Brief Report

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Novel missense mutation affecting the LIM-A domain of LMX1B in a family with Nail-Patella syndrome

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Summary **Nail-patella syndrome (NPS) is a rare autosomal dominant disease characterized by developmental defects of dorsal limb structures, the kidney, and the eye, that manifest as dysplastic nails, hypoplastic or absent patella, elbow dysplasia, iliac horns, glomerulopathy, and adult-onset glaucoma, respectively. This disorder is inherited in an autosomal dominant mode and is caused by heterozygous loss-of-function mutations in the *LMX1B* gene, which encodes the LIM homeodomain transcription factor LMX1B. In this study, we report the clinical findings of a Spanish family, from the Canary Islands, with three affected members who displayed varying phenotypes. DNA sequence analysis identified a novel heterozygous missense mutation in *LMX1B*, c.305A>G, p.(Y102C), that segregated with the disease. The tyrosine residue affected by the mutation is highly conserved in evolution, and is located in the LIM-A domain, next to one of the cysteine residues involved in zinc binding, suggesting that p.(Y102C) affects LMX1B function by disturbing its interactions with other proteins. Our results expand the mutation spectrum of *LMX1B* and provide insight into the molecular mechanisms of NPS pathology.**

Keywords: *LMX1B*, nail-patella syndrome, missense mutation, transcription factor, zinc finger

1. Introduction

Nail-patella syndrome (NPS; OMIM #161200), also known as hereditary osteoonychodysplasia, is a rare autosomal dominant disease characterized by nail malformations, absent or hypoplastic patellae, dysplasia of the elbows and dorsal ilium, nephropathy, and, in some cases primary open-angle glaucoma (*1*). Other common findings in NPS are hyperpigmentation of the central part of the iris (Lester's sign), ocular hypertension, and sensorineural hearing loss. Furthermore, some patients present with renal involvement, ranging from asymptomatic proteinuria to nephrotic syndrome and sporadically end-stage renal failure (*2-4*).

The disease is caused by heterozygous loss-of-function mutations in the gene *LMX1B*, located on chromosome 9q34, which encodes the LIM-homeodomain transcription factor LMX1B (*5-7*). Molecular studies in *Lmx1b* knock out (KO) mice have shown that *Lmx1b* plays an important role in dorso-ventral patterning of limb development, morphogenesis and function of the podocytes and the glomerular basement membrane, and development of the anterior segment of the eye (*8-11*). Some of these findings indicate that the skeletal phenotype of NPS is the consequence of a defect in developmental patterning. More recent studies in an inducible podocyte-specific *Lmx1b* KO mouse have shown that deletion of this gene in fully differentiated podocytes causes proteinuria and deregulation of the actin cytoskeleton (*12*). This indicates an essential role of *Lmx1b* in maintenance of

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differentiated podocytes in adult kidneys. The LMX1B protein contains two N-terminal zinc-binding LIM domains, LIM-A and LIM-B, which mediate protein-protein interactions, a homeodomain important for DNA binding, and a C-terminal glutamine-rich region that could be involved in transcriptional regulation (*1*). Activation of transcription by LMX1B requires its interaction with other transcription factors. Different *LMX1B* mutations that cause NPS have been identified, including mainly missense and nonsense mutations, small deletions and insertions, splice site mutations, and a few large gene deletions (*3,13-15*). These mutations are generally located in the homeodomain or in the LIM domains and affect conserved amino acid residues (*16*). It has not been possible to establish a correlation between phenotype and genotype in NPS patients. In fact, significant phenotypic variability at the individual, intrafamilial, and interfamilial level has been reported for different NPS symptoms (*2*). However, specific mutations in the central homeodomain of LIMX1B seem to be associated with proteinuria and nephropathy without the NPS skeletal defects (*2,4*).

In the present study, we report the clinical findings of a Spanish family with three NPS affected members, and the identification of a novel heterozygous *LMX1B* missense mutation that segregates with the disease and disturbs the LIM-A domain of LMX1B.

2. Subjects and Methods

2.1. *Patients*

The index case, a 7-year-old boy, was the second child of a non-consanguineous marriage, who presented at birth with a vertical astragalus foot, joint hypermobility and muscular hypotonia. In successive consultations, nail dystrophy and Lester's sign in his right eye were observed (Figures 1A and 1B). His father also showed clinical signs compatible with NPS including dystrophic nails and bilateral elbow dysplasia, not achieving extension of elbows. Consequently, clinical and genetic studies were also requested for his father and his 9-year-old brother. This clinical study included radiology, renal ultrasound, and urine analysis. Informed written consent for the genetic analysis was obtained from the patients' parents. The Ethics Committee of Nuestra Señora de Candelaria University Hospital (Santa Cruz de Tenerife, Spain) approved this study.

2.2. *Mutation analysis*

After obtaining written informed consent, genomic DNA of patients and relatives was extracted from peripheral blood samples using the Gen Elute Blood Genomic DNA kit (Sigma-Aldrich, St. Louis, MO,

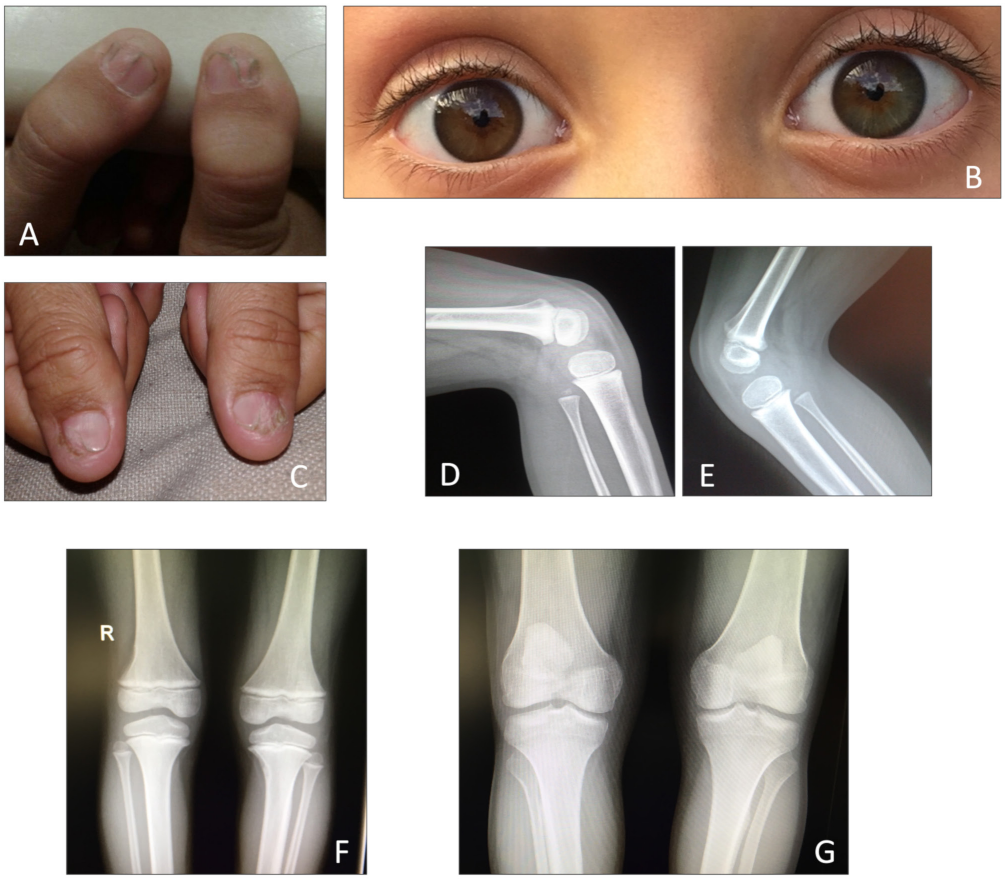

Figure 1. Clinical features of patients. (A and C) Dystrophic nails in index case and his father, respectively; **(B)** Lester's sign in index case; **(D and E)** Absent patella in index case; **(F)** Absent patella the patient's brother; **(G)** Presence of kneecaps in the father.

USA) following the manufacturer's instructions. The eight coding exons and the flanking intronic sequences of *LMX1B* were amplified by polymerase chain reaction (PCR) using intronic primers previously described (*17*). PCR products were purified with the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequence reactions were purified with Performa® DTR Gel Filtration Cartridges (EdgeBio BioSystems, Gaithersburg, Maryland, USA), and analyzed on a 3500 Series Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Mutations were identified by comparison to the *LMX1B* reference sequence NG_017039.1 (*https://www.ncbi.nlm.nih.gov*), and confirmed by sequencing additional independent amplification products. We examined several databases, including Exome Aggregation Consortium (ExAC, *http://exac.broadinstitute.org*), 1000 Genomes Project (TGP, *http://www.internationalgenome.org*), Human Gene Mutation Database (HGMD, *http://www.hgmd.cf.ac.uk/ac/index.php*) and ClinVar (*https://www.ncbi.nlm.nih.gov/clinvar/*), to verify that the mutation detected in our patients was not a common variant and to confirm that it was novel. Online bioinformatics tools PolyPhen (*http://genetics.bwh.harvard.edu/pph2*), SIFT (*http://sift.bii.a-star.edu.sg/www/SIFT_seq_submit2.html*), Align GVGD (*http://agvgd.iarc.fr*), MutPred2 (*http://mutpred.mutdb.org*) and Mutation Taster (*http://www.mutationtaster.org*) were used to predict the pathogenicity of the mutation. Default settings were used for all programs. The protein sequence of human LMX1B (isoform 2 containing 402 amino acids) was obtained from the NCBI database (accession number NP_001167618.1). Human Splicing Finder v3.1 (HSF) was used to predict the effect of the new mutation on exonic splicing regulatory sequences (*http://www.umd.be/HSF3/*).

3. Results and Discussion

Radiological examination revealed bilateral agenesis of the patellae in the index case and his brother, and confirmed the existence of kneecaps in their father (Figures 1D to 1G). Urinalysis did not reveal proteinuria in any of the cases, and renal ultrasound examination showed normal kidneys. Therefore, affected members of the family studied here displayed the typical characteristics of NPS including dysplastic nails, the most constant characteristic of NPS, absent patella and elbow dysplasia. However, they showed varying phenotypes as has been described for other families with NPS (*13,14,17*). The index case presented nail dysplasia and bilateral agenesis of patellae, while his bother only showed absence of patella, and his father displayed dystrophic nails and elbow dysplasia. The proband showed the Lester's sign, which is present in around 50% of the NPS patients (*1*). Approximately 10% of NPS patients will develop glaucoma and a smaller percentage has ocular hypertension (*1*), but these features were lacking for now in this family. The prognosis of NPS patients is established by their renal manifestations, which occur in approximately 40% of NPS patients and ranges from asymptomatic proteinuria to occasional renal failure (*2,4*). These manifestations may take many years to appear. Renal involvement was not present in any of our patients. It is worth noting that the mutation identified in our study is located in de LIM-A domain, since NPS patients with *LMX1B* mutations affecting the homeodomain showed significantly higher frequency of nephropathy and higher values of proteinuria than those carrying mutations in the LIM domains (*2*). The factors responsible for the phenotypic heterogeneity in NPS are basically unknown but it is possible that there are genetic variants that modify the interaction of LMX1B with other proteins giving rise to different clinical manifestations. Only a few cases of NPS have been reported in Spain (*18-20*) and, in general, pediatricians are not familiar with this disease.

Sequencing analysis of the proband revealed a novel heterozygous mutation, c.305A>G, in exon 2 of *LMX1B* (Figure 2A). The mutation segregated with the disease in the family; it was also present in heterozygous state in the patient's brother and father (Figures 2A and 2B). This new *LMX1B* variant implies the substitution of tyrosine 102 for cysteine, p.(Y102C), and it was not found in databases such as ExAC, TGP, HGMD and ClinVar. To obtain an estimation of the mutation pathogenicity, we evaluated the change of tyrosine for cysteine at position 102 of the LMX1B protein with five different bioinformatics tools. All of them predicted that the mutation affects protein function (Table 1). This amino acid residue is highly conserved among eight different species (human, mouse, cat, chicken, fugu, pufferfish, zebra fish, fruit fly and worm) and among other LIM proteins (Figure 2C). The novel variant described here was submitted to ClinVar and was included with the accession number NM_001174146.1 (*https://www.ncbi.nlm.nih.gov/clinvar/variation/587694/*).

LIM-homeodomain proteins contain two cysteine-rich zinc-binding LIM domains near the amino terminus that are involved in interactions with other cofactors for cooperative transcriptional regulation of genes in a tissue-specific manner (*21*). Most LIMX1B mutations affect the LIM domains or the homeodomain by altering amino acids that are essential for the binding of zinc or amino acids essential for DNA binding, respectively. Functional studies of a few *LMX1B* mutations have shown reduced transcriptional activity and decreased DNA-binding ability, resulting in the partial or complete loss of LMX1B function (*6,15,22*). These and other results suggest that the main pathogenic

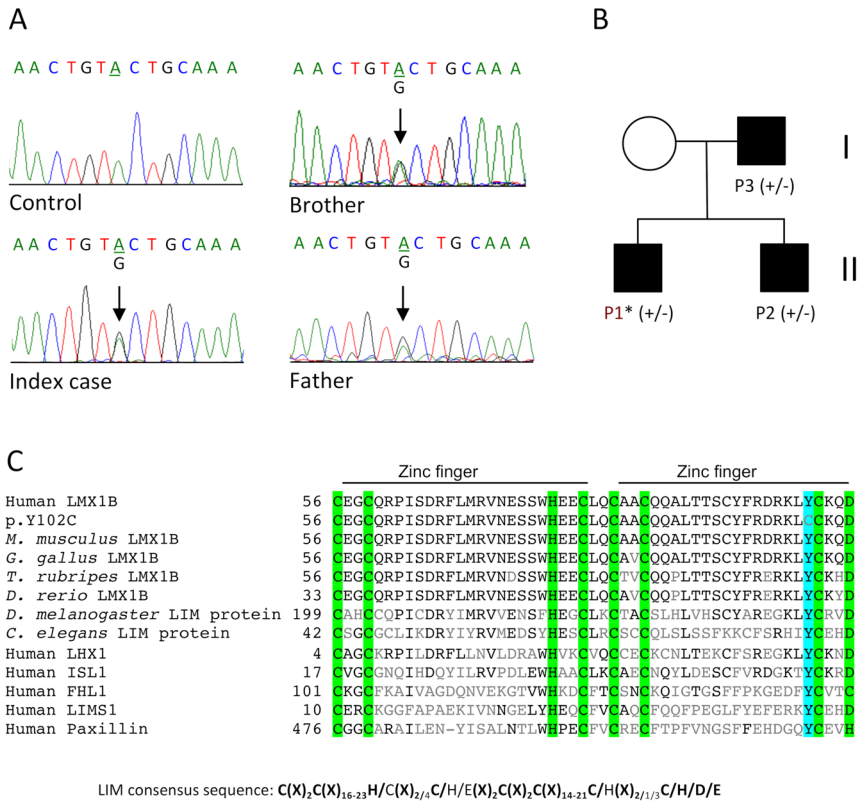

Figure 2. Mutation analysis of the family with NPS and evolutionary conservation of tyrosine 102. (A) Electropherograms showing the partial sequence of *LMX1B* exon 2 in affected members of the family and a control. The arrows indicate the location of the identified heterozygous missense mutation c.305A>G, p.(Y102C). **(B)** Pedigree of the family. Filled and open symbols represent affected and normal individuals, respectively. Circles and squares indicate females and males, respectively. The index case is marked with an asterisk. -, mutant *LMX1B* allele; +, normal *LMX1B* allele. **(C)** Protein alignment showing that tyrosine 102 (highlighted in light blue) in the LIM-A domain of LMX1B is totally conserved among species and among other LIM proteins suggesting that it is important for the zinc finger structure and function. The eight highly conserved zinc-binding residues (cysteine, histidine and aspartic acid) are highlighted in green. Since the less conserved spacer regions vary in size, non-conserved amino acid residues G87 and I88 of C. *elegans* LIM protein, S33, T56, C57 of ISL1, Y117 and V135 of FHL1, S26, G55 of LIMS1, and P521 of paxillin, were deleted to facilitate the alignment of the zinc-binding residues. Black and grey letters represent conserved and nonconserved residues, respectively. The LIM consensus sequence is shown at the bottom of the figure, where X represents any amino acid (*21*).

Table 1. Bioinformatics predictions of pathogenicity for mutation p.(Y102C)

Tool	PolyPhen-2[1]	SIFT[2]	Align GVGD[3]	MutPred[4]	MutationTaster[5]
Score	0.991	0.00	Class C65	0.919	194
Prediction	Probably damaging	Affects function	Affects function	Deleterious	Disease causing

[1]The PolyPhen-2 score ranges from 0.0 to 1.0. Values closer to 1.0 are more confidently predicted to be deleterious. Variants with scores in the range 0.0 to 0.15 are predicted to be benign, while variants with scores in the range 0.15 to 1.0 are possibly damaging. Variants with scores in the range 0.85 to 1.0 are more confidently predicted to be damaging. [2]The SIFT probability score ranges from 0 to 1.0. Amino acid substitutions with scores < 0.05 are predicted to be deleterious (scores closer to 0 are more confidently predicted to be deleterious). Variants with scores in the range 0.05 to 1.00 are predicted to be tolerated (scores very close to 1.0 are more confidently predicted to be tolerated). [3]Align-GVGD classifies variants in seven risk grades (C0, C15, C25, C35, C45, C55, C65) with C65 most likely to interfere with function and C0 least likely. [4]The general score of MutPred2 ranges from 0.0 and 1.0, with a higher score indicating a greater propensity to be pathogenic. [5]The MutationTaster score ranges from 0.0 to 215. It is taken from the Grantham Matrix for amino acid substitutions and reflects the physicochemical difference between the original and the mutated amino acid.

mechanism causing NPS is haploinsufficiency (*7,15,23,24*). Tyrosine residue 102 of LMX1B is located in the second zinc-binding motif of the LIM-A domain, next to cysteine 103, which is one of the four amino acid residues involved in zinc binding (Figure 3). Several missense mutations in the LIM-A domain of LIMXB, including p.(C103W) and p.(D106G), that lead to substitutions of amino acids essential for the binding of zinc have been identified in NPS patients (Figure 3) (*14,16*). Similar mutations affecting highly conserved cysteine residues within the LIM-B domain have also been reported (*3,13*). Therefore, we suggest that p.(Y102C) disturbs the binding of zinc and the function of LMX1B.

Previous studies in other genes have shown that some exonic mutations can be damaging by altering pre-

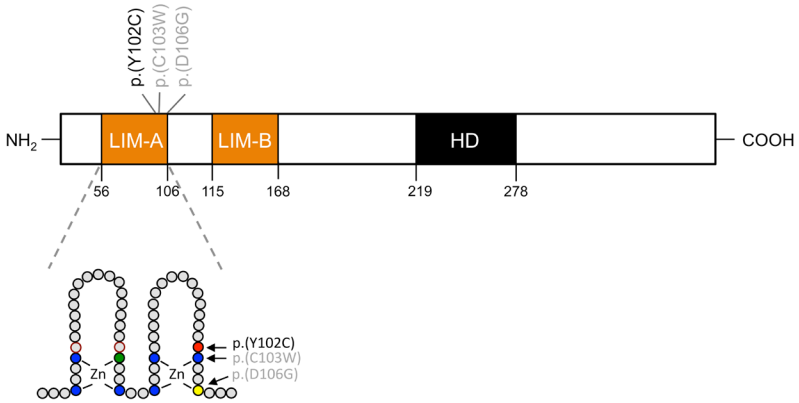

Figure 3. Schematic representation of LMX1B. The two protein-binding LIM domains (orange boxes) located near the N terminus and the DNA binding homeodomain (HD) (black box) are shown. The position of mutation p(Y102C) identified in this study is indicated. Two previously reported missense mutations, p.(C103W) and p.D106G, affecting zinc-binding residues of the LIM-A domain are also indicated (grey letters). Numbers beneath the boxes denote amino acid residues. Below is a diagram showing the structure of the two zinc fingers present in LIM-A. Blue, green and yellow circles indicate conserved zinc binding residues, cysteine, histidine and aspartic acid, respectively. The mutated residue, tyrosine 102, is identified in red. Non-conserved residues are represented as grey circles. Mutations p.(C103W) and p.D106G are also indicated.

mRNA splicing (*25-27*). Since mutation c.305A>G is located in exon 2, twenty-two nucleotides away from the donor splice site of intron 2, we analyzed its potential effect on splicing. Results obtained with HSF showed that the A to G change (underlined) creates a potential exonic splicing silencer (5'-ACTGTGCTG) and could, therefore, alter *LMX1B* pre-mRNAsplicing. This will have to be investigated further using RNA from a patient or cell lines expressing the mutant *LMX1B*.

In conclusion, we identified a novel *LMX1B* missense mutation, c.305A>G, p.(Y102C), in a family with NPS. We suggest that this mutation, located in the highly conserved LIM-A domain of LMX1B, affects the formation of a zinc-binding motif and disturbs the interaction of the protein with other transcription factors. Mutation p.(Y102C) expands the spectrum of *LMX1B* mutations. The identification of missense mutations within the LIM domains may help elucidate the function of these domains.

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