Homozygous Nonsense Mutations in TWIST2 Cause Setleis Syndrome

Turgut Tukel,1,5,7 Dražen Šošić,2,5 Lihadh I. Al-Gazali,3 Mónica Erazo,1 Jose Casasnovas,4 Hector L. Franco,4 James A. Richardson,2 Eric N. Olson,2 Carmen L. Cadilla,4,6 and Robert J. Desnick1,6,*

The focal facial dermal dysplasias (FFDDs) are a group of inherited developmental disorders in which the characteristic diagnostic feature is bitemporal scar-like lesions that resemble forceps marks. To date, the genetic defects underlying these ectodermal dysplasias have not been determined. To identify the gene defect causing autosomal-recessive Setleis syndrome (type III FFDD), homozygosity mapping was performed with genomic DNAs from five affected individuals and 26 members of the consanguineous Puerto Rican (PR) family originally described by Setleis and colleagues. Microsatellites D2S1397 and D2S2968 were homozygous in all affected individuals, mapping the disease locus to 2q37.3. Haplotype analyses of additional markers in the PR family and a consanguineous Arab family further limited the disease locus to ~3 Mb between D2S2949 and D2S2253. Of the 29 candidate genes in this region, the bHLH transcription factor, TWIST2, was initially sequenced on the basis of its known involvement in murine facial development. Homozygous TWIST2 nonsense mutations, c.324C>T and c.486C>T, were identified in the affected members of the Arab and PR families, respectively. Characterization of the expressed mutant proteins, p.Q65X and p.Q119X, by electrophoretic mobility shift assays and immunoblot analyses indicated that they were truncated and unstable. Notably, Setleis syndrome patients and Twist2 knockout mice have similar facial features, indicating the gene’s conserved role in mammalian development. Although human TWIST2 and TWIST1 encode highly homologous bHLH transcription factors, the finding that TWIST2 recessive mutations cause an FFDD and dominant TWIST1 mutations cause Saethre-Chotzen craniofaciostosis suggests that they function independently in skin and bone development.

The focal facial dermal dysplasias (FFDDs) are a group of inherited syndromes characterized by distinctive bitemporal scar-like depressions resembling forceps marks.1 Three subtypes have been delineated on the basis of their clinical features: type I FFDD, or Brauer syndrome (MIM 136500), is inherited as an autosomal-dominant trait, and most affected patients have only the characteristic bitemporal lesions; type II FFDD (MIM 227260) is the autosomal-recessive form of Brauer syndrome without additional features;1 and type III FFDD, or Setleis syndrome (MIM 227260), first described in patients from consanguineous Puerto Rican (PR) families,3 is characterized by bilateral temporal marks and additional facial features, including an aged-leonine appearance, absent eyelashes on both lids or multiple rows on the upper lids, absent Meibomian glands, slanted eyebrows, thin clefting, and other nonfacial manifestations (Figure 1A). The disease is panethnic, having been described in White, Hispanic, Asian, and American Indian patients from North America, Europe, Japan, the Middle East, and Samoa.3,7–10 The mode of inheritance of Setleis syndrome has been variably reported as autosomal dominant (e.g.,11,12), autosomal recessive with variable expressivity and decreased penetrance in families in which a parent had minimal to mild facial dysmorphia or in sporadic cases in which neither parent had manifestations (e.g.,7,13–16), and autosomal recessive (e.g.,1,3,5,17). These reports suggest that Setleis syndrome is genetically heterogeneous, perhaps reflecting the interactive nature of the underlying gene defects.

Histologically, the bitemporal lesion is a mesodermal dysplasia with near absence of subcutaneous fat and with skeletal muscle almost contiguous with the epidermis,6 suggesting insufficient migration of neural crest cells into the frontonasal process and the first branchial arch.4 To date, the genetic bases of the FFDDs have not been identified. Here, we report that homozygous TWIST2 (MIM 607556) nonsense mutations, identified by positional cloning, cause Setleis syndrome. TWIST2 is a member of the bHLH transcription factor family first described in mice (Dermo1),18 whose function has been characterized in knockout (KO) mice.19

For identification of the Setleis syndrome locus, a genome scan was performed on five affected individuals and 26 family members from the original consanguineous family from the San Sebastian and Lares regions of Puerto Rico described by Setleis et al.3 (Figure 2). Informed consent was provided by the subjects and/or their parents, and each subject was examined and provided blood samples. Several affected individuals provided skin biopsies and were photographed. Genomic DNAs were isolated

1Department of Genetics and Genomic Sciences, Mount Sinai School of Medicine of New York University, New York, NY 10029, USA; 2Department of Molecular Biology, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA; 3Department of Pediatrics, Faculty of Medicine and Health Sciences, United Arab Emirates University, Al Ain, United Arab Emirates; 4Department of Biochemistry, University of Puerto Rico School of Medicine P.O. Box 365067, San Juan, PR 00936-5067, USA

2These authors contributed equally to this work

3These authors contributed equally to this work

4Deceased

*Correspondence: robert.desnick@mssm.edu

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with the Puregene Isolation Kit according to the manufacturer's instructions (Gentra Systems, Minneapolis, MN, USA). Cultured fibroblasts were established from skin biopsies by standard procedures. Peripheral blood and/or cultured fibroblasts also were obtained from patients and members of four unrelated families with FFDD or Setleis syndrome phenotypes. These included an Arab family and three United States families of Hispanic, German Italian, and Cherokee Indian ancestry.

Linkage analysis was performed by genotyping 501 microsatellite markers at a 10 cM density (Genome-Wide Screening Set, version 9, Single Chromosome Set, Invitrogen Life Technologies, Carlsbad, CA, USA) in genomic DNAs from the five affected members of the PR family as previously described. Microsatellite assays were analyzed on an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA) with GeneScan Analysis software (version 3.1.2) and Genotyper software (version 2.5) (Perkin-Elmer-Cetus, Norwalk, CT, USA). Homozygous markers in all five affected individuals were then genotyped in all available family members for determination of their segregation and for haplotype analyses. Additional markers (D2S2344, D2S206, D2S1279, D2S2348, D2S2973, D2S1397, D2S2968, D2S2285, D2S2253, D2S125, D2S395, and D2S140) were genotyped, and the critical region was further narrowed by haplotype analyses of other putative FFDD families, including two informative affected first cousins from a consanguineous Arab family.
from the United Arab Emirates.8 Linkage calculations were made with the SimWalk2 program21 and were confirmed with the Linkmap program.22

Only microsatellites D2S1397 and D2S2968 were homozygous in all affected individuals from the PR family, mapping the disease locus to 2q37.3 (Figure 3A). Saturation of the region with additional markers further defined the locus to 17 cM (~7 MB) between D2S206 and D2S2253 (LOD score \( Z = 5.22 \) at D2S1397). These markers were analyzed in three unrelated Setleis syndrome families. Only the consanguineous Arab patients8 were homozygous for the region, and their haplotypes further narrowed the centromeric boundary, reducing the critical region to 8 cM (~3 MB) between D2S2949 and D2S2253 (Figures 4A–4D), which localized the Setleis syndrome critical region between 238,061,150 and 241,439,288 bp on the chromosome 2 physical map. Inspection of the 29 genes in this region suggested TWIST2 as an initial candidate for sequencing on the basis of the spatial and temporal dermal facial expression pattern of the murine ortholog during embryogenesis.18 The promoter, exonic, adjacent intronic, and 3’ untranslated regions of the TWIST2 gene were amplified via PCR from genomic DNAs of these patients and sequenced on an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems).

Sequencing of TWIST2 revealed homozygous nonsense mutations in the PR (c.486C>T [p.Q119X]) and Arab (c.324C>T [p.Q65X]) patients (Figure 3C), which segregated in respective family members in an autosomal-recessive pattern with full penetrance. Both nonsense mutations predicted early truncation of the TWIST2 transcription factor; the c.486C>T mutation truncated the C-terminal region after the bHLH motif, and the c.324C>T mutation eliminated the bHLH region and the C terminus (Figure 3D). Sequencing of TWIST2 in five other unrelated familial and/or sporadic Setleis syndrome and FFDD patients did not identify disease-causing mutations, emphasizing the genetic heterogeneity of the bilateral temporal lesions that characterize the Setleis syndrome and FFDD phenotype.

For characterization of the mRNA levels and truncated proteins expressed by these mutations, Myc-tagged constructs of wild-type and mutated (c.324C>T and c.486C>T) TWIST2 cDNAs were prepared in the pCINEO vector and used for transient transfection of HeLa cells. Quantitative PCR revealed approximately 3-fold to 4-fold decreased amounts of the c.324C>T and c.486C>T fibroblast mRNAs compared to wild-type levels (not shown). The expressed TWIST2 c.324C>T and c.486C>T mutant proteins in HeLa cells were unstable, consistent with the decreased amounts of the p.Q119X protein and even less of the p.Q65X mutant protein, detected by immunoblot analysis (Figure 3E). The instability of both mutant transcripts and their truncated proteins is consistent with nonsense-mediated decay and might explain why the patients with different mutations had similar phenotypes.

Twist2 KO mice on a 129 genetic background develop cachexia and die within days after birth as a result of
elevated proinflammatory cytokines. However, Twist2 KO mice on a 129/C57 mixed background have a milder phenotype, some attaining adulthood and living a normal life span. Analysis of the 129/C57 KO mice revealed a facial phenotype similar to that of Setleis syndrome patients, including thin skin and sparse hair with bilateral
pronounced alopecic areas between the ears and the eyes resembling the bitemporal forceps marks in humans. They also had absent eyelashes, narrow snouts, protruding chins, short anterior-posterior head diameters, and low-set dysmorphic ears (Figures 1B and 1C, Table 1).

Histologically, the bilateral alopecic areas in the KO mice were similar to the defects in Setleis syndrome patients, including the hypoplastic dermis and absence of epidermal appendages and subcutaneous fat (Figure 1D). In mice, Twist2 is expressed in the dermis, not the epidermis, and the alopecic areas are located in the region with predominantly mesoderm and not neural-crest-derived dermis. The KO mice also have absent/hypoplastic Meibomian glands (Figure 1E), as seen in Setleis syndrome patients. Development of eyelids in mammals is governed by bidirectional signaling between surface epithelium and underlying neural-crest-derived mesenchyme. Mice with Wnt1-Cre-mediated deletion of Twist2 do not show an eyelid phenotype or bilateral head skin lesions (data not shown), indicating that unlike Twist1, Twist2 does not function in the cranial neural crest. We suggest that Twist2 is involved in cranial dermal mesenchyme development, while indirectly regulating overlying epidermis and induction of its appendages. Furthermore, murine Twist2 and human TWIST2 may regulate mesenchymal signal(s) for migration and/or differentiation of neural crest cells involved in Meibomian gland and eyelash development. Thus, Twist2 KO mice provide an excellent model for the investigation of its role in facial development.

The murine Twist2 and human TWIST2 proteins are 100% identical, whereas their TWIST1 (MIM 601622) proteins have 92% identity. Human TWIST1 and TWIST2 proteins are 98% identical in the ~100 residues in the bHLH region and C terminus (Figure 5). The main difference between these two human proteins lies in the N terminus, where TWIST2 lacks two glycine-rich regions found in TWIST1. Throughout their coding regions, the identity and similarity are 65% and 71%, respectively. In mice, Twist1 is essential for embryonic viability and cranial neural tube formation. In humans, TWIST1 haploinsufficiency causes Saethre-Chotzen syndrome (MIM 101400). The bHLH proteins of the TWIST subfamily form heterodimers with ubiquitous E proteins, such as E12, as well as homodimers. For determining the DNA-binding properties of the TWIST2 mutated proteins, EMSA studies were conducted with the wild-type and mutant proteins p.Q65X and p.Q119X, as previously described. Relative positions of TWIST homodimers, TWIST2/E12 heterodimers, and supershifted complexes are shown in Figure 3F. A 32P-labeled E box containing oligonucleotide derived from the proximal region of the human TNF-α promoter was used as a probe (5'-GGGCCGACTACCGCTTCCTCAGATGAGCTCATGGGTTT-3'). This probe is part of an evolutionarily conserved DNA region upstream of the TNF-α transcription start site that has been shown previously to bind both mouse Twist1 and Twist2 proteins. The gel mobility shift assays revealed that the human Twist2 protein bound to the DNA probes containing a conserved E box, both as homodimers and as heterodimers (Figure 3F). However, neither mutant protein was able to bind a DNA probe as a homodimer. Mutant protein p.Q119X, but not p.Q65X, bound DNA as a heterodimer, albeit at reduced levels in comparison to full-length TWIST2 (Figure 3F). The outcome of this binding is unclear, because the C terminus contains an important inhibitory domain that can repress target gene transcription. Therefore, the p.Q119X mutant protein could cause aberrant expression of target genes, given that its trans-activating effects presumably differ from those of the wild-type protein. The p.Q65X protein likely represents a true loss-of-function mutant. Notably, the mouse phenotypes of the Twist1 null mutant and the mutant with C-terminal mutations (“Charlie Chaplin” strain) differ significantly. We speculate that dominant-negative mutations in TWIST2 or partner proteins could cause autosomal-dominant inheritance of FFDD.

In summary, Setleis syndrome results from TWIST2 homozgyous nonsense mutations that affect TWIST2 protein stability and DNA binding, providing the first genetic basis for an FFDD syndrome. The similar facial dysmorphia in Twist2 KO mice demonstrates the conservation of TWIST2 function in mammalian facial dermal development. Thus, Twist2 KO mice represent an appropriate animal model for the study of the molecular mechanism(s) leading to the FFDD syndromes. Our results suggest that TWIST2 binding partners or other dermal-development transcription factors may be defective in other Setleis syndrome patients or FFDD syndromes.
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Web Resources

The URL for data presented herein is as follows:


References


