

## Mutations in the Gene Encoding the Wnt-Signaling Component R-Spondin 4 (RSPO4) Cause Autosomal Recessive Anonychia

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Anonychia is an autosomal recessive disorder characterized by the congenital absence of finger- and toenails. In a large German nonconsanguineous family with four affected and five unaffected siblings with isolated total congenital anonychia, we performed genomewide mapping and showed linkage to 20p13. Analysis of the *RSPO4* gene within this interval revealed a frameshift and a nonconservative missense mutation in exon 2 affecting the highly conserved first furin-like cysteine-rich domain. Both mutations were not present among controls and were shown to segregate with the disease phenotype. *RSPO4* is a member of the recently described R-spondin family of secreted proteins that play a major role in activating the Wnt/ $\beta$ -catenin signaling pathway. Wnt signaling is evolutionarily conserved and plays a pivotal role in embryonic development, growth regulation of multiple tissues, and cancer development. Our findings add to the increasing body of evidence indicating that mesenchymal-epithelial interactions are crucial in nail development and put anonychia on the growing list of congenital malformation syndromes caused by Wnt-signaling-pathway defects. To the best of our knowledge, this is the first gene known to be responsible for an isolated, nonsyndromic nail disorder.

Anonychia (MIM 206800) is defined as the absence of finger- and toenails.<sup>1</sup> Usually, anonychia and its milder phenotypic variant hyponychia occur as a feature of genetic syndromes and may be associated with significant limb anomalies. Well-known examples are Coffin-Siris syndrome (MIM 135900), different ectodermal dysplasias (MIM 129490, MIM 300291, MIM 224900), nail-patella syndrome (MIM 161200), and brachydactyly type B (MIM 113000). Most of these syndromes are transmitted in an autosomal dominant mode. In contrast, isolated, nonsyndromic anonychia without additional features is a rare entity that may present either as a so-called partial autosomal dominant form that affects only the thumbs<sup>2</sup> or—in its most severe, autosomal recessively inherited variant—with involvement of all digits and toes.<sup>3</sup> So far, no gene or locus has been reported for nonsyndromic anonychia.

We describe a large, nonconsanguineous German family with four children afflicted with isolated total congenital anonychia (fig. 1). Both parents and five siblings were unaffected. Further family history was unremarkable. Affected individuals were of normal height and proportionate stature and were not different in any aspect, besides anonychia, from their healthy siblings. In any affected individual, anonychia had been present since birth. X-rays of the right upper limb and foot of individual II-8 showed normal results (fig. 2). No additional malformations were found on clinical examination of affected siblings, neither of ectodermal structures nor of sensory organs. There was

no report of inner abnormalities in any affected individual, and an additional abdominal ultrasound performed in individual II-8 showed normal results. All siblings were of normal intelligence. The youngest affected sibling is 36 years old, the eldest currently 47 years old. All affected individuals have healthy children with normal nails.

We obtained blood samples after receiving informed consent from all individuals of this family (who were all adults) and extracted genomic DNA using standard procedures. To identify the causative disease locus in this family, we undertook a genomewide linkage scan using a panel of microsatellite markers with an average distance of 10 cM (Weber panel Version 10 [Research Genetics]), an ABI3100 sequence analyzer, and Genotyper software v3.7 (Applied Biosystems) (table 1). We achieved marker saturation by use of existing and newly generated microsatellite markers. We performed two-point LOD score calculations by use of the LINKAGE program package, with help from the computer programs LINKRUN and MKS, with an autosomal recessive, fully penetrant model. We identified two regions with multipoint LOD scores  $>2$ , on chromosome 10 and chromosome 20 (fig. 3). The candidate interval on chromosome 10 comprised 27.7 Mb between the flanking markers *D10S1214* and *D10S1221*. The candidate region on chromosome 20p could be further refined by typing additional microsatellite markers from the draft human genome sequence to a 7.8-Mb region on chromosome 20p13 flanked by *D20S1155* and the newly established STR marker *AL021879*. At about the same time,

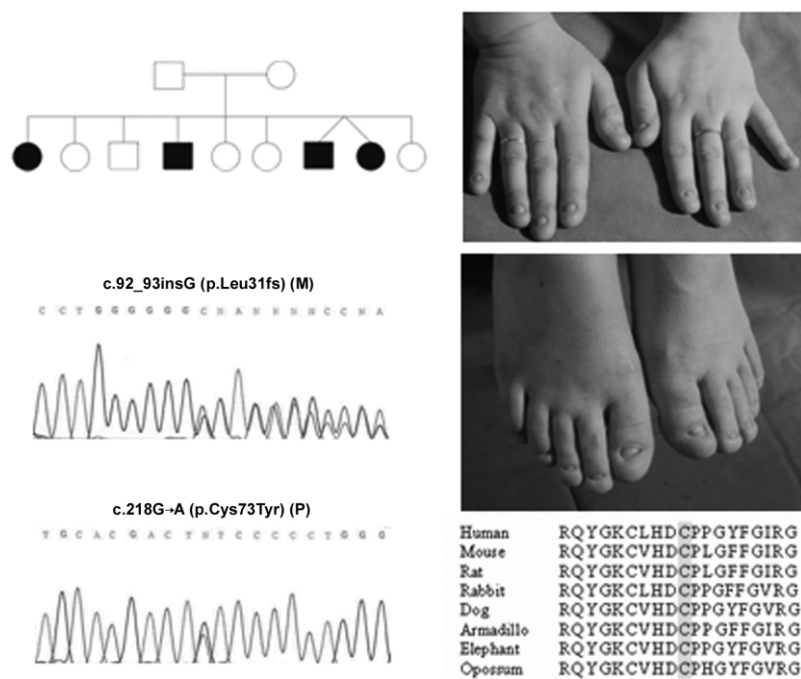
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**Figure 1.** *Top left*, Pedigree of the German nonconsanguineous family with isolated total congenital anonychia, with four affected and five unaffected siblings. *Top right* and *middle right*, Hands and feet of affected individual II-8, showing isolated total congenital anonychia. *Middle left*, Maternally transmitted guanine nucleotide insertion leading to a frameshift and premature truncation of the corresponding protein (c.92\_93insG, p.Leu31fs). *Bottom left*, paternal nonconservative *RSPO4* missense mutation c.218G→A leading to an amino acid exchange from cysteine to tyrosine at position 73 (p.Cys73Tyr). *Bottom right*, Multiple protein sequence alignment, generated with the program ClustalW with use of translation from genome assemblies and expressed sequences. Sequence comparison shows that the identified *RSPO4* missense mutation c.218G→A (p.Cys73Tyr) affects an amino acid (displayed on a grayish background) that is highly conserved in evolution.

consanguineous pedigrees with autosomal recessively inherited anonychia were reported to be linked to the same region on chromosome 20.<sup>4</sup>

Given the multitude of genes in the chromosome 20p13 region, we aimed to further narrow the candidate interval. Under the hypothesis of distant parental consanguinity, we additionally performed a genomewide screening for homozygous regions in an affected individual of the family (individual II-7 in fig. 1) by use of the 100K Affymetrix SNP set of arrays (table 2). With a Perl script (available from the authors on request), genotypes from the SNP array and STR analysis were merged and sorted according to their physical positions. Homozygous stretches of  $\geq 10$  adjacent SNPs in the candidate region were detected, and all genes or ESTs contained in these homozygous segments were analyzed by direct sequencing. However, no change was identified that cosegregated with the disease phenotype.

**Table 1. LOD Score Data for Chromosomes 10 and 20**

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

Thus, we next prioritized genes within the mapped critical interval on 20p13 for mutational screening, on the basis of putative function and expression data, using the University of California–Santa Cruz (UCSC) human genome database. Initially, we designed primers (see Primer3 Web site) for the entire coding and adjacent intronic regions of the *SOX12* (MIM 601947), *TCF15* (MIM 601010), *SCRT2* (GenBank accession number NM\_033129), *RSPO4* (GenBank accession number NM\_001029871), *SNPH* (GenBank accession number NM\_014723), *TGM3* (MIM 600238), *TGM6* (GenBank accession number NM\_198994), *ADRA1D* (MIM 104219), and *BMP2* (MIM 112261) genes and performed direct sequencing using genomic DNA of an affected individual (individual II-7 in fig. 1) on an ABI PRISM 310 genetic analyzer (Applied Biosystems). Primer sequences are available on request.

No convincing change was identified in any of the other candidates; however, we detected two mutations in exon 2 of the *RSPO4* gene and confirmed segregation of the changes with the phenotype in this family. Neither mutation has been described previously in public domain databases. As shown in figure 1, the maternally transmitted mutation was a guanine nucleotide insertion leading



**Figure 2.** X-ray of the right hand of affected individual II-8, showing no skeletal alteration or any degenerative or inflammable joint process.

to a frameshift and premature truncation of the corresponding protein (c.92\_93insG, p.Leu31fs) (GenBank accession number NP\_001025042). Sixty healthy German controls tested by *Bgl*I restriction enzyme analysis were negative for this change, further corroborating its pathogenicity. On the paternal allele, the nonconservative missense mutation c.218G→A, which leads to an amino acid exchange from cysteine to tyrosine at position 73 (p.Cys73Tyr), was identified. The affected residue is highly conserved in evolution, was not found among 300 German white control chromosomes tested by *Hpy*CH4III restriction enzyme analysis and, thus, is thought to be pathogenic too.

*RSPO4* spans ~44 kb of genomic DNA and comprises five exons that encode a secreted protein of 234 aa.<sup>5</sup> The R-spondin protein family was only recently described and comprises four independent gene products in mammals that are widely expressed and are predicted to share a common organization with substantial structural homologies.<sup>6</sup> It is noteworthy that each of the five exons constitutes a predicted structural domain. Although the N-terminal signal peptide sequences (exon 1) share only

relatively weak conservation, the two adjacent cysteine-rich furin-like domains encoded by exons 2 and 3 and the single thrombospondin (TSP-1) motif that is encoded by exon 4 display significant similarity among protein family members. Finally, the C-terminal basic region encoded by exon 5 is of varying length and scores as a nuclear localization signal.

The R-spondin proteins were recently shown to play a major role in activating the Wnt/ $\beta$ -catenin signaling pathway.<sup>5,7</sup> Wnt signaling is known to be evolutionarily conserved and pivotal for embryonic development by regulation of cell morphology, proliferation, and motility.<sup>5,8,9</sup> Its inappropriate activation has been reported in several human cancers.<sup>10</sup> All four human R-spondin family members, as well as a splice variant of *RSPO4* (GenBank accession number NM\_001040007), were recently shown to be able to induce proliferation of intestinal epithelium.<sup>5</sup> The functions of R-spondins essentially overlap with those of the canonical Wnt ligands by binding to the Frizzled (Fzd) family receptors (MIM 606143) and the low-density lipoprotein-related receptor (LRP) 5 (MIM 603506) or LRP6 coreceptor (MIM 603507), ultimately leading to gene activation by  $\beta$ -catenin (MIM 116806) and the T-cell factor (TCF)-lymphoid enhancer factor (LEF) family transcription factor complex (MIM 153245). A minimum of 10 different Fzd-related receptors are known in the human genome, and it might be hypothesized that a greater degree of selectivity is achieved through specific responses of cells to Wnt or R-spondin ligands that may be dictated by the available, possibly cell-line- or tissue-specific Fzd receptor(s) on the cell surface.<sup>11</sup> Moreover, it has recently been shown that mouse R-spondin proteins increase the activity of Wnt ligands, possibly through a direct interaction.<sup>7</sup> There is also evidence of independent receptor-signaling pathways of R-spondin proteins that may intersect those of Wnt only at the  $\beta$ -catenin level.<sup>5</sup> This adds to the growing body of evidence that a wide range of soluble ligands is capable of activating the  $\beta$ -catenin transduction complex, expanding well beyond the canonical Wnt-Fzd system. On the other hand, as noted above, R-spondins contain furin-like domains. Furin is a subtilisin family serine protease that is involved in the processing of many different proteins (MIM 136950).<sup>12</sup> Notably, the Dickkopf (DKK) proteins (MIM 605189), regulators of Wnt activity, possess furin cleavage sites<sup>13</sup>; thus, it is conceivable that the R-spondin family may indirectly regulate Wnt-pathway activity through regulation of DKK activation.

The figure is available in its entirety in the online edition of *The American Journal of Human Genetics*.

**Figure 3.** Genomewide linkage data generated using a panel of microsatellite markers. The legend is available in its entirety in the online edition of *The American Journal of Human Genetics*.

**Table 2. Screen for Homozygous Regions in Affected Individual II-7 for the Critical Region on Chromosome 20, Performed Using the 100K Affymetrix SNP Set of Arrays**

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

It is noteworthy that both *RSPO4* mutations detected in our family affect the highly conserved first furin-like cysteine-rich domain. Experimental data suggest that a shortened *RSPO4* protein comprising just the two furin-like regions is sufficient for  $\beta$ -catenin stabilization, further supporting the harmful character of the identified mutations. Evidence of the pathogenicity of the missense change c.218G→A (p.Cys73Tyr) is striking, particularly given that the cysteine residues within the furin-like domains are strictly conserved among R-spondins and in evolution (fig. 1).<sup>5</sup> *RSPO4* mutation analysis in further families may answer the intriguing question of whether mutations in domains other than the highly conserved furin-like regions result in a similar clinical phenotype or allow for a residual protein function, conceivably giving rise to a milder expression pattern with hyponychia. Likewise, it may also be hypothesized that *RSPO4* mutations in other functionally important regions might lead to a more severe phenotype, with skeletal involvement and reduction defects of distal limbs.

This hypothesis fits the long-standing argument of bone-dependent nail formation and, in particular, the observation that nail development depends on correct dorsoventral polarization.<sup>14</sup> Embryonic development of the human fingernails begins at the 8th or 9th wk of gestation and ends by the 20th wk, with completion of the toenails lagging ~4 wk behind.<sup>15</sup> Recent molecular data have indicated a crucial role for epithelial-mesenchymal interaction at different developmental stages in terms of proper nail development. These critical first steps require expression of *Wnt7a* (MIM 601570), *Noggin* (MIM 602991), *Bmp4* (bone morphogenetic protein 4 [MIM 112262]), and *Lmx1b* (MIM 602575), among others.<sup>16,17</sup> Mutations in *LMX1B* cause nail-patella syndrome (MIM 161200), and it has been suggested that the LIM-domain homeobox protein *Lmx1b* is required for dorsal specification and hence for nail development.<sup>18</sup> Indeed, it turns out that *Wnt-7a* induces the expression of *Lmx1b* in the dorsal limb mesenchyme. In the ventral limb bud, *Engrailed-1* (MIM 131290) represses *Wnt-7a* expression.<sup>19</sup> Thus, it is conceivable that, in the absence of *RSPO4*, *Wnt-7a* activity in the developing limb is reduced. In that scenario, the establishment of dorsal-ventral polarity in the developing limb would be disturbed, and the nail would finally fail to be specified. Pertinent confirmation of this idea could be obtained, for instance, by conditional knockout experiments in mice.

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## Web Resources

The URLs for data presented herein are as follows:

ClustalW, <http://www.ebi.ac.uk/clustalw/>  
GenBank, <http://www.ncbi.nlm.nih.gov/Genbank/>  
Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/>  
Primer3 Web site, [http://frodo.wi.mit.edu/cgi-bin/primer3/primer3\\_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)  
UCSC Genome Browser, <http://genome.ucsc.edu/>

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