A novel loss-of-function mutation in TTF-2 is associated with congenital hypothyroidism, thyroid agenesis and cleft palate

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Thyroid dysgenesis is the most common cause of congenital hypothyroidism (CH) and its genetic basis is largely unknown. Here, we describe the second homozygous missense mutation in TTF-2 (or FOXE1), a transcription factor that has been implicated in thyroid development. Two male siblings, born to consanguineous parents, presented with CH, athyreosis and cleft palate and were found to be homozygous for a mutation corresponding to a serine to asparagine substitution at codon 57 (S57N) in the forkhead DNA binding domain of TTF-2. Their heterozygous parents were unaffected and this mutation was not found in 31 unrelated cases of athyreosis or normal controls. Consistent with its location, the S57N TTF-2 mutant protein showed impaired DNA binding and partial loss of transcriptional function. Such incomplete loss of TTF-2 function may account for the absence of choanal atresia and bifid epiglottis in our patients, anomalies which were present together with CH and cleft palate in two other individuals with the only other, more deleterious, TTF-2 mutation (A65V) described previously. Our observations support the role of TTF-2 in both thyroid and palate development but suggest phenotypic heterogeneity of this syndromic form of CH.

INTRODUCTION

Congenital hypothyroidism (CH) is the most common neonatal endocrine disorder, being found at a rate of 1 in 3000–4000 live births (1) and results in severe neurodevelopmental impairment if treatment is delayed. Consequently, most countries operate a neonatal screening programme to enable early detection of cases and therapeutic intervention. CH is most commonly (85% of cases) due to defects in thyroid development leading to glandular dysgenesis. Thyroid dysgenesis comprises either complete agenesis (35–40%), ectopic (55–60%), or hypoplastic (5%) development of the gland. Remaining dyshormonogenetic causes of CH are associated either with a goiter or a normal-sized thyroid gland (2–4). The pathogenesis of thyroid dysgenesis is largely unknown. Whilst most cases are sporadic, up to 2% of patients with thyroid dysgenesis have a family history of the condition, suggesting the existence of genetic factors which could contribute to the disorder (5). Further evidence in support of a genetic basis for CH is provided by the higher incidence of non-thyroidal congenital anomalies (cardiac and renal malformations and hip dislocation) and chromosomal defects in infants with this disorder than in the general population (6–8).

Thyroid transcription factor 2 (TTF-2) is a member of the forkhead/winged helix-domain protein family (9), many of which are key regulators of embryonic development. The mouse gene is located on chromosome 4 (9) and the human gene (known as TTF-2, FKHL15 or FOXE1) on chromosome 9q22 (10). Both the mouse and human gene contain a single coding exon (9–12). TTF-2 regulates the transcription of target genes such as thyroglobulin and thyroid peroxidase by binding to specific regulatory DNA sequences in their promoters via its forkhead DNA binding domain (13–15). In the rat, TTF-2 is expressed in the thyroid primordium at the onset of its development and TTF-2 expression continues during migration of the thyroid diverticulum (9,10). Homozygous null mice with targeted disruption of TTF-2, exhibit cleft palate and thyroid malformation consisting of either thyroid agenesis or thyroid ectopy (16). We recently described the first human TTF-2 gene defect in two siblings born with congenital hypothyroidism due to thyroid agenesis together with other anomalies including cleft palate, choanal atresia, bifid epiglottis and spiky hair (12).
Both individuals were homozygous for a missense mutation (A65V) in a highly conserved residue within the forkhead domain. The mutant TTF-2 protein exhibited complete loss of DNA binding and transcriptional function.

Here, we describe a second family with two affected siblings, born to consanguineous parents, who presented with CH, thyroid dysgenesis (TD), cleft palate and spiky hair. They are homozygous for a novel missense mutation (S57N) in the forkhead domain of TTF-2. Functional studies in vitro indicate that the S57N mutant retains some specific DNA binding and transcriptional activating function. Unlike the cases described previously, our patients had an incomplete clinical phenotype, lacking choanal atresia and bifid epiglottis, which may indicate partial preservation of TTF-2 function in vivo.

RESULTS

Clinical features

Two male sibling probands (Fig. 1A, IV-5 and IV-11) were born at term after normal pregnancies to first cousin parents (Fig. 1A, III-1 and III-2) of Tunisian origin. They were both born with CH and extensive clefting of both soft and hard palate (Fig. 1B) in the absence of any other non-thyroidal congenital midline anomalies. Both are known to have normal karyotypes.

Proband IV-5 was born before the establishment of routine neonatal screening for CH in France and therefore did not have the condition diagnosed until he was 4 months of age when he presented with features of frank hypothyroidism including developmental delay and poor growth (length 3 SD below mean). Measurement of circulating thyroid hormones showed a low total T₄ of 9 nM/l (reference range 50–137.5 nM/l). Knee radiographs showed severe delay in bone maturation with absent ossification centers in the superior tibial epiphyses and very short, dysplasic distal femoral epiphyses. ⁹⁹Tc scanning and ¹²³I scanning at 5 months (before treatment) and 11y respectively, both showed no uptake of tracer in neck regions corresponding to the line of thyroid migration, suggesting thyroid agenesis. Thyroid autoantibodies are absent. Following his delayed diagnosis thyroxine replacement therapy was commenced but his thyroid status has been erratic. Consequently, he has mental retardation in addition to a significant psychiatric problem necessitating prolonged admissions to psychiatric institutions. Despite repeated operations on his palate, velopharyngeal incompetence remains.

His brother, proband IV-11, was noted to have hypotonia, prolonged jaundice and poor weight gain in the neonatal period (birth weight 4.5 kg; birth length 55 cm) and CH was diagnosed shortly after birth by the neonatal screening programme. Additional dysmorphic clinical features noted at birth include hypertelorism, low-set posteriorly rotated ears, a low posteriorly rotated ear, low-set posteriorally rotated ears, a low posteriorly rotated ear and spiky hair (Fig. 1C and D). His heel prick TSH level was 110 μU/ml (normal range <20 μU/ml) and his serum TSH and total T₄ concentrations were 200 μU/ml (reference range 0.5–4.5 μU/ml) and 28 nM/l (reference range 50–137.5 nM/l), respectively at 3 days of age. He was started on thyroxine therapy at diagnosis but did have some psychomotor retardation and delay in bone age in infancy. Recently, his thyroid status was reassessed following thyroxine withdrawal for 14 days: the TSH rose to 20 μU/ml (reference range 0.27–4.2 μU/ml), circulating thyroid hormones decreased, FT₄ to 6.8 pm/l (reference range 11.5–23 pm/l) and FT₃ to 2.3 pm/l (reference range 2.5–5.8 pm/l) and serum thyroglobulin was undetectable at <0.4 ng/ml. Ultrasound and ¹²³I scanning failed to detect any thyroid tissue. As his brother, he has persistent velopharyngeal incompetence despite surgery.

The parents and remaining siblings in this family are euthyroid and have no congenital anomalies. There is no family history of thyroid dysfunction.

Haplotyping and linkage analysis of TTF-2

Initially, genetic linkage between the TTF-2 locus and CH with cleft palate was sought and the two affected siblings and their parents were genotyped for polymorphic markers flanking the gene (Fig. 2A). The two hypothyroid siblings (IV-5 and IV-11) are homozygous for a TTF-2 haplotype, which is present in both parents (III-1 and III-2), who are heterozygous for this allele (Fig. 1A). Based on the likelihood of linkage to this locus, SS0CP analysis and direct sequencing of TTF-2 was undertaken.

SSCP

SSCP analysis was performed with TTF-2 amplicon 2 (Fig. 2B), which encompasses the proximal part of the forkhead domain. The two affected siblings were homozygous and their parents heterozygous for abnormally migrating bands which were absent in both unaffected controls (n = 18) and unrelated cases of athyrosis (n = 13) (Fig. 2C). Accordingly, direct sequencing of this region of TTF-2 was undertaken in probands IV-5 and IV-11 to confirm the presence of a mutation.

Identification of a mutation in the TTF-2 gene

Direct sequencing of the coding region of the TTF-2 gene revealed a single nucleotide substitution (AGC to AAC) at position 169, corresponding to a serine to asparagine mutation at codon 57 (S57N) in the predicted protein sequence (Fig. 2D). Both probands were homozygous for this mutation and the parents were heterozygous, consistent with autosomal recessive inheritance of the mutation in this family. DNA from the unaffected siblings was not available for analysis. Sequencing of the remainder of the coding region of the TTF-2 gene in the affected individuals revealed no other abnormalities (data not shown).

Functional characterization of mutant TTF-2

DNA binding activity. The S57N mutation identified in TTF-2 localizes to the forkhead domain of the protein which mediates DNA binding. Furthermore, an alignment of amino acid sequences within forkhead domains of a number of proteins indicated that the residue corresponding to Ser 57 in TTF-2 is highly conserved in all members of this family (Fig. 3), suggesting that this amino acid is functionally significant.

In view of the location of this mutation, we first tested the DNA binding properties of the S57N TTF-2 mutant in comparison with both wild type TTF-2 and the A65V mutant.
Figure 1. (A) Pedigree of kindred. The affected subjects and their parents were tested for dinucleotide polymorphic microsatellite markers in the region of the TTF-2 gene (9q22.3) and the haplotypes are shown below the corresponding individual. The results of thyroid function tests (obtained before the institution of L-thyroxine treatment) are also aligned with each individual. Normal values were from 0.5–4.5 for TSH and from 50–137.5 for T4. Subject number for each generation is on the top left and the age (in years) is on the top right of each symbol. The TTF-2 haplotype suggests linkage to the TTF-2 locus. (B) Extensive cleft palate in proband IV-11 which was similarly present in his brother, proband IV-5. (C and D) Photographs of the proband IV-11 at age 8 months illustrating dysmorphic features included hypertelorism, low-set posteriorly rotated ears, a low posterior hair line and spiky hair.
Figure 2. (A) Microsatellite markers used to determine TTF-2 haplotypes. They are known markers in the region of 9q22.3, each separated by a distance of 0.5 to 1.6 cM and altogether covering a distance of 3.2 cM. (B) Schematic representation of the TTF-2 gene and the localization of the primers used for SSCP and sequencing analysis with the position of the two described TTF2 mutations. The box corresponds to the forkhead domain. (C) SSCP results from the two probands (P), their parents (M = mother, F = father), 2 control subjects without CH (C1 and C2) and two other subjects with thyroid dysgenesis (TD1 and TD2). The solid arrow indicates the normal band and the broken arrow indicates the abnormal band with altered migration pattern present in a homozygous state in the probands, in a heterozygous state in the parents and absent from all the controls. (D) Direct sequencing of the TTF-2 gene from a proband, a parent and a normal control. The sequence of a section of the forkhead domain of TTF-2 from the proband contains a single nucleotide substitution (AGC to AAC) at position 169, corresponding to a serine to asparagine mutation at codon 57 (S57N) in the predicted protein sequence. The parents’ sequence indicates a heterozygous change at the same position.
described previously (12), using electrophoretic mobility shift assays (EMSA). As described previously, in vitro-translated wild-type TTF-2 protein complexed specifically with DNA corresponding to a TTF-2 binding site whereas the A65V mutant showed negligible binding (Fig. 4A). With the S57N TTF-2 protein, DNA binding was markedly attenuated but not completely abolished (Fig. 4A). We extended these observations by testing the DNA binding properties of wild type or mutant TTF-2 expressed in cells. The 293 human kidney fibroblast cells were transfected with FLAG epitope-tagged wild type or mutant TTF-2 expression vectors and nuclear extracts were prepared. With wild type TTF-2 containing cells, a specific DNA-protein complex was clearly formed (Fig. 4B, closed solid arrowhead) whose mobility could be retarded by incubation with an anti-FLAG antibody to form a DNA-protein complex (Fig. 4B, open arrowhead) whose mobility could be retarded by incubation with an anti-FLAG antibody. In contrast, the S57N TTF-2 mutant preserved some function, achieving ~75% of the maximal transcriptional response attained with wild type TTF-2 (Fig. 5).

**DISCUSSION**

In a family with parental consanguinity, we have shown that a combination of congenital anomalies including thyroid agenesis, cleft palate and spiky hair in two siblings are linked to the TTF-2 locus. Subsequent sequence analysis of the TTF-2 gene revealed the two probands to be homozygous for a missense (serine 57 to asparagine) mutation in a highly conserved residue within the forkhead DNA binding domain of this transcription factor. Both parents were heterozygous for this mutation but were euthyroid and devoid of congenital defects. Functional studies indicate that the S57N TTF-2 mutant retains some DNA binding and transcription regulatory activity.

The forkhead family of transcription factors, defined by a conserved central region of 110 residues mediating DNA binding, was discovered over a decade ago with the description of the *Drosophila* forkhead gene (17) and of the HNF-3 protein family in rodents (18). Since then, their importance as key regulators of embryogenesis and cellular differentiation has been increasingly recognized. Studies in mice show that an interplay between transcription factors including TTF-2 appears to be critical in early thyroid organogenesis (9). TTF-2 and other transcription factors such as TTF-1 and PAX8 are all expressed at the onset of the formation of the thyroid primordium on embryonic day 8–8.5. The expression of the three genes continues throughout the migration of the thyroid diverticulum until embryonic day 15 when migration is complete and thyroid follicular cell differentiation begins (9–10, 19–20). Knockout mouse models are informative and homozgyous TTF-2-null mutant mice are born with thyroid malformation consisting of either complete agenesis or ectopic sublingual gland development together with cleft palate (16).

We have previously reported the only other human TTF-2 gene mutation identified to date. In a Welsh family, two male siblings born to unrelated parents, presented with congenital hypothyroidism due to thyroid agenesis and other anomalies including cleft palate, choanal atresia and bifid epiglottis together with spiky hair (21). They were homozygous for a different missense mutation (A65V), also involving a highly conserved residue within the forkhead DNA binding domain (Fig. 2B). Functional studies indicate that the A65V mutation is highly deleterious, with the mutant protein exhibiting a complete lack of DNA binding and transcriptional activation (Figs 4 and 5) (12). In the family described in this report, the probands also presented with congenital hypothyroidism. Detailed investigation of one sibling (IV-11), showing immediate hypothyroidism following thyroxine withdrawal, absent circulating serum thyroglobulin and no thyroid tissue on ultrasound and iodine scanning, is consistent with complete agenesis of the gland. Whilst both siblings exhibited cleft palate, they lacked the other cervical midline defects (choanal atresia, bifid epiglottis) seen in our first family. When compared directly in functional studies, the S57N TTF-2 mutation found in this second family is less deleterious, preserving some DNA

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**Transcriptional activity.** The transcriptional properties of wild type and mutant TTF-2 were tested by cotransfection assays in the 293 cell line, using a reporter gene containing concatamerized TTF-2 binding sites (15), together with either wild type or mutant TTF-2 expression vectors (Fig. 5). Transfection of increasing amounts of wild type TTF-2 expression vector induced reporter gene activity in a dose-dependent manner and the A65V TTF-2 mutant exhibited negligible transcriptional activity as reported previously (12). In contrast, the S57N TTF-2 mutant preserved some function, achieving ~75% of the maximal transcriptional response attained with wild type TTF-2 (Fig. 5).

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**Figure 3.** Amino acid alignment of forkhead domain proteins showing conservation of the residues corresponding to serine at codon 57 and alanine at codon 65 in TTF-2 (boxed).

<table>
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<td>AKPPSYISLITMAQQ</td>
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binding and transcriptional activity. It is tempting to speculate that such residual mutant TTF-2 function may account for the incomplete clinical phenotype, with lack of choanal atresia and bifid epiglottis in affected subjects in this second family.

The association of CH with cleft palate alone in the absence of other congenital anomalies is infrequent (Cleft Lip and Palate Association (UK) database; 8,22,23). Mutations in the TTF-2 gene have not been found in cases with CH and cleft palate only or in patients with cleft palate or thyroid dysgenesis alone (unpublished personal data) (22,24–26). Together with the clinical features in the four affected individuals found to date, these observations suggest that the phenotype of human TTF-2 gene defects is likely to be a specific combination of multiple anomalies including thyroid dysgenesis, cleft palate and spiky hair together and possibly with other midline craniocervical defects.

Although thyroid dysgenesis is the major underlying cause of CH, defects in thyroid transcription factor genes have been implicated in only a minority of cases so far and usually in association with extrathyroidal anomalies, in keeping with their wider developmental role in non-thyroidal tissues during embryogenesis. This suggests that either combinations of defects in these transcription factor genes during development or abnormalities in downstream target genes whose expression are limited to the thyroid, constitute the genetic basis of isolated thyroid dysgenesis or cleft palate.

Figure 4. DNA binding studies. (A) In vitro-translated wild-type and S57N or A65V mutant TTF-2 protein bound to an oligonucleotide probe corresponding to the TTF-2 binding site in the thyroglobulin promoter (solid arrowhead). S57N displays reduced binding and A65V negligible binding compared with wild-type TTF-2. (B) Nuclear extracts from 293 cells transfected with pFLAG expression vectors and incubated with anti-FLAG antibody (+) or no antibody (−). The solid arrowhead indicates a specific complex between wild-type or mutant TTF-2 and the oligonucleotide probe, whereas the blank arrow denotes this complex supershifted by the anti-FLAG antibody. Note the weaker S57N-probe complex that is also supershifted with the addition of anti-FLAG antibody whereas there is again negligible binding seen with A65V. A non-specific complex is seen in reactions incubated with unprogrammed reticulocyte lysate (mock) as well as programmed lysates. (C) Western blot of the same nuclear extracts used in B, showing the FLAG–TTF-2 protein (solid arrowhead). Protein molecular weight markers are indicated at left.
SUBJECTS AND METHODS

Subjects

Twenty two pedigrees with CH associated with thyroid dysgenesis were selected for linkage analysis to the TTF-2 gene. From the pedigree described in this report (Fig. 1A), referred to us by colleagues at Grenoble in France, linkage to the TTF-2 locus was established in two siblings with CH due to thyroid dysgenesis associated with cleft palate and spiky hair. CH was diagnosed in the younger proband by the French neonatal screening programme. The patients were clinically evaluated and underwent radiological and biochemical investigations by the team in Grenoble.

Haplotyping and linkage analysis

Genomic DNA was prepared from peripheral blood lymphocytes of the two affected brothers and their parents, from 13 unrelated cases of athyreotic CH and 18 normal controls. Informed consent was obtained from the adult patients or the parents of the children. The study was reviewed and approved by the Paris/Saint Louis ethical committee.

Four dinucleotide microsatellite markers (D9S1809, D9S1786, D9S180, and D9S1690) in the region 9q22.3 spanning a distance of 3.2 cM were used to haplotype the TTF-2 alleles in order to determine whether there was linkage of the CH and cleft palate phenotype to the TTF-2 locus (Fig. 2A).

SSCP

The entire coding-region (a single exon) was amplified by PCR in 25 μl reactions using 7 primer pairs producing overlapping amplicons (Fig. 2B). The expected length and annealing temperature for each amplicon is as follows: F1: 5’-ctgagctctccgcagaagg-3’, R1: 5’-agctgtagggcggcttcc-3’ (272 bp, 60°C); F2: 5’-ggtacctcgaaggaagac-3’, R2: 5’-gaaaagcagctgttgaagtt-3’ (272 bp, 60°C); F3: 5’-ggccgctctatgcaggctac-3’, R3: 5’-gtgacgagcctagtgtagggag-3’ (254 bp, 58°C); F4: 5’-ggtacgggatctggtaga-3’, R4: 5’-cggctgatctgcatggat-3’ (260 bp, 60°C); F5: 5’-cggcgggaagtctggagaa-3’, R5: 5’-gtgacgagcctagtgtagggag-3’ (254 bp, 60°C); F6: 5’-ggtcggcggctgatctgctac-3’, R6: 5’-tcggcggctgatctgctg-3’ (221 bp, 60°C); F7: 5’-cggcgtctgctgatctgctg-3’, R7: 5’-ggtcggcggctgatctgctg-3’ (299 bp, 58°C).

PCR buffer contained 20 mM Tris-HCl pH 8.0, 50 mM KCl, 2.5 mM MgCl2, 5% dimethylsulfoxide (DMSO), 160 μM dNTPs, 0.4 μM 32P-dCTP (2500 Ci/mM), 0.25 U of AmpliTaq DNA polymerase (Invitrogen, Cergy Pontoise, France), 10 μM of each primer and 200 ng of genomic DNA. The following PCR conditions were used: 95°C for 5 min, then 35 cycles of 95°C for 40 s, annealing temperature (see above) for 40 s, and 72°C for 1 min, followed by 72°C for 7 min on a GeneAmp 9700 thermal cycler (Perkin-Elmer, Foster City, CA).
SSCP analysis was performed by diluting PCR products two fold in a buffer containing 98% formamide, 8 mM EDTA, 0.1% bromophenol blue and 0.1% xylene cyanol, denatured at 95°C for 7 min and loaded onto a non-denaturing gel (25% MDE gel solution from Tebu, 60% TBE 1×). The gels were run at room temperature and at 4°C at 7 W constant power for 13–15 h, then dried on the glass plate and exposed for 48–72 h on Kodak films (Amersham Pharmacia Biotech, Saclay, France).

Direct sequencing

When anomalous SSCP migration patterns were observed, genomic DNA was amplified using the same primer pairs used for SSCP (see above) and both forward and reverse strands were sequenced by the fluorometric method (DyeDeoxy Terminator Cycle Sequencing kit, Applied Biosystems, Appleria, Courtboeuf, France).

Electrophoretic mobility shift assays

Wild-type TTF-2 cDNA was amplified by PCR using genomic DNA from a normal control and cloned as a BamHI–XhoI fragment into pcDNA3 (Invitrogen) and pFLAGCMV2 eukaryotic expression vectors (Sigma Aldrich, Saint Quentin Fallavier, France). Mutant TTF-2 was generated by site-directed mutagenesis of the wild-type TTF-2 template using a standard protocol (Stratagene, Amsterdam, The Netherlands). All constructs were verified by sequencing. Equal amounts of in vitro translated (TNT, Promega, Charbonnières les bains, France) wild-type or mutant TTF-2 protein were incubated for 30 min at room temperature with 32P-labelled oligonucleotide probe corresponding to the TTF-2 binding site in the thyrolobulin promoter 5'-gaggaggtctcgtgacatcgagataaaagttggacca-3' (27) in buffer (20 mM Hepes, 10% glycerol, 2 mM dithiothreitol, pH 7.8) with KCl added at concentrations of 100, 150, and 200 mM in the presence of 1 μM poly (dI-dC). The protein–DNA complexes were then resolved on a 6% polyacrylamide gel. Alternatively, nuclear extracts were prepared (28) from 293 EBNA cells 36–48 h after transfection with pFLAG expression vectors (10 μg). Nuclear extract protein (1 μg) was incubated with oligonucleotide probe in the presence of anti-FLAG monoclonal antibody M2 (3 μg; Sigma Aldrich) or an unrelated monoclonal antibody.

Western-blot analysis

Nuclear extract proteins were resolved by 10% SDS–PAGE and electroblotted onto Hybond ECL membrane (Amersham). As a positive control, FLAG epitope-tagged wild-type TTF-2 protein was in vitro translated from a pcDNA3 vector. The blot was probed with a 1 : 1000 dilution of monoclonal anti-FLAG antibody M2, and developed using enhanced chemiluminescence (Amersham).

Transient transfection assays

293 EBNA cells (ECACC No: 85120602) were grown in Dulbecco's modified Eagle medium (supplemented with 10% fetal bovine serum and 1% penicillin, streptomycin, and fungizone; Gibco-BRL). A reporter gene containing concatamerized TTF-2 binding sites from the rat thyroid peroxidase promoter linked to the thymidine kinase promoter and luciferase which has been described previously was used (12). Cells were transfected in 24-well plates with 1 μg reporter gene and 100 ng of transcription factor expression vector by a 4 h exposure to calcium phosphate. After 36–48 h, cells were harvested and luciferase and β-galactosidase assays were performed as previously described (28). Luciferase values were normalized to β-galactosidase activity from the internal control plasmid pOSβgal (29) and represent the mean ± SEM of at least three independent experiments, each performed in triplicate.

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