurt), B. Klare (Munich), B. Leumann (Zurich), R. Mallmann (Bonn), H. Neumann (Freiburg), W. Rascher (Essen), H. Ruder (Erlangen), K. Schärer (Heidelberg), H.-J. Stolpe (Rostock), and R. Waldherr (Heidelberg).

Received June 10, 1993; revision received August 23, 1993.

Address for correspondence and reprints: Dr. Friedhelm Hildebrandt, University Children's Hospital, Mathildenstrasse 1, D-79106 Freiburg, Germany.

0002-9297/93/5306-0012$02.00

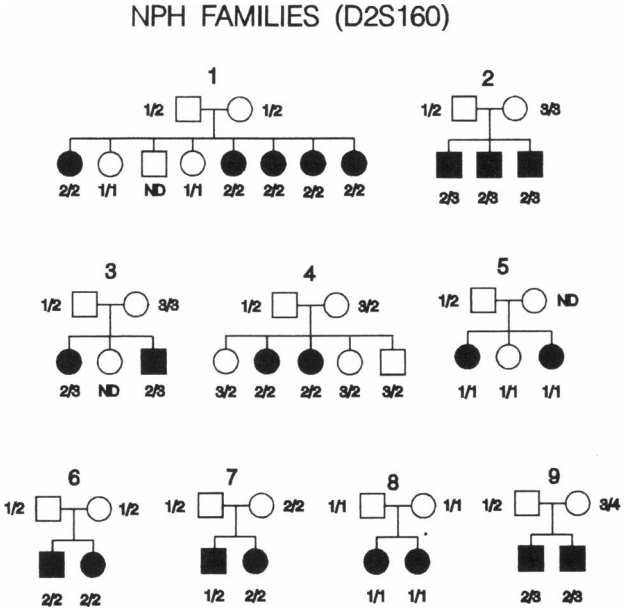

Figure 1 Pedigrees of nine NPH families used in the linkage study, and genotypes produced by the marker AFM220ze3 at locus D2S160. ND = not determined.

ease: early peritubular lymphohistiocytic infiltrations merge into a chronic sclerosing tubulo-interstitial nephropathy with dilated tubules and cysts occurring primarily at the cortico-medullary border (Waldherr et al. 1982). Electron microscopy reveals thickening, splitting, and attenuation of the tubular basement membrane (TBM). Therefore, a defect of TBM composition has been suggested as the primary genetic defect (Cohen and Hoyer 1986). Since there has been no unequivocal demonstration of a biochemical defect in NPH or SLS, linkage analysis has been employed to map and isolate a gene for the diseases (Antignac et al. 1993). All cases of NPH exhibiting extrarenal involvement that have been described seem to follow an autosomal recessive mode of inheritance and are identical with respect to renal histology and the clinical course of renal impairment. An autosomal dominant disease with identical histology but slower progression into renal failure has been described in the United States (Gardner 1971; Avasthi et al. 1976) and has been termed "medullary cystic disease of the autosomal dominant type." The different clinical entities that share a similar finding on renal histology have been summarized in the so-called nephronopthisis complex. Whether the different disease entities of this disease complex are attributable to gene locus heterogeneity or multiple allelism in one and the same gene is currently not known.

We have excluded 40% of the entire human genome from linkage with a disease locus for NPH or SLS by examination of 134 microsatellite markers. After initial demonstration of linkage on chromosome 2, by Antignac et al. (1993), linkage to this region was confirmed by examination of nine families with NPH, and the map of the NPH region was refined by definition of flanking markers and the demonstration of linkage of these markers to markers with unique cytogenetic assignment.

Patients and Methods

Patients

Blood samples were obtained after informed consent from nine multiplex families with NPH (22 affected individuals and 8 unaffected individuals). For calculation of linkage to a disease locus, only families with at least two affected children were used. There was no known consanguinity in these families. Seven families were from Germany, one was from the United States, and one was from Switzerland. The diagnosis of NPH was made, and a pedigree was obtained (fig. 1) by a pediatric nephrologist who was the primary physician

Table 1

Pairwise Z Values for an NPH Locus with Microsatellites on Chromosome 2

		Z AT θ =								
LOCUS	NAME	.0	.01	.04	.10	.15	.20	.30	Z_{max}	θ at Z_{max}
CD8A	CD8A	$-\infty$	−1.90	−.28	.53	.71	.73	.51		
D2S135.....	AFM172xc3	$-\infty$	−2.00	.12	1.10	1.29	1.25	.85		
D2S176.....	AFM262xb5	$-\infty$	4.86	5.03	4.52	3.93	3.27	1.89	5.05	.03
D2S160.....	AFM220ze3	$-\infty$	4.11	4.35	3.97	3.46	2.88	1.66	4.35	.04
D2S121.....	AFM087xa1	$-\infty$	2.12	2.98	3.04	2.74	2.31	1.33		
IL1A	IL1A	$-\infty$	.94	1.33	1.31	1.14	.93	.48		
D2S110.....	AFM016yc5	$-\infty$	−6.87	−3.00	−.92	−.27	.02	.14		

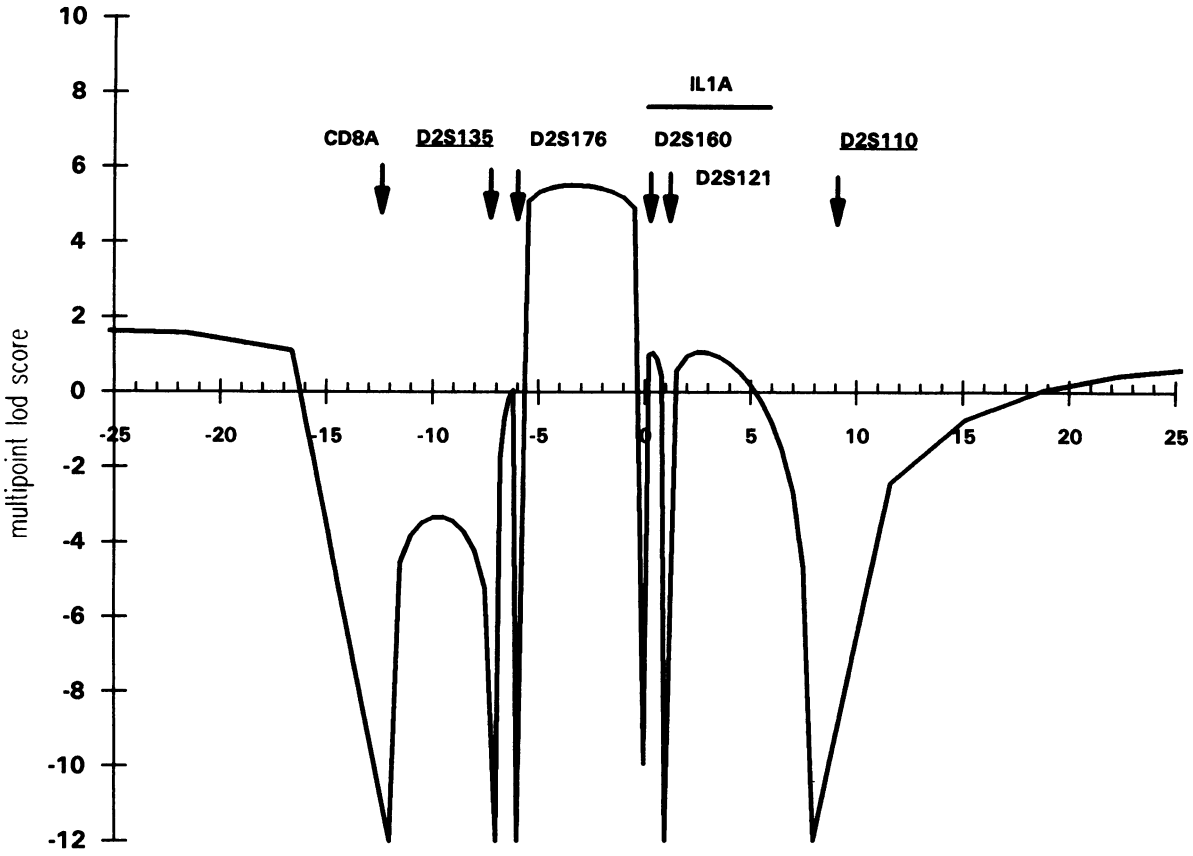

Figure 2 Multipoint *Z* values for an NPH locus on chromosome 2. The *X*-axis represents genetic intermarker distances (in cM) as published elsewhere (Weissenbach et al. 1992). Above the *X*-axis the symbols of microsatellite markers are drawn relative to the calculated intermarker distances. D2S160 is positioned at map position 0. Multipoint *Z* values calculated with each of two markers and an NPH disease locus are shown as a continuous line. The two markers D2S135 and D2S110 that are flanking the NPH region are underlined.

of this proband. Diagnostic criteria were the development of ESRD after a history of polyuria, polydipsia, and anemia. In all NPH families a kidney biopsy result consistent with NPH was obtained. Unaffected siblings were included in the study only if they had reached 14 years of age with no clinical evidence of NPH.

Linkage Analysis

Genomic DNA was isolated by standard methods (Maniatis et al. 1987) either directly from the blood samples or after Epstein-Barr virus transformation of peripheral blood lymphocytes (Steel et al. 1977). Highly polymorphic microsatellite markers (Litt and Luty 1989; Weber and May 1989) that localize on chromosome 2 (Sukathme et al. 1985; Lafage et al. 1989; Weissenbach et al. 1992) were examined by PCR amplification followed by PAGE and autoradiography. PCR was performed in a volume of 10 μl containing 30 ng of template DNA, 6 pmol of primers, 0.2 mM each of dATP, dGTP, and dTTP, 2.5 μM dCTP, 0.1 μCi ^{32}P-adCTP/μl, 10 mM Tris-HCl (pH7.3), 50 mM KCl, 0.001% gelatin (w/v), and 0.3 U of *Thermus aquaticus* DNA polymerase (Perkin Elmer Cetus). The PCR reactions were done in heat-resistant multiwell microtitration plates. After initial denaturation at 94°C for 4 min, 27 cycles were performed at 94°C for 30 s, 55°C for 40 s, and 72°C for 40 s, followed by a 6-min final extension step at 72°C. The amplified fragments were separated by electrophoresis in 8% denaturing polyacrylamide sequencing gels. Microsatellite results were interpreted independently by two investigators.

Genetic linkage analysis was performed using MLINK, ILINK, and LINKMAP subroutines of the computer program LINKAGE 5.01 (Lathrop et al. 1984). This program allows for calculation of lod score (*Z*) values for different recombination fraction (θ) values, a maximum *Z* (Z_{max}), and multipoint Z values. On the basis of clinical and pedigree data, an autosomal

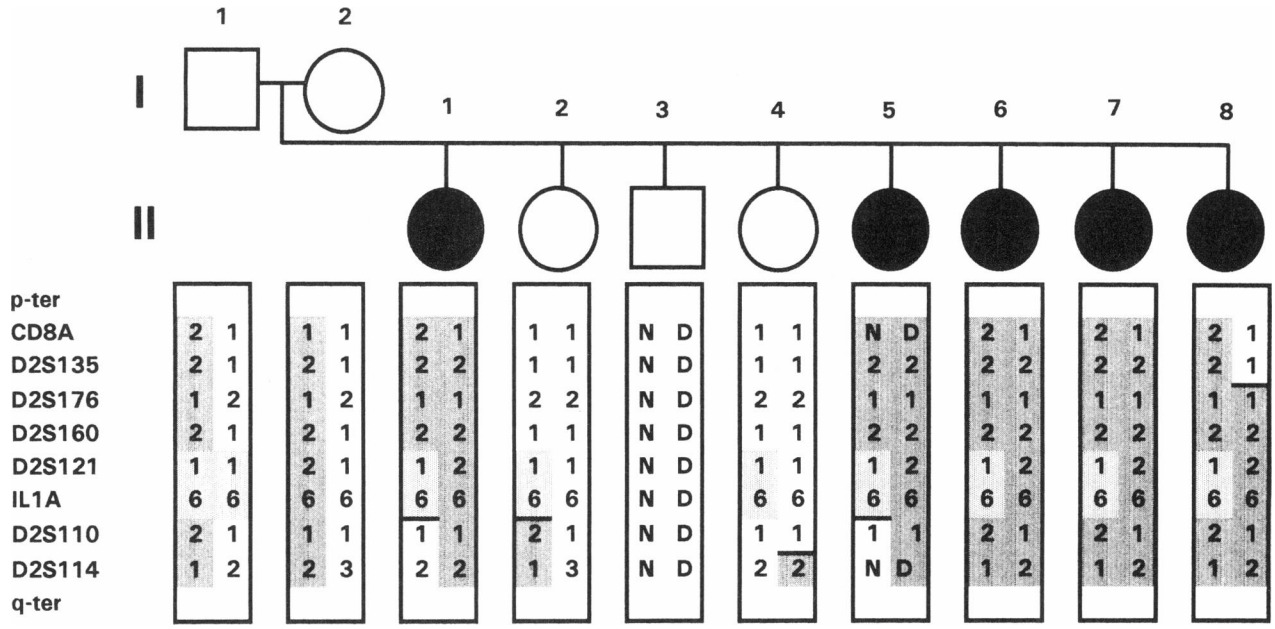

Figure 3 Haplotypes of family 1. The paternal and maternal haplotypes that are associated with the mutant NPH allele are shaded in darker gray. Recombination events (horizontal bars) in marker AFM172xc3 at D2S135 and in marker AFM016yc5 at D2S110 identify those markers as flanking the NPH region. Since for D2S121 and IL1A the paternal haplotypes are uninformative, it is undetermined where between D2S160 and D2S110 the recombination events have occurred; a lighter gray shading is used in the paternal alleles of D2S121 and Il1A to indicate this fact.

recessive mode of inheritance was assumed for the linkage analyses. The gene frequency of NPH was set at .001 (Potter et al. 1980), and penetrance was set at 100% for affected individuals and at 0% for unaffected individuals. The "Z_{max} − 1 support interval" was defined as the θ values intersecting the Z curve at $Z_{max} - 1$ (Conneally et al. 1985). Exclusion-mapping results were evaluated using the program LODVIEW (Hildebrandt et al., in press).

Results

In nine NPH families and eight SLS families (data not shown) 134 microsatellite markers on all autosomes were tested for linkage to an NPH or SLS locus. Under the hypothesis of a single locus for NPH and SLS, a total of 1,450 cM was excluded by the criterion of $Z < -2$, which is equivalent to approximately 40% of the human genome (data not shown).

Linkage Analysis with Chromosome 2 Markers

With all nine NPH families that were available, linkage of a gene locus for NPH with the most informative marker AFM220ze3 at D2S160 was confirmed with $Z_{max} = 4.35$ at $\theta_{max} = .04$. Genotypes obtained in these kindreds with marker AFM220ze3 at locus D2S160 are shown for NPH families, in figure 1. For NPH families one recombination of an NPH locus with this locus was observed in family 7, and family 8 was not informative (see fig. 1). The highest Z value was obtained with marker AFM262xb5 at locus D2S176 ($Z_{max} = 5.05$ at $\theta_{max} = .03$). Two-point Z values of microsatellite markers with an NPH locus on chromosome 2 are given in table 1.

Multipoint Z values were calculated with each of two adjacent markers versus a disease locus for nine NPH families, by using genetic intermarker distances as published elsewhere (Weissenbach et al. 1992) (fig. 2). Three-point Z values between the locus pairs D2S135 and D2S176, D2S176 and D2S160, D2S160 and D2S121, and D2S121 and D2S110 are shown in figure 2. The Z_{max} was 5.52 and occurred in the interval between D2S176 and D2S160, at map position −3.6, i.e., 3.6 cM p-terminal to D2S160. The $Z_{max} - 1$ support interval is spanning 5.8 cM from map position −0.1 cM to −5.9 cM.

Definition of Flanking Markers and Cytogenetic Assignment of the NPH Region

In a large NPH family that for D2S176 yielded $Z_{max} = 2.66$ at $\theta_{max} = .0$, a recombination event between the

marker AFM172xc3 at locus D2S135 and an NPH locus was detected. Three recombinations were detected between marker AFM016yc5 at locus D2S110 and an NPH locus (fig. 3). Since a $Z > 2$ can be considered significant linkage in a candidate region, these data identify marker AFM172xc3 at D2S135 and marker AFM016yc5 at D2S110 as being flanking markers to the NPH region, thereby defining the interval for an NPH locus to be a region of approximately 15 cM.

The microsatellite marker CD8A has been uniquely assigned to 2p12 (Sukathme et al. 1985). By calculation of three-point intermarker linkage data, using markers CD8A, AFM172xc3 at D2S135, and AFM262xb5 at D2S176 in 20 two-generational families, we demonstrated linkage of CD8A to AFM172xc3 at locus D2S135 ($Z_{max} = 3.05$ at $\theta_{max} = .046$). It is known from the literature that CD8A maps 4.7 cM p-terminal to D2S25 (NIH/CEPH Collaborative Mapping Group 1992) and that, in turn, D2S25 maps 4.2 cM p-terminal to AFM172xc3 at D2S135 (Murray et al. 1993). Since marker AFM172xc3 at D2S135 is a flanking marker to the NPH region, the cytogenetic band 2p12 is thus defined as the p-terminal border of the NPH region. Likewise, IL1A has been uniquely assigned to D2q13 (Lafage et al. 1989). In 20 two-generational families, two-point linkage for IL1A with marker AFM220ze3 at D2S160 resulted in $Z_{max} = 5.49$ at $\theta_{max} = .025$, and two-point linkage for IL1A with marker AFM087xa1 at D2S121 resulted in $Z_{max} = 6.08$ at $\theta_{max} = .024$. Therefore, ILA1 is closely linked to D2S160 and D2S121. Since the q-terminal flanking marker AFM016yc5 at locus D2S110 has been mapped 7 cM q-terminal to D2S121 (Weissenbach et al. 1992), the NPH candidate region extends from 2p12 across the centromere to 2q13 or adjacent bands.

Discussion

Since there is no strong hypothesis on a protein defect responsible for NPH, and since there is no available information on a cytogenetic defect, the best approach toward isolation of an NPH gene was considered to be the use of positional cloning by linkage analysis. Our results confirm linkage of a gene locus for NPH, on chromosome 2, with marker AFM220ze3 at locus D2S160, as reported by Antignac et al. (1993). Significant linkage was also demonstrated for additional markers in the region, specifically markers AFM262xb5 at locus D2S176 and AFM087xa1 at D2S121, which have previously not been examined.

An important prerequisite for physical mapping of a disease gene for NPH is the definition of markers that are flanking an NPH locus. Flanking markers can be defined by the demonstration of recombination between such markers and a disease locus in a large kindred. This kindred has to be large enough to yield significant linkage between polymorphic markers and a disease locus. In a candidate region—i.e., a region where a strong hypothesis exists for linkage with a disease gene—$Z > 2$ is considered significant for linkage, in contrast to the approach of a random search of the whole genome, where multiple testing is performed with >100 markers and where, therefore, criteria for significant linkage have to be more stringent ($Z > 3.0$) (Ott 1991). Finding a family that can contribute $Z > 2$ as a rule poses a problem in autosomal recessive disease, since pedigrees tend to be small. The availability of a large NPH family gave us the opportunity to use this kindred for the definition of flanking markers. The region flanked by D2S135 and D2S110 spans approximately 15 cM (Weissenbach et al. 1992). In family 1 no recombinations of an NPH locus have been observed with markers AFM262xb5 at D2S176 and AFM087xa1 at D2S121. Once additional markers are available, for the intervals D2S135–D2S176 and D2S121–D2S110, flanking markers delimiting the region more closely may potentially be defined. The definition of flanking markers for the NPH region provides a framework for additional genetic and physical mapping of the NPH region.

Linkage was demonstrated to exist between marker CD8A and the flanking marker AFM172xc3 at D2S135. While 2p12 could unequivocally be determined as the p-terminal border, no unique cytogenetic assignment was possible for the q-terminal border. However, with the information that the interval of approximately 13 cM between the markers with unique cytogenetic assignment CD8A and IL1A corresponds to six cytogenetic bands, the interval from IL1A to the flanking marker AFM016yc5 at D2S110, which spans approximately 8 cM, may correspond to a comparable number of cytogenetic bands. Therefore, the q-terminal cytogenetic definition of the NPH region has not yet been clearly defined. Our results indicate that the candidate region for NPH is not restricted to the short arm of chromosome 2p as suggested elsewhere (Antignac et al. 1993). This cytogenetic assignment may help to identify candidate genes for NPH once such candidates have been assigned to the region.

Acknowledgments

We wish to thank Helga Salm for excellent technical assistance. F.H. was supported by Deutsche Forschungsgemein-

schaft grant Hi 381/3-1 and by the state of Baden-Württemberg. We are indebted to the German Microsatellite Marker Consortium (Chairman, A. Reis, Berlin) for sharing microsatellite primers and related information and to Dr. J. Weissenbach (Genethon, Paris) for communicating primer sequences and mapping data prior to publication. We are grateful to Dr. T. Wienker for helpful discussion.

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