Nail-Patella Syndrome: Identification of Mutations in the LMX1B Gene in Dutch Families

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Abstract. Nail-patella syndrome is an autosomal dominant disorder characterized by dysplasia of finger nails, skeletal anomalies, and, frequently, renal disease. It has recently been shown that this disorder is caused by putative loss-of-function mutations in a transcription factor (LMX1B) belonging to the LIM-homeodomain family, members of which are known to be important for pattern formation during development. These proteins contain two tandem LIM domains, which are cysteine-rich, zinc-binding domains that facilitate the interaction with other transcription factors: a homeodomain that has DNA-binding activity and a transcriptional activation domain. The identification of the LMX1B gene; seven different mutations, including one novel variant, were identified. Three of the mutations are very likely to result in truncated LMX1B proteins, three are predicted to influence sequence-specific DNA binding, and one is presumed to prevent the formation of a stable protein by abolishing the Zn(II) binding site of the protein. Although there was a remarkable high incidence of renal disease in one of the families, the nephropathy was not seen in all affected family members and the severity of renal impairment varied significantly among the patients. This indicates that the incidence and severity of nephropathy within this family cannot be attributed to the LMX1B genotype. In addition, evidence of a correlation between other characteristics of the NPS phenotype and specific mutations has not been found.

Nail-patella syndrome (NPS) (MIM 161200), also known as hereditary osteo-onychodysplasia, is a rare autosomal dominant disorder that is characterized by nail and bone abnormalities and, frequently, renal disease. It has recently been shown that this disorder is caused by putative loss-of-function mutations in the LMX1B gene. The involvement of this gene in NPS was subsequently confirmed by others (11,12). Mutations in LMX1B were also found in families with NPS and glaucoma (10). LMX1B belongs to a family of highly related LIM-homeodomain transcription factors that are involved in pattern formation during development. These proteins contain two tandem LIM domains, which are cysteine-rich, zinc-binding domains that facilitate the interaction with other transcription factors: a homeodomain that has DNA-binding activity and a transcriptional activation domain (13). The identification of the
LMX1B gene as being responsible for NPS was anticipated by investigations of the role of LMX1B homologous genes in limb development. In the chicken, it had been shown that lmx1 specifies the dorsal cell fate of the limb. Thus, absence of lmx1 expression leads to the development of biventral limbs (14). Mice with a homozygous deletion of exon 3 to 7 in the mouse lmx1 homologue, LmX1b, exhibit nail dysplasia and skeletal defects similar to those observed in NPS, such as iliac hypoplasia, aplasia of the patellae, and joint abnormalities marked by osseous misarticulations. In addition, ultrastructural examination of the kidneys showed prominent irregular thickening of the GBM with occasional regions of membrane discontinuity, reminiscent of the renal findings in NPS (15).

In this report, we present the identification of seven different mutations, including one novel variant, in the LMX1B gene in eight Dutch families with NPS and discuss the presumptive effect of these mutations on the function of the LMX1B protein, as well as the absence of convincing genotype–phenotype correlations.

Materials and Methods

Patients

We studied three large families with NPS, two smaller families with NPS, and three families with sporadic cases of NPS. The clinical phenotype of affected individuals in the eight families is summarized in Table 1. The two largest families (families 3 and 8) were studied more than 10 yr ago, at that time with the aim of determining the presence or absence of nephropathy (16). Recently, most of the patients of family 8 were seen again for molecular genetic analysis and confirmation of the absence/presence of other major NPS signs. Some patients that were included in the study of 10 yr ago had died, and three additional patients had received diagnoses. We discovered that one recently identified patient who was a presumed sporadic patient belongs to family 3. We were not able to examine all of the affected individuals of this family again; therefore, we have the data on only nail, patellar, and iliac signs from three patients. The nephropathy data from families 3 and 8 were taken from the original paper (16). We also were not able to examine clinically all patients from family 4.

In the families with NPS, we had previously confirmed linkage to the 9q34.1 region, and haplotype analysis in two of the larger families had proved helpful in narrowing the region encompassing the NPS gene to a 1 cM interval (8,9).

Mutation Detection Analysis

Genomic DNA was extracted from whole blood according to the procedure described by Miller et al. (17). To find mutations in the LMX1B gene, we performed single-strand conformation polymorphism (SSCP) analysis. The different LMX1B exons were amplified by PCR using standard PCR buffer in a total volume of 25 µl, containing 50 ng of genomic DNA, 15 pmol of each appropriate primer (Table 2), 1 U of Taq polymerase (Life Technologies, Breda, The Netherlands) and 0.5 mM of each dNTP. Amplifications were performed in a Thermal Cycler (Perkin Elmer, Norwalk, CT). Samples were denatured at 92°C for 5 min and then subjected to 35 cycles of amplification: 1 min at 95°C, 1 min at annealing temperature (Table 2), 1 min at 72°C. SSCP analysis was performed using the Genephore Gelsystem (Amersham Pharmacia Biotech, Roosendaal, the Netherlands). PCR products were loaded on precast, ready-to-use GeneGel Excel 12.5/24 polyacrylamide gels (Pharmacia). After electrophoresis, gels were silver stained in an automated gel stainer using the Plus-one DNA Silverstaining Kit (Pharmacia), according to the protocol of the manufacturer. Samples were run in dupple at 5°C and 15°C, respectively, to increase sensitivity. When a band shift was detected, the corresponding fragment was bidirectionally sequenced to reveal the nature of the mutation. In case no mutations were found through SSCP analysis, all exons were sequenced. Automated DNA sequencing was performed on an ABI PRISM 377 (PE Biosystems, Nieuwkerk ald Yssel, The Netherlands) using dye terminator chemistry.

Results

Human genomic DNA from the probands of five families and of three sporadic patients was screened for mutations in the LMX1B gene by PCR-SSCP of exons 2 to 8, followed by sequencing of amplified DNA fragments that had resulted in

<table>
<thead>
<tr>
<th>Family</th>
<th>No. of Affected Members</th>
<th>Nail Hypo/Dysplasia</th>
<th>Absent/Small Patellae</th>
<th>Elbow Immobility</th>
<th>Iliac Horns</th>
<th>Eye Abnormalities</th>
<th>Nephropathy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1&lt;sup&gt;s&lt;/sup&gt;</td>
<td>1/1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1/1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1/1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0/1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0/1&lt;sup&gt;d&lt;/sup&gt;</td>
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</tr>
<tr>
<td>2</td>
<td>5</td>
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<td>1</td>
<td>2/5</td>
<td>Unknown</td>
</tr>
<tr>
<td>3</td>
<td>14</td>
<td>3/3</td>
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<td>2/2</td>
<td>1/1</td>
<td>0/2</td>
<td>0/11</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>2/2</td>
<td>2/2</td>
<td>2/2</td>
<td>1/1</td>
<td>1/1</td>
<td>0/1</td>
</tr>
<tr>
<td>5</td>
<td>1&lt;sup&gt;s&lt;/sup&gt;</td>
<td>1/1</td>
<td>1/1</td>
<td>1/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
</tr>
<tr>
<td>6</td>
<td>1&lt;sup&gt;s&lt;/sup&gt;</td>
<td>1/1</td>
<td>1/1</td>
<td>1/1</td>
<td>0/1</td>
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<td>3</td>
<td>3/3</td>
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<tr>
<td>8</td>
<td>33</td>
<td>23/24</td>
<td>20/24</td>
<td>20/24</td>
<td>17/23</td>
<td>5/12</td>
<td>13/30</td>
</tr>
</tbody>
</table>

<sup>a</sup> Numbers refer to the families in which mutations in the LMX1B gene are presented in Table 3.

<sup>b</sup> Eye abnormalities include hyperpigmentation of the iris, cataract, and/or glaucoma.

<sup>c</sup> Number of patients in which symptom is present<sup>d</sup> number of patients examined.

<sup>s</sup> Sporadic.

Unknown = not examined.

Table 1. Summary of clinical features of NPS patients
SSCP band shifts. We identified mutations in all of them (Table 3). Two patients from different families (families 3 and 4) share the same R198X mutation. The seven different mutations were all single base-pair substitutions, consisting of four missense mutations, two nonsense mutations, and 1 splice-site mutation. One of the identified mutations (A249P) has not been described previously. Of the remaining six mutations, three (Q64X, C95Y, splice-site mutation) have been found in one other family, and the other three (R198X, R200Q, A213P) are more frequently occurring mutations and have been found in five, six, and four families, respectively (10–12, 18). Both nonsense mutations (R198X and Q64X) are predicted to cause premature termination of translation, resulting in truncation of the \( LMX1B \) protein. The G to C transversion and the G to A transition in exon 4, leading to an A213P and an R200Q missense mutation, respectively, in the homeodomain of the \( LMX1B \) protein, have been shown to have a negative effect on DNA binding (11). The G to A transition in exon 3, resulting in a C95Y missense mutation in the second LIM domain, may abolish the Zn(II) binding site and prevent the formation of a stable protein. The splice-site mutation (672+1G→A) will probably lead to a shorter \( LMX1B \) mRNA as a result of exon skipping. Because \( LMX1B \)-expressing tissue, such as kidney, was not available, we were not able to confirm this presumed effect of this mutation. In families 2, 3, 4, 7, and 8, the mutations cosegregate with the NPS phenotype. The seven mutations were not found in a panel of 100 control chromosomes. In addition to these putative loss-of-function mutations, five common polymorphisms, two silent mutations (Ser219Ser; Glu124Glu), and three intron polymorphisms (IVS 2, +7 G→C; IVS 4, −49 C→T; IVS 8, −49 C→A) were found in our series.

**Discussion**

In our group of Dutch families with NPS, we identified seven different mutations in the \( LMX1B \) gene, including one novel variant, which brings the total number of different mutations identified to 65 (10–12, 18, 19). Approximately 43% of these 65 mutations are localized in the homeodomain, 40% in the LIM1 domain, and the rest in the LIM2 domain. Remarkably, mutations in the transcriptional activation domain in the C-terminus of the \( LMX1B \) protein have not yet been found.

Three of the mutations identified in our study are frequently occurring mutations. R198X has been found in 4%, R200Q in 5%, and A213P in 3% of all families tested. Although they did not show their data, McIntosh et al. (12) concluded, on the

**Table 2. Primers flanking \( LMX1B \) exons**

| Exon | Forward Primer | Reverse Primer | \( T_a (°C) \)
|------|----------------|----------------|----------
| 2    | cgctgacggcgcggcttc | gctcgagtcggccgg | 58       |
| 3    | ggcagaggtgccccctcg<sup>b</sup> | tccagacccccccagc | 61       |
| 4    | gtgcacagagcggacaggg | gtgtgcgggactacgcttg | 61       |
| 5    | ccacacccacctcctcccg | acctgatgctctggctgcc | 59       |
| 6    | ggcagccagacacctaggg | tctgcgccagctacccctg | 55       |
| 7    | ctggcagccagtaggggt<sup>b</sup> | atgcctgcagccccctagg | 63       |
| 8    | gtcgagccagcccatctgt<sup>b</sup> | ggaetctgcagctggataga<sup>a</sup> | 55       |

<sup>a</sup> \( T_a \) = annealing temperature.

<sup>b</sup> Primer has previously been reported (11).

**Table 3. Mutations identified in NPS patients**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Nucleotide&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Domain</th>
<th>Putative Effect</th>
<th>Recurrent/ New</th>
<th>Family Number&lt;sup&gt;b&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>Q64X</td>
<td>C190T</td>
<td>LIM1</td>
<td>PTC</td>
<td>Recurrent&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1</td>
</tr>
<tr>
<td>C95Y</td>
<td>G284A</td>
<td>LIM2</td>
<td>Zn (II) binding</td>
<td>Recurrent&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2</td>
</tr>
<tr>
<td>R198X</td>
<td>C592T</td>
<td>HD</td>
<td>PTC</td>
<td>Recurrent&lt;sup&gt;d,e&lt;/sup&gt;</td>
<td>3,4</td>
</tr>
<tr>
<td>R200Q</td>
<td>G599A</td>
<td>HD</td>
<td>DNA binding</td>
<td>Recurrent&lt;sup&gt;e&lt;/sup&gt;</td>
<td>5</td>
</tr>
<tr>
<td>A213P</td>
<td>G637C</td>
<td>HD</td>
<td>DNA binding</td>
<td>Recurrent&lt;sup&gt;e&lt;/sup&gt;</td>
<td>6</td>
</tr>
<tr>
<td>A249P</td>
<td>G745C</td>
<td>HD</td>
<td>DNA binding</td>
<td>New</td>
<td>7</td>
</tr>
<tr>
<td>672+1G→A</td>
<td>672+1G→A</td>
<td>HD</td>
<td>Loss of exon 4, frameshift, PTC</td>
<td>Recurrent&lt;sup&gt;e&lt;/sup&gt;</td>
<td>8</td>
</tr>
</tbody>
</table>

<sup>a</sup> Nucleotide numbering follows GenBank AFO57135.

<sup>b</sup> Numbers refer to the families presented in Table 1.

<sup>c</sup> (18).

<sup>d</sup> (10).

<sup>e</sup> (12).
basis of haplotype analysis with polymorphic microsatellite markers from the 9q34 region, that these mutations recur in unrelated individuals. Haplotype analysis in our Dutch cohort has been performed only in the larger families, at the time when the gene responsible for NPS was not yet identified, to narrow the chromosomal region encompassing the disease gene. The patients carrying the R198X, R200Q, and A213P mutations did not belong to these previously analyzed families, either because they were sporadic patients or because the family was too small or only a few family members were available for study. Therefore, although unlikely, we cannot exclude the possibility that our patients are related to any of the other families carrying a similar mutation. The high rate of recurrence of the R198X and R200Q mutations might be explained by the fact that these mutations are the result of a transition at a CpG dinucleotide, which is a mutational hot spot because of the tendency for the cytosine to be methylated and subsequently deaminated to thymine. The recurrence of the A213P mutation, however, remains unexplained.

On the basis of the nature of the mutations and/or the mutations’ localization in functionally important domains of the LMX1B protein, together with the cosegregation of the mutations with the NPS phenotype within the families and the absence of these mutations in 100 control chromosomes, it is very likely that these seven mutations are indeed harmful mutations and not innocuous polymorphisms. Thus, the two nonsense mutations and the splice-site mutation are predicted to result in truncated LMX1B proteins. The four remaining mutations are missense mutations, and three of these amino-acid substitutions are localized in the homeodomain of the protein, where they are likely to have an effect on DNA binding. R200Q and A213P were shown to reduce and abolish DNA binding, respectively, in in vitro DNA binding studies (12). The homeodomain-DNA interactions of homeobox genes have been intensively studied in many species (for review, see reference (20). In the course of the evolution, the amino-acid sequence of the homeodomain has been conserved to a high degree, reflecting the importance of this sequence for establishing DNA binding. Because it is known which amino acids in the homeodomain are important for DNA-binding specificity, direct DNA-backbone contacts, and secondary structure, the putative effects of the identified mutations in the homeodomain of LMX1B on DNA binding may be predicted. The R200Q substitution affects a highly conserved amino acid located in the flexible N-terminal arm of the homeodomain, which is important in establishing contact to the minor groove of the DNA and as such significantly contributes to the high DNA-binding affinity of the homeodomain. The alanine substitution in the first helix of the homeodomain (A213P) is likely to disturb the formation of a three-dimensional structure that is determined by the folding of the α helices I, II, and III into a tight globular structure (21). Because this three-dimensional structure of the homeodomain ensures the positioning of the third (recognition) helix in the major groove of the DNA, where it establishes contact with both strands of the DNA, it can be predicted that disturbance of the three-dimensional structure by the A213P mutation will indirectly disrupt direct DNA contact with the third helix and therefore DNA binding. The substitution of an alanine in the third helix of the homeodomain (A249P) is likely to hinder the base-specific contact with DNA in the major groove and as such the specificity of DNA binding.

From the function and expression studies of lmx-1 in chicken and Lmx1b in mice, respectively, it can be deduced that the skeletal defects seen in NPS reflect a disruption of normal dorsoventral patterning of the limb during development (14,15). Although it is known from these animal studies that Lmx1b is expressed in fetal and adult kidney tissue, the exact role of this gene in the development of the kidney is much less clear. On the basis of the renal manifestations in NPS, it is likely that Lmx1b has an important role in patterning and cell differentiations in the kidney also. Recent studies by Morello et al. (22) showed that Lmx1b localizes to the glomeruli of E15.5 and newborn wild-type mice. In addition, these investigators demonstrated absence of α3 (IV) and α4 (IV) collagen expression in Lmx1b−/− mutant kidneys. It is widely known that during development, there is a switch from an α1/α2 to an α3/α4 network of collagen IV expression in the GBM (23). Therefore, on the basis of the findings in Lmx1b mutant kidneys, it was postulated that Lmx1b might play an integral role in this coordinated transcriptional switch (22). For humans with a heterozygous mutation in LMX1B, however, the situation may be much less clear. Thus, previous immunologic studies of the GBM using MCA1 antibody, which recognizes the NC1 domain of α3 (IV), gave conflicting results: normal labeling in three patients and no GBM labeling in two others (24,25).

Consistent with the findings of other investigators (11,12,18), we have not found convincing evidence of a correlation between the presence and severity of certain features of NPS and the type or location of the LMX1B mutation. The absence of a genotype–phenotype correlation is not surprising given that there is not only interfamilial but also intrafamilial variability in the expression and severity of symptoms. Family 8 is a very good example of intrafamilial phenotypic variability. It is intriguing that a large percentage of the patients within this family have nephropathy. Nevertheless, renal abnormalities are not present in all affected individuals of this family; in addition, the severity of the nephropathy varies among the patients, ranging from very mild proteinuria in nine patients to end-stage renal disease in four of them. Patients from a family reported by McIntosh et al. (12), who carry the same splice-site mutation (672 +1G→A) as patients in family 8, do not have nephropathy. Therefore, the high incidence and the significant difference in severity of nephropathy within family 8 cannot be attributed to the LMX1B genotype. Recently, Farley et al. (26), on the basis of genetic studies in a very small family with NPS and quantified variable expression of orthopaedic symptoms, found support for the old hypothesis of Renwick (27), who proposed that the severity of NPS symptoms is modulated by the allele contributed by the unaffected parent. We believe that more genetic studies in clinically well characterized large families with NPS are necessary to evaluate the possible in-
fluence of modifying alleles on phenotypic expression and variability.

GBM abnormalities similar to those seen in NPS have been observed in several families without bone or nail abnormalities (28). It will be of interest to determine, by \( \text{LMX1B} \) mutation analysis, whether these cases represent partial expression of NPS or an independent genetic form of GBM disease. In case this NPS-like nephropathy turns out not to be due to \( \text{LMX1B} \) mutations, the possibility that genes encoding proteins that interact with \( \text{LMX1B} \) are involved should be investigated. We are performing yeast-two-hybrid analysis with the ultimate aim of identifying these genes.

Acknowledgment

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