Two novel variants in the thyroxine-binding globulin (TBG) gene behind the diagnosis of TBG deficiency

Rita Domingues¹, Maria João Bugalho¹,², António Garrão², José Manuel Boavida² and Luís Sobrinho²
¹Centro de Investigação de Patobiologia Molecular and ²Serviço de Endocrinologia do Instituto Português de Oncologia de Lisboa, Lisbon, Portugal

(Correspondence should be addressed to R Domingues, Centro de Investigação de Patobiologia Molecular, Instituto Português de Oncologia de Lisboa, R. Prof. Lima Basto, 1099-023 Lisboa Codex, Portugal; Email: rdomingues@ipolisboa.min-saude.pt)

Abstract

Objective: Search for germline mutations in the thyroxine-binding globulin (TBG) gene of two unrelated Portuguese females of Caucasian origin in whom the diagnosis of TBG deficiency was suspected because of suppressed TSH despite marginally low total thyroxine and tri-iodothyronine.

Design and Methods: Screening for germline mutations was conducted by non-radioactive PCR-SSCP analysis. The variants documented by this approach were characterized by sequencing. Moreover, in order to define whether they were mutations or polymorphisms we looked for the same variants analysing 100 alleles at random. To achieve this goal we used, alternatively, restriction analysis and the minisequencing method with an automated capillary electrophoresis system and fluorescent-labelled dideoxynucleotides.

Results and Conclusions: Two novel variants, one in each patient, were identified. One, involved codon 23 TCA → TAA and the other, codon 223 CAA → TAA. Analysis of 50 DNA samples, randomly chosen, revealed that all were homozygous for the wild variant at codon 23. One of them was heterozygous for the variant CAA → TAA at codon 223. This sample was found to correspond to a Caucasian female in whom serum TBG proved to be not detected. Since both variants identified result in stop codons likely to induce truncated TBG proteins, they are probably responsible for the TBG phenotype observed in the individuals studied.

European Journal of Endocrinology 146 485–490

Introduction

Thyroxine binding globulin (TBG) is the major thyroid hormone transport protein, with greater affinity for thyroxine (T₄) than for tri-iodothyronine (T₃) (1–3). It is synthesized in the liver as a 54 kDa glycoprotein consisting of 395 amino acids and four oligosaccharide chains (4–6). Its gene is located on the long arm of the Xq22.2 (7) and consists of one non-coding and four coding exons (8).

Inherited TBG abnormalities, either excess or deficiency, have been described (9–12). Familial TBG variants follow an X-linked inheritance pattern (7, 13) except for a single-family (14).

TBG deficiency is defined as complete (TBG-CD) or partial (TBG-PD) depending on serum values (15, 16). Diagnosis is generally fortuitous and depends on the finding of low levels of total T₄ and T₃ (TT₄, TT₃) together with normal or low thyrotropin (TSH) values. TBG measurements as well as exclusion of pituitary disease will further confirm the diagnosis. Since TBG deficiencies are inherited in an X-linked fashion, they are easily recognized in males (7) whereas women carrying both the normal and mutated alleles have TBG levels overlapping the normal range.

Incidence of TBG-CD is more common in Japanese (1:1200 to 1:1900) (17, 18) than in Caucasians (1:5000 to 1:13 000) (19, 20).

The molecular basis for TBG deficiency consists of mutations on the TBG gene (Table 1) as documented in different studies (10, 21–35).

In the present study we looked for mutations in the TBG gene in two unrelated females in whom a previous diagnosis of TBG deficiency was established. Two novel variants in exons 1 and 2 were identified. Both variants resulted in stop codons inducing truncated proteins.

Patients and methods

Patients

Patient A was a 57-year-old woman with a slow-growing multinodular goitre and complaints of sporadic tachycardia. Patient B was a 54-year-old woman...
previously submitted to partial thyroidectomy because of a large goitre and, since then, under therapy with levothyroxine (200 μg/day). Both were otherwise normal.

Thyroid function was initially evaluated by measurements of TT₃, TT₄ and TSH. In both cases, suppressed TSH levels co-existed with a marginally low TT₄. This unexpected pattern led to the hypothesis of TBG deficiency and justified a re-evaluation including free T₃ (FT₃), free T₄ (FT₄) and TBG measurements. At the same time, computed tomography scans of sella turcica were performed and were considered normal. Patient B was studied under levothyroxine therapy and after levothyroxine withdrawal. Individual data are shown in Table 2. Later on, a scintiscan of patient A revealed a hot nodule with partial suppression of the remaining gland.

**Methods**

**Amplification of genomic DNA** High molecular weight DNA from white blood cells was prepared by a manual method adapted from Bowtell (36).

Since the great majority of mutations previously described were located in exons 1 and 2, our attention was focused on these exons. Ten different primers were used (Table 3) from which four have been previously reported (28). Primers were designed according to GenBank sequence (GenBank accession number L13470). Approximately 100 ng genomic DNA were amplified in a 40 μl reaction containing 20 mmol/l Tris–HCl (pH 8.4), 50 mmol/l KCl, 1.5 mmol/l MgCl₂, 0.2 mmol/l dNTPs, 2 units of Taq polymerase and 1 μmol/l of appropriate PCR primers (Table 3). Genomic DNA was denatured for 5 min at 94°C prior to 35 cycles of denaturation for 45 s at 94°C, annealing for 45 s at 58–60°C (Table 3) and extension for 45 s at 72°C followed by a 7 min 72°C polishing step. The reactions were run on an UNO II thermocycler (Biometra, Göttingen, Germany).

**Non-radioactive PCR-SSCP analysis** PCR products were heat denatured and separated on a non-denaturing 8% polyacrylamide gel at 40 W for 4 h, at 4°C (37). Gels were silver stained.

**Sequencing** PCR purified products were either sequenced directly using the Sequenase Version 2.0 kit (USB, Cleveland, OH, USA) or subcloned into pGEM-T Easy Vector (Promega, Madison, WI, USA) and subsequently sequenced using the fmol DNA Sequencing System kit (Promega).

Table 1 Summary of previously reported mutations in TBG gene.

<table>
<thead>
<tr>
<th>Type</th>
<th>Name</th>
<th>Exon</th>
<th>Codon</th>
<th>Base change</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBG-CD</td>
<td>Yonago</td>
<td>1</td>
<td>28, 29:fs51aaX</td>
<td>GACTT → GAATT</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>Negev</td>
<td>1</td>
<td>38:fa51aaX</td>
<td>ACT → AC</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>CD6</td>
<td>1</td>
<td>165:fs168aaX</td>
<td>GTT → GT</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Kankakee</td>
<td>2</td>
<td>188:fs195aaX</td>
<td>Insertion of one G</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>Buffalo</td>
<td>3</td>
<td>Trp280Stop</td>
<td>TGG → TAG</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>Japanese</td>
<td>4</td>
<td>352:fs374aaX</td>
<td>CTT → TT</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>San Diego</td>
<td>1</td>
<td>Ser23Thr</td>
<td>TCA → ACA</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>Gary</td>
<td>1</td>
<td>Ile96Asn</td>
<td>ATC → AAC</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Montreal</td>
<td>1</td>
<td>Ala13Pro</td>
<td>GCC → CCC</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>Slow</td>
<td>1</td>
<td>Asp171Asn</td>
<td>GAC → AAC</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Aborigine</td>
<td>2</td>
<td>Ala191Thr</td>
<td>GCA → ACA</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Quebec</td>
<td>4</td>
<td>His331Tyr</td>
<td>CAT → TAT</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Japanese</td>
<td>4</td>
<td>Pro363Leu</td>
<td>CCT → CTT</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>Poly</td>
<td>3</td>
<td>Leu283Phe</td>
<td>TTG → TTT</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>Chicago</td>
<td>3</td>
<td>Tyr309Phe</td>
<td>TAT → TTT</td>
<td>35</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Other TBG variants</th>
<th>Poly</th>
<th>Chicago</th>
</tr>
</thead>
</table>

Table 2 Individual patients’ data. Laboratory tests at first visit: A₀ and B₀. Subsequent tests: A₁ – basal conditions; B₁ – under levothyroxine therapy; B₂ – after withdrawal of levothyroxine. A refers to patient A, B refers to patient B. —, not determined.

<table>
<thead>
<tr>
<th>Patient</th>
<th>TT₄</th>
<th>TT₃</th>
<th>TSH</th>
<th>FT₄</th>
<th>FT₃</th>
<th>TBG</th>
</tr>
</thead>
<tbody>
<tr>
<td>A₀</td>
<td>58</td>
<td>0.9</td>
<td>&lt;0.02</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A₁</td>
<td>59</td>
<td>1.2</td>
<td>&lt;0.02</td>
<td>19</td>
<td>5.5</td>
<td>196</td>
</tr>
<tr>
<td>A₂</td>
<td>68</td>
<td>0.8</td>
<td>&lt;0.02</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B₀</td>
<td>68</td>
<td>0.9</td>
<td>&lt;0.02</td>
<td>27</td>
<td>4.4</td>
<td>104</td>
</tr>
<tr>
<td>B₁</td>
<td>50</td>
<td>0.9</td>
<td>2.1</td>
<td>13</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>B₂</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal range</td>
<td>69–142 nmol/l</td>
<td>0.8–2.4 nmol/l</td>
<td>0.2–3.2 μmol/l</td>
<td>10–23 pmol/l</td>
<td>3.4–7.2 pmol/l</td>
<td>271–625 nmol/l</td>
</tr>
</tbody>
</table>
Restriction analysis Since the variant TCA → TAA at codon 23 destroys a restriction site for FokI we used restriction analysis not only to confirm sequencing data but also to seek the same variant in 50 DNA samples randomly chosen. Restriction enzyme digests were analysed on ethidium bromide-stained 10% polyacrylamide gels.

Minisequencing method The variant CAA → TAA at codon 223 does not destroy or generate a restriction site. To screen for this variant in 50 DNA samples randomly chosen we have used the minisequencing method with a specific primer and fluorescent-labelled dideoxynucleotides. A primer extension reaction was performed starting from the purified amplified target (exon 2, fragment 1) using a primer that was exactly one base short of the polymorphic site (5′-ccccttgagcacatc-3′). Fluorescent-labelled dideoxynucleotides are complementarily incorporated according to the sequence. Interruption of the reaction occurs after only one incorporation. Reactions were performed using the SnaPshot ddNTP Primer Extension Kit (Applied Biosystems, Foster City, CA, USA). The separation of the products obtained was performed using the ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The peak signal was analysed with the GeneScan Analysis Software.

TBG measurements TBG was assayed by RIA using a commercial kit (GammaDab TBG 125I RIA Kit; Incstar, Stillwater, MN, USA), sensitivity 25 nmol/l, normal range 271–625 nmol/l (Table 2).

Results Non-radioactive PCR-SSCP analysis of exons 1 and 2 of the TBG gene revealed two types of variants. Results obtained are shown in Fig. 1.

Nucleotide sequence analysis revealed a C → A point mutation (Fig. 2) at nucleotide 4446 (patient A) resulting in an early stop at codon 23 and a C → T point mutation (Fig. 3) at nucleotide 6095 (patient B) resulting in an early stop at codon 223. Moreover, sequence analysis clearly showed that mutations were heterozygous.

The codon 23 variant was confirmed by restriction analysis with FokI (Fig. 2). The variant at codon 223 was further confirmed by an alternative and straightforward method to detect single-nucleotide polymorphisms (SNPs), as described in Methods (Fig. 4).

Both mutations occurred throughout the coding region and are therefore likely to result in a truncated TBG protein.

Analysis of 50 DNA samples, randomly chosen, revealed that all were homozygous for the wild variant at codon 23. One of them, however, was heterozygous for the variant CAA → TAA at codon 223. Review of the clinical record of this patient revealed that it corresponded to a female patient diagnosed as having...
hypothesis. Under therapy with levothyroxine, TSH normalized despite persistent low levels of TT₃ and TT₄. Measurements of serum TBG, FT₃ and FT₄ (respectively 104 nmol/l, 4 pmol/l and 16 pmol/l) supported the diagnosis of TBG deficiency.

Discussion

In the present report we describe two mutations in the TBG gene that have not been previously reported. They were identified in two Portuguese females of Caucasian origin having in common low levels of serum TBG. Whether they carry a complete or partial deficiency is questionable since only a commercial assay for TBG measurement was used.

Since the TBG gene is located on chromosome X, inherited TBG abnormalities are generally fully manifested in hemizygous males and, therefore, are easily diagnosed. In heterozygous women, on the other hand, they are moderately expressed and are diagnosed less frequently. A possible explanation for the different phenotype expression among deficient women is the way in which X-inactivation occurs (38).

TBG deficiency is usually detected in euthyroid individuals. Our patients presented with subclinical thyrotoxicosis. Besides low levels of TT₄ and TT₃ they had suppressed TSH. In one case this was secondary to a toxic goitre and in the other it was induced by levothyroxine therapy.

Molecular studies of TBG gene disclosed two novel variants. Both variants resulted in stop codons likely to induce a truncated TBG protein as the cause for the observed phenotype.

Screening for mutations was performed by SSCP analysis under conditions established by us. The variants we now report, Ser23Stop and Gln223Stop, result in proteins of 22 and 222 amino acids as compared with normal TBG with 395 amino acids (39). At a cellular level a truncated TBG protein may be associated with an impaired intracellular transport and subsequently with low or undetected levels of serum TBG (40, 41). The possibility that the carboxyl terminus appears to be important for the transport from the rough endoplasmic reticulum to the Golgi apparatus (42) further supports this finding.

A missense mutation has been previously reported at codon 23 (33). The variant at codon 223 had not been previously documented.

Screening for the same variants, among 50 DNA samples at random, enabled us to find one more individual heterozygous for the Gln223Stop variant. Review of clinical and biochemical data of this case disclosed a deficiency of TBG. Apparently, this individual

---

**Figure 2** (a) DNA direct sequencing analysis of exon 1 PCR products from germline DNA of patient A. This individual is heterozygous for the mutation TCA → TAA at codon 23. (b) Ethidium bromide-stained polyacrylamide gel of FokI restriction digestion. As a consequence of the above mutation a FokI site is lost. Lane 1, uncut PCR products (224 bp); lane 2, negative control (digestion produces bands of 93, 33 and 98 bp); lane 3, germline DNA from patient A (for the mutant allele, restriction digestion produces bands of 93 and 131 bp). The band of 33 bp is not shown. MW denotes the lane containing the 100 bp ladder marker (Gibco BRL, Gaithersburg, MD, USA).

**Figure 3** DNA sequence in the TBG codon 223 region of cloned exon 2 amplicons from patient B. Codon 223 is CAA in the normal allele (a) and TAA in the mutant allele (b).
and patient B are unrelated. However, we cannot definitely exclude a common ancestor since we did not perform haplotype studies.

The presence of the two described mutations in all three patients with low TBG levels and in none of 49 random samples supports the view that there is a causal relationship between phenotype and genotype.

Acknowledgements

Supported by grant from the Ministério da Saúde (DR 898/98 – Projecto 114) and by Programa Operacional “Ciência, Tecnologia, Inovação”/Quadro Comunitário de Apoio III POCTI/QCAIII, Portugal.

References


Figure 4 SNP analysis on the ABI PRISM310 Genetic Analyzer System. Results of a minisequencing reaction for the CAA → TAA variant at codon 223. Top: heterozygotic type, two different peaks occur in the electropherogram. Bottom: homozygotic wild type, one peak occurs. C (cytosine), peak not shaded; T (thymine), shaded peak.


Flink IL, Bailey TJ, Gustafson TA, Markham BE & Morkin E. Complete amino acid sequence of human thyroxine-binding globulin deduced from cloned DNA: close homology to the serine proteases. PNAS 1986 83 7708–7712.


Received 21 August 2001
Accepted 19 November 2001