Phenotypic Homogeneity and Genotypic Variability in a Large Series of Congenital Isolated ACTH-Deficiency Patients with *TPIT* Gene Mutations


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**Context:** Congenital isolated ACTH deficiency (IAD) is a rare disease characterized by low plasma ACTH and cortisol levels and preservation of all other pituitary hormones. This condition was poorly defined before we identified *TPIT*, a T-box transcription factor with a specific role in differentiation of the corticotroph lineage in mice and humans, as its principal molecular cause.

**Objective:** We have enlarged our series of IAD patients to better characterize the phenotype and the genotype of this rare disease.

**Design:** Each exon of the *TPIT* gene was amplified and sequenced in IAD patients without any identified cause. A functional analysis of each new *TPIT* mutation was performed.

**Results:** We described the largest series of 91 IAD patients and identified three distinct groups: neonatal onset complete or partial IAD or late onset IAD. We did not identify any *TPIT* mutation in patients with partial or late-onset IAD. However, we found a *TPIT* mutation in 65% of patients with neonatal-onset complete IAD. These patients are homozygous or compound heterozygous for *TPIT* mutations, and their parents are healthy heterozygous carriers. We identified nine new mutations: four missense, one one-nucleotide deletion, three splice-site mutations, and one large deletion. *TPIT* mutations lead to loss of function by different mechanisms, such as non-sense-mediated mRNA decay, abnormal mRNA splicing, loss of *TPIT* DNA binding or protein-protein interaction defects.

**Conclusion:** *TPIT* mutations are responsible for two thirds of neonatal-onset complete IAD but cannot be detected in partial or late-onset IAD. (J Clin Endocrinol Metab 97: E486–E495, 2012)
ACTH deficiency is characterized by low plasma ACTH levels resulting in low cortisol production by adrenal glands. It is a major cause of secondary adrenal insufficiency, a condition that can be life threatening if not diagnosed and treated properly. Acquired ACTH deficiency is secondary to glucocorticoid therapy or medical condition such as pituitary tumors, inflammatory lesions, radiation therapy, pituitary surgery or trauma. In contrast, etiologies of congenital ACTH deficiency are less documented. Mutations in transcription factors involved in the early steps of pituitary development such as Prophet of Pir-1, LIM c homeobox-3, LIM c homeobox-4, Sry-box 3, and homeobox ES cells expressed 1 lead to variable phenotypes of congenital combined pituitary hormone deficiencies including ACTH deficiency (1–4). ACTH is derived from the processing of the precursor polypeptide proopiomelanocortin (POMC) by proconvertase-1. POMC gene mutations have been identified in patients with red hair pigmentation, early-onset severe obesity, and ACTH insufficiency (5). Proconvertase-1 mutant patients have a very complex phenotype including abnormal glucose homeostasis, ACTH deficiency but elevated plasma POMC levels, childhood obesity, and hypogonadism (6). Although these congenital defects cause a wide range of phenotypes, they cannot explain congenital isolated ACTH deficiency (IAD), a rare clinical condition characterized by low plasma ACTH and cortisol levels and preservation of all other pituitary hormones. This condition is most often diagnosed in sporadic cases from birth to adulthood and was not well defined before we identified TPIT mutations as the principal molecular cause (7–9).

T-box pituitary-restricted transcription factor (TPIT) is a T-box transcription factor restricted to pituitary POMC-expressing cells in mice and humans. TPIT is essential for both POMC gene transcription and terminal differentiation of POMC-expressing cells. TPIT is also a marker of corticotroph adenomas because it is present only in this subtype of pituitary adenomas (10). Tpit-deficient mice have only a few remaining pituitary POMC-expressing cells, with very low ACTH levels, adrenal hypoplasia, and undetectable corticosterone levels (11). We previously showed that human TPIT mutations are responsible for 60% of neonatal-onset IAD (9). We now extend our series from 27 to 91 patients with IAD, including patients with partial or late-onset IAD, in which we did not identify any TPIT mutation. TPIT mutations were found in 37 of 57 (65%) of patients with neonatal-onset and complete IAD; the present study defined the molecular basis of TPIT loss of function for nine new mutations, including four splicing mutations. This large series revealed that the 21 different TPIT gene mutations identified in 29 unrelated families from around the world are responsible for neonatal congenital IAD with a very homogenous clinical presentation.

### Patients and Methods

#### Patients

Patients were included on the basis of IAD in the absence of identified cause, such as cranial trauma, previous corticosteroid therapy, or lymphocytic hypophysitis. The age of onset of ACTH deficiency was not taken into consideration for inclusion in this study at one center, whereas recruitment of nonneonatal IAD patients was stopped at the other, after the analysis of the first nine nonneonatal IAD patients. The proportion of neonatal- vs. juvenile-onset IAD cannot be inferred from our cohort. After obtaining informed consent from their parents, we collected DNA samples from patients belonging to unrelated families that originated from different countries.

#### Hormone studies

Hormone assays were performed using several commercial kits, and normal values for each center were used as references. Plasma ACTH and cortisol concentrations were measured at 0800 h using RIA kits. Complete ACTH deficiency was defined as low baseline ACTH levels associated with basal cortisol levels less than 60 nmol/liter without any response to spontaneous hypoglycemia and/or to a 250-µg ACTH test. Partial ACTH deficiency was defined as low or inappropriately normal baseline ACTH levels associated with basal cortisol levels between 60 and 220 nmol/liter and/or stimulated cortisol between 300 and 500 nmol/liter. Other pituitary functions were evaluated by measuring basal prolactin, free T4, TSH, LH, FSH, testosterone or estradiol, and IGF-I levels in all patients and GH during hypoglycemia or stimulation test (n = 46).

#### Radiological imaging

Pituitary magnetic resonance imaging (MRI) was performed in 12 patients, using precontrast coronal spin-echo T1-weighted images, followed by postgadolinium T1-weighted imaging.

#### Genomic analysis of the TPIT gene

TPIT exons were PCR amplified and sequenced with flanking intronic primers. Amplification reactions were performed and the products purified as previously described (8). Internal primers were used for direct sequencing using ABI 3130xl genetic analyzer (Applied Biosystems, Foster City, CA).

#### Cell culture, transfections, and plasmids

GH3 and αT3 cells were cultured, transfected and harvested as previously described (8). New Tpit mutant expression vectors were generated using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). We generated a Tpit minigene that contains the flanking 100 bp of the first or fifth intron of human TPIT inserted into an expression plasmid that contains the human TPIT cDNA. We also amplified the full-length intron 1 (9.6 kb) or intron 5 (4.5 kb) and cloned it into the minigene.
construct. Results are presented as the mean ± SEM of three independent experiments done in duplicate.

**Protein extraction and Western blotting**

293T cells (3 × 10⁶) were transfected in 100-mm dish with 6 µg of appropriate expression vectors. We prepared total cell extracts 48 h later as described previously (12). αT3 cells (6 × 10⁵) were transfected in six-well-plates cells with 1 µg of expression vector. Whole-cell extracts were performed 48 h later by adding 200 µl of 1× Laemml buffer on cells, and the lystate was boiled for 5 min. Thirty micrograms of nuclear extract or 20 µl of whole-cell extract were analyzed by Western blotting as described previously (13) using homemade rabbit anti-Tpit antibody (1:1000), rabbit anti-RNA polymerase II (sc-899, 1:500), or mouse anti-α-tubulin (sc-32293, 1:5000; Santa Cruz Biotechnology, Santa Cruz, CA) and horseradish peroxidase-conjugated antirabbit or mouse IgG (1:20000; Sigma-Aldrich, St. Louis, MO). The result was revealed by chemiluminescence using an ECL + kit (GE Healthcare Biosciences Corp., Piscataway, NJ). In vitro-translated protein expression was assessed by the same method on a 2-µl reaction aliquot.

**Gel retardation assays**

Band shift experiments on the palindromic T-box consensus binding site were obtained following the probe-labeling and binding protocols described previously (8).

**Quantitative PCR (Q-PCR) on genomic DNA**

Primers amplifyng fragments of about 100 bp were designed in human TPIT exons and in different regions of the TPIT locus. MYOD1 and β-ACTIN genes were used as controls to normalize DNA concentration. PCR reactions using 2.5 ng genomic DNA were prepared, run, and analyzed according to the method described in the Quantitect SYBR green PCR kit (QIAGEN, Valencia, CA).

**PCR for splicing analysis and junction sequencing**

αT3 cells were transfected as above, and mRNA were purified with the RNeasy minikit (QIAGEN). cDNA were produced with Superscript III (Invitrogen, Carlsbad, CA) and primer oligo(dT)₂₀ following the manufacturer’s protocol. Primers were designed in exon or intron sequences of the human TPIT gene and combined in PCR using Taq polymerase (Invitrogen).

**Junction sequencing**

We used the same mRNA as above and produced cDNA with Superscript III (Invitrogen) and an antisense gene specific primer in exon 2. PCR amplification and purification were done as previously described with a sense primer in exon 1 and an antisense primer in exon 2. The same primers were used for direct sequencing.

**Results**

We analyzed a large panel of 91 IAD patients. Twenty-two of these had juvenile-onset IAD, whereas the remainder presented in the neonatal period (Table 1). The mean age at presentation of juvenile patients was 9 ± 6.7 yr (range 2–29 yr), and we did not find TPIT mutation in any, confirming our previous results (8). Among the 69 patients with neonatal-onset IAD, 57 had complete ACTH deficiency with low baseline plasma ACTH (6.9 ± 3.5 pM/liter, range 1–20 pM/liter) and cortisol (28 ± 10 nM/liter, range 20–55 nM/liter) levels, and 12 had partial ACTH deficiency (ACTH 23.1 ± 11.3 pM/liter; range 10–46 pM/liter; cortisol 106 ± 48 nM/liter, range 20–216 nM/liter). We found TPIT gene mutations in 37 of 57 patients with complete neonatal IAD (65%). The TPIT mutation group was comprised of 19 males (51%) and 18 females. Familial cases (i.e. more than one IAD patient in the family) were found in 64% of cases and consanguinity was found in 42% of cases (Table 1). Neonatal death occurred in 25% of these families. All patients had very low plasma ACTH (6.2 ± 3.3 pM/liter) and cortisol (24 ± 6 nM/liter) levels, with weak or no response to an iv CRH test. They all presented with severe neonatal hypoglycemia episodes associated with seizures in 53% of

| TABLE 1. Clinical and biochemical characteristics of neonatal-onset IAD patients with and without TPIT mutations and juvenile-onset IAD patients |
|--------------------------------------------------|--------------------------------------------------|--------------------------------------------------|
| **Neonatal IAD with TPIT mutations** | **Neonatal IAD without TPIT mutations** | **Juvenile IAD** |
| 37 patients | 32 patients | 22 patients |
| 29 families | 27 families | 22 families |
| Sex ratio (male/female) | 19/18 | 17/15 | 10/12 |
| Familial cases | 18/28 (64%) | 7/22 (32%) | 3/22 (14%) |
| Consanguinity | 10/24 (42%) | 3/22 (14%) | 1/22 (5%) |
| Neonatal death in the family | 7/28 (25%) | 5/16 (31%) | 0 |
| Induced labor | 2/13 (15%) | nd | nd |
| Neonatal hypoglycemia | 37/37 (100%) | 24/26 (92%) | 0 |
| Seizures | 16/30 (53%) | nd | nd |
| Prolonged cholestatic jaundice | 21/34 (62%) | 5/17 (29%) | 0 |
| Complete IAD | 37/37 (100%) | 20/32 (62%) | 14/22 (64%) |
| Normal pituitary MRI | 11/11 (100%) | nd | nd |

nd, Not determined.
cases. Neonatal hypoglycemia was reported even in the three cases in which an IAD diagnosis was made later in childhood (at 2, 2, and 6 yr, respectively). Most of these patients (62%) also suffered from prolonged cholestatic jaundice. In two of 13 cases (15%), the labor was induced at 42 wk of gestation, but in the other cases, normal term delivery was reported (Table 1). Two patients had partial and transient GH deficiency. GH reserve tested in another 44 patients was normal, and we did not report any growth retardation in our cohort. Pituitary MRI was normal. Because diagnosis and glucocorticoid treatment were initiated early, there was no evidence of mental retardation or psychomotor development defects for most patients. However, some sporadic clinical anomalies were documented: three cases of mental retardation, two of them likely due to frequent episodes of severe hypoglycemia associated with seizures; one case of congenital cardiomyopathy; two cases of Arnold-Chiari type I malformation in the same family; one case of triple X syndrome; one patient with mild dystrophic features such as flat nasal bridge, convergent squint, simple ears, short broad neck; and one case of unilateral choana atresia. In four of 12 sporadic patients who had neonatal partial ACTH deficiency, we found a monoallelic TPIT gene variant (6 bp insertion at the 3′-end of intron 1) that appears to be a polymorphism rather than a mutation as discussed below. Overall, we identified 21 different TPIT gene mutations in 37 patients with neonatal and complete IAD belonging to 29 families (Table 1 and Fig. 1). Most of these IAD patients (35 of 37) are homozygous or compound heterozygous for a TPIT loss-of-function mutation, and their parents are all unaffected heterozygous carriers. In two patients with complete neonatal IAD, we found only one TPIT mutant allele in coding sequences. This study revealed nine new mutations. These mutations are located throughout the TPIT gene but affect mainly the T-box domain of the protein (Fig. 1). There are four missense mutations, one one-nucleotide deletion, one large 20-kb deletion encompassing exon 1, and three splicing site mutations (see Figs. 1, 2, and 4).

Missense mutations
We identified four new missense mutations located in exon 2 affecting the T-box domain that stretches between amino acids 40 and 222 and is essential for DNA binding (Figs. 1 and 2A). Mutations R100H, H125Y, G132V, and K146R were inserted into expression vectors encoding Tpit and the plasmids were transfected in tissue culture cells to compare transcriptional activity and DNA-binding ability. All these mutant Tpit proteins did not exhibit significant, if any, activation of a Tpit/Pitx1 reporter gene compared with wild-type Tpit (Fig. 3A). In addition, we did not observe any monomeric nor dimeric binding on a palindromic consensus T-box binding site (Fig. 3B). This loss of DNA-binding ability is consistent with the location of these mutated residues in the T-box region of Tpit.

New large deletion mutation
To date, neonatal IAD linked to TPIT gene mutations have always been inherited in a recessive mode. Thus, most IAD patients are homozygous or compound heterozygous for a TPIT gene mutation and their parents are heterozygous carriers and unaffected. However, in some patients we found only one mutated allele. To identify heterozygous large deletions that would not be detected by direct sequencing of TPIT coding regions...
in putative compound heterozygous patients, we used a new method based on Q-PCR. We designed primers to amplify regions of about 100 bp overlapping each TPIT exon and then performed Q-PCR on genomic DNA. We tested all putative combined heterozygous patients in which we previously found a TPIT mutation in only one allele. We validated this method by confirming the already described mutation nt247del37bp for which we counted only one copy of exon 2 (data not shown). More interestingly, we detected only one copy of exon 1 in a heterozygous patient carrying the monoallelic nt782delA TPIT mutation inherited from her mother (Fig. 4, A and B). To map this deletion, we scanned the TPIT locus from −20 kb to +10 kb with a series of

![DNA sequence and pedigree of unpublished patients with TPIT mutations. A, Missense mutations in the TPIT coding sequence. B, Heterozygous and homozygous splicing site mutations. Black symbol represents the mutant allele. In cases of compound heterozygous patients, the second mutant allele is illustrated in the gray color to distinguish the two mutations.](https://academic.oup.com/jcem/article-abstract/97/3/E486/2536594)
primer pairs. We thus delimited a large deletion extending from −14 kb to +6 kb that presumably came from the paternal grandfather (father’s genomic DNA was not available) (Fig. 4, B, C, and D). This patient is therefore a compound heterozygote with a 1-bp deletion (nt782delA) and a large 20-kb deletion, which is consistent with the neonatal IAD phenotype observed in this patient.

**Splicing site mutations**

We identified three new intronic TPIT gene mutations: one affecting the first nucleotide of the splice site at the 5′-end of the first intron (IVS1 + 1 G→A); the second one affects the first nucleotide of the splice site at the 5′-end of the fifth intron (IVS5 + 5 G→A). According to the splice database (SpliceDB), 98.7% of known expressed sequence tag sequences contain canonical GT-AG junctions and 0.56% have noncanonical GC-AG splice site pairs (14). We also identified a mutation that affects the fifth nucleotide of the splice site at the 5′-end of the fifth intron (IVS5 + 5 G→A) in 2 unrelated patients and a monoallelic 5-bp duplication (IVS1–15 ins5bp) in 4 unrelated patients (Fig. 2B) at the 3′-end of the first intron between the branch site and the acceptor site in 4 patients with partial neonatal IAD. To be sure that this sporadic heterozygous mutation (not found in 50 control alleles) could be responsible for the partial phenotype observed in these patients (as opposed to other unidentified genomic defect), we completed an *in vitro* assessment for the typical IVS1 + 1 G→A as a control and IVS1–15 ins5bp, using a TPIT minigene (Fig. 5A). As expected, we found that the IVS1 + 1 G→A mutant protein is devoid of transcriptional activity with an absence of protein expression, even if mRNA levels are equivalent. Conversely, we showed that IVS1–15 ins5bp was more efficient than wild-type Tpit to transactivate a reporter gene (Fig. 5B). This was correlated to higher levels of mRNA for IVS1–15 ins5bp compared with wild-type Tpit (Fig. 5C and D). To assess splicing anomalies, we performed PCR on cDNA using primers flanking exon 1 and 2 (Fig. 5E). Using primer set no. 2, we observed that only the longest 742-bp (unspliced) fragment was detected for either the wild type or IVS1–15 ins5bp (Fig. 5F). Splicing efficiency was completely abrogated for IVS1 + 1 G→A mutant and 10 times more frequent for IVS1–15 ins5bp compared with the wild type (Fig. 5G). Direct sequencing of exon 1 and 2 junction confirmed that splicing process was correctly performed in IVS1–15 ins5bp (Fig. 5H). IVS1 + 1 G→A mutant junction could not be sequenced because splicing never happens. Each of these experiments (except Fig. 5H) was also done with full-length intron 1 constructs and generated equivalent results (data not shown). According to these experiments, only IVS1 + 1 G→A caused splicing defect. This result is consistent with the location of this mutation that affects the consensus donor splicing site of intron 1. The insertion of intron 1 could result in
an mRNA with a stop codon 64 bp after the 3’end of exon 1, which could lead to nonsense-mediated mRNA decay (15, 16) or to the generation of an 88-amino acid truncated protein (P88X) unrecognized by our TPIT antibody. IVS1–15 ins5bp increased in vitro Tpit splicing, suggesting that this insertion was not responsible for the phenotype of partial IAD observed in our patients. The IVS5 mutations were predicted to affect splicing, and this was indeed observed when tested using the full-length intron 5 construct because there was no IVS5 + 1 and IVS5 + 5 protein expression compared with the wild type (data not shown).

Discussion

This study defined three groups of IAD patients: juvenile-onset IAD, neonatal-onset partial IAD, and neonatal-onset complete IAD. Patients with juvenile-onset IAD and/or partial IAD were not associated with a TPIT gene mutation, as previously described in a subset of patients (8, 17). The genetic investigation of neonatal-onset IAD patients highlighted a high frequency of TPIT mutations (65%) in the subset that had complete ACTH deficiency. Complete neonatal IAD is characterized by a very homogeneous phenotype and inherited by an autosomal recessive mode. Prolonged neonatal jaundice observed in more than half of the IAD patients is most often associated with cholestatic hepatitis. In our series, two patients had hepatic needle biopsy revealing accumulation of biliary pigments in the liver cells and giant cells cholestatic hepatitis. In most cases of our series and in literature, this cholestasis improved shortly with glucocorticoid replacement (18, 19). Thus, it might be attributable to hypocortisolism, but the exact pathogenic mechanism remains unclear. The study cohort included limited associated signs or symptoms. Psychomotor development was normal in all patients treated with appropriate glucocorticoid replacement. A high rate of neonatal death (25%) is still observed despite an increased level of awareness of this rare condition since its first description. Low maternal estriol plasma levels during pregnancy should alert pediatricians and help reduce this high neonatal mortality (20). In our series, only two patients were born after an induced labor at 40 wk gestation. Other parturitions were normal, spontaneous (from 34 to 39 wk gestation), or by cesarean section in one case of twins. This observation confirms that the role of fetal cortisol in labor induction remains secondary in humans (21).

To date, we have identified 21 different mutations in 29 different families. Although TPIT mutations are found throughout the TPIT protein, they are mainly in the T box, resulting in DNA binding defects. Only the M86R mutation located within the T box caused protein-protein interaction defects (22). One mutation (nt782delA) was
Intronic mutations affect transcriptional activity. Transfection assays in 
IVS1/H11001-dependent reporter, whereas IVS1–15 ins5bp increases the Tpit transcriptional potential. C, Protein expression levels vary, depending on intronic mutations. The IVS1 + 1 G>A mutant did not yield detectable Tpit, whereas IVS1–15 ins5bp protein is overexpressed. CHX, Cycloheximide. D, Intronic mutations do not affect mRNA transcript levels assessed by Q-PCR in αT3 expressing mutated transcripts. Ctl, Control; WT, wild type. E, Experimental design to demonstrate that the splicing of mRNA is affected by intronic mutations. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase. F, PCR on cDNA samples show the variation in the ratio of spliced vs. unspliced messenger. G, Splicing site mutations affect splicing efficiency, IVS1 + 1 G>A abrogates splicing, whereas there is a 10-fold increase with IVS1–15 ins5bp. H, Sequencing of the mRNA splice junction between exon 1 and exon 2 in putative splicing mutants. The junction sequence is normal for the IVS1–15 ins5bp mutant. IVS1 + 1 G>A mRNA is not spliced so the junction cannot be sequenced.

**FIG. 5.** New mutations in intronic sequences cause mRNA splicing abnormality. A, Schematic representation of IVS1 + 1G>A and IVS1–15 ins5bp intronic mutations. B, Intronic mutations affect transcriptional activity. Transfection assays in αT3 cells show that IVS1 + 1 G>A mutation completely abrogates activation of the POMC Tpit/PitxRE-dependent reporter, whereas IVS1–15 ins5bp increases the Tpit transcriptional potential. C, Protein expression levels vary, depending on intronic mutations. The IVS1 + 1 G>A mutant did not yield detectable Tpit, whereas IVS1–15 ins5bp protein is overexpressed. CHX, Cycloheximide. D, Intronic mutations do not affect mRNA transcript levels assessed by Q-PCR in αT3 expressing mutated transcripts. Ctl, Control; WT, wild type. E, Experimental design to demonstrate that the splicing of mRNA is affected by intronic mutations. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase. F, PCR on cDNA samples show the variation in the ratio of spliced vs. unspliced messenger. G, Splicing site mutations affect splicing efficiency, IVS1 + 1 G>A abrogates splicing, whereas there is a 10-fold increase with IVS1–15 ins5bp. H, Sequencing of the mRNA splice junction between exon 1 and exon 2 in putative splicing mutants. The junction sequence is normal for the IVS1–15 ins5bp mutant. IVS1 + 1 G>A mRNA is not spliced so the junction cannot be sequenced.
patients with a similar IAD phenotype and normal TPIT coding sequences may have altered regulatory sequences. TPIT regulatory sequences have not yet been defined, although we have attempted to do so in transgenic mice. These studies (unpublished) indicated that critical sequences are likely quite distant from Tpit coding sequences. Because the mouse regulatory sequences have not yet been identified, we have not performed any PCR amplification of human upstream or intronic TPIT sequences. Our group recently characterized a novel pituitary-specific POMC enhancer, with preferential activity in corticotroph cells (24). This enhancer is highly conserved and the human enhancer was found to be hormone responsive. It is possible that this enhancer might play a role in the maintenance of POMC expression; hence, its inactivation could have jeopardized corticotroph function in IAD patients. We investigated this enhancer for putative mutations in 34 IAD patients without TPIT mutations (10 neonatal IAD, six partial IAD, 18 juvenile onset IAD), but we did not find any mutation or polymorphism.

In conclusion, we described the largest series of 91 patients with IAD showing a clear phenotype-genotype relationship between neonatal onset of complete IAD and TPIT mutations. We characterized nine new TPIT loss-of-function mutations, confirming the essential role of TPIT in corticotroph development. Moreover, our work allowed the improved clinical definition of IAD patients by identifying three distinct groups of juvenile, neonatal onset, complete, and partial IAD.

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