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Mutations in the fourth β-propeller domain of LRP4 are associated with isolated syndactyly with fusion of the third and fourth fingers

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Abstract

Isolated hand syndactyly is a common limb malformation with limited known genetic etiology. We used exome sequencing to discover two novel variants, chr11 g.46896373C>G; p.D1403H and chr11 g.46893078G>T; p.Q1564K, in *LRP4* in a child with isolated bilateral syndactyly of the third and fourth fingers. Each variant was inherited from a different parent and neither parent was affected. Variants in *LRP4* have been previously associated with syndactyly in Cenani-Lenz syndactyly syndrome and Sclerosteosis 2, but have not been reported in individuals with isolated syndactyly. LRP4 inhibits LRP6/LRP5-mediated activation of canonical Wnt signaling and mediates sclerostin-dependent inhibition of bone formation. p.D1403H and p.Q1564K are located within the fourth β-propeller of the extracellular protein domain that has yet to be associated with human disease. Functional analyses of p.D1403H and p.Q1564K show that they significantly decrease LRP4's inhibition of Wnt signaling. These results suggest that variants in the fourth β-propeller of the extracellular protein domain may cause a phenotype distinct from previously characterized *LRP4* variants.

Keywords

LRP4; Syndactyly; Wnt signaling; limb malformations

Syndactyly is a malformation of the digits in which adjacent fingers and/or toes fail to separate (i.e., remain fused) during limb development. Syndactyly can be complete (i.e., the

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entire length of a digit) or partial, bony or cutaneous, and may affect only the phalanges or involve the metacarpal/metatarsal and/or carpal/tarsal bones as well. Syndactyly can occur as an isolated trait or as part of a multiple malformation syndrome. It is the one of most common of all congenital hand deformities with an incidence of at least 3–10 in 10,000 live births and is twice as common in males, as well as in persons of European ancestry (Castilla et al. 1980, Canale ST 2008, Jordan et al. 2012, Malik 2012). Syndactyly is a heterogeneous phenotype with inter- and intra-familial variability and often differs between the left and right side in the same individual. A number of subtypes of non-syndromic syndactyly have been delineated based upon morphological and radiological characteristics. Most of these segregate in an autosomal dominant pattern but autosomal recessive and X-linked syndactyly have also been reported (Malik 2012). The genetic basis of several types of isolated syndactyly have been identified but the genes underlying isolated syndactyly in many families remain to be discovered (Malik 2012).

One gene underlying syndactyly is low-density lipoprotein receptor-related protein 4 (*LRP4*; MIM# 604270; RefSeq: NR_038909.1). Specifically, variants in *LRP4* are responsible for Cenani-Lenz syndrome (CLS; MIM# 212780) and Sclerosteosis 2 (MIM# 614305). CLS is characterized by bilateral complex syndactyly of the hands (carpal, metacarpal and digital synostoses, disorganization of the carpal bones, digital reduction) and feet (toe syndactyly, metatarsal fusion, absent metatarsals), renal abnormalities, and dysmorphic facial features (Cenani et al. 1967, Temtamy et al. 1978, Harpf et al. 2005, Li, Pawlik, et al. 2010). Either homozygous or compound heterozygous variants in *LRP4* can cause CLS (Li, Pawlik, et al. 2010). Sclerosteosis 2 is a severe sclerosing bone dysplasia characterized by progressive skeletal overgrowth, syndactyly, facial asymmetry and hearing loss caused by variants in regions encoding the highly conserved residues in the third β-propeller of the extracellular domain of LRP4 protein (Leupin et al. 2011, Fijalkowski et al. 2016). In addition, homozygous variants in *Lrp4* can cause syndactyly in both mice and cattle (Johnson et al. 2005, Johnson et al. 2006, Simon-Chazottes et al. 2006, Drogemuller et al. 2007).

The Low-density lipoprotein receptor-related protein 4 (LRP4) protein, also known as MEGF7, is a member of the highly conserved LDL receptor-related protein (LRP) family which has diverse functions in developmental processes, lipoprotein trafficking and cell signaling (Herz et al. 2009). LRP4 is composed of an extracellular domain containing a signal peptide, eight LDLa domains (class A repeats), four β-propeller domains (class B repeats, or YWTD domains) and a domain for O-linked oligosaccharide modification. The intracellular domain contains an NPxY motif and a PDZ-interacting motif at the C-terminus. In addition, LRP4 has six EGF-like domains (Shen et al. 2015) (Fig. 1). One of the pathways in which *LRP4* and other members of the LDLR-related protein family are involved in, is the Wnt signaling pathway. Wnt is known to be involved in numerous aspects of embryonic development and has been shown to act as a morphogen (Clevers 2006). Binding of WNT ligands to *LRP5* and *LRP6* leads to the stabilization of β-catenin and downstream transcriptional activation (Gong et al. 2001, Li et al. 2005, Clevers 2006, Mani et al. 2007). Limb malformations are thought to be caused by abnormal Wnt and β-catenin signaling in the developing limb bud (Baron et al. 2013). LRP4 was shown to inhibit LRP6/LRP5-mediated activation of canonical Wnt signaling (Johnson et al. 2005, Li et al. 2005). In the bone, LRP4 functions as a specific facilitator of sclerostin-mediated inhibition of Wnt1/β-

catenin signaling and therefore mediates sclerostin dependent inhibition of bone formation (Leupin et al. 2011, Xiong et al. 2015). Loss of LRP4 function leading to excessive Wnt and β-catenin signaling underlies both CLS and Sclerosteosis 2 pathophysiology (Logan et al. 2004, Johnson et al. 2005, Li, Pawlik, et al. 2010, Fijalkowski et al. 2016).

We report a novel phenotype consisting of isolated bilateral syndactyly of the third and fourth fingers due to compound heterozygous variants in *LRP4*. These variants are in the region encoding the fourth β-propeller of the extracellular protein domain of LRP4. Functional analyses of mutant LRP4 suggests that Wnt signaling shows reduced inhibition due to these mutations.

All methods and ethics approvals are described in the online Supplementary Materials and Methods. The proband is a female with bilateral hand anomalies born at term after a normal pregnancy. A prenatal ultrasound was unremarkable but postnatally, syndactyly of the third and fourth fingers resulting in oligodactyly was found. Specifically, the bones of the proximal phalanges of the 3rd and 4th digits were fused with a single middle and distal phalanges and a single nail (Fig. 2A and B). Five metacarpals and five proximal phalanges were present in both hands. The third proximal phalanx of the right hand was small. The creases of the proximal, middle and distal phalanges were hypoplastic. The thumbs and index fingers were normal. The 5th digits had mild clinodactyly at the middle phalanx. The wrists and forearms were normal, as were both feet. An abdominal ultrasound did not reveal any abnormalities of the kidneys or urinary tract.

At age 7 years, she was noted two missing upper canine teeth by radiography. Examination at 10 years of age revealed no abnormal facial asymmetry, normal hearing and no additional health problems. Her height was 58 inches (90th percentile) and weight was 93 pounds (75–90th percentile). Her head circumference was 21.5 inches (~98th percentile). Her psychomotor development was normal. The parents and her brother do not have limb malformations (Fig 2C). There is no family history of congenital anomalies. Initial work-up included a karyotype which was normal and an array CGH (with 2867 informative BAC probes) which was normal: 1–22(3002BAC)x2, X(198BAC)x2, Y(22BAC)x0.

Exome sequencing was performed on the proband and her parents. Under a recessive model of inheritance, candidate variants were identified in four genes: FAT atypical cadherin 2 (*FAT2*; MIM #604269), keratin 10 (*KRT10*; MIM# 148080), TMEM*110-MUSTN1* read through and LDL receptor related protein 4 (*LRP4*). Because of the phenotypic overlap between CLS and the findings in our proband, *LRP4* was prioritized as the top candidate gene. Variants in FAT2, KRT10, and TMEM*110-MUSTN1* have not been associated with limb malformations.

The proband is compound heterozygote for two non-synonymous variants in *LRP4*, GRCh37 chr11 g.46896373C>G (p.D1403H) in exon 28 (RefSeq NM_002334.3) and GRCh37 chr11 g.46893078G>T (p.Q1564K) in exon 31. Both variants were confirmed by Sanger sequencing. g.46896373C>G was inherited from her father and g.46893078G>T was inherited from her mother (Fig. 2D). A healthy brother of the proband was also Sanger sequenced and does not carry any of these mutations (Fig. 2D). Neither variant has been

reported previously in gnomAD r2.0.2, ExAC v1.3 (Lek et al. 2016), ESP (Tennessen et al. 2012), 1000 Genomes (Abecasis et al. 2010), or ClinVar (Landrum et al. 2016) and both had CADD scores (Kircher et al. 2014) of >20 (p.D1403H 29.9, p.Q1564K 23.2). p.D1403H and p.Q1564K are located in the fourth β-propeller domain of the LRP4 protein (Fig. 1) in which no variants underlying human phenotypes have been reported previously. The p.D1403H mutation changes a negatively charged amino acid to a positively charged one and is predicted to decrease the stability of the protein by MUpro (Cheng et al. 2006) [support vector machine (SVM) −0.236 and neural network −0.715 confidence scores]. The p.Q1564K mutation changes an uncharged amino acid to a positively charged one and is predicted by MUpro to increase the stability of protein by SVM (confidence score 0.192) and decrease stability by neural network (confidence score.−0.523). Combined, these observations suggest that mutations of LRP4 encoding this domain might be associated with isolated syndactyly.

To test whether p.D1403H and p.Q1564K perturb LRP4 function, we measured Wnt signaling activity *in vitro*. HEK293T cells were transfected with the following plasmids: LRP6, Wnt1, Top Flash along with the following Lrp4 alleles: 1) wild-type; 2) p.D1403H mutation; 3) p.Q1564K mutation; 4) p.C160Y mutation. p.C160Y underlies CLS and has been previously shown to increase Wnt signaling (Li, Pawlik, et al. 2010). As shown in past experiments (Li, Pawlik, et al. 2010), Wnt1 was able to significantly activate LRP6-mediated β-catenin signaling and the addition of Lrp4 antagonized this activation, reducing Wnt signaling by 50%. When the wild-type *Lrp4* is replaced with either the p.D1403H or the p.Q1564K mutation, we observe a decrease in the inhibition of Wnt signaling (Fig. 2E). This reduction is similar to the one observed when the *Lrp4* wild-type plasmid was substituted with the CLS associated p.C160Y positive control mutation (Fig. 2E). These results suggest that these mutations lead to increased Wnt signaling.

Our results describe a family with isolated syndactyly of the hands due to compound heterozygous missense variants in *LRP4* in a region encoding for the fourth β-propeller domain of MEGF7. Pathogenicity of these variants is suggested by the high CADD scores and functional assessment demonstrates that these variants result in loss of function of *LRP4* inhibition which in turn results in increased Wnt signaling. Both variants reported in our family resulted in inhibition of Wnt signaling comparable to the previously reported mutation p.C160Y, a variant associated with CLS.

It is unclear whether this family represents expansion of a known phenotype (e.g., CLS) or a novel phenotype caused by variants in *LRP4*. CLS is associated with a wide phenotypic spectrum (Seven et al. 2000, Temtamy et al. 2003, Jarbhou et al. 2008). At the severe end of the spectrum is a lethal form with mesomelic limb reductions, oligosyndactyly, genitourinary malformations caused by compound heterozygosity for truncating mutations in *LRP*4 (Lindy et al. 2014). Other severe phenotypes with additional features such as hearing loss, high arched palate, cleft-lip, enamel hypoplasia, supernumerary nipples short stature, and hypoplastic shoulder joint have been reported (Seven et al. 2000, Kariminejad et al. 2013, Afzal et al. 2017). On the mild end of the spectrum, homozygosity for the missense variant, p.L953P, in *LRP4* was found in a large family with a mild CLS phenotype restricted mainly to the upper limbs and kidneys (Khan et al. 2013). Lower limb malformations were

either mild or absent and there were no radioulnar synostosis or craniofacial involvement. It is also worth noting that a case of CLS with isolated, nearly symmetrical hands and feet anomalies typical for CLS, with fusion and disorganization of metacarpal and phalangeal bones, was previously reported (Elcioglu et al. 1997). While this pedigree was suggestive of autosomal recessive inheritance of *LRP4* it was not sequenced for mutations in this gene. In general, dysmorphic facial features and renal anomalies are present in the majority of individuals with CLS.

Isolated syndactyly as well as syndatctyly with dental anomalies due to *LRP4* variants has been observed in animal models as well. Homozygous variants in the *Lrp4* gene have been found in various breeds of cattle with isolated congenital syndactyly (Drogemuller et al. 2007, Johnson et al. 2006). Two of the variants reported in cattle, G907R and G1199S, are located within the second and third beta-propeller structures (Drogemuller et al. 2007). In *LRP4*-deficient mice polysyndactyly of the fore- and hind limbs was found in all mice and partly penetrant abnormalities of tooth development in some of the mice (Johnson et al. 2005) (Simon-Chazottes et al. 2006).

In conclusion, we report a person with isolated syndactyly due to compound heterozygote non-synonymous variants in *LRP4*. These variants are predicted to alter amino acid residues in the 4^{th} β-propeller of the extracellular domain of MEGF7 and reduce the ability of *LRP4* to inhibit Wnt signaling, similar to variants that result in syndactyly in CLS. Accordingly, our findings suggest that either isolated syndactyly is a rare presentation of CLS or that some LRP4 variants cause a novel phenotype consisting of non-syndromic syndactyly. Distinguishing between these two possibilities will require identification and robust phenotyping of additional individuals with pathogenic variants in *LRP4*. This emphasizes the need for broad and open sharing of genetic data and phenotypic information from families with rare Mendelian conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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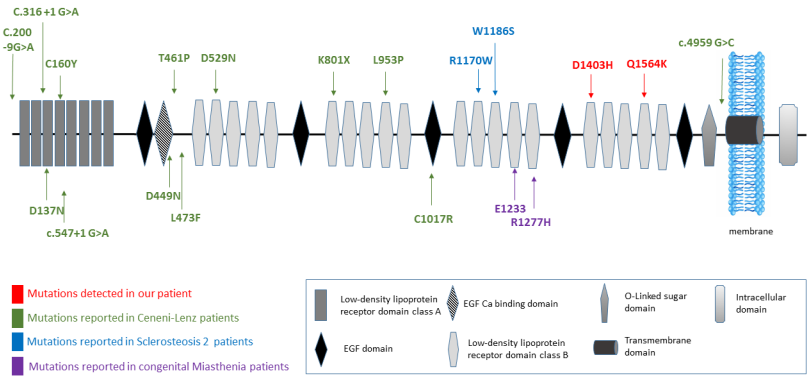

Figure 1.

The LRP4 protein and the domains in which mutations are located both for previously reported mutations in *LRP4* related syndromes and the ones found in our patient.

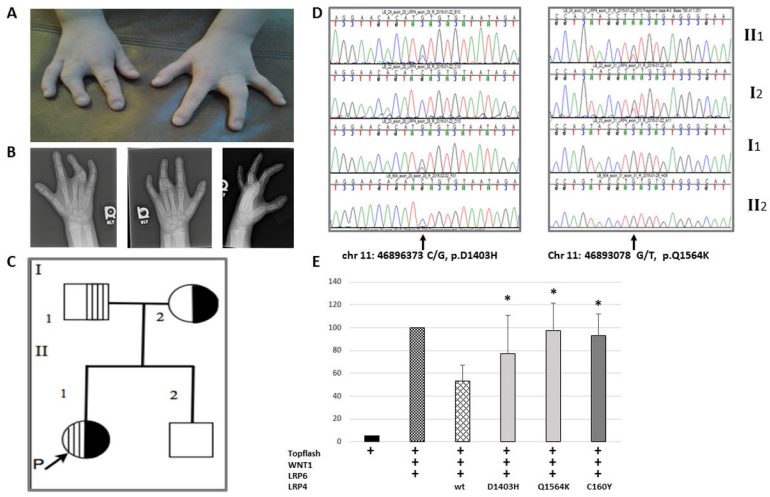

Figure 2.

Clinical picture and radiographs of the patient with syndactyly, family pedigree and sequencing and luciferase results. (**A**) A picture showing the proband's hands. In both hands, the middle and ring finger are completely fused with a single nail. Neither hand had any flexion of the joined fingers. (**B**) X-Ray of right and left hands followed by a lateral view of the left hand. The bones of the proximal phanlanges of the 3rd and 4th digits are fused with a single middle and distal phalanges and a single nail. Five metacarpals and five proximal phalanges are present in both hands. The third proximal phalanx of the right hand is small. The creases of the proximal, middle and distal phalanges are hypoplastic. The thumbs are normal as is the index finger. The 5th digit has mild clinodactyly at the middle phalanx. (**C**) Pedigree of the family. The arrowhead points to the proband. (**D**) Sequencing chromatogram of the family members. The proband (II1) is a compound heterozygote of the two variants on chromosome 11 46896373 C/G, p.D1403H and 46893078 G/T, p.Q1564K (GRCh37/hg19). Her parents (I1 and I2) are each a carrier of one the variants. The healthy brother (II2) does not carry any of these variants. (**E**) Luciferase reporter assays to assess β-catenin signaling. Both alterations (chr11 g.46896373C>G; p.D1403H and g.46893078G>T; p.Q1564K) decreased the inhibition of Lrp4 on LRP6 –mediated Wnt signaling similar to the C160Y positive control as determined by luciferase measurements using the Top Flash reporter vector. Experiments were performed in two separate days with four technical replicates each day (N=8). Asterisks represents statistically significant values compared to wild-type (wt) Lrp4 ($p<0.05$; t-test, one sided, type 2). Error bars represent one standard deviation.