Published in final edited form as:
Hum Mutat. 2014 August ; 35(8): 945–948. doi:10.1002/humu.22581.

A Novel ZRS Mutation Leads to Preaxial Polydactyly Type 2 in a Heterozygous Form and Werner Mesomelic Syndrome in a Homozygous Form

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Abstract

Point mutations in the zone of polarizing activity regulatory sequence (ZRS) are known to cause human limb malformations. Although most mutations cause preaxial polydactyly (PPD), triphalangeal thumb (TPT) or both, a mutation in position 404 of the ZRS causes more severe Werner mesomelic syndrome (WMS) for which malformations include the distal arm or leg bones in addition to the hands and/or feet. Of more than 15 reported families with ZRS mutations, only one homozygous individual has been reported, with no change in phenotype compared with heterozygotes. Here, we describe a novel point mutation in the ZRS, 402C>T (AC007097.4:g. 105548C>T), that is transmitted through two Mexican families with one homozygous individual. The homozygous phenotype for this mutation, WMS, is more severe than the numerous heterozygous individuals genotyped from both families who have TPT and PPD. A mouse transgenic enhancer assay shows that this mutation causes an expansion of the enhancer's expression domain in the developing mouse limb, confirming its pathogenicity. Combined, our results identify a novel ZRS mutation in the Mexican population, 402C>T, and suggest that a dosage effect exists for this ZRS mutation.

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Disclosure statement: The authors declare no conflict of interest.

Additional Supporting Information may be found in the online version of this article.

Keywords

ZRS; enhancer; *SHH*; preaxial polydactyly; Werner mesomelic syndrome

Limb malformations are the second most common human congenital abnormality with a prevalence of one in every 500 births [Moore and Persaud, 1998]. Some of these malformations are the result of defects in signaling pathways. The orientation of the cells during development is determined by the gradient accumulation of proteins and other signaling molecules. In the anterior–posterior (AP) axis of the developing upper limb, changes in expression can result in malformations including preaxial polydactyly (PPD), triphalangeal thumbs (TPTs), and others. The pattern of the AP axis is determined by the expression of sonic hedgehog (*SHH*; MIM #600725) at the posterior side of the limb bud, in a region called the zone of polarizing activity (ZPA) [Saunders and Gasseling, 1968].

Shh limb expression is controlled by a *cis*-regulatory enhancer that is conserved from humans to fish known as the ZPA regulatory sequence (ZRS; MIM #605522); an 800 base pair sequence within intron 5 of *LMBR1* (MIM #605522), which is nearly 1 megabase upstream of the *SHH* gene [Lettice et al., 2003]. Point mutations at over 15 locations and a 13 base pair (bp) insertion in the ZRS have been shown to cause PPD (reviewed in [VanderMeer and Ahituv, 2011]). Analysis of some of these mutations shows that they appear to increase the expression domain of *Shh* in the posterior ZPA to include the anterior portion of the limb bud.

ZRS point mutations are associated with a wide spectrum of hand malformations including PPD type II (PPD2; MIM #174500), TPT type I (TPT1; MIM #174500), and Werner mesomelic syndrome (WMS) (tibial hypoplasia-polysyndactyly-triphalangeal thumb, THPSTPT; MIM# 188770). TPT is characterized by a long, finger-like thumb with three phalanges. TPT can occur as an isolated malformation or in association with polydactyly and syndactyly. Heterozygous mutations at position 404 in the ZRS cause WMS, which is characterized by hypoplastic tibias, shortening of radioulnar bones, PPD of toes, and TPT with or without polysyndactyly [Lettice et al., 2003; Furniss et al., 2008; Wieczorek et al., 2009]. The mechanism to explain this more severe phenotype remains unknown.

Here, we report two Mexican families with a novel point mutation in the ZRS that causes PPD and TPTs. Interestingly, one individual is homozygous for the mutation and has WMS, suggesting a dosage effect. Analysis of this new ZRS mutation in an *in vivo* mouse enhancer assay was performed and showed that it causes expanded reporter gene expression from the ZPA to an ectopic anterior limb region, validating the pathogenesis of the mutation.

All members of the participating families provided written informed consent. This study was approved by the ethics committee of each of the respective institutions and was conducted according to the principles established in the Declaration of Helsinki. DNA was obtained from blood or saliva samples. Subjects in both families were sequenced at the ZRS region (chr7:156,583,564–156,585,727; UCSC Genome Browser; http://genome.ucsc.edu; hg19; g. 104811 105583 in reference sequence AC007097.4) and mutations were confirmed with replicate sequencing reactions. Unrelated normal individuals were also genotyped for this

mutation (see Supporting Information). Transcription factor binding sites (TFBSs) were screened using TRANSFAC for 15 base pairs centered on the mutation [Kel et al., 2003]. Copy number at the ZRS was confirmed by qPCR as previously published [Sun et al., 2008]. The genome-wide SNP microarray genotype analysis using OmniExpress Beadchips (Illumina, San Diego, CA) was performed on the proband from family A and affected from family B at the Microarray facility at Cedars-Sinai Medical Center. The data were analyzed with GenomeStudio software.

In the first of the two families, family A, the proband (Fig. 1A, individual V/29) has mesomelic shortening of upper and lower extremities due to hypoplastic tibiae and hypoplastic radioulnar bones. She also has bilateral PPD of toes with two extra toes in both sides and bilateral proximally placed hypoplastic thumbs (Fig. 1D–F, Supp. Fig. S1) and rudimentary thumbs; they are shortened and laterally deviated (Fig. 1D). She has mesomelic shortening of lower extremities with bowed tibias and bilateral dimples. Her feet are shortened and have five remaining toes; two extra toes were removed on each side and she has a gap with a surgical scar between the first and second toe. Her X-rays show short TPTs, mild shortening and fusion of radioulnar bones, fusion of metacarpal bones, and hypoplastic tibiae with bowing of the fibula bilaterally (Fig. 1D–F). She was the only subject with lower limb malformations.

Both parents have abnormal thumbs. The father (Fig. 1A, IV/30) has bilateral TPTs and a preaxial remnant (Supp. Fig. S1). Radiography showed a small proximal extra digit and TPTs in both sides (Fig. 1B). The mother (Fig. 1C, IV/31) has digitalization of thumbs with an extra crease in the distal interphalangeal space (Supp. Fig. S1). Radiography reveals bilateral elongated distal phalanx of the first finger (Fig. 1C). The proband has a 5-year-old sibling reported to have digitalization of thumbs who was not available for examination.

Family history reveals multiple members in both sides with digitalization of thumbs and TPTs with and without polydactyly, but no other member with shortening of the limbs. A pedigree of six generations was constructed from the reported family history (Fig. 1A). The proband’s parents deny consanguinity, however, both paternal and maternal grandparents were from the same small village in Mexico.

Family B is a Mexican family of which five generations was ascertained (Fig. 1G). The most common phenotype in this family was digitalization of thumbs and TPTs (Fig. 1H). Two subjects had TPT on one hand and PPD on the other hand (Supp. Fig. S1). Another two subjects had unilateral PPD, and two more had only radioulnar synostosis. No involvement of the feet was observed in any subject. DNA samples were collected from the family in generations IV and V, consisting of the father (IV/17) with radioulnar synostosis, unaffected mother, three affected siblings with bilateral TPTs, and one unaffected son.

We screened a 2.1 kilobase (kb) region in both families (chr7:156,583,564–156,585,727; UCSC Genome Browser; http://genome.ucsc.edu; hg19; g.104811 105583 in reference sequence AC007097.4) encompassing the ZRS and the preZRS (pZRS), an adjacent region where mutations cause polydactyly in dogs [Park et al., 2008]. Mutations within the ~800 bp ZRS are numbered according to their position within the original region defined by Lettice

et al. (2003). This numbering begins at g.106333 in reference sequence NG 009240.1 (chr7: 156,584,570; UCSC Genome Browser; http://genome.ucsc.edu; hg19). In family A, the parents of the proband (IV/30 and IV/31) are heterozygous for a novel g.106735C>T mutation within with reference sequence NG 009240.1 (position 402 of the ZRS sequence; chr7: 156,584,168, hg19), whereas the proband is homozygous for this mutation (Fig. 2A) (here named ZRS 402C>T) with a normal copy number as confirmed by qPCR (data not shown). In family B, the father and three affected subjects are heterozygous for the same mutation. The unaffected sibling did not have the mutation. This mutation is not found in dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) or in the 1000 Genomes Project database (http://www.1000genomes.org/) and was not detected in 118 chromosomes from 59 unrelated Mexican individuals (see Supporting Information and Supp. Fig. S2). This novel mutation has the following ClinVar database (www.ncbi.nlm.nih.gov/clinvar/) accession number: RCV000114312.1. Haplotype analysis determined that the mutation is likely to have arisen in a common ancestor with both families sharing a 0.5 Mb haplotype around the ZRS. TFBS analysis using TRANSFAC [Kel et al., 2003] did not reveal changes to TFBSs in this database (data not shown).

A transgenic mouse assay was used to determine the impact of ZRS402C>T on the normal ZPA-specific expression pattern of the ZRS [Lettice et al., 2003]. The mutant allele ZRS402C>T was inserted into a human ZRS-pZRS-HSP68-LacZ enhancer assay vector by site-directed mutagenesis (QuikChange Lightning; Agilent, Santa Clara, CA). This vector uses a minimal promoter and allows for LacZ expression driven by an enhancer [Pennacchio et al., 2006]. Transgenic mouse embryos were generated by Cyagen Biosciences and screened at mouse embryonic day 11.5. All mouse work was approved by the UCSF institutional animal care and use committee. ZRS mutant embryos were compared with the wild-type ZRS-HSP68-LacZ mice from a study previously published by our laboratory [Laurell et al., 2012]. The wild-type ZRS sequence recapitulates *Shh* expression; LacZ is restricted to the posterior of the limb bud (Fig. 2D). The ZRS402C>T transgenic mice show ectopic anterior expression in six of six LacZ-positive transgenic embryos (Fig. 2B and C, Supp. Fig. S3). The anterior expression of LacZ in the ZRS402C>T embryos is consistent with other ZRS mutations in this assay [Lettice et al., 2003; Furniss et al., 2008; Lettice et al., 2008; Laurell et al., 2012]. It is worth noting that these *in vivo* assays are qualitative because of uncontrollable variables including variation in expression level between embryos, likely the result of copy number or site of integration differences. No other consistent expression patterns were observed in these embryos.

Our findings suggest that the ZRS402C>T mutation is the likely cause of the limb phenotypes in these families. It segregates with all assayed affected individuals, is not found in multiple human variation databases or 59 unrelated Mexican individuals and leads to ectopic expression of the ZRS in mouse E11.5 embryos. The phenotype is transmitted in an autosomal dominant manner with variable expression among the heterozygotes. Although many ZRS mutations have invariable phenotypic presentation [Semerci et al., 2009; Albuisson et al., 2010; Farooq et al., 2010; Laurell et al., 2012], there are others where point mutations have variable phenotypes among affected individuals, similar to what is seen here.

There is also at least one family whose mutation leads to incomplete penetrance [Gurnett et al., 2007].

Human mutations in the ZRS are dispersed over 600 bp and cause multiple limb phenotypes [VanderMeer and Ahituv, 2011]. There has been only one homozygous individual reported and this individual had no change in phenotype compared to heterozygotes in the same family [Furniss et al., 2008]. Here, we identified an additional homozygous individual with a more severe phenotype, WMS, compared with numerous heterozygous individuals with the same mutation both from their family and another family. This is the only individual with a phenotype in the lower limbs and more severe anomalies in the upper limbs. Interestingly, the ZRS mutations that have been reported to cause WMS are heterozygous mutations at ZRS404, two bases away from this ZRS402 mutation. A less likely explanation for the severe phenotype in the proband is variable penetrance of this mutation; individuals with isolated TPT and PPD have been identified in other WMS families with heterozygous ZRS404 mutations. There is no clear relationship between the location of ZRS mutations and the severity of the phenotypes they cause, but mutations may disrupt TFBS. For this mutation, no candidate TFBS changes were predicted based on the TRANS-FAC database. It is possible that alternative TFBS were not detected or that there is differential binding of TFs whose binding motifs are not known. It is also possible that these mutations interfere with looping of the ZRS to the *Shh* promoter or the chromatin domain changes that are related to *Shh* expression, but the mechanisms of these interactions are still poorly understood [Amano et al., 2009].

The 402C>T mutation adds to our understanding of the ZRS. The homozygous phenotype shows that the presence of two mutated copies of the ZRS may cause a more severe congenital limb abnormality. The addition of a second point mutation that can cause WMS further supports the hypothesis that mutations in the ZRS near 402 and 404 can lead to a more severe limb phenotype. Expanding the known cohort of mutations and phenotypes will help researchers investigate the molecular methods that allow distal enhancers to regulate gene expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Contract grants sponsors: NICHD awards (R01HD059862, P01HD22657); NHGRI awards (R01HG005058, 1R01HG006768); NIGMS award (GM61390); NIDDK award (1R01DK090382); NINDS award (1R01NS079231); Shriners Hospitals for Crippled Children Research Grant – Clinical and Molecular Studies of Hand and Feet Malformations; National Natural Science Foundation of China (30871355); National Institutes of Health Fogarty International Center (D43 TW006176 – International Collaborative Genetics Research Training Program); UCLA Intercampus Medical Genetics Training Program.

We thank the families for participating in this research project and are grateful to Stacy Jones for technical assistance, Dr. John W. Belmont from Baylor College of Medicine for providing the Mexican control DNAs, and Prof. Xue Zhang from Peking Union Medical College for technical discussion.

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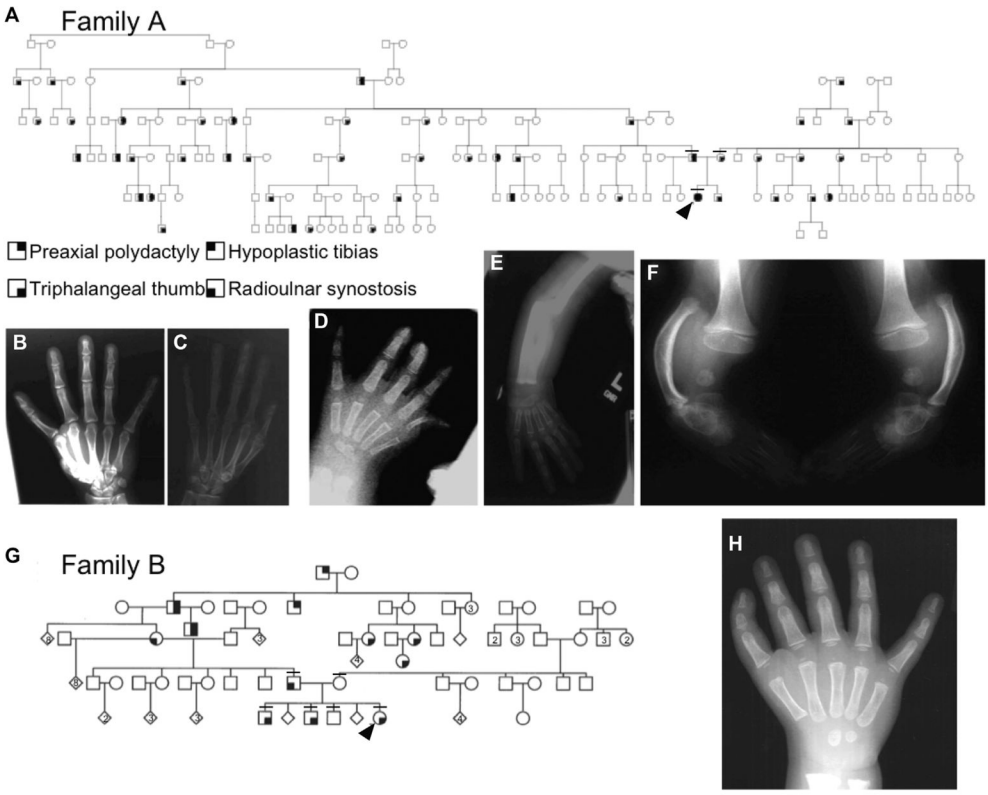

Figure 1.

A: The pedigree of family A contains 154 individuals; 31 have isolated TPT, 14 have PPD with TPT and only one (V/29) has WMS. **B**: The proband's father (IV/30) has bilateral TPTs and a preaxial remnant with a small bone that he is able to manipulate. **C**: The proband's mother (IV/31) has digitalization of thumbs with an extra crease in the distal interphalangeal space and bilateral elongated distal phalanx of first fingers. **D–F**: The proband (V/29) was born with PPD and after surgery has rudimentary thumbs. She has mild shortening and fusion of radioulnar bones, fusion of metacarpal bones and bilateral hypoplastic tibias with bowing of the fibula. **G**: The five-generation pedigree of family B contains two individuals with PPD, six individuals with isolated TPT, two individuals with PPD and TPT, and two individuals with mild radioulnar synostosis. **H**: The proband and siblings have bilateral TPTs, V/11 is shown here.

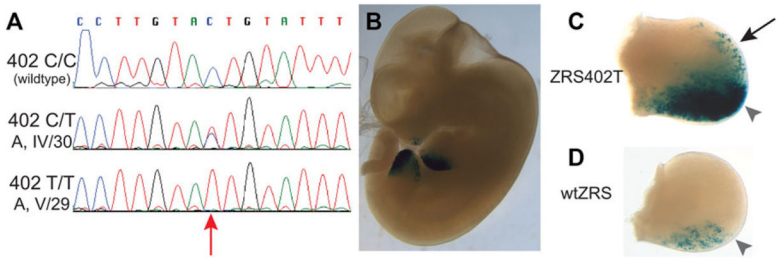

Figure 2.

A: Chromatogram traces show the heterozygous mutation in the father (IV/30) and the homozygous mutation in the proband of family A compared with a wild-type control sample. The site of the mutation is indicated by the red arrow. **B**: The ZRS402C>T mutant transgenic enhancer assay shows the expanded anterior expression of LacZ beyond the posterior of the limb buds of a representative E11.5 mouse embryo. **C**: The isolated right hindlimb clearly shows enhancer expression beyond the normal ZPA region (indicated by the gray arrowhead) toward the anterior part of the limb (arrow). **D**: A control wild-type ZRS mouse assay shows LacZ confined to the normal ZPA region (gray arrowhead).