Human Molecular Genetics, 1998, Vol. 7, No. 7 **1091–1098**

ARTICLE

Loss-of-function mutations in the LIM-homeodomain gene, *LMX1B*, in nail–patella syndrome

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Received April 2, 1998; Revised and Accepted April 24, 1998

DDBJ/EMBL/GenBank accession nos AF059572–AF059575

Nail–patella syndrome (NPS) is an inherited developmental disorder most commonly involving maldevelopment of the fingernails, kneecaps and elbow joints. NPS exhibits wide variation in phenotypic expression within and among families with respect to these features. Other skeletal abnormalities such as hip dislocation and club foot have also been reported in some individuals with NPS. There is an association between NPS and renal disease, and between NPS and open-angle glaucoma (OAG), but it is not known whether mutations in a single gene cause the observed skeletal, renal and ophthalmic abnormalities. Recently, *LMX1B*, a transcription factor of the LIM-homeodomain type with homologs that are important for limb development in vertebrates, was mapped to the same general location as NPS at 9q34. We sequenced a large segment of *LMX1B* from the genomic DNA of probands from four families with NPS and OAG, and identified four mutations: two stop codons, a deletion causing a frameshift and a missense mutation in a functionally important residue. The presence of these putative loss-of-function mutations in the DNA of individuals with NPS indicates that haploinsufficiency of *LMX1B* underlies this disorder. These findings help to explain the high degree of variability in the NPS phenotype, and suggest that the skeletal defects in NPS are a result of the diminished dorsoventral patterning activity of LMX1B protein during limb development. The results further suggest that the NPS and OAG phenotypes in the families studied result from mutations in a single gene, *LMX1B*.

INTRODUCTION

Nail–patella syndrome (NPS; OMIM 161200) is an autosomal dominant disorder characterized by pleiotropic developmental defects of dorsal limb structures such as the nails, patellae (kneecaps), elbows and bony outgrowths of the dorsal ilium termed iliac horns. Individuals exhibiting some of the now classic signs of NPS were first described in 1820 and have been reported regularly for over a century (1–3). Indeed, one of the first autosomal linkages discovered in humans was between the NPS locus and the ABO blood group (4), and the first three locus autosomal linkage group in humans included NPS (5).

NPS is highly penetrant, but exhibits variable severity of expression within and among families (6–11). Kneecaps range from slightly hypoplastic to absent, and fingernails may be absent or simply brittle and fissured. Additional skeletal defects have been described that include foot and ankle abnormalities such as clubfoot, and dislocation or subluxation of the hip or of the radial head (12). Renal abnormalities accompany NPS in some families (6), and open-angle glaucoma (OAG) has been found to co-segregate with NPS in other families (11), but the specific genetic relationship among renal disease, OAG and the classic NPS phenotype has not been determined.

Linkage studies have refined the location of the NPS gene to a 1–2 cM interval at 9q34 (10,13). Recently, a LIM-homeodomain gene, *LMX1B*, was mapped to the same general location as NPS (14,15). LIM-homeodomain proteins are a family of transcription factors, frequently involved in pattern formation during development (16,17), that contain two LIM domains (LIM1 and LIM2), distinguished by characteristic patterns of conserved amino acids. Each LIM domain binds two ions of Zn(II), with the LIM1 domain located N-terminal to the LIM2 domain, followed by a homeodomain and a transcriptional activation domain. LIM domains of LIM-homeodomain proteins facilitate interactions with other transcription factors, leading to synergistic activation of transcription (17–19). Provocatively, *LMX1B* homologous genes have been shown to play an important role in dorsal–ventral patterning during chicken and mouse limb development (20–22), making *LMX1B* an appealing positional candidate for the gene mutated in NPS. We describe here independent loss-of-function mutations in *LMX1B* that cosegregate with NPS and OAG in families.

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Table 1. Primer sequences for *LMX1B* amplification from human genomic DNA

Exon	Sense primer	Antisense primer	Product size (bp)
2	AGGACTGGGACGGACTAG	ACCCAGGCACCAAATC	650
2	CATCTCCGACCGCTTCCT	ACCCAGGCACCAAATC	418
3	GGCAGGAGTGGCCTCTG	AGTGCGCGTGTGCATCT	449
4	GATGAGGGAGTGAGGCGT	TGGAGGGATGTCCCTACC	536
5 + 6	GGTAGGGACATCCCTCCA	CCTTTGTCCCTAGCCCTG	563
7	CTTCAGCCAGAGTGGGGT	ACAGGATGGCCTGCTGAC	473
8	GTCAGCAGGCCATCCTGT	GGAGCTCTGCATGGAGTAGA[a]	259

[a]Hamster sequence.

RESULTS

Sequence and gene structure of human *LMX1B*

To determine the precise location of *LMX1B* with respect to NPS, a PCR assay that amplifies a segment of genomic DNA from the gene (15) was tested against a collection of large insert DNA clones containing genetic markers within the NPS genetic inclusion interval between *D9S60* and adenylate kinase (*AK1*) (10; J.E. Richards, M.V. Clough and I. McIntosh, unpublished data). Two bacterial artificial chromosome (BAC) clones scored positive for the assay. One of the clones also contained *D9S112*, a marker previously shown to be tightly linked to NPS [LOD = 27 at a recombination fraction of 0.00 (10)]. Oligonucleotide primers based on the hamster (accession no. U61141) and human (accession nos T12579, T12628) partial cDNA sequence of *LMX1B* were used to determine a nearly complete sequence and genomic structure for the gene (Fig. 1).

The gene comprises at least eight exons in a genomic region with high GC content, and encodes a typical LIM-homeodomain protein of 379 amino acids. The putative LMX1B protein is 93% identical and 96% similar to the protein encoded by chicken *Lmx1*, a gene involved in dorsoventral patterning during limb development (20,21), and 99% identical to the protein encoded by hamster *Lmx1b* which, together with the basic helix–loop–helix (bHLH) transcription factor E47/Pan-1, synergistically promotes transcription (23).

Mutation screen of *LMX1B* in NPS/OAG families

We searched for mutations in *LMX1B* among affected members of four unrelated families with NPS and OAG (Fig. 2). Each of the four families included multiple individuals who met the standard criteria for a diagnosis of NPS, with characteristic patellar and fingernail abnormalities (see Materials and Methods). Many, but not all, of the individuals with NPS also had OAG, and no at-risk family member had OAG but not NPS. OAG exhibits an age-dependent penetrance, and it is generally the younger family members who have NPS but not OAG. The NPS phenotype in two of the families (UM:47 and UM:65) was localized previously to 9q34 by linkage analysis, which also showed that the OAG and NPS phenotypes were tightly linked [LOD = 2.98 at a recombination fraction of 0.00 (11)].

To search for mutations, exons 2–7 and most of exon 8, along with adjoining intronic sequences, were amplified from the genomic DNA of probands and sequenced (Table 1). Mutations were found in all four families (Fig. 3), and no other NPS families were searched for mutations. The proband of family UM:47 was heterozygous for a G→T transversion in exon 3 that results in a cysteine to phenylalanine missense mutation (Cys95Phe) in the LIM2 domain of the protein. The proband of family UM:65 was heterozygous for a 2 bp deletion in exon 2 (233delTG), predicting a severely truncated protein that lacks part of the LIM1 domain, all of the LIM2 and homeodomains, and that contains a stretch of 44 N-terminal amino acid residues generated from an incorrect reading frame. The proband of UM:68 was heterozygous for a C→T transition in exon 2 that creates a premature stop codon (Gln59Ter), predicting an even shorter protein than that posited for UM:65. The proband of UM:310 was heterozygous for a C→T transition in exon 4 that creates a premature stop codon near the beginning of the homeodomain (Arg208Ter). The mutations found in the probands of UM:47, UM:65 and UM:68 co-segregate with the NPS phenotype in the respective families (Fig. 2). The stop codon identified in the proband of UM:310 is also present in the proband's affected daughter, but additional family members were not available for testing. The mutations were not found among a collection of either 108 (UM:65, UM:68), 114 (UM:310) or 112 (UM:47) control chromosomes. No other mutations were found in the remainder of the gene examined, for any of the four families, but two common polymorphisms were identified that result in synonymous substitutions (Fig. 1).

DISCUSSION

Haploinsufficiency of *LMX1B* is the likely cause of NPS

The nature of the mutations that we have found indicates that loss-of-function of one allele of *LMX1B* is the likely cause of NPS with OAG in three of the four families (UM:65, UM:68 and UM:310), and may be the cause in the fourth family (UM:47), in which a gain-of-function mechanism is an alternative explanation. The presence of premature stop codons in three of the families (UM:65, UM:68 and UM:310) probably leads to a severe reduction in the levels of mutant transcripts, as compared with the normal alleles (24,25), which would result in very little, if any, synthesis of abnormal proteins. If produced, it is unlikely that such abnormal proteins would be stable, or that they would retain remnants of function. The most severe mutations predict truncation of the protein in the LIM1 domain (UM:65 and UM:68), with or without the addition of incorrect amino acid residues. The stop codon in the proband of UM:310 predicts

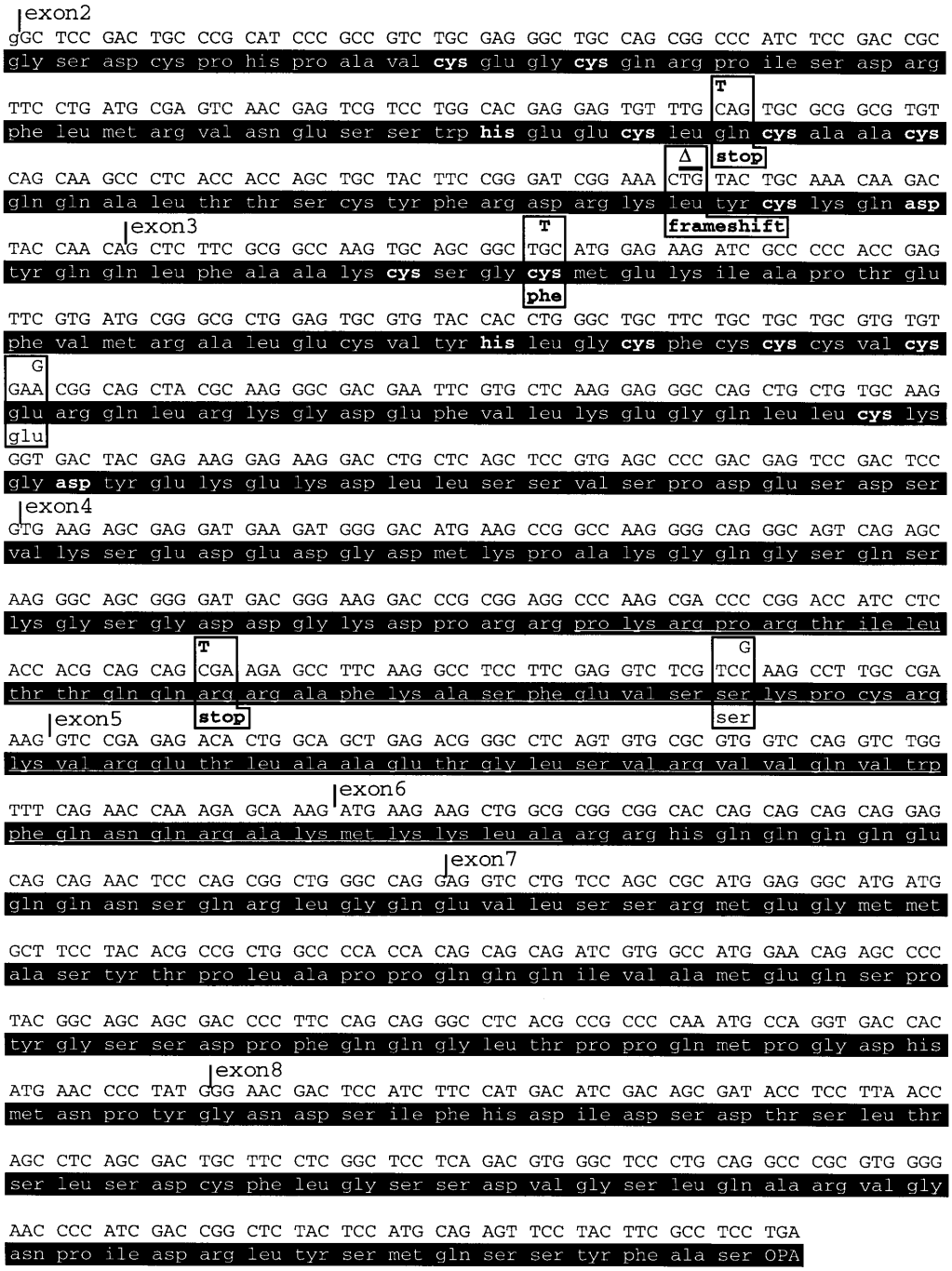


Figure 1. *LMX1B* genomic structure, coding sequence and mutations. The complete coding sequence and deduced amino acid sequence of *LMX1B* are shown. Sequence was derived from a BAC clone containing the gene. Exons are numbered consecutively, beginning with the first coding exon. Additional non-coding exons may exist 5′ to the first coding exon. The positions of introns 1–7 are marked by small vertical lines next to the exon numbers. Exon 2 encodes the LIM1 domain and exon 3 encodes the LIM2 domain. The Zn(II)-binding residues characteristic of LIM domains are in bold. The homeodomain (underlined residues) is encoded by exons 4–6. The location of six sequence variants discovered through a mutation screen of four families with NPS are shown as boxed codons and residues. The nature of a sequence variant is shown above the nucleic acid sequence and the consequence of a variant is shown below the amino acid sequence. Four mutations are shown in bold and two synonymous substitutions are shown in standard type. The correspondence between mutations and NPS families referred to in the text is as follows (top to bottom): UM:68, exon 2 stop codon; UM:65, exon 2 frameshift; UM:47, exon 3 cysteine to phenylalanine; and UM:310, exon 4 stop codon.

truncation of the protein just past its midpoint, removing the bulk of the homeodomain and all of the activation domain.

The consequences of the cysteine to phenylalanine mutation in family UM:47 are less obvious, but may be just as severe. The affected cysteine residue is conserved in all known LIM2 domains and is one of four residues that form one of the two Zn(II)-binding sites (16). Replacement of the corresponding cysteine with histidine in a related LIM2 domain abolishes the

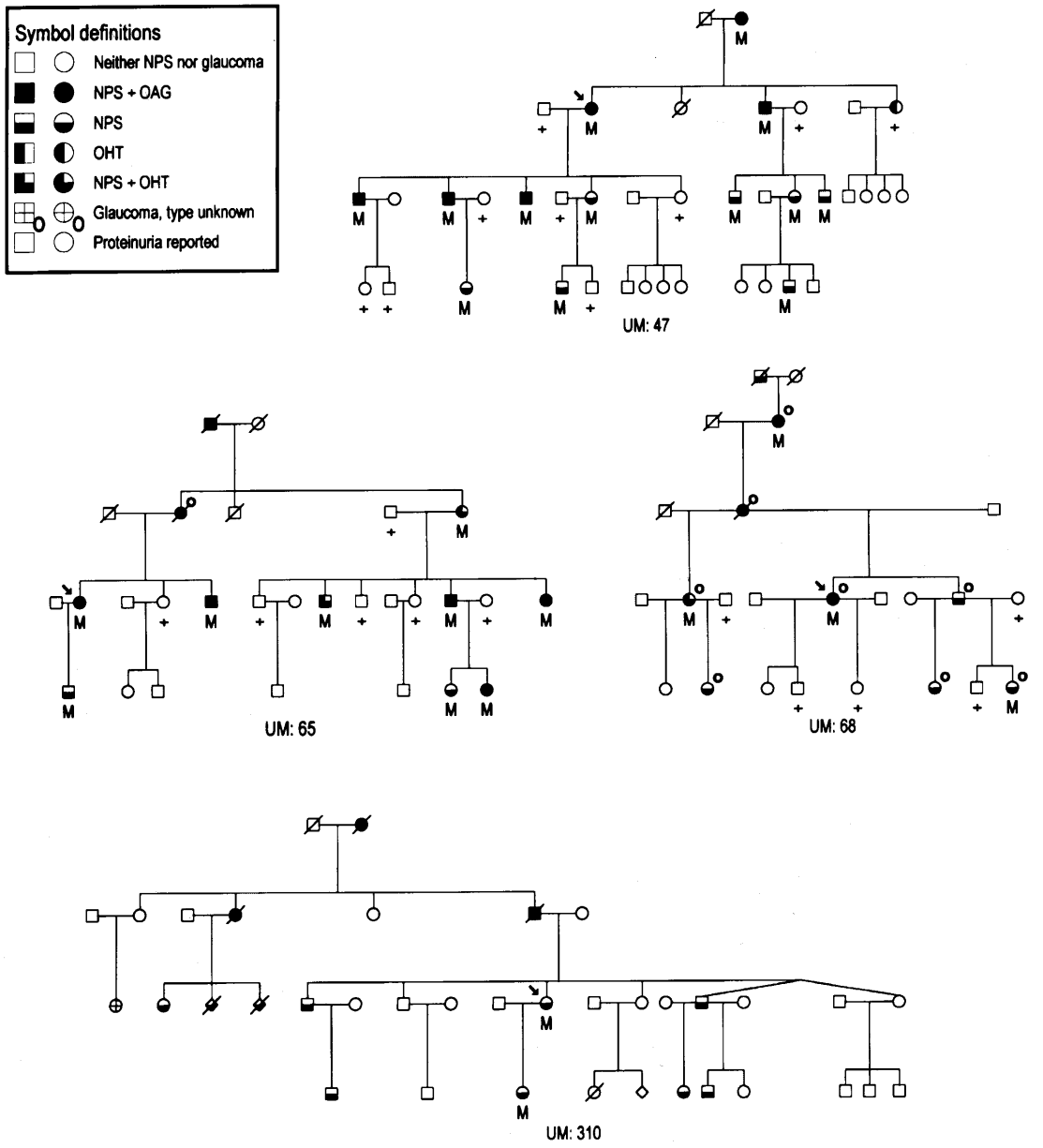
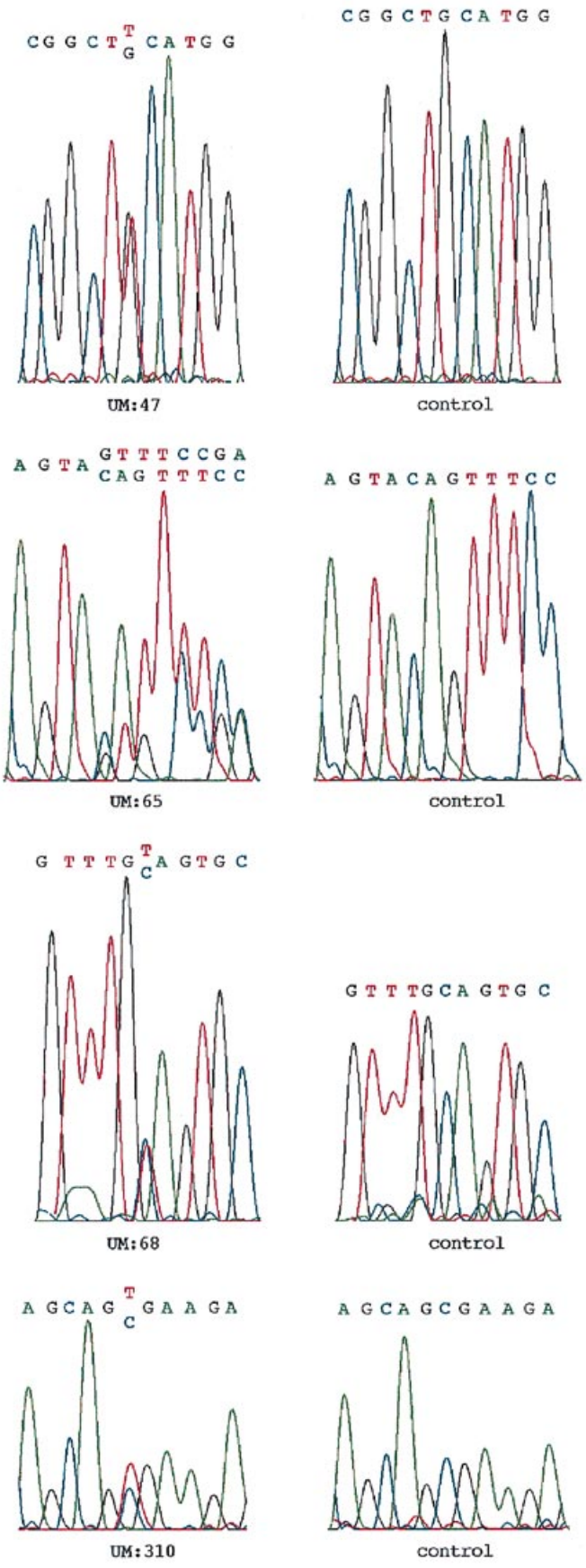


Figure 2. Pedigree structure and co-segregation of NPS in four families. M, mutation present along with the normal allele; +, mutation not present and only the normal allele detected. Symbols for phenotype designations are presented in the key. The diagonal arrow indicates a proband. Diagonal lines mark deceased individuals. OAG exhibits age-dependent penetrance, as illustrated by all four pedigrees. Non-penetrance of OAG has been reported in some families but has not been reported for NPS.

Zn(II)-binding site and may prevent formation of a stable domain (26). Replacement by phenylalanine is expected to be even more disruptive to the structure than replacement by histidine which, in principle, might still ligate a metal ion. Loss of function is therefore a plausible explanation for the mode of action of the missense mutation in family UM:47.

Mutations in *LMX1B* explain the association of NPS with OAG and renal disease

Individuals with what are now regarded as the typical characteristics of NPS were first described >175 years ago, yet only in the past year has a connection been made between OAG and NPS (11). The two phenotypes could result from mutations in a single gene, or from mutations in more than one gene, as Hawkins and Smith noted in 1950 when they described a family with NPS and renal dysplasia (6). Because OAG and NPS are tightly linked in the families studied, if a second gene is involved, it must be genetically close to the NPS locus (11). The observation of parology between 9q33–q34 and 1q21–q24 lends credence to this hypothesis (14). Chromosome band 1q23–q24 is the location of a gene, *myocilin*, involved in inherited OAG (27), and it is possible that a *myocilin*-related gene located at 9q33–q34, distinct



from the NPS gene, might be responsible for the OAG in the families studied.

On the other hand, OAG alone (without NPS) has not been localized to 9q33–q34 in any families, and no at-risk individual in the four families studied had OAG alone without NPS. Furthermore, NPS is a rare disorder, and the occurrence of four families with NPS and OAG, in the absence of OAG alone, cannot easily be ascribed to coincidence. Moreover, additional families with NPS and OAG have been identified (28). A founder effect for a rare chromosome carrying mutations in closely linked NPS and OAG genes could explain these observations, but we see no evidence for a founder effect in the families studied. All four mutations are different, and alleles on the affected chromosome for microsatellite markers located very close to *LMX1B* (i.e. on the same BAC clone), or more distant from the gene, provide no indication of a conserved haplotype in families UM:47, UM:65 and UM:68 (data not shown).

In contrast, our results provide substantial support for a single gene model. The putative loss-of-function mutations we observe indicate that *LMX1B* can be added to a growing list of homeodomain genes, haploinsufficiency of which leads to a variety of developmental defects in humans [e.g. *PAX2* (29), *PAX3* (30), *PAX6* (31) and *RIEG* (32); reviewed in ref. 33]. As with NPS, wide phenotypic variability, within and between families, is a hallmark of several of these diseases, some of which also affect the eye or kidney (34,35). Thus, the long-standing connection of renal disease with NPS, and the very recent observation of co-segregation of OAG and NPS, are best explained by mutations at a single locus in the context of variable expressivity. The observed variability may result from stochastic processes during development, from the effects of different mutant alleles, or from the segregation of modifier loci. Analysis of phenotypes in a large number of NPS families with characterized mutations may help to address this issue. The wide phenotypic variability characteristic of NPS also raises the possibility that mutations in *LMX1B* may be found in a subset of individuals with OAG or renal disease alone, lacking obvious limb defects.

Disruption of dorsoventral patterning explains the limb defects in NPS

Studies of the roles of the *LMX1B* homologs in dorsoventral patterning during limb development in chick and mouse provide insight into the limb abnormalities observed in NPS (20–22). These studies have shown that *Lmx* expression in the dorsal mesenchyme is a major determinant of dorsal cell fate in the distal limb. The dorsal limb ectoderm expresses a secreted factor, Wnt-7a, that controls expression of *Lmx1* (chick) and *Lmx-1b* (mouse) within the distal, dorsal limb mesenchyme, and ectopic expression of *Wnt-7a* or *Lmx1* results in dorsalization of normally ventral limb structures. Conversely, mice homozygous for a *Wnt-7a* null allele have reduced

Figure 3. *LMX1B* mutations in individuals with NPS and OAG. Electropherograms are shown that demonstrate mutations in the genomic DNA of four individuals with NPS, three of whom also have OAG (probands of families UM:47, UM:65 and UM:68). For UM:47, UM:68 and UM:310, heterozygous bases were detected, while for UM:65, a region of double sequence was detected due to a two base deletion in one of the alleles. Sequence from the sense strand is shown for UM:47, UM:68 and UM:310, and sequence from the antisense strand is shown for UM:65. The corresponding segments of *LMX1B* in which mutations were found were sequenced from a control individual, unaffected with NPS or OAG, and these are shown in the right hand column for comparison.

expression of *Lmx-1b* in the distal, dorsal limb (22), and exhibit a phenotype in which ventral structures are partially duplicated on the dorsal half of the paw (36). One feature of this phenotype is variable truncation of nails. By analogy, nail abnormalities in NPS may reflect varying degrees of ventralization of the dorsal aspect of the fingertips. Interestingly, there is an anterior–posterior polarity to the pattern of nail involvement in NPS; thumbnails are always involved to some extent, with less severe changes observed on the fingers of the ulnar half of the hand (10,12). This may reflect a requirement for higher levels of *LMX1B* activity in the determination of dorsal cell fate in the anterior half of the distal hand. Consistent with this hypothesis, *Lmx-1b* expression in a *Wnt-7a* null mouse is more severely diminished in the anterior, as compared with the posterior, dorsal mesenchyme near the lateral margin of the limb bud (22). Similarly, the skeletal defects observed in NPS probably reflect a requirement for high levels of LMX1B protein activity in determining dorsal characteristics in the knees, elbows, feet and pelvis.

The role of *LMX1B* in eye and kidney development is unknown, but, based on its role in limb development, *LMX1B* is probably involved in patterning tissues in these organs. Mutations in genes encoding transcription factors have been shown to disrupt mammalian kidney or eye development (37,38) and, in particular, several LIM-homeodomain proteins are known to be necessary for proper formation of these organs in vertebrates (39–41). LIM-homeodomain proteins function by binding to, and acting synergistically with, other transcription factors (17,19,23). It will be of interest to determine whether LMX1B protein acts in concert with the same, or different, transcription factors while functioning in the developing limbs, eyes and kidneys.

MATERIALS AND METHODS

Pedigrees

Multiple members of all four families studied displayed fingernail and patellar defects typical of NPS. As illustrated in Figure 2, NPS and OAG were found together in older members of the families studied, while many of the younger members exhibited only NPS. This is probably because NPS is highly penetrant, beginning at birth, while OAG exhibits age-dependent penetrance. However, we cannot be certain that all individuals with NPS in these families will go on to develop OAG. As far as we could determine through records and/or direct examination, NPS-associated glaucoma results from an open-angle mechanism and is usually indistinguishable from typical adult primary OAG, except for a broader range in age at onset.

The realization that OAG is a feature of NPS began with examination of individuals in family UM:47 (11). Surprisingly, typical features of NPS were present in all members of this four generation family of German ancestry who had glaucoma, as well as in members who did not have OAG. Nail findings ranged from mild to severe, including ridging, splitting, aplasia and hypoplasia. Joint function also varied, with limitation of range of motion in the elbow joint in some, but not all, family members. Patella findings included subluxation, dislocation, hypoplasia and aplasia. Some individuals demonstrated altered gait even after corrective surgery. Iliac horns were detected by palpation in some family members. Proteinuria was not present. Age at onset of glaucoma ranged from 18 to 41 years (mean 32 years). Maximum intraocular pressure observed ranged from 30 to 43 mmHg (mean 36.7 mmHg). The mildly elevated intraocular pressure and narrow but open angles (grade 1–2) present in the individual with ocular hypertension (OHT) is an ocular phenotype distinct from that found in the rest of the family. Haplotyping indicates that this individual carries the non-recombinant haplotype across a large region surrounding the genetic inclusion interval in this family (data not shown), and thus cannot be a recombinant separating the NPS gene from a nearby OAG gene.

OAG was also the basis for recruitment of family UM:65 (11). Typical NPS signs were found during examination of members of this four generation family of French and Irish ancestry. Although gait did not appear to be as severely affected as in UM:47, significant skeletal anomalies were reported, including extra or missing vertebrae, hand bones and foot bones. The proband reported that she and two of her affected cousins were diagnosed with spina bifida occulta and that the proband's mother had Arnold Chiari deformity not accompanied by spina bifida. Three affected family members had anal stenosis. Split, ridged, hypoplastic and aplastic nails were observed in different family members. Elbow function varied from full to a limited range of motion. Kneecaps were hypoplastic or aplastic. Proteinuria was not present in family members screened, but medical records indicate that the proband's mother had proteinuria and kidney disease. The affected proband and six other individuals with glaucoma (two of them deceased) also had NPS. Age at onset of glaucoma ranged from birth to 54 years of age (mean 24 years). The maximum recorded intraocular pressure ranged from 23 to 55 mmHg (mean 36.5 mmHg). Pigment dispersion was observed in one individual with glaucoma and in one individual with OHT (11).

Members of family UM:68 reported that NPS in their family could be traced to a Cherokee Indian ancestor. Information was obtained from medical records and reports of family members. NPS findings ranged from mild splitting to hypoplasia or aplasia of the fingernails, and elbow joint function ranged from 'almost normal' in some individuals to a severely limited range of motion in others. Knees were described as hypoplastic with some cases of dislocation of the kneecap. Leg length discrepancy, joint pain and hearing loss were also reported in individual affected family members. Age at onset of OAG ranged from 40 to 77 years of age (mean ~56 years of age). In contrast to the other families, all of the individuals with NPS in UM:68 were reported to have proteinuria.

UM:310 is a Caucasian family, two members of which recently joined our study. They reported an extensive family history of typical NPS as shown in Figure 2. One individual reported 'apparently normal' patellae and the other reported hypoplastic patellae. These individuals reported no proteinuria or known family history of proteinuria. Similarly to the other families, the severity of fingernail anomalies reportedly ranged from mild ridging to hypoplasia and aplasia. Elbow mobility was variable. Glaucoma was reported for three deceased members of UM:310, but the study participants do not report glaucoma. Onset of glaucoma was reported at 40–50 years of age (mean 45 years). No medical records or self-report were available for one female individual marked in Figure 2 as having glaucoma of unknown type. She is descended from an at-risk family member who is reported to have neither NPS nor glaucoma. An intriguing possibility is that her glaucoma results from the same mutation in *LMX1B* that caused NPS (with or without glaucoma) in her relatives, and that she and her mother reflect an even greater range in phenotypic variation than so far suspected. Alternative explanations are also possible, such as incomplete information or

that her glaucoma is due to one of a number of other causes. Efforts are underway to further characterize this family.

Identification of BACs

BAC clones (Human BAC DNA Pools Release II; Research Genetics, Huntsville, AL) previously identified as containing markers from the NPS genetic inclusion interval were screened by PCR for the presence of a genomic fragment from *LMX1B*. Primers used were 5′ GCA GCG GGG ATG ACG GGA AGG 3′ and 5′ CTG GAC CAC GCG CAC ACT GAG G 3′ reported by Iannotti *et al.* (15).

Genomic structure determination

Primers designed from human or hamster exon sequences were used to sequence DNA from BAC clone 265A2 directly using BigDye terminator chemistry and an ABI semi-automated sequencing machine. BAC DNA was purified over a Qiagen column according to the manufacturer's instructions. Sequence was determined on both strands.

Mutation detection

Oligonucleotide primers based on genomic sequence were used to amplify individual exons from human genomic DNA by PCR (see Table 1). Products were gel purified and sequenced on both strands using d-rhodamine dye terminator chemistry. PCR was carried out for 33–40 cycles using an annealing temperature of 62°C in a buffer containing 10% dimethylsulfoxide (DMSO) (42). Genomic DNA purified from peripheral blood lymphocytes was used as template. As a control, exons were amplified and sequenced from the DNA of a 74-year-old individual without NPS or OAG. The 2 bp deletion found in the proband of family UM:65 was verified by cloning PCR products from exon 2, and sequencing multiple copies of the normal and mutant alleles.

Allele-specific hybridization

Screening for the presence of mutations was carried out by allele-specific oligonucleotide (ASO) hybridization. Genomic DNA, PCR amplified from family members and normal controls, was denatured and bound to Hybond N+ (Amersham) in a dot-blot format. The concentration of DNA in each PCR product was evaluated by running an aliquot on an acrylamide or agarose gel, and the volume of each product spotted onto the filter was adjusted as necessary to produce a near uniform amount of DNA in each dot. The blot was then hybridized to oligonucleotides (Table 2) that were end-labeled with ^{32}P using DNA kinase. Hybridization was carried out at the temperature indicated in Table 2 for 1 h in 2.5× SSPE (0.0.375 M NaCl, 0.025 M $NaH_2PO_4 \cdot H_2O$, 4.5 mM EDTA), 0.1% polyvinylpyrrolidone, 0.1% ficoll, 0.1% bovine serum albumin, 1 mM Tris pH 8.0, 1 mM EDTA, 0.1 mg/ml salmon sperm DNA, 0.5% SDS. The filter was rinsed twice at room temperature in 1× SSPE, 0.1% SDS, then washed for 15 min in 2.5× SSPE, 0.1% SDS, for 15 min in 1× SSPE, 0.1% SDS, and for 15 min in 0.5× SSPE, 0.1% SDS. Washes were carried out at a temperature specific for each oligonucleotide (Table 2). Filters were hybridized to the mutant oligonucleotide first, and subsequently stripped and hybridized to the control oligonucleotide to assess the amount of DNA bound to the filter and to ensure that there were no homozygous mutants. Oligonucleotide sequences were designed so that the point of mismatch was located at the center.

ACKNOWLEDGEMENTS

We thank the families for their participation in this study and Catherine Downs for assistance with gathering information on the families. This study was supported in part by grants from the National Institutes of Health [EY11405 (D.V.), EY09580 and EY11671 (J.E.R.), EY07003 (UM-CORE), AR44702 (I.M.)], the Glaucoma Research Foundation (J.E.R.), the Helen Van Arnam Glaucoma Research Fund (J.E.R.), and the March of Dimes Birth Defects Foundation (D.V.).

Table 2. ASO sequences and conditions

Oligonucleotide	Sequence	Hybridization temperature (°C)	Wash temperature (°C)
UM:65			
mutant	TCGGAAACTACTGCAAAC	50	53
control	TCGGAAACTGTACTGCAAAC	55	59
UM:68			
mutant	GAGTGTTTGTAGTGCGCGG	55	63
control	GAGTGTTTGCAGTGCGCGG	58	63
UM:47			
mutant	GCAGCGGCTTCATGGAGAA	55	63
control	GCAGCGGCTGCATGGAGAA	55	63
UM:310			
mutant	ACGCAGCAGTGAAGAGCCT	55	61
control	ACGCAGCAGCGAAGAGCCT	58	63

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