Report

Homozygous *WNT3* Mutation Causes Tetra-Amelia in a Large Consanguineous Family

Stephan Niemann,[1] Chengfeng Zhao,[3] Filon Pascu,[2] Ulrich Stahl,[2] Ute Aulepp,[1] Lee Niswander,[4] James L. Weber,[3] and Ulrich Müller[1]

Institutes of [1]Human Genetics and [2]Pathology, Justus-Liebig-University, Giessen, Germany; [3]Center for Medical Genetics, Marshfield Clinical Research Foundation, Marshfield, WI; and [4]Howard Hughes Medical Institute, Developmental Biology Program, Sloan-Kettering Institute, New York

Tetra-amelia is a rare human genetic disorder characterized by complete absence of all four limbs and other anomalies. We studied a consanguineous family with four affected fetuses displaying autosomal recessive tetra-amelia and craniofacial and urogenital defects. By homozygosity mapping, the disease locus was assigned to chromosome 17q21, with a maximum multipoint LOD score of 2.9 at markers D17S931, D17S1785, D17SS1827, and D17S1868. Further fine mapping defined a critical interval of ~8.9 Mb between D17S1299 and D17S797. We identified a homozygous nonsense mutation (Q83X) in the *WNT3* gene in affected fetuses of the family. *WNT3*, a human homologue of the *Drosophila wingless* gene, encodes a member of the WNT family known to play key roles in embryonic development. The Q83X mutation truncates WNT3 at its amino terminus, suggesting that loss of function is the most likely cause of the disorder. Our findings contrast with the observation of early lethality in mice homozygous for null alleles of *Wnt3*. To our knowledge, this is the first report of a mutation in a *WNT* gene associated with a Mendelian disorder. The identification of a *WNT3* mutation in tetra-amelia indicates that WNT3 is required at the earliest stages of human limb formation and for craniofacial and urogenital development.

Mutations in genes involved in limb development have been identified in various malformation syndromes (Tickle 2002). However, the genetic basis of limb agenesis is still unknown.

Tetra-amelia (MIM 273395 and MIM 301090), the complete absence of the extremities, occurs extremely rarely. To date, it has been described in four families, where it appears to follow an autosomal recessive mode of inheritance. In all families, tetra-amelia was associated with craniofacial, nervous system, pulmonary, skeletal, and urogenital anomalies (Zimmer et al. 1985; Gershoni-Baruch et al. 1990; Rosenak et al. 1991; Zlotogora et al. 1993; Başaran et al. 1994).

We studied a consanguineous Turkish family of Ara-

maic descent with autosomal recessive tetra-amelia (fig. 1). In eight pregnancies, tetra-amelia was diagnosed four times prenatally, and pregnancies were terminated between the 13th and 20th wk of gestation. Cytogenetic studies showed that three of the affected fetuses were female, and one was male. Autopsy was possible in three of the four affected fetuses (fetuses IV:1, IV:4, and IV:7) and revealed various anomalies in addition to complete absence of all four limbs (fig. 2). In the first affected fetus (fetus IV:1; fig. 2A and 2D), a female, these findings were cleft lip with cleft palate; gastroschisis; diaphragmatic defect with malposition of the right lung (which was bilobular); agenesis of the left kidney, left supra-adrenal gland, and spleen; a malformed uterus, with only a right, rudimentary ovary and salpinx; and only one umbilical artery. In the second affected fetus (fetus IV:4; fig. 2B and 2E), a female, findings were a protrusion and cataract of the left eye, microphthalmia of the right eye, cleft lip with cleft palate, malformed nose with a single naris and choanal atresia, hypoplasia of the pelvis, and atresia of the urethra, vagina, and anus. In the third affected fetus (fetus IV:7; fig. 2C and 2F), a male, findings
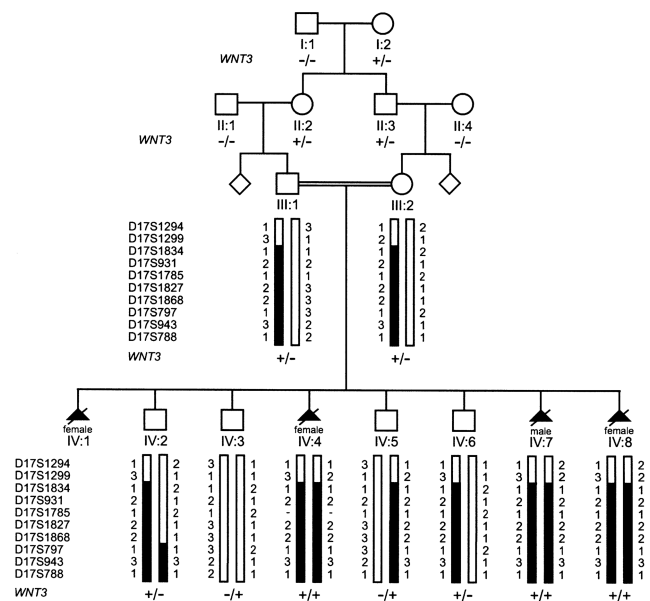
Figure 1 Pedigree, haplotype, and *WNT3*-mutation status of tetra-amelic family. Individuals' numbers are listed below the pedigree symbols. Genotyped markers from the chromosome 17q21 region are shown to the left of generations III and IV, and individuals' allele numbers for each marker are given next to the bar. Black bars represent haplotype segregating with the tetra-amelia gene. *WNT3* is located between markers D17S1299 and D17S1834. The mutation genotypes of *WNT3* are shown below individuals' numbers (generations I and II) or below haplotypes (generations III and IV). A plus sign (+) indicates mutation Q83X; a minus sign (−) indicates wild-type allele.

were agenesis of the left kidney and left supra-adrenal gland, hypoplasia of the pelvis, persistence of cloaca, and no external genitalia.

Mice in which both copies of the *Fgf10* gene have been disrupted by gene targeting fail to develop limbs (Sekine et al. 1999). On the basis of the phenotypic similarities between *Fgf10*$^{-/-}$ mice and the affected subjects in the family in our study, *FGF10* appeared to be an attractive candidate gene for limb agenesis. Sequence analysis of all three *FGF10* exons in an affected subject, however, did not reveal a mutation.

We then performed homozygosity mapping with 378 autosomal polymorphic markers with an average spacing of ~10 cM (Center for Medical Genetics [screening set version 10]) for individuals III:1, III:2, IV:2, IV:3, IV:5, IV:6, IV:7, and IV:8. Informed consent was obtained from all family members involved in this study, and ethical approval was given by the local ethics committee. Following the initial genome screen, individual markers were genotyped in fetus IV:4, from whom formalin-fixed paraffin sections were available (fig. 1). Under the assumption of an autosomal recessive mode of inheritance, selected informative markers were analyzed for fetus IV:4

when identical alleles were shared homozygously by fetuses IV:7 and IV:8, when one copy of this allele was present in both parents, and when no more than one copy of the allele was found in the unaffected individuals. This approach did not identify a shared homozygous haplotype by the above criteria in fetuses IV:4, IV:7, and IV:8, and it ruled out chromosome 5p12, to which *FGF10* has been assigned. At marker D17S1299, fetuses IV:4, IV:7, and IV:8 were heterozygous for the same alleles (fig. 1), and the MLINK program of the LINKAGE software package (Lathrop and Lalouel
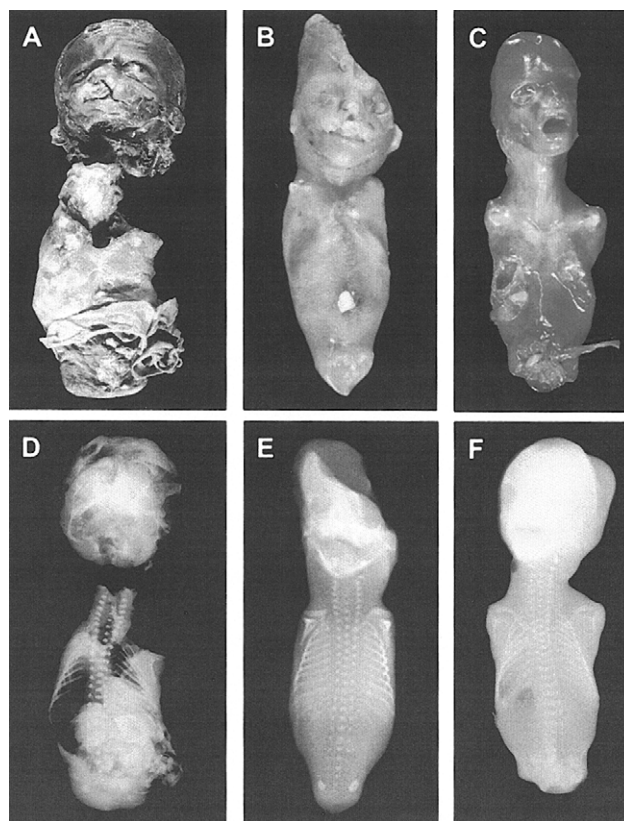
Figure 2 Complete limb agenesis, urogenital defects, and other anomalies in affected fetuses. Postmortem photographs (*A–C*) and radiographs (*D–F*) demonstrate complete absence of all four limbs, without defects of scapulae and clavicles. *A* and *D,* Tetra-amelic fetus IV:1 (female) at 19 wk of gestation, crown-to-rump length 17 cm. Note cleft lip with cleft palate. The separation of the head from the trunk and the multiple lesions on the fetus were iatrogenic. The poorly preserved caudal region did not allow for assessment of external genitalia. *B* and *E,* Tetra-amelic fetus IV:7 (female) at 16 wk of gestation, crown-to-rump length 10.5 cm. Note protrusion and cataract of the left eye, microphthalmia of the right eye, malformed nose, hypoplasia of the pelvis, and undefined vaginal and anal regions, with atresia of the urethra, vagina, and anus. Parts of the head were damaged during abortion. *C* and *F,* Tetra-amelic fetus IV:7 (male) at 20 wk of gestation, crown-to-rump length 15.5 cm. Note hypoplasia of the pelvis, persistence of the cloaca, and no external genitalia.

1984) had yielded a positive two-point LOD score. We subsequently typed additional markers surrounding marker D17S1299. For multipoint LOD score analysis, we used the HOMOZ linkage program (Kruglyak et al. 1995) and assumed autosomal recessive inheritance with full penetrance, a disease gene frequency of 0.0001, and uniform marker allele frequencies. A maximum LOD score at $\theta = 2.9$ was found at markers D17S931, D17S1785, D17SS1827, and D17S1868 (table 1). Further fine mapping and haplotype examination narrowed the tetra-amelia locus to a critical region of ~8.9 Mb of homozygous sequence between markers D17S1299 and D17S797 on chromosome 17q21 (fig. 1).

Obvious candidate genes in the critical interval were the *HOXB* cluster, *WNT3,* and *WNT9B*. The gene encoding WNT3 appeared particularly interesting. *WNT* genes, the vertebrate relatives of the *Drosophila wingless* gene (Klingensmith and Nusse 1994), encode a family of highly conserved, secreted signaling molecules that play major roles in many developmental processes (Wodarz and Nusse 1998). WNTs act as ligands for the frizzled family of transmembrane receptors. In mice, Wnt3 signaling has been implicated in the formation of the primary body axis and has recently been shown to be critical for limb growth (Liu et al. 1999; Barrow et al. 2003). *WNT3* consists of five exons and encodes a protein of 355 amino acids. Sequence analysis of the *WNT3* coding region in the affected subjects revealed a nonsense mutation, Q83X (C→T transition at position 366 [GenBank accession number NM_030753]) (fig. 3). The analyzed fetuses (fetuses IV:7 and IV:8) had homozygous mutations, whereas both parents and the unaffected subjects IV:2, IV:5, and IV:6 had heterozygous mutations. The mutation was also observed in individual I:2, who introduced the mutation into the family, and in individuals II:2 and II:3 but not in individuals I:1, II:1, and II:4. Furthermore, this mutation was absent in 170 chromosomes from the German population and was absent in 176 chromosomes from the Turkish population. The nonsense mutation at codon 83 of *WNT3* in the family in our study creates a premature stop codon. This stop codon putatively results in a truncated protein of only 82 amino acids (including the signal peptide of 21 amino acids), with no other known functional domains, instead of 355 amino acids of the mature peptide. Hence, loss of function of both copies of *WNT3* in the affected subjects of the family in our study is the most likely mechanism of the mutation.

The established models of limb initiation in vertebrates have stressed the role of two members of the fibroblast growth factor (FGF) superfamily; that is, FGF8 and FGF10 (Cohn et al. 1995; Martin 1998). It has been proposed that limb outgrowth depends on an FGF8/FGF10 positive regulatory loop. According to this model, FGF10 produced in the prospective limb mesoderm induces *Fgf8*

Table 1

LOD Scores for Markers at 17q21

| Marker	Genetic Distance[a] (cM)	Physical Location[b] (Mb)	LOD at $\theta = 0$	
			Multipoint	Two-Point
D17S1294[c]	50.74	28.2	$-\infty$	$-\infty$
D17S1299[c]	62.01	38.9	$-.097$	1.102
D17S1834	64.16	45.3	2.733	1.210
D17S931	66.85	45.3	2.900	1.177
D17S1785	66.85	46.3	2.901	1.102
D17S1827	66.85	46.9	2.902	2.908
D17S1868	64.16	47.5	2.902	2.908
D17S797	66.85	47.8	$-\infty$	$-\infty$
D17S943	68.44	48.2	$-\infty$	$-\infty$
D17S788	73.62	50.6	-1.145	.604

[a] Position according to the Marshfield map (Center for Medical Genetics).

[b] Position according to University of California Santa Cruz Genome Bioinformatics.

[c] Markers used in the genome screen.

expression in cells of the surface ectoderm that later form the apical ectodermal ridge (AER) (Johnson and Tabin 1997; Niswander 2003). The AER, running along the distal margin of the limb bud, is critical for the outgrowth of the limb. Once the limb bud has developed, FGF8 produced by cells of the AER maintains *Fgf10* expression in the underlying mesenchyme, which in turn maintains *Fgf8* expression. This model has been tentatively expanded by work in chickens that postulates that the FGF8/FGF10 loop is mediated by Wnt/β-catenin signaling, both upstream and downstream of FGF10. First, Wnt signaling through β-catenin appears to be required for the restriction and maintenance of *Fgf10* expression in the limb mesoderm and, hence, is needed for limb induction. Second, FGF10 is suggested to induce Wnt3a signaling through the β-catenin pathway in the ectoderm; this leads to activation of *Fgf8* expression (Kawakami et al. 2001). Recently, this view has been supported by studies in mice in which the conditional removal of Wnt3 in the limb ectoderm leads to failure of AER formation and to limb defects that, in the most-severe cases, cause limb agenesis (Barrow et al. 2003). Our work on human tetra-amelia disorder supports and extends a role for Wnt3/WNT3 signaling in the initiation of limb development.

Our data are consistent with a model in which WNT3 is required in the limb ectoderm for initiation of AER formation. Moreover, our work may indicate that WNT3 also acts within the mesoderm. Loss of Wnt3 in the mouse ectoderm leads to variable effects, ranging from normal limbs to agenesis, whereas loss of β-catenin in the ectoderm has a more consistent and severe effect (Barrow et al. 2003). This may indicate redundancy in Wnt function in the limb ectoderm. The complete penetrance and complete absence of all four limbs, as well as the pelvic hypoplasia observed upon loss of WNT3
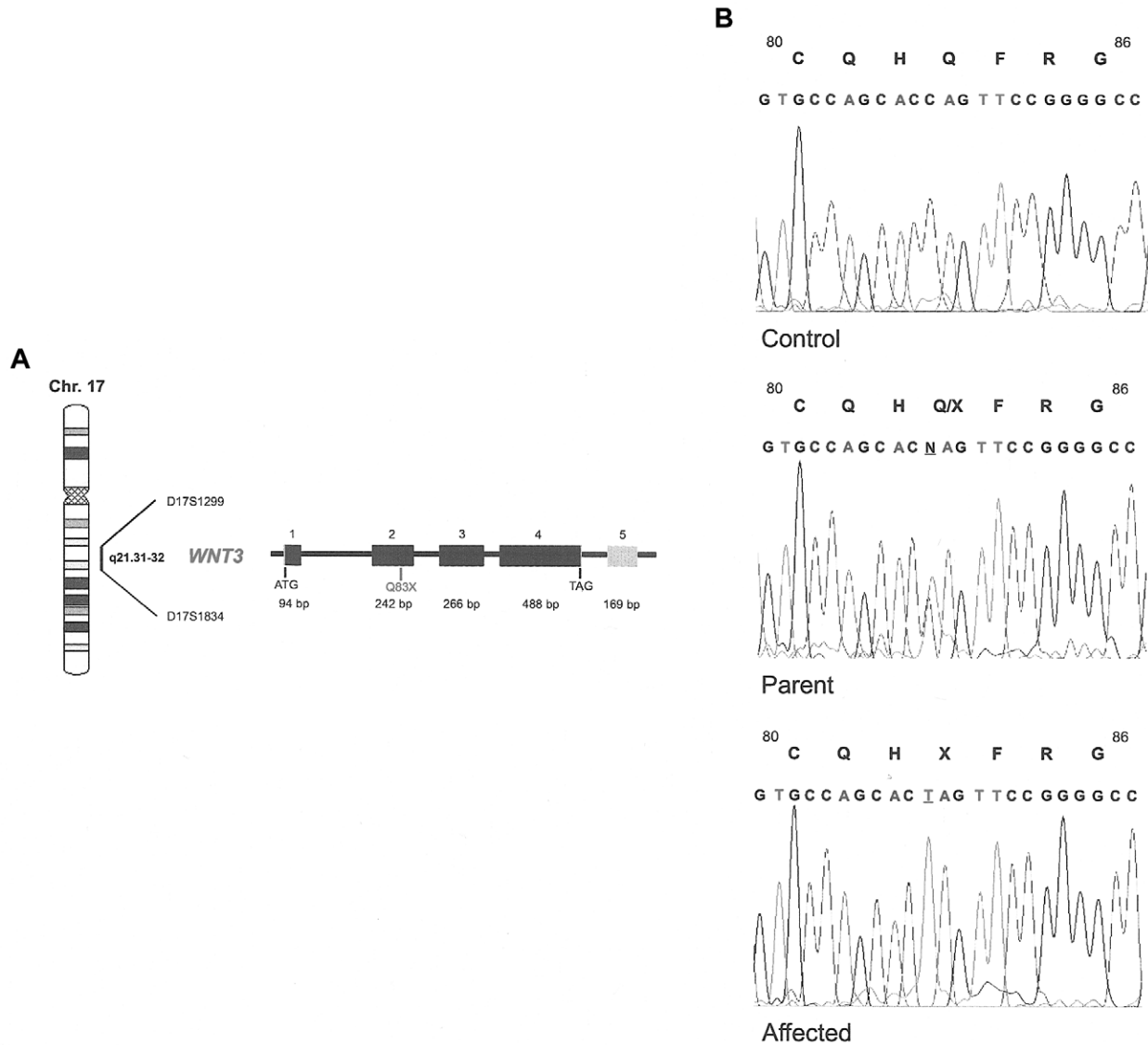
Figure 3 *WNT3* nonsense mutation associated with tetra-amelia. (See online version for color.) *A,* Location of *WNT3* on chromosome 17q21.31-32 and genomic organization of *WNT3*. The dark boxes represent exons, with the coding region illustrated in black and the 5′ and 3′ UTRs in gray. Positions of the initiation codon, the termination codon, and the nonsense mutation in exon 2 are indicated. *B,* Electropherograms of *WNT3* exon 2 in a control individual (*top*), one parent (*middle*), and an affected fetus (*bottom*). DNA sequence analysis revealed a homozygous C→T substitution at nt 366 (from the translation start site) in the tetra-amelic fetus, causing a Q83X mutation. The parent is heterozygous with respect to the Q83X mutation.

function in humans, lead to the suggestion that WNT3 may also be required earlier within the mesoderm to restrict and maintain FGF10 expression. Our discovery of *WNT3* as a limb-inducing gene in humans provides new ways to understand the earliest stages of limb development (Martin 2001; Tickle and Münsterberg 2001).

It is intriguing that gastrulation occurs normally upon loss of human WNT3, whereas mice homozygous for null alleles of *Wnt3* fail to gastrulate and thus never develop limbs (Liu et al. 1999). Under the assumption of func-

tional inactivity of the truncated protein, this suggests that other WNT proteins act to regulate gastrulation and axis formation of the human embryo and that the phenotypic differences between the *Wnt3*$^{-/-}$ mice and the affected subjects in the family in our study are species specific. Different members of the Wnt family may exert identical roles in different species. Indeed, the key ligands that are postulated to regulate the activation of *Fgf8* in the limb ectoderm differ between mice (Wnt3) and chickens (Wnt3a) (Kawakami et al. 2001; Barrow et al. 2003).

Our work indicates that WNT3 is the key signal in the human limb. Because of the early gastrulation defect in *Wnt3$^{-/-}$* mice and the lack of other conditional knockout mutants, the role of Wnt3 in tissues other than the limbs has not yet been studied. Loss of WNT3 function in the tetra-amelic fetuses causes urogenital, craniofacial, and skeletal malformations, demonstrating that WNT3 signaling is required for the embryonic development of these organs and structures. The urogenital anomalies in the affected fetuses included uterine and ovarian malformations, agenesis of kidney, and genital defects. In fact, two (fetuses IV:4 and IV:7) of the three autopsied fetuses displayed defects of the external genitalia. This could not be analyzed in the damaged fetus (fetus IV:1). The presence of urogenital defects in the limbless fetuses provides novel evidence that WNT signaling is involved in the earliest steps of limb formation and urogenital development in humans. Wnt signaling has been studied in mouse urogenital development, and Wnt4 and Wnt11 were shown to play key roles in kidney development (Stark et al. 1994; Majumdar et al. 2003). It is possible that, in humans, WNT3 signaling may regulate functions of urogenital development that are controlled by other Wnt ligands in mice.

To our knowledge, this is the first report of a mutation in a member of the *WNT* superfamily associated with a Mendelian disorder and the first description of a limb-inducing gene in humans. The discovery of the molecular basis of tetra-amelia demonstrates a pivotal role for WNT3 in several aspects of human embryonic development. It will be important to explore whether other mutations in *WNT3* cause other human malformation syndromes.

Acknowledgments

Electronic-Database Information

The accession number and URLs for data presented herein are as follows:

Center for Medical Genetics, http://research.marshfieldclinic.org/genetics/ (for marker screening set version 10)
GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for the human *WNT3* genomic sequence [accession number NM_030753])
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for tetra-amelia)
University of California Santa Cruz Genome Bioinformatics, http://genome.ucsc.edu/

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