

A Nonsense Mutation in *MSX1* Causes Witkop Syndrome

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Witkop syndrome, also known as tooth and nail syndrome (TNS), is a rare autosomal dominant disorder. Affected individuals have nail dysplasia and several congenitally missing teeth. To identify the gene responsible for TNS, we used candidate-gene linkage analysis in a three-generation family affected by the disorder. We found linkage between TNS and polymorphic markers surrounding the *MSX1* locus. Direct sequencing and restriction-enzyme analysis revealed that a heterozygous stop mutation in the homeodomain of *MSX1* cosegregated with the phenotype. In addition, histological analysis of *Msx1*-knockout mice, combined with a finding of *Msx1* expression in mesenchyme of developing nail beds, revealed that not only was tooth development disrupted in these mice, but nail development was affected as well. Nail plates in *Msx1*-null mice were defective and were thinner than those of their wild-type littermates. The resemblance between the tooth and nail phenotype in the human family and that of *Msx1*-knockout mice strongly supports the conclusions that a nonsense mutation in *MSX1* causes TNS and that *Msx1* is critical for both tooth and nail development.

Introduction

Witkop syndrome, also known as “tooth and nail syndrome” (TNS [MIM 189500]) or “nail dysgenesis and hypodontia” was first described by Witkop in 1965 as a rare autosomal dominant disorder (Witkop 1965). The incidence has been estimated to be ~1–2:10,000 (Witkop 1990). It belongs to the ectodermal dysplasias (EDs), a heterogeneous group of disorders characterized by defects in at least two ectodermally derived organs, such as teeth, nails, hair, and sweat glands (Slavkin et al. 1998). TNS can be distinguished from other types of EDs by the fact that the abnormalities involve only teeth and nails (Hudson and Witkop 1975). Although a few reported cases have sparse or fine hair in addition to tooth and nail defects (Chitty et al. 1996), almost all individuals affected with TNS were reported to have normal hair, sweat glands, and ability to tolerate heat. Affected individuals have a variable number and variable types of congenitally missing permanent and/or primary teeth, which frequently results in lip eversion due to loss of occlusion in the vertical dimension. Nails are generally

thin, slow-growing, brittle, and spoon-shaped (koilonychia), but, in some instances, marked longitudinal ridges and pitting are the only main features. Toenails are usually more severely affected than fingernails. In rare cases, nails spontaneously separate from the nail beds or are absent at birth. The nail defects, however, are alleviated with age and may not be easily detectable during adulthood.

The expressivity of tooth and nail defects is highly variable (Witkop 1965; Hudson and Witkop 1975). Previous reports of families affected with TNS and sporadic cases show variability of the pattern of congenitally missing teeth and varying degrees of nail abnormalities. Nevertheless, congenitally missing teeth and nail dysplasia are the consistent diagnostic features in individuals with TNS (Giansanti et al. 1974; Hudson and Witkop 1975; Murdoch-Kinch et al. 1993; Hodges and Harley 1999).

Gene mutations associated with TNS have not been reported. Mutations in *ED1* and *GJB6* that are responsible for X-linked anhidrotic ectodermal dysplasia and autosomal dominant hidrotic ectodermal dysplasia (HED or Clouston syndrome), respectively (Kere et al. 1996; Lamartine et al. 2000), are unlikely to be causing TNS, since *ED1* is located on the X chromosome and phenotypes associated with mutations in *GJB6* include nail dystrophy, partial or total alopecia, and palmar-plantar hyperkeratosis, but sweat glands and teeth are normal. *MSX1*, however, is an excellent candidate gene for TNS, since mutations in *MSX1* were shown to be

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associated with tooth agenesis in two unrelated families (Vastardis et al. 1996; van den Boogaard et al. 2000). In addition, *Msx1* was expressed in developing murine tooth buds and nail beds (Mackenzie et al. 1991; Reginelli et al. 1995). Indeed, we found linkage between TNS and markers surrounding the *MSX1* locus and showed that a nonsense mutation (S202X) in *MSX1* cosegregated with the TNS phenotype in a three-generation family. Further analysis of *Msx1*-knockout mice revealed disruption of nail development as well as tooth development. On the basis of the data from the human family affected with TNS and the *Msx1*-knockout mice, we conclude that a nonsense mutation in *MSX1* is responsible for TNS.

Subjects and Methods

Pedigree and Diagnosis

The family with TNS was first described in 1997 (Stimson et al. 1997). After the proband was contacted and every member (8 affected and 11 unaffected) agreed to participate in the study, informed consent forms and either venous blood samples or buccal-cell samples were obtained from every member. The procedures were performed in accordance with the protocol approved by the Harvard Medical School/Harvard School of Dental Medicine Committee on Human Studies. We confirmed the diagnosis on the basis of clinical history, clinical examination, and evaluation of either panoramic and/or complete periapical dental radiographs of every member.

MSX1 Linkage Analysis

We extracted genomic DNA from either venous blood or buccal cells using the PUREGENE DNA isolation kit (Gentra System). Linkage analysis was performed by use of fluorescently labeled polymorphic microsatellite markers surrounding the *MSX1* locus (4p16.1). The markers used were *D4S412*, *D4S432*, *D4S3023*, and *D4S2935*, purchased from either Research Genetics or Applied Biosystems. The markers were PCR-amplified in a 10- μ l reaction volume, using 20 ng of genomic DNA as template, 3 nmol of dNTPs, 1.5 mM MgCl₂, 4 pmol of each primer, 0.5 U *Taq* DNA polymerase (Sigma), 50 mM KCl, and 10 mM Tris HCl buffer (Sigma). PCR conditions were as follows: 95°C for 3 min, 94°C for 1 min, 55°C for 50 s, and 72°C for 50 s, with a final extension of 10 min after 30 cycles. PCR products were analyzed by 4% denaturing PAGE, using an ABI PRISM 377 DNA sequencer. Data were collected and analyzed using GENESCAN 3.1 and GENOTYPER 2.0 software (Applied Biosystems). Two-point LOD scores were calculated using the MLINK option of the LINKAGE package, version 5.10, and FASTLINK, version 4.0P, under

the assumption of .001% disease-gene frequency and 95% penetrance.

Mutation Analysis of MSX1

To generate templates for direct DNA sequencing, we PCR amplified *MSX1* exons 1 and 2, including flanking intronic sequences from 20 ng of genomic DNA of one affected (II-7) and one unaffected (II-8) individual. Primers used to amplify exon 1 were 361-F and 920-R, as described elsewhere (Vastardis et al. 1996). Primers used to amplify exon 2 were 500-F (5'-AGGCACTTGGCGGCACTCA-3') and 1250-R (5'-CACTTTTTGGCAGGGATCAGACTTC-3'). We used *Taq* DNA polymerase (Sigma) and PreMix G of MasterAmp PCR amplification kit (Epicentre Technologies) which contains 1.5 mM Mg²⁺ and 4 \times Betaine to amplify exon 1. For exon 2, we used *Taq* DNA polymerase (Sigma) and the buffer supplied by Sigma for the PCR reactions. The conditions for PCR were 95°C for 3 min, 94°C for 1 min, 63°C (for exon 1) or 58°C (for exon 2) for 50 s, and 72°C for 1 min, with a final extension of 30 min after 30 cycles.

We purified the PCR products using the PCR purification kit (Qiagen) and sequenced the products using both forward and reverse primers for each exon. In addition, we designed internal primers 643-F (5'-CCTGCACCTCCGCAAACAC-3') and 1044-R (5'-ACATGCTGTAGCCCATGTTGG-3') to sequence exon 2. We used BigDye Terminator Mixes (Applied Biosystems) for sequencing reactions and performed the reactions in 4% denaturing polyacrylamide gels on the ABI 377 DNA sequencer.

Restriction-Enzyme Analysis

We PCR amplified genomic DNA of every member in the pedigree, using primers 643-F and 1044-R for the analysis in a 50- μ l reaction and using the same conditions as described above for linkage analysis, except that the annealing temperature was 61°C. The PCR products were subjected to *NheI* digestion at 37°C for 3 h, by incubation of the DNA products with 5 U of *NheI* (New England BioLabs) in a 30- μ l volume, according to the recommendations of the manufacturer. We analyzed the digestion products by 3% NuSieve GTG agarose gel electrophoresis in 1 \times TBE buffer at 5 v/cm for 3 h.

Postimplantation Embryos and Genotyping

Wild-type, *Msx1*^{+/-}, and *Msx1*^{-/-} embryos were collected from matings of *Msx1*^{+/-} \times *Msx1*^{+/-} mice maintained in a N8-9 BALB/c background and were fixed in 4% paraformaldehyde/PBS for ~24 h. Genotyping of the collected embryos was performed as described by Bei and Maas (1998).

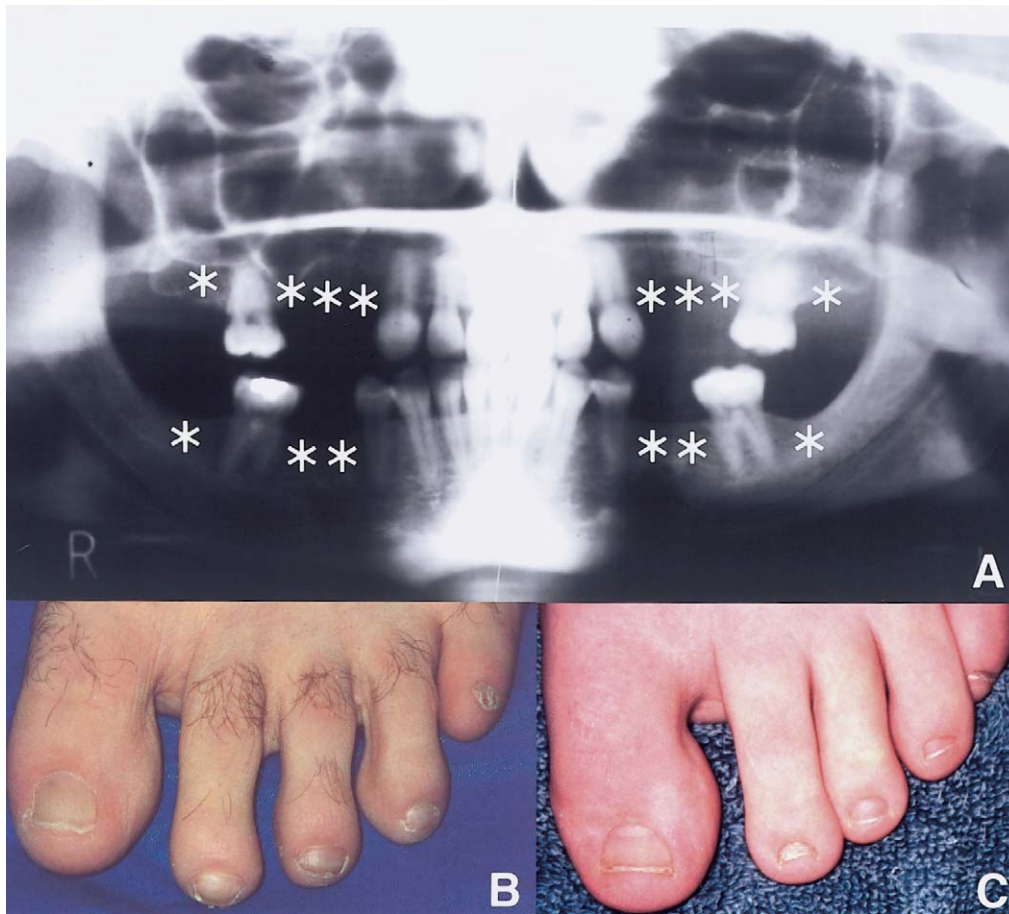


Figure 1 TNS phenotype. *A*, Panoramic radiograph of individual II-5 at age 33 years. Asterisks (*) indicate congenitally missing permanent teeth. This individual has a total of 14 congenitally missing teeth. *B*, Toenail dysplasia in individual III-7. Note that the fifth toenail is hypoplastic and more affected than the others. *C*, Toenail dysplasia of individual III-6. All nail plates are concave and hypoplastic. Note that the fifth toenail is almost absent.

Histology

After fixation, forelimbs and hindlimbs were excised from wild-type, heterozygous, and homozygous *Msx1* mutant embryos. The tissues were dehydrated through increasing concentrations of ethanol and were embedded in paraffin wax. Tissues were serially sectioned by microtome at 5 μm , and every fourth section was grouped onto the same slide. A subset of the slides was stained with hematoxylin and eosin, according to standard procedures.

In Situ Hybridization

Msx1 riboprobes were transcribed from 500-bp fragment of *Msx1* cDNA. In situ hybridization on the paraffin sections with [^{33}P]-UTP-labeled *Msx1* riboprobes was carried out as described elsewhere (Hartmann and Tabin 2000). Slides were hybridized overnight at 60°C in a humidified chamber. Sections were exposed to emul-

sion for 1 wk at 4°C and were developed using Kodak D-19 developer. Slides were counterstained with 0.1% toluidine blue. *Msx1* expression was analyzed under dark-field microscopy.

Results

Pedigree and Diagnosis

We analyzed a three-generation family with TNS (Stimson et al. 1997) (fig. 2*B*). Of 20 members, 9 were affected. Affected individuals had 11–28 congenitally missing permanent teeth (oligodontia) (fig. 1*A*; table 1) and dysplastic toenails and/or fingernails (fig. 1*B* and 1*C*). Sweat glands and hair were normal in all affected individuals. The severity of the phenotype in the family was quite variable. The predominant tooth types affected were premolars, first molars, and third molars (table 1). In a few cases (e.g., III-1 and III-6), other tooth

Table 1**Summary of Phenotype in Family with TNS**

| PEDIGREE ID AND DENTAL ARCH | CONGENITALLY MISSING PERMANENT TEETH ^a | | | | | | | | | | | | | | | | NAIL DYSPLASIA ^b | PROMINENT MAXILLARY FRENUM ^b | |
|-----------------------------------|---|---|---|---|---|---|---|---|------|---|---|---|---|---|---|---|--------------------------------|---|---|
| | Right | | | | | | | | Left | | | | | | | | | | |
| | 8 | 7 | 6 | 5 | 4 | 3 | 2 | 1 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | | | |
| I-2: | | | | | | | | | | | | | | | | | | | |
| Maxillary | * | | * | * | * | | | | | | * | * | | | | | * | - | + |
| Mandibular | * | | * | * | | | | | | | | * | * | * | | | * | | |
| II-2: | | | | | | | | | | | | | | | | | | | |
| Maxillary | * | | * | * | * | | | | | | * | * | * | | | | * | + | + |
| Mandibular | * | | * | * | | | * | | | | | * | * | * | | | * | | |
| II-3: | | | | | | | | | | | | | | | | | | | |
| Maxillary | * | | * | * | * | | | | | | * | * | * | | | | * | + | + |
| Mandibular | * | | * | * | | | | | | | | * | * | * | | | * | | |
| II-5: | | | | | | | | | | | | | | | | | | | |
| Maxillary | * | | * | * | * | | | | | | * | * | * | | | | * | + | + |
| Mandibular | * | | * | * | | | | | | | | * | * | * | | | * | | |
| II-7: | | | | | | | | | | | | | | | | | | | |
| Maxillary | * | | | * | * | | | | | | * | * | | | | | * | + | + |
| Mandibular | * | | * | * | | | | | | | | * | | | | | * | | |
| III-1: | | | | | | | | | | | | | | | | | | | |
| Maxillary | * | | * | * | * | * | * | | | | * | * | * | * | * | | * | - | + |
| Mandibular | * | | * | * | * | * | * | | | | * | * | * | * | * | | * | | |
| III-6: | | | | | | | | | | | | | | | | | | | |
| Maxillary | * | * | * | * | * | * | * | | | | * | * | * | * | * | | * | + | + |
| Mandibular | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * | | |
| III-7: | | | | | | | | | | | | | | | | | | | |
| Maxillary | * | | * | * | * | | | | | | * | * | * | | | | * | + | + |
| Mandibular | * | | * | * | | | | | | | | * | * | * | | | * | | |

^a * = Congenitally missing tooth; 1 = central incisor; 2 = lateral incisor; 3 = canine; 4 and 5 = first and second premolars, respectively; and 6, 7 and 8 = first, second, and third molars, respectively.

^b - = absent; + = present.

types (e.g., incisors and canines) were also absent. Permanent teeth that were present appeared smaller in the mesiodistal dimension and had shorter root lengths than normal teeth. All primary teeth were reported to be normal in size, shape, and number, except those of individual III-6, whose mandibular right primary central and lateral incisor were fused. Retained primary teeth in affected adults were ankylosed and submerged. Maxilla and mandible appeared to be smaller than normal. Toenails generally were more affected than fingernails. The nails were concave and easily broken. Affected members reported that they rarely had to cut their toenails. The fifth toenails appeared to be more affected than others (fig. 1B and 1C). They were hypoplastic and, in a few cases, were almost absent (fig. 1C). It is also interesting to note that all affected members of this family—except individual II-6, whose diagnosis could not be confirmed—had a prominent maxillary frenum, requiring frenectomy for accommodation of dental prostheses. No orofacial cleft or any other craniofacial abnormalities were present in affected members, except that individual III-6 had a small head circumference. Interestingly, individual III-6 is the daughter of the affected member II-5, who was married to an unrelated individual (II-6)

with TNS (his DNA, unfortunately, is not available for analysis). She was severely affected, with 28 congenitally missing permanent teeth and dysplasia of both fingernails and toenails.

Linkage Analysis of the Family with TNS and MSX1 Mutation Analysis

On the basis of previous expression studies and reports of human mutations, the homeobox-containing transcription factor MSX1 was selected as a candidate for TNS. To test whether TNS is linked to the MSX1 locus, we performed a two-point linkage analysis between polymorphic microsatellite markers surrounding the MSX1 locus (4p16.1) and the TNS phenotype. We found strong linkage (marker D4S412 gave a LOD score of 2.98) between MSX1 and TNS, supporting the hypothesis that a mutation in MSX1 is the cause of TNS.

To search for a mutation in MSX1 that could cause TNS, we sequenced MSX1 coding regions and their flanking intronic sequences in DNA from one affected (II-7) and one unaffected member (II-8). MSX1 consists of two exons separated by a 1.6-kb intron (Hewitt et al. 1991). We found heterozygosity for a C→A trans-

version at nucleotide position 605 (as counted from the A of the translational start codon within the coding region) in exon 2 of the affected individual, but not in the control individual (fig. 2A). This C→A transversion resulted in a replacement of serine codon 202 by a stop codon in the homeodomain region of *MSX1*. Since the S202X mutation created an *NheI* restriction site, we performed restriction-enzyme analysis by digesting PCR-amplified genomic DNA with *NheI* to find out whether the mutation cosegregated with the TNS phenotype. The results showed that all affected members, but none of the unaffected members, were heterozygous for the mu-

tation (fig. 2B). In addition, the mutation was not present in 132 normal control chromosomes from unrelated unaffected individuals, allowing an estimation of allele frequency to be <.01.

The *NheI* analysis revealed that individual III-6 was heterozygous for the mutation (fig. 2B). To test whether she may represent a compound heterozygote, with two different mutated alleles of *MSX1* inherited from her affected parents, we sequenced both exons 1 and 2 and their flanking intronic sequences from her genomic DNA. We found no mutation other than the S202X mutation in the *MSX1* coding regions. However, we did

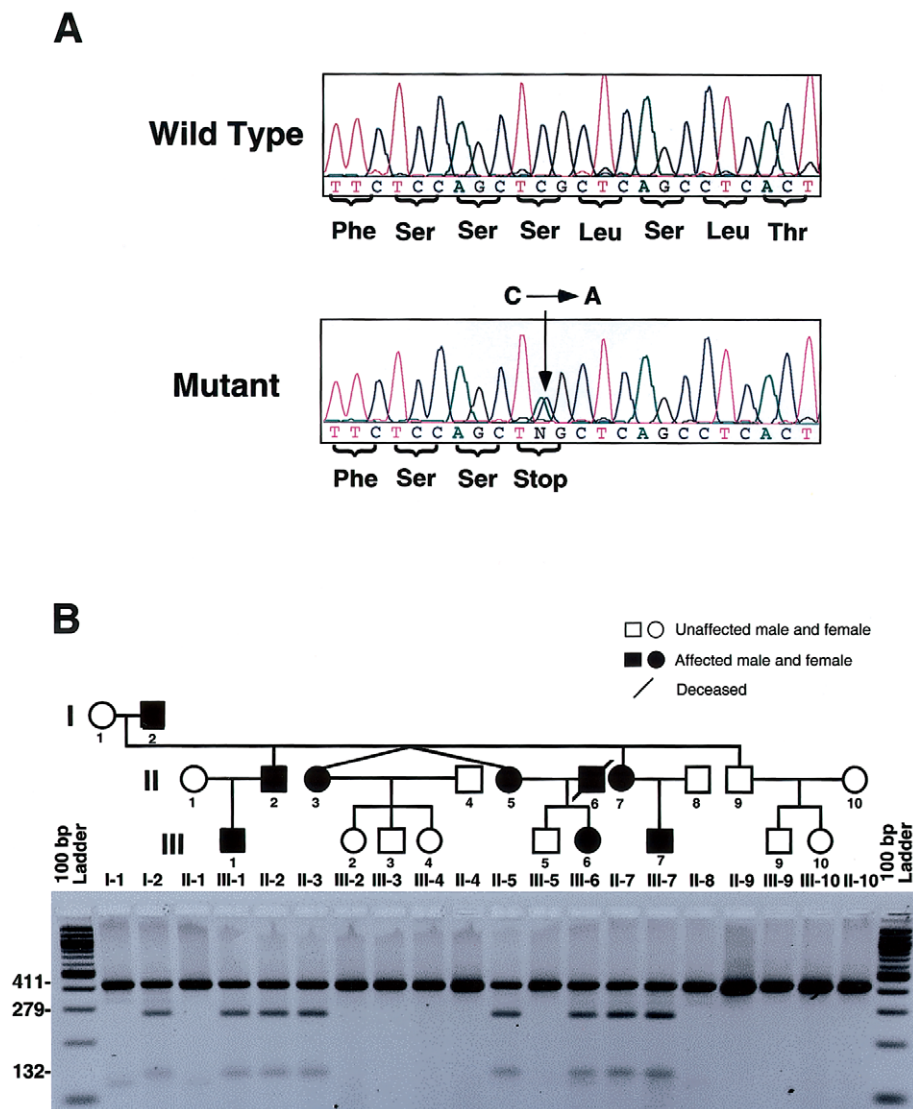


Figure 2 *MSX1* mutation analysis. A, DNA and amino acid sequence of part of exon 2 of *MSX1* in unaffected (wild type; II-8) and affected (mutant; II-7) individuals. C→A transversion at nucleotide position 605 in mutant sequence results in a premature stop codon in the homeodomain of *MSX1*. B, TNS pedigree and restriction-enzyme analysis of PCR-generated DNA fragments of exon 2 of *MSX1*. The S202X mutation cosegregates with the TNS phenotype. The PCR fragments of 411 bp in length were subjected to *NheI* digestion. The mutant fragment was cleaved into 279-bp and 132-bp fragments, whereas the wild-type fragment was not.

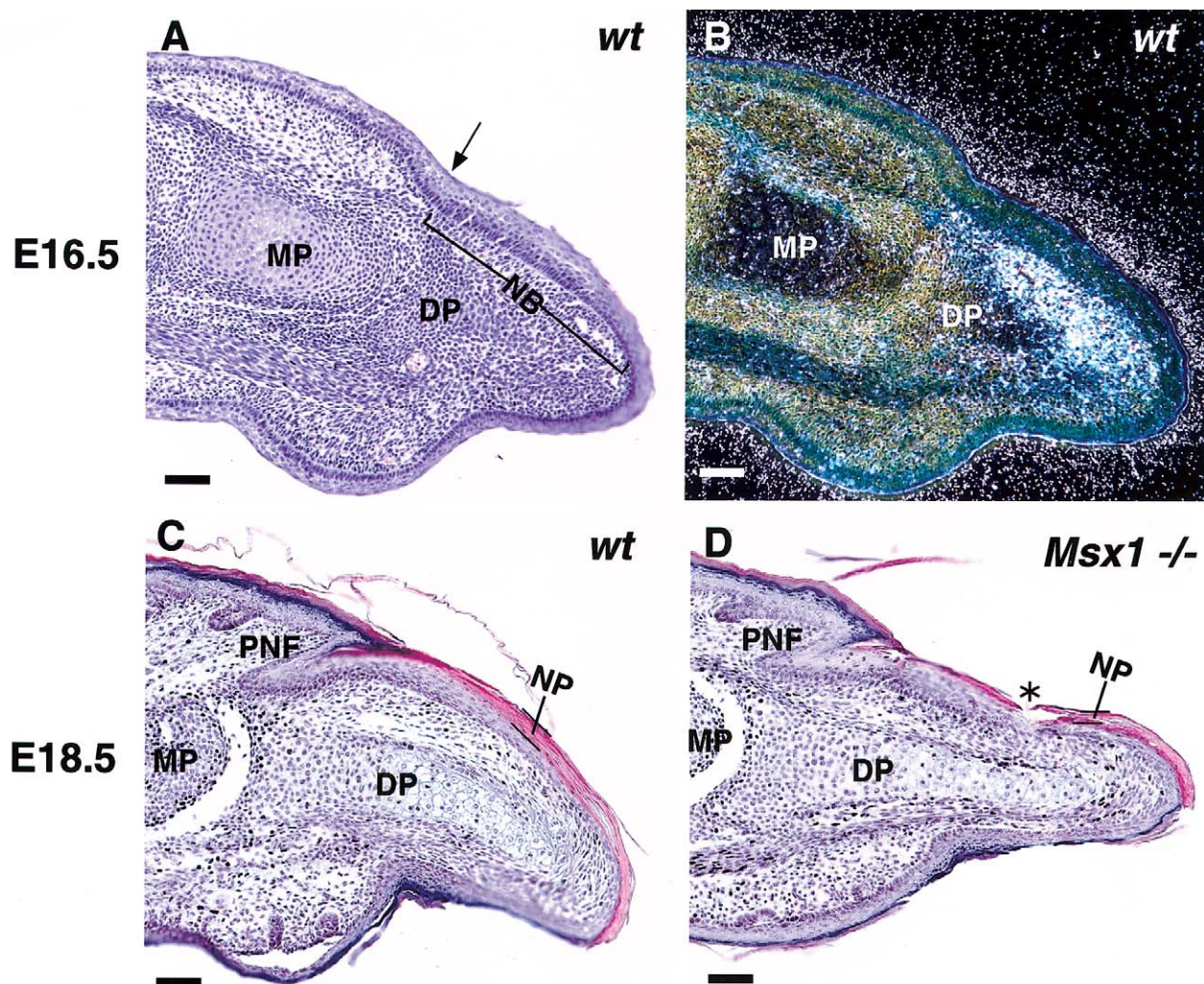


Figure 3 Histology and in situ hybridization of developing mouse nails. **A**, In wild-type embryos at E16.5, the epithelial layers of the nail bed above distal phalanx are thickened. Arrow indicates epithelial invagination site where the future proximal nail fold forms. **B**, *Msx1* is expressed only in mesenchyme subjacent to the epithelium of the nail bed. **C** and **D**, At E18.5, the wild-type nail plate is longer and thicker than that of *Msx1*-null mice. Pitting defects are observed in some areas of the epithelium (*asterisk*). NB = nail bed; PNF = proximal nail fold; NP = nail plate; DP = distal phalanx; MP = middle phalanx. *Bar*, 50 μ m.

not exclude the possibility that she might carry a mutation in regulatory regions of *MSX1*. Another possibility is that she could have inherited the wild-type allele from her affected father and her severe phenotype could be due to variable expressivity which is commonly observed in TNS.

Nail Phenotype in Msx1-Knockout Mice

Interestingly, the TNS phenotype partially overlapped that of homozygous *Msx1* knockout mice. These mice died at birth and exhibited several craniofacial abnormalities including arrested tooth development at bud stage and cleft secondary palate. Heterozygous *Msx1* mutant mice, however, were normal (Satokata and Maas 1994). To find out whether nail development in *Msx1*

mutant mice was affected, we analyzed the distal limbs of E16.5 and E18.5 wild-type and *Msx1* knockout embryos. In E16.5 wild-type embryos, the dorsal epithelium above the distal interphalangeal joint began to invaginate to form a proximal nail fold, and *Msx1* was expressed in the mesenchyme subjacent to the thickened epithelium of the nail bed (fig. 3A and 3B). In *Msx1*-null mice, however, epithelial invagination did not occur at this stage (data not shown). Moreover, at E18.5 the wild-type nail plate consisted of a smooth, homogeneous layer of keratinized epithelium; in contrast, the nail plate in mutant embryos was thinner, was irregular in shape, and contained pitting defects (fig. 3C and 3D). Histological analysis of developing nails in the heterozygous mice at E16.5 and E18.5 revealed no abnormalities (data

not shown). It appears that, at least in mice, inactivation of one *Msx1* allele is not sufficient to produce any obvious phenotypic abnormalities. However, the mesenchymal expression of *Msx1* during the early development of teeth and nails, and the coexistence of tooth and nail defects in human and mouse *Msx1* mutants, suggest that a common developmental mechanism underlies both defects.

Discussion

We have provided genetic evidence that a nonsense mutation in the homeodomain of *MSX1* is responsible for TNS in a three-generation family. We strongly believe that this mutation (S202X) is the cause of the disorder, on the basis of several findings. First, there is complete cosegregation between the 605(C→A) nucleotide substitution and the TNS phenotype in the family, and the nucleotide change is not present in 132 chromosomes derived from unrelated normal individuals. Second, the expression domains of *Msx1* in developing teeth and forming nail beds of mice correlate with the tooth and nail defects in TNS (Mackenzie et al. 1991; Reginelli et al. 1995). Third, *Msx1*-null mice exhibit tooth agenesis and defective nail plates, a phenotype that is similar to that of the family with TNS. Finally, the mutation is located in the homeobox coding region in exon 2 and results in a stop codon at amino acid position 37 of the homeodomain. The putative missing part of the protein is known to be important for protein stability (helix II) (Hu et al. 1998) and DNA binding (helix III) (Isaac et al. 1995). We predict that the truncated protein, which lacks part of the homeodomain and the entire C-terminal region, very likely is not properly folded, is unstable, or is unable to bind to DNA.

We hypothesize that the S202X mutation produces a dominant phenotype of TNS through haploinsufficiency, rather than a dominant-negative mechanism. Since S202X *MSX1* has an incomplete homeodomain that is required for DNA binding, it is likely that, even if S202X *MSX1* is synthesized as a stable protein fragment, it cannot effectively bind to DNA. This hypothesis is supported by a study demonstrating that a missense mutation (R196P) in the homeodomain of *MSX1* causes familial tooth agenesis (FTA). Functional and biochemical analyses of R196P *MSX1* confirmed that the mutant protein had no biological function, and haploinsufficiency is the most likely pathogenic mechanism of FTA (Hu et al. 1998).

An interesting aspect of the tooth phenotype in this family is the pattern of missing permanent teeth. The *MSX1* mutation reported here seems to affect only specific types of permanent teeth—namely, premolars, first molars, and third molars. Other types of permanent teeth (e.g., maxillary central incisors and primary teeth) are not affected. Since basic genetic mechanisms in-

involved in tooth development are known to be conserved between different tooth types (Stock et al. 1997; Keranen et al. 1998), the timing of *MSX1* expression, in combination with the presence or absence of functional redundancy from other genes during tooth morphogenesis, could be critical for the development of certain types of teeth.

Our data suggest that *Msx1* is important for nail development. Although the nail matrix (germinative epithelium located underneath the proximal nail fold) is known to produce nail plates, previous studies show conflicting results about whether the nail bed contributes to nail plate formation (Johnson et al. 1991). Here we demonstrate that *Msx1*-specific expression in the nail bed mesenchyme is important for nail plate thickness and integrity. These data, combined with the nail plate phenotype in *Msx1*-null mice suggest that nails, as well as teeth, develop via epithelial-mesenchymal interactions. Further studies are needed to elucidate the role of *Msx1* during nail plate development.

We describe here for the first time a mutation responsible for TNS. Previously reported *MSX1* mutations (MIM 106600) include R196P and S105X (Vastardis et al. 1996; van den Boogaard et al. 2000), which were associated with isolated tooth agenesis and tooth agenesis combined with oral clefting, respectively. The S105X mutation, which results in a more proximal truncation of *MSX1* than the S202X mutation reported here, is also likely to cause the phenotype via haploinsufficiency. Clinical variability between affected individuals in these families, all of whom are heterozygous for mutations that lead to loss of function of the mutant allele, could be explained by effects of modifier genes. Since *Msx1* is known to interact with several other regulatory proteins—for example, TBP (TATA-binding protein), DLX (distalless homeobox protein), and *MSX2* (muscle segment homeobox 2 protein) (Zhang et al. 1996, 1997)—and perhaps with many other, yet-unidentified gene products, the interplay between these genes could result in different phenotypic outcomes in different environments. In the context of *MSX1* mutations, the presence of both overlapping (tooth) and nonoverlapping (clefting and nail) phenotypes suggests that tissue-specific modifier loci influence the expression of these phenotypes. An analogous role for modifier loci in other EDs could help explain the enormous phenotypic diversity observed in the ≥ 150 distinct syndromes that constitute this class of developmental defects.

Acknowledgments

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Genbank, <http://www.ncbi.nlm.nih.gov/GenBank/> (for *MSX1* [previously known as *HOX7*] genomic sequence [accession numbers M76731 and M76732])

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for TNS [MIM 189500] and tooth agenesis [MIM 106600])

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