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CONGENITAL HYPOTHYROIDISM AND THOX2 MUTATIONS

INACTIVATING MUTATIONS IN THE GENE FOR THYROID OXIDASE 2 (THOX2) AND CONGENITAL HYPOTHYROIDISM

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ABSTRACT

Background Several genetic defects are associated with permanent congenital hypothyroidism. Immunologic, environmental, and iatrogenic (but not genetic) factors are known to induce transient congenital hypothyroidism, which spontaneously resolves within the first months of life. We hypothesized that molecular defects in the thyroid oxidase system, which is composed of at least two proteins, might be involved in the pathogenesis of permanent or transient congenital hypothyroidism in babies with defects in iodide organization, for which the oxidase system is required.

Methods Nine patients were recruited who had idio pathic congenital hypothyroidism (one with permanent and eight with transient hypothyroidism) and an iodide-organization defect and who had been identified by the screening program for congenital hypothyroidism. The DNA of the patients and their relatives was analyzed for mutations in the genes for thyroid oxidase 1 (THOX1) and 2 (THOX2).

Results The one patient with permanent and severe thyroid hormone deficiency and an iodide-organization defect had a homozgyous nonsense mutation in the THOX2 gene that eliminates all functional domains of the protein. Three of the eight patients with mild transient congenital hypothyroidism and a partial iodide-organization defect had heterozygous mutations in the THOX2 gene that prematurely truncate the protein, thus abolishing its functional domains.

Conclusions Biallelic inactivating mutations in the THOX2 gene result in complete disruption of thyroid-hormone synthesis and are associated with severe and permanent congenital hypothyroidism. Monoallelic mutations are associated with milder, transient hypothyroidism caused by insufficient thyroidal production of hydrogen peroxide, which prevents the synthesis of sufficient quantities of thyroid hormones to meet the large requirement for thyroid hormones at the beginning of life. (N Engl J Med 2002;347:95-102.)

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Congenital hypothyroidism is the most common congenital endocrine disorder, affecting 1 in every 3000 to 4000 newborns. Neonatal screening programs allow its early detection and treatment, thus preventing the cognitive and motor impairment caused by lack of thyroid hormone during the early postnatal phase of brain development. The need for thyroid hormone supplementation can be permanent or transient.

The cause of permanent congenital hypothyroidism of primary origin has been linked to defects in proteins involved in the synthesis of thyroid hormones and to defects in transcription factors involved in the development of the thyroid gland. Overall, these cases represent a small percentage of the population with congenital hypothyroidism, and the cause of the vast majority of cases remains unknown.

Transient congenital hypothyroidism can be caused by iodine deficiency, exposure to excess iodine in the perinatal period (e.g., from the use of iodinated disinfectants or contrast agents), or fetal exposure to either maternally derived thyroid-blocking antibodies or antithyroid drugs taken by pregnant women with thyroid autoimmune disease. Congenital thyroid dysfunction can also be a consequence of premature birth or, in rare cases, of protein-losing nephrosis. However, in about 20 percent of patients with transient disease, the cause remains elusive. Sporadic reports have suggested that transient congenital hypothyroidism might be related to mild thyroid dys hormogenesis.

The generation of hydrogen peroxide is a critical step in the synthesis of thyroid hormones. Hydrogen peroxide is used as a substrate by thyroid peroxidase in the incorporation of iodine into thyroglobulin, an essential step of thyroid hormonogenesis as organification. The thyroid oxidase 1 (THOX1) and 2 (THOX2) proteins have recently been identified as components of the hydrogen peroxide generation system of the thyroid. The level of expression of the THOX2 gene, as determined by serial analysis of gene expression, is at least five times as high as that of THOX1. The THOX1 and THOX2 genes encode two very similar proteins that are inserted in the apical membrane of the thyroid follicular cell. The structure of these proteins includes seven putative transmembrane domains, four NADPH-binding sites and one flavine adenine dinucleotide (FAD)–binding site, and (unlike other human oxidases) two EF-hand motifs.
Most inborn errors of thyroid hormone synthesis are caused by defects in iodide organification. To date, patients with thyroid-organification defects have been shown to harbor mutations in the genes encoding thyroid peroxidase, thyroglobulin, and pendrin.27–29 We tested the hypothesis that mutations in the thyroid oxidase system are the molecular basis for apparently idiopathic cases of congenital hypothyroidism with an iodide-organification defect.

METHODS

Selection of Patients

Patients with congenital hypothyroidism were selected for genetic screening after written informed consent had been obtained from the parent or guardian. The inclusion criterion was the presence of an iodide-organification defect, as determined by a positive intravenous perchlorate test (discharge, 10 percent or more) in the neonatal period. Patients who had iodide-organification defects of known cause were excluded. The causes included complete iodide-organification defects with mutations in the thyroid peroxidase gene27; Pendred’s syndrome or mutations in the PDS gene, which encodes the iodide transporter pendrin28; and biochemical indicators of thyroglobulin-synthesis defects or mutations in the thyroglobulin gene.31 Patients who had transient congenital hypothyroidism of known cause were also excluded. The causes included maternal thyroid autoimmune disease, maternal use of antithyroid drugs during pregnancy, an excess or shortage of iodine, and premature birth.

Evaluation of Clinical Data

Data on gestational age, mode of delivery, birth weight, and documented use of iodinated products were collected from clinical files. In the Dutch screening program for congenital hypothyroidism, the total thyroxine in a filter-paper blood spot is determined, normally within the first week of life. When thyroxine values are less than or equal to −0.8 SD of the mean value on the standard daily distribution curve, thyroxine is measured. When the screening results are abnormal, plasma thyrotropin, total thyroxine, free thyroxine, total triiodothyronine, thyroxine-binding globulin, and thyroglobulin are determined. Urinary excretion of iodine is determined within the first three weeks of life.32 Before the start of thyroxine treatment, when dysshormonogenesis is suspected, the uptake of iodine-123 by the thyroid is measured, followed by the administration of sodium perchlorate. Thyroid hormone therapy is monitored by periodic determinations of plasma thyrotropin and free thyroxine levels, and the thyroxine dose is adjusted accordingly. Therapy is stopped in patients with suspected transient hypothyroidism when they reach the age of three years. Four weeks later, thyrotropin, thyroxine, and triiodothyronine are measured. All these measurements obtained from the study patients were compared with those of 44 patients with congenital hypothyroidism who had a complete iodide-organification defect due to mutations in the thyroid peroxidase gene.

Perchlorate-Discharge Test

The perchlorate challenge was performed according to a protocol adapted for neonates.33 After intravenous administration of 0.9 MBq (25 μCi) [131I]sodium iodide, thyroidal uptake of the isotope was monitored every 30 minutes with a gamma camera and a pinhole collimator. At 120 minutes, 100 mg of sodium perchlorate was given intravenously, and the decrease in radioactivity in the thyroid was determined at 150 and 180 minutes. The percent discharge of iodine from the thyroid gland was calculated as the ratio between the uptake 60 minutes after perchlorate administration and the uptake just before perchlorate administration, multiplied by 100. A discharge value above 10 percent indicates failure to retain the administered radioiodine, usually because of a defect in organification.

Identification of the Genomic Organization of the THOX1 and THOX2 Genes

The GenBank data base was screened with the THOX1 and THOX2 complementary DNA sequences (AF230495 and AF230496, respectively). From three human genomic clones (contigs) on chromosome 15 (AC009700.4, AC12255.4, and AC051619), the intron–exon boundaries of the THOX genes were analyzed, and the number of coding exons was determined.

Detection of Mutations

After written informed consent had been obtained, genomic DNA was isolated from the venous blood of patients and first-degree relatives, together with 100 control subjects of white, black, and Asian origin. The complete coding region of the human THOX1 and THOX2 genes, including intron–exon boundaries, was amplified from genomic DNA with use of the polymerase chain reaction (PCR) and sense and antisense primers designed on the basis of the genetic sequences. The PCR fragments were analyzed on an Agilent 1100 HPLC system, equipped with a Zorbax double-stranded DNA temperature-controlled column and a Diode array detector.33,34 The oligonucleotide sequences, PCR-amplification process, and conditions used in chromatography are described in Supplementary Appendix 1 (available with the full text of this article at http://www.nejm.org). Samples showing an aberrant chromatographic pattern were directly sequenced with fluorescent dideoxynucleotide primers (Big Dye, Perkin Elmer Applied Biosystems) on an automated DNA sequencer (ABI S1000, Perkin Elmer Applied Biosystems).

The relatives of the patients and the 100 controls underwent genotyping by heteroduplex analysis, sequencing, or digestion of the respective DNA-amplified PCR products with appropriate restriction enzymes, according to the specifications of the manufacturers. The sponsors of this study had no involvement in the design of the study; in the collection, analysis, and interpretation of data; or in the writing of the report.

RESULTS

Pattern of Congenital Hypothyroidism in Selected Patients

From an original cohort of 45 patients with severe congenital hypothyroidism at screening and a complete iodide-organification defect, 44 patients were excluded because of mutations in the thyroid peroxidase gene, leaving 1 patient of this group in the study (Patient 1 in Table 1). From an original cohort of 15 patients who had mild hypothyroidism at screening that proved to be transient during follow-up and a partial iodide-organification defect, 4 were excluded because of iodine intoxication (1 patient), putative thyroglobulin-synthesis defects (2 patients), or Pendred’s syndrome (1 patient). Another three of these patients were not available for genetic testing. Thus, eight patients in this group were studied (Patients 2,
3, and 4 and the five patients with a partial organification defect). Table 1 shows the clinical data from these patients and their status with respect to mutations in the THOX1 and THOX2 genes.

At screening, Patient 1 had thyroxine levels below the limit of detection and very high thyrotropin levels. Subsequent diagnostic procedures showed a properly located gland with a high uptake of iodine-123 and complete discharge of iodide in the perchlorate test. This patient requires continued thyroid hormone therapy.

At screening, Patient 2 had mildly decreased thyroxine and elevated thyrotropin levels, whereas Patients 3 and 4 had thyroxine values in the low-normal range (0.8 and 1.1 SD below the mean value of the daily distribution curve, respectively) and hyperthyrotropinemia. Routine determination of thyrotropin in blood spots at thyroxine levels below 0.8 SD of the

<table>
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<th>TABLE 1. Thyroid Function in Patients with Iodide-Organification Defects and Mutations in the THOX2 Gene.*</th>
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*Plus–minus values are means ±SD. NA denotes not applicable, and dashes not available. To convert values for thyroxine to nanomoles per liter, multiply by 1.5; and to convert values for urinary iodine to nanomoles, multiply by 7.87. To convert values for thyroxine-binding globulin to nanomoles per liter, multiply by 18.5; to convert values for thyroglobulin to picomoles per liter, multiply by 1.5; and to convert values for urinary iodine to nanomoles, multiply by 7.87.
†The values listed are those used in our institution if not otherwise stated.
‡Data and cutoff levels were obtained from the Dutch screening program for congenital hypothyroidism.
§Values are from Fisher.
¶Values are from Nelson et al.
‖Values are from Vulsma et al.
**Values were as measured in healthy adults two hours after the administration of radioiodide.
††Values are from McDougall and Cavalieri.
‡‡This uptake study was performed when the patient was three years of age.
§§These mutant proteins are prematurely truncated at the amino acid positions indicated.
¶¶This frame-shift mutation at position 965 truncates the protein at position 994.
|||The mutations have been described by Bakker et al.
Figure 1. Mutations in the Thyroid Oxidase 2 Gene (THOX2) in Patients with Permanent and Transient Congenital Hypothyroidism.

Sequencing chromatograms of genomic DNA from control subjects and patients are shown. Arrows indicate the positions of identified mutations. Single chromatogram peaks (C1300T) indicate homozygosity at the mutant locus. Two overlapping peaks at the same locus (C2056T and C2101T) denote heterozygous mutations. Double overlapping patterns of chromatogram peaks (2895–2898del) represent heterozygous frame-shift mutations. The C1300T, C2056T, and C2101T nucleotide changes induce premature stop codons (TGA or TAG) that truncate the corresponding proteins. The 4-bp deletion (GTTC) induced by the 2895–2898del mutation causes a shift in the reading frame that leads to a stop codon (TGA) after the coding of 29 aberrant amino acids.
The mean value of the daily distribution curve allowed the detection of hyperthyrotropinemia and subsequent referral of Patients 3 and 4. Iodine-123-uptake studies in each case showed a properly located gland with a partial (40 to 66 percent) discharge of iodide in the perchlorate test. After several adjustments in dosage, these three patients were given very low doses of thyroxine (mean, 1.3 µg per kilogram of body weight per day), and after they reached the age of three years, therapy was stopped for diagnostic purposes. All of them remained euthyroid during the follow-up period of 12 months.

The same mild and transient phenotype of congenital hypothyroidism was present in the other five patients with a partial iodide-organification defect in whom no mutations in the THOX genes were identified. The phenotype of this subgroup clearly differed from that of patients who had severe congenital hypothyroidism with complete iodide-organification defects due to mutations in the gene for thyroid peroxidase (Table 1).

**Screening for Mutations of the THOX1 and THOX2 Genes**

The open reading frames of the THOX1 and THOX2 genes are divided among 33 exons, spanning 36 and 22 kb, respectively, on the long arm of chromosome 15. All 33 coding exons for both genes were PCR-amplified from genomic DNA of the patients.

Analysis of PCR products by denaturing high-performance liquid chromatography showed multiple aberrant patterns. Most of them were also present in normal control alleles and are considered nonfunctional polymorphisms. The samples corresponding to the aberrant chromatographic patterns of exon 11 (Patient 1), exon 16 (Patients 2 and 3), and exon 21 (Patient 4) of the THOX2 gene were directly sequenced, revealing three different single-nucleotide changes and a 4-bp deletion. Patient 1 was homozygous for the mutation, and Patients 2, 3, and 4 were heterozygous (Fig. 1). These changes were absent in 100 control alleles. In Patient 1, exon 11 of THOX2 had a homozygous substitution of thymine for cytosine at position 1300 (C1300T) that generates a premature termination signal (R434X). Patient 2 was heterozygous for the C2056T mutation in exon 16 of THOX2, which also generates a premature stop codon instead of the incorporation of a glutamine (Q686X). Patient 3 was heterozygous for the C2101T nonsense mutation in exon 16 of THOX2, which changes arginine 701 into a premature termination signal (R701X). Patient 4 had a monoallelic deletion of GTTC at position 2895 (2895–2898del) in exon 21 of THOX2 that introduces a frame shift generating a termination signal in exon 22 (S965fsX994). Southern blotting found no evidence of chromosomal deletions in the THOX genes (data not shown).

**Figure 2.** Functional Domains and Mutations of the Thyroid Oxidase 2 (THOX2) Protein.

Arrows indicate the places where mutant proteins are prematurely truncated. The frame shift induced by the S965fsX994 mutation codes for 29 aberrant amino acids before truncation. The relative position of calcium-binding (EF-hand), flavine adenine dinucleotide (FAD)–binding, and NADPH-binding motifs are indicated.
All four \textit{THOX2} mutations should induce premature stop codons that delete the predicted functional hydrogen peroxide–generating domains and are considered inactivating mutations (Fig. 2).

**Pedigree Analysis**

The parents of Families 1 and 2 (from Turkey) and Family 3 (from Surinam) settled in the Netherlands before the 1980s, and most of their descendants participated in the Dutch screening program for congenital hypothyroidism. Family 4 has a white Dutch background. The parents were all born before the screening program was instituted. A total of 17 persons from two generations were available for hormonal and genetic testing. The levels of thyrotropin, thyroxine, free thyroxine, triiodothyronine, and thyroglobulin and the thyroid size were normal in every member of the four families except for the patients (data not shown). The genotypes of the available family members were determined by direct sequencing, restriction-site analysis, or heteroduplex analysis (Fig. 3).

In Family 1, the index patient (Subject II-1 in Fig. 3), the product of a consanguineous marriage, was homozygous for the C1300T mutation in \textit{THOX2}. Her father (Subject I-1), mother (Subject I-2), and brother (Subject II-2) were heterozygous and had normal thyroid function.

In Family 2, the index patient (Subject II-3 in Fig. 3) and her father (Subject I-1) were heterozygous for the C2056T \textit{THOX2} mutant allele, whereas the mother (Subject I-2) was homozygous for the wild-type allele. The patient’s older brother (Subject II-1) did not carry the mutation and had normal screening values. Subject II-2 was born in Turkey and was not available for screening.

In Family 3, the index patient (Subject II-2 in Fig. 3), her brother (Subject II-1), and her mother (Subject I-2) had the \textit{THOX2} C2101T mutation. The results of screening of the patient’s brother (Subject II-1) for congenital hypothyroidism were reported to be normal. Retrieval of these results after 10 years showed a blood-spot thyroxine value of 12 µg per deciliter (154 nmol per liter), corresponding to −0.6 SD of the mean value of the daily thyroxine-distribution curve, just above the cutoff level for thyrotropin determination in the Dutch screening program. This borderline blood-spot thyroxine value might have

**Figure 3. Segregation Analysis of Mutations in the Thyroid Oxidase 2 Gene (\textit{THOX2}) in Three Families with Permanent or Transient Congenital Hypothyroidism.**

Squares indicate male family members, and circles female family members. Arrows indicate index patients, the solid symbol a patient with permanent congenital hypothyroidism and a complete iodide-organification defect, the hatched symbols patients with transient congenital hypothyroidism and partial iodide-organification defects, and the stippled symbol a patient with a thyroxine-screening result 0.6 SD below the reference value. Gels show restriction-fragment–length polymorphism (RFLP) and heteroduplex analyses of mutations. Polymerase-chain-reaction (PCR) fragments were stained with ethidium bromide. Fragments corresponding to the mutant alleles are indicated by asterisks. The \textit{THOX2} C1300T and C2101T mutations introduce a \textit{Dde}I site, and an \textit{Ava}II site is destroyed by the C2056T mutation. The 2895–2898del mutation cannot be distinguished by restriction-site analysis, but in this case the presence of the mutated allele results in a heteroduplex PCR fragment with an aberrant pattern on agarose gel.
caused a false negative screening result. At 10 years of age, Subject II-1 was euthyroid and performed normally at school. His target height was 173.5 cm, and he was growing at –2.0 SD of the mean value on the Dutch standard growth curve, whereas his sister (Subject II-2), with a target height of 159 cm, was growing at –0.8 SD of the standard curve.

In Family 4, the index patient (Subject II-2 in Fig. 3) inherited the THOX2 2895–2898del mutation from her father (Subject II-1). Her older brother, who had normal screening results, did not carry the deletion.

**DISCUSSION**

The generation of hydrogen peroxide is an essential step in the synthesis of thyroid hormones. Over the past three decades, a few cases of thyroidal hydrogen peroxide deficiency have been described, but the molecular basis of these defects has not been investigated.39-41

We studied the genomic organization and screened the THOX1 and THOX2 genes for mutations in patients with congenital hypothyroidism and iodide-organification defects. Mutations in the THOX2 gene were present in one patient with a complete iodide-organification defect and permanent congenital hypothyroidism and in three of eight patients with partial iodide-organification defects and transient congenital hypothyroidism. All mutations resulted in premature stop codons that delete the NADPH- and FAD-binding sites of the THOX2 protein. Functional studies of gp91phox, a protein of the phagocyte oxidase system that is homologous with the THOX proteins, show that truncation of these sites leads to complete loss of activity.32

The patient with severe and permanent congenital hypothyroidism was homozygous for a THOX2 inactivating mutation. This finding proves that abolishing functional THOX2 protein completely blocks thyroid hormone synthesis.

The three patients with a milder and transient form of the disease were heterozygous for three other inactivating mutations, suggesting that insufficiency of the THOX2 protein within the thyroid oxidase complex was the underlying mechanism of disease. However, the neonatal euthyroid profile of Subject II-2 in Family 1, who was heterozygous for the C1300T mutation, might indicate the existence of dominant negative properties in mutant proteins 2, 3, and 4 that are not present in mutant protein 1. In contrast to mutant 1, mutants 2, 3, and 4 retain the hydrophobic stretch of the first transmembrane domain of the THOX2 protein, which might allow the insertion of the protein in the membrane and aberrant interactions with other components of the oxidase system.43 It is tempting to speculate that these putative other components are involved in the molecular basis of the transient congenital hypothyroidism of the patients in our study who did not have THOX1 or THOX2 mutations.

Partial insufficiency of the THOX2 protein, resulting in diminished hydrogen peroxide production, is present only during the first weeks or months of postnatal life, when the requirement for thyroid hormones is large. On the basis of evidence that transient congenital hypothyroidism and hyperthyrotropinemia are associated with impaired intellectual development in children, we advise treatment of these patients as soon as possible after birth, as well as thyroid-function tests in newborn siblings of children with transient congenital hypothyroidism. Furthermore, it is important to follow these patients for subclinical or overt hypothyroidism, goiter, or both in adolescence and adulthood, especially during pregnancy, when the need for thyroxine increases. If the expression of this genetic disorder recurs during pregnancy, the neurologic development of the offspring can be hampered.45,46

In conclusion, biallelic and monoallelic inactivating mutations in the THOX2 gene are associated with permanent and transient congenital hypothyroidism, respectively. These findings prove that the THOX2 protein is an essential component of the thyroid system of hydrogen peroxide generation. Furthermore, they represent to our knowledge the first demonstration that transient congenital hypothyroidism can be genetically determined and show that thyroid dys hormonogenesis is involved in the transient form of congenital hypothyroidism.

**REFERENCES**


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