New insights in the human thyroglobulin structure
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A premature stop codon in thyroglobulin mRNA results in familial goiter and moderate hypothyroidism.

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ABSTRACT

Impaired thyroglobulin (Tg) synthesis is one of the putative causes for dyshormonogenesis of the thyroid gland. This type of hypothyroidism is characterized by intact iodide trapping, normal organification of iodide, usually low serum Tg levels in relation to high TSH and when untreated the patients develop goiter.

In thyroid tissue from a thirteen year old patient, suspected of a thyroglobulin synthesis defect, the Tg mRNA was studied. The complete coding region of 8307 bp was directly sequenced and revealed a homozygous point mutation: a C886T transition in exon 7. Upon translation this mutation would result in a stop codon at amino acid position 277 replacing the arginine residue. A Tg cDNA construct containing the mutation was expressed in rabbit reticulocyte lysate resulting in a truncated protein of 30 kDa. Expression in the presence of microsomal membranes resulted in a gel shift of this Tg molecule indicating glycosylation ability.

Two other siblings had a clinical presentation like the index patient while their parents were unaffected. Additional RFLP analysis of the pedigree verified that the homozygous nonsense mutation cosegregated with the clinical phenotype.

Clinically, hypothyroidism was not severe in the affected siblings because the truncated Tg glycoprotein was still capable of thyroid hormonogenesis.
INTRODUCTION
Primary congenital hypothyroidism (CH) is caused by disorders of thyroid gland development (80%) or dyshormonogenesis (20%). In thyroid dyshormonogenesis a mutation is expected in one of the genes encoding a key protein involved in the biosynthesis of thyroid hormones (1, 2).

One of these proteins is thyroglobulin (Tg), the predominant glycoprotein (660 kDa) of the thyroid gland which functions as matrix protein in thyroid hormonogenesis. Catalyzed by thyroid peroxidase (TPO), tyrosine residues in the Tg molecule are iodinated and subsequently some specific ones are coupled to form mainly $T_4$ and some $T_3$ (2, 3). The human Tg gene, located on chromosome 8 (8q24.2-8q24.3), is over 300 kb and contains over 37 exons (4, 5, 6). We recently revised the Tg mRNA sequence that was originally reported in 1987 (7). This revealed 8307 nucleotides of coding sequence (instead of 8304) of which 66 triplets (instead of 67) encode a tyrosine residue (8). Up till now nineteen polymorphisms have been identified in the thyroglobulin gene locus of which 8 result in amino acid residue variation (8, 9, 10). Furthermore, at least 12 alternative splice products have been identified in normal Tg mRNAs (10-16). Beside these wildtype variations, some mutations in the Tg gene have been identified in animal models and in man resulting in aberrant Tg protein expression and linked to subsequent impaired thyroid hormone synthesis.

In Afrikander cattle a homozygous nonsense mutation, Arg697OPA (exon 9), results in the expression of a truncated Tg protein of 75 kDa. In this case also an alternatively spliced mRNA lacking exon 9 sequence is observed, encoding a Tg protein of 250 kDa (17, 18). In Dutch goats a homozygous nonsense mutation, Tyr296AMB, results in a truncated Tg protein product (40 kDa in vivo) and causes hypothyroidism with goiter (19, 20). Furthermore, in a mouse model, congenital goiter (cog/cog) is linked to the Tg locus (21) and the mutation has recently been identified as Leu2366Pro (22).

So far only in three patients with congenital hypothyroidism and goiter a mutation in the Tg gene has been elucidated. A homozygous mutation at the acceptor splice site of intron 3 results in the in frame deletion of exon 4 sequences (nt 275 - 478) from the mRNA which results in an aberrant Tg protein lacking hormonogenic site Tyr130 (15). A homozygous in frame mRNA deletion is described of 138 bp (nt 5552 - 5789)(23). The preferential accumulation of a Tg mRNA alternative splice product with an in frame deletion of 171 bp (nt 4529 - 4699, exon 22) has also been reported, linked to a homozygous nonsense mutation at position 1510 (13).

In the present study, we have identified a homozygous nonsense mutation in the
thyroglobulin mRNA of a moderately hypothyroid patient with goiter.

MATERIAL AND METHODS

Patients

Figure 1 shows the pedigree of a Brazilian family with goiter with three affected siblings (Table 1).

Patient IV-2: female, first examined at the age of 17. She developed normally and had menarche at 15 yr of age. Her height was 149 cm and her weight 43.5 kg. The thyroid gland was diffusely enlarged (65 g; normal: 7.4 → 2.2 g) and ultrasonography indicated a nodule of 15 mm in the left lobe with micro calcifications. She has two unaffected children (V-1, V-2) from a consanguineous marriage.

Patient IV-6 (index patient): male, first examined at the age of 13. At presentation, he showed clinical signs of hypothyroidism and stunted growth (bone age 7 yr). His mental function was normal. The thyroid gland was diffusely enlarged (60 g; normal: 7.4 → 2.2 g) and ultrasonography indicated a nodule of 13 mm in the right lobe.

Patient IV-10: male, first examined at the age of 2. He developed slowly and showed retarded growth (bone age 3 months). His mental function appeared to be normal. Ultrasonography of the thyroid gland indicated a diffuse heterogeneous goiter of 13.5 g (normal: 4.1 → 1.1 g).

Patients IV-2 and IV-6 were subjected to subtotal thyroidectomy to correct compressive symptoms and received total thyroxine supplementation since.

Both anti-TPO and anti-Tg antibody tests were repeatedly negative in all three patients. No data were available on iodine intake and urinary secretion.

Microscopic examination of the goitrous tissue revealed the classic macro follicular pattern with dilated follicles lined with high columnar cells and follicular lumen devoid of colloid. Immunostaining for Tg indicated the presence of Tg-related antigens only inside the cells.

RNA isolation and complementary DNA preparation, genomic DNA isolation

Total RNA was isolated from goitrous (patient IV-6) and control thyroid tissue using TRizolReagent (Life Technologies BV). cDNA was synthesized using random hexamers and reverse transcriptase according to standard procedures. Genomic DNA of patient IV-6 was isolated from one of the TRizol fractions and genomic DNA of the indicated family members was isolated from white blood cells by the SDS-proteininase K method (24).
Table 1 Clinical and laboratory data from the affected siblings

<table>
<thead>
<tr>
<th>No</th>
<th>Year of birth</th>
<th>TT₄ nM</th>
<th>TT₃ nM</th>
<th>TSH mIU/l</th>
<th>Plasma Tg µg/l: basal / 48h*</th>
<th>% RAI uptake: at 2 / at 24h**</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV-2</td>
<td>1970</td>
<td>82.3</td>
<td>2.6</td>
<td>13</td>
<td>1.5 / 1.6</td>
<td>55 / 54</td>
</tr>
<tr>
<td>IV-6</td>
<td>1976</td>
<td>38.6</td>
<td>1.5</td>
<td>112</td>
<td>3.5 / 4.0</td>
<td>54 / 71</td>
</tr>
<tr>
<td>IV-10</td>
<td>1985</td>
<td>25.7</td>
<td>1.2</td>
<td>96</td>
<td>2.8 / 5.6</td>
<td>8 / 35</td>
</tr>
<tr>
<td>Normal Range</td>
<td>70 -160</td>
<td>1.1-3.1</td>
<td>&lt; 4.5</td>
<td></td>
<td>12 ± 6.5 / 34 ±16</td>
<td></td>
</tr>
</tbody>
</table>

* plasma Tg levels: basal and 48h after 10 IU of bovine TSH im (mean and SD from ref. 27)
** administration of perchlorate in radioactive iodide uptake studies (RAI) had no effect in any of the siblings

Figure 1 Family pedigree of index patient.
Chapter 5

DNA amplification

PCR amplification (25) was performed using 100 ng cDNA as template in a total reaction volume of 25 µl.

For nucleotide sequencing, fragments of 500 bp (with 20-70 bp overlap) were amplified with 2.5 units of AmpliTaq DNA polymerase (Perkin Elmer) using the protocol: 2 min 94°C; 35 cycles of 15 sec 94°C, 1 min 60°C, 1 min 72°C; 10 min 72°C. The human Tg specific oligonucleotides (synthesized on Expedite Nucleic Acid Synthesis System; Millipore) coupled to M13 tags are already described (10).

Reactions were electrophoresed on 0.8% agarose gel and purified using the Quiaquick DNA gel extraction kit (Quiagen).

For determination of alternative splice products, a cDNA fragment ranging from exon 4 to exon 9 (nt 400 - 1350) was amplified with 2.5 units of AmpliTaq DNA polymerase (Perkin Elmer) using the protocol: 2 min 94°C; 35 cycles of 15 sec 94°C, 1 min 57°C, 1.5 min 72°C; 10 min 72°C. M13 linked oligonucleotides 2F (nt 400) and 3R (nt 1350) were used (10).

For subcloning purposes, the same conditions and oligonucleotides were used.

For RFLP analysis, genomic DNA was amplified using the protocol: 5 min 95°C; 35 cycles of 1 min 95°C, 1 min 57°C, 1.5 min 72°C; 10 min 72°C, 2.5 units of AmpliTaq DNA polymerase (Perkin Elmer), and the following oligonucleotides: (794) 5' TGGACCTTCCTTCCACCTTCACTG 3' and (1002) 5' CCTTCCGTCTGGCACTGCA 3'.

Nucleotide sequence analysis

DNA amplification resulted in 20 fragments of approximately 500 bp covering the entire thyroglobulin cDNA of patient IV-6.

Both the sense and antisense strand were sequenced either using the M13 tags linked to the PCR fragments with the Big Dye Primer Cycle Sequencing Kit or using Tg specific oligonucleotides with the Big Dyedehcy terminator Cycle Sequencing Kit, depending on the GC content of the fragment (both kits from Applied Biosystems / Perkin Elmer). After electrophoresis on a sequencing gel the samples were analyzed on the ABI Prism 377 DNA sequencer and aligned to the Tg cDNA sequence (8,10) using AutoAssembler software (Applied Biosystems / Perkin Elmer).

Determination of alternative splice products

The coding region of nt 400 to 1350 (exon 4 to 9) was amplified on mRNA of patient IV-6 and of wildtype and the reactions were run on a 1.2 % agarose gel stained with ethidium bromide.
Subcloning of mutated Tg fragment
The TGI construct (8) containing wildtype Tg nucleotides -6 to 2110 in the pcDNA3 plasmid (TG-WT) was restricted with Bsu36 I thereby deleting the wildtype sequence from nt 686 to 1137 and purified from a 0.8% agarose gel. The amplified product of 950 bp, containing the mutation at nt position 886 was also restricted with Bsu36 I. The mutant fragment of 451 bp was purified from a 1.2% agarose gel and ligated into the digested TG-WT resulting in TG-M. By automatic sequencing using Tg specific primers the nt sequence was validated.

The protocols used for digesting with Bsu36 I (Biolabs) and for ligation with T4 ligase (Boehringer Mannheim; rapid DNA ligation kit) were according to the manufacturer. For gel purification, the Quiaquick DNA gel extraction kit (Quiagen) was used.

Standard heat shock transformation was performed with DH5α competent cells (Gibco BRL).

Expression of Tg in Rabbit Reticulocyte Lysate and analysis
For in vitro transcription and translation a TnT7 T7 Coupled Reticulocyte Lysate System was used (Promega) providing rabbit reticulocyte lysate, a reaction buffer, T7 RNA polymerase and an amino acid mixture lacking cysteine. 25 μl reactions were done as recommended adding Rnasin7 Ribonuclease Inhibitor (Promega), a mixture of 35S-methionine and 35S-cysteine (ICN Pharmaceuticals; Tran35S-label). To obtain glycosylation, Canine Pancreatic Microsomal Membranes (CPMM) were added. In each reaction 300 ng plasmid DNA was used as template (TG-WT or TG-M or positive control). Positive control 1 was used to check for expression of a protein of 61 kDa molecular weight. Control sample 2 was used to check for glycosylation.

Incubation was done at 30°C for 90 min. Aliquots of 5 μl (minus CPMM) or 10 μl (plus CPMM) of reticulocyte lysate reactions together with a mol wt marker (Biolabs; Rainbow general) were subjected to SDS PAGE according to Laemmli's method (17.5%) and the gel was dried afterwards. The radioactive signal of expressed proteins was detected using a Phosphorimager and Image Quant software (Molecular Dynamics). Protein products from 40 μl reticulocyte lysate reactions, were immunoprecipitated using a rabbit polyclonal antibody specific for human Tg (26) coupled to Protein A-Sepharose CL-4B (Pharmacia Biotech) and were analyzed identically.

Restriction Fragment Length Polymorphism analysis
The mutation detected by nucleotide sequencing at position 886 created an AlwN I recognition site. This enzyme was used according to the manufacturers protocol to screen for the presence of the mutation in the amplified genomic DNA Tg fragment
of the indicated family members (III-1, III-2, IV-6, IV-10, IV-2, V-1, V-2) and of a wildtype control. The samples were run on 2.5% agarose gel and stained with ethidium bromide.

RESULTS
The index patient (IV-6), suspected to have a defect in thyroglobulin synthesis as a cause for hypothyroidism, was subjected to subtotal thyroidectomy and thyroid tissue was available to screen for Tg mutations. RT-PCR was performed on the total RNA isolated from this tissue resulting in 20 overlapping fragments of 500 bp each, covering the total coding region of 8307 bp. Direct sequencing revealed a cytosine to thymidine mutation at nt position 886 (Fig 2A). Its position in the gene near the end of exon 7 is schematically given in Fig 2B and the supposed amino acid sequence after translation is also shown. Instead encoding for an arginine residue on position 277, the triplet harboring the mutation encodes a stopcodon.

Since the C886T transition induces a AlwNI restriction site, carriership for the mutation could be established using RFLP analysis. Therefore a fragment ranging from exon 7 to 8 (including intron 7 of 200 bp) was amplified on genomic DNA, isolated from blood of different family members: III-1, III-2, IV-6 (index patient), IV-10, IV-2, V-1 and V-2 (Fig 1). The wildtype amplified fragment of 400 bp was not digestible by AlwNI. The AlwNI digestion of the mutated fragment resulted in two fragments of about 300 and 100 bp (Fig 2C). All affected siblings (IV-6, -10, -2) showed two fragments after digestion (300 and 100 bp) and are homozygous for the mutation. Both parents (III-1, -2) and the youngest offspring (V-1, -2) showed three fragments after digestion (400, 300 and 100 bp) and are heterozygous (carriers). To determine whether the nonsense mutation caused alternative splicing of exon 7, a cDNA fragment ranging from exon 4 to 9 was amplified from mRNA of patient IV-6 and a wildtype control (data not shown). No difference in either splicing nor abundance of the amplified product was detected.

Figure 2 Screening for mutation in Tg mRNA (next page).
A: Part of wildtype and mutated (patient IV-6) thyroglobulin mRNA sequence. The arrow points to the homozygous C886T transition. B: Schematic drawing of thyroglobulin mRNA. Top: coding region from nt 1 - 8307 (italics) as an open box, 5' and 3' untranslated regions are indicated as solid lines. Middle: part of the Tg gene showing exon 7 and exon 8 (introns as dashed lines). Bottom: coding nt sequences and corresponding aa sequences are shown (AlwNI recognition site is underlined). C: Agarose electrophoresis and ethidium bromide visualization of AlwNI RFLP analysis. Shown are the mol wt marker lane (bp), wildtype control plus or minus AlwNI, respectively and the PCR amplified genomic DNA fragment (exon 7 - 8) of several family members after digestion. The open arrow indicates undigested wildtype fragment and the filled arrows indicate mutant fragments resulting from AlwNI digestion.
PREMATURE STOP IN TG mRNA

A

B

C

MW marker  wildtype -  wildtype +  III-1  III-2  IV-6  IV-10  IV-2  V-1  V-2

492  369  246  123
The mutation was expected to result in a truncated Tg molecule upon translation, still harboring putative N-glycosylation sites. To examine translation and putative glycosylation in relation to the mutation, a cell free in vitro transcription / translation system (rabbit reticulocyte lysate + T7 RNA polymerase) was used in which glycosylation conditions could be established by addition of microsomal membranes (CPMM). For comparison two expression constructs were used containing the first 2110 bp of coding Tg sequence: wildtype (TG-WT) and mutant (TG-M). $^{35}$S labeled methionine and cysteine were incorporated enabling visualization of the expressed protein after SDS PAGE using the Phosphorimager. The apparent mol wts proteins expressed from TG-WT and TG-M were respectively 76,000 and 30,000 (Fig 3A). After addition of CPMM both TG-WT and TG-M proteins were glycosylated observed by a shift in apparent mol wt, although the expression of the TG-WT was low (Fig 3B). The controls 1 and 2 provided by the manufacturer showed that both translation (Fig 3A, lane control 1) and glycosylation (Fig 3B, lane control 2) occurred. To validate that the expressed proteins were human thyroglobulin fragments, the reaction samples were immunoprecipitated with an anti-human-Tg polyclonal. Specific recognition of the Tg wildtype and mutant proteins with and without glycosylation is shown in Fig 3C and 3D respectively.

![Figure 3](image-url) In vitro expression of wildtype and mutant Tg fragment.

Incubations of rabbit reticulocyte lysates with wildtype (TG-WT) or mutant (TG-M) or control (provided by the kit) templates or no template (none) were performed with $^{35}$S labeled amino acids. After SDS PAGE proteins were visualized using the Phosphorimager. Open arrows indicate wildtype Tg proteins and filled arrows indicate mutant Tg proteins. Panel A and C correspond with the arrows on the left. In the reactions shown in panel B and D microsomal membranes were added to provide glycosylation (corresponding with arrows on the right). Panel C and D show the electrophoresis of immunoprecipitated samples. The mol wt marker bands are indicated (kDa).
In this paper we present the results of studies conducted in a family in which goiter and hypothyroidism occur. Three siblings out of ten from a consanguineous marriage showed thyroid function abnormalities (Fig 1), in accordance with a Mendelian pattern of inheritance of an autosomal recessive mutation. The affected family members all showed goiter with moderate hypothyroidism. Serum Tg levels were relatively low despite the high serum TSH levels and did not increase after exogenous bovine TSH stimulation (27). The absence of an iodide organification defect was based on the results of the radioactive iodide uptake studies (RAI): a high and rapid uptake and no iodide washout effect by administered perchlorate ions (Table 1). These characteristics indicated a defect in thyroglobulin synthesis.

After sequencing the total Tg cDNA of patient IV-6, a homozygous nonsense mutation was determined which resulted in an AlwNI recognition site (Fig 2). RFLP analysis demonstrated that both parents were heterozygous for this mutation and that all three affected siblings carried the same mutated Tg alleles. The mutation is a cytosine to thymidine transition at nt position 886 in exon 7 creating a stop codon at amino acid Arg277. The change occurs in a CpG dinucleotide, and can be caused by deamination of a methylated cytosine to thymine (28). Furthermore, in general the CGA arginine codon is considered 'hot spot' for mutations (29).

RNA transcripts containing a premature stop codon may be relatively unstable, because the untranslated part of the messenger is not protected by ribosomes (13, 19). However, we have no indication that the mRNA of patient IV-6 was very unstable as after total RNA isolation and RT-PCR amplification of 500 - 950 bp fragments, the results of normal and patient’s thyroid tissues were similar with respect to the quantity of the generated products. The amount of tissue however was not sufficient to perform a Northern blot.

No differences were detected in expression of the described alternative transcripts (10) compared to normals (data not shown). After RT-PCR amplification of a fragment from nt 400-1350 (exon 4 to 9) no differences in product length and abundance were detected between patient and wildtype samples. This excluded an alternative splice event of the Tg RNA, as is described for Afrikaner cattle (17) and human (13) where a nonsense mutation at amino acid residue 687 and residue 1510 respectively, results in a relatively increased expression of a smaller messenger RNA species lacking the mutated exon. The specific skipping of exons containing a nonsense mutation has also been described elsewhere (30).

Upon translation the mutated Tg transcript generated a Tg protein, validated by
immunoprecipitation with a specific anti-human-Tg antibody, with an apparent mol wt of 30,000 (Fig 3). This was in good accordance with the predicted mol wt of 30,778 (276 amino acid residues). Three putative N-linked glycosylation sites are still present in this truncated protein of which two have been shown to be glycosylated in the mature protein (Asn57 and Asn179) (31). The use of microsomal membranes in the *in vitro* expression assay indicated that the aberrant Tg protein could indeed be glycosylated.

It has been reported that the phenotypic expression of defective Tg protein varies considerably when different families or affected siblings within the same family are compared (32). Although the laboratory tests were performed at a younger age in patient IV-10 than in patients IV-2 and IV-6, it seems that the consequences of the defective Tg synthesis in patient IV-2 are less severe. Apparently the goiter is able to compensate the hypothyroid status with a somewhat elevated TSH. Her brothers show diminished total T<sub>4</sub> levels while their total T<sub>3</sub> levels are in the normal range (Table 1).

Thyroid hormone synthesis involves a two step modification of tyrosine residues. Iodination and subsequent coupling take place at the apical membrane of the cell and both reactions are catalyzed by thyroid peroxidase (2, 3). The specific iodinated tyrosine residues that are involved in the coupling reaction can either accept (hormonogenic sites) or donate iodinated phenyl groups. The most important acceptor site in all vertebrate species examined is at Tyr5, while priority for hormonogenesis at the other acceptor sites Tyr1291, Tyr2554 and Tyr2747 varies among species (2). So far for human Tg, three potential donor sites have been identified (Tyr130, Tyr847, Tyr1488)(33). The truncated form of Tg described here, harbors both the acceptor Tyr5 and the donor Tyr130 residues. This feature as well as its size and ability to become glycosylated makes it comparable to the truncated Tg product in the goitrous Dutch goats. In these animals the glycosylated Tg fragment (mol wt of 40,000) was able to synthesize T<sub>4</sub> *in vivo* and the amounts produced were comparable to normal when 1 mg iodide / day was administered, although goiter remained (34). It has been reported that also in man oral administration of excess iodine can correct partially the hypothyroid condition in patients with defective Tg synthesis (35). Therefore iodide administration may in part explain the variability in the clinical presentation of the affected individuals within this family.

In conclusion, molecular analysis of a family with hereditary hypothyroidism and goiter reveals a novel autosomal recessive mutation in the thyroglobulin gene. The
mutation is a C to T transition at nt position 886 in exon 7, creating a stop codon at amino acid Arg277. The expressed truncated Tg protein has a mol wt of 30,778, can be glycosylated and is still able to produce thyroid hormone.

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