

# TBG deficiency: description of two novel mutations associated with complete TBG deficiency and review of the literature

Deborah Mannavola · Guia Vannucchi ·  
Laura Fugazzola · Valentina Cirello · Irene Campi ·  
Giorgio Radetti · Luca Persani · Samuel Refetoff ·  
Paolo Beck-Peccoz

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**Abstract** Thyroxine-binding globulin (TBG) is the main thyroid hormone transport protein in serum. Inherited TBG defects lead to a complete (TBG-CD) or a partial (TBG-PD) deficiency and have a diagenic transmission, being clinically fully expressed only in hemizygous males and in homozygous females. In the present study, seven patients from two unrelated families with TBG-CD were studied and two novel TBG mutations were documented. In particular, a T insertion at the 5' donor splice site of exon 0, between nucleotides 2 and 3 at the beginning of intron 1 (g.IVS1+2\_3insT) was found in one family and was named TBG-Milano. The other novel mutation is a T deletion at nucleotide 214 of exon 1, which leads to a frameshift at codon 50 with a premature stop codon at position 51

D. Mannavola · G. Vannucchi · L. Fugazzola · V. Cirello ·  
I. Campi · L. Persani · P. Beck-Peccoz (✉)  
Institute of Endocrine Sciences, University of Milan,  
Pad. Granelli Via F. Sforza, 35,  
Milan 20122, Italy  
e-mail: paolo.beckpeccoz@unimi.it

D. Mannavola · G. Vannucchi · L. Fugazzola · V. Cirello ·  
I. Campi · P. Beck-Peccoz  
Fondazione Policlinico Istituto di Ricovero e Cura a Carattere  
Scientifico di natura pubblica (IRCCS),  
Milan, Italy

G. Radetti  
Pediatric Division, Ospedale Regionale di Bolzano,  
Bolzano, Italy

L. Persani  
Istituto Auxologico Italiano-IRCCS,  
Milan, Italy

S. Refetoff  
Departments of Medicine and Pediatrics and the Committee on  
Genetics, The University of Chicago,  
Chicago, USA



DEBORAH MANNAVOLA received her Ph.D. in Molecular Medicine from the University of Milan. She has completed a Trainship in Endocrinology and Thyroid Research at the University of Chicago, Division of Biological Sciences, section of Endocrinology and Thyroid Unit study. She is presently a Research Assistant for the Endocrine Unit at the Fondazione Policlinico-IRCCS in Milan, Italy.

PAOLO BECK-PECCOZ is currently Full Professor of Endocrinology at the University of Milan and Chief of the Endocrine Unit in the Department of Medicine at the Fondazione Policlinico-IRCCS in Milan, Italy. He is also Editor-in-Chief of the European Journal of Endocrinology.

(c.214delT, P50fsX51) and was named TBG-Nikita. According to the X-linked transmission of the defect, females harboring the mutation showed a reduction in TBG levels with normal TSH and total thyroid hormone values at the lower limit of normal. Males harboring either TBG-Milano or TBG-Nikita, showed normal TSH values and low levels of total thyroid hormones and lacked TBG. In conclusion, we report two novel mutations of the TBG gene associated with a complete TBG defect. The first mutation lies at the 5' donor splice site of exon 0 and

probably alters the start of translation, while the second is a single nucleotide deletion and leads to a premature stop codon.

**Keywords** Thyroxine-binding globulin defects · TBG-CD · TBG-PD · TBG gene mutations · Thyroid hormones

## Introduction

Thyroid hormones circulate in the blood mainly bound by proteins. Thyroxine-binding globulin (TBG) is the most relevant one, as it binds about 75% of circulating T4 and 70% of T3 [1]. TBG (MIM+314200) is a 54-kDa glycoprotein synthesized in the liver and consists of a coding polypeptide with a leader sequence containing 20 amino acids and four oligosaccharide chains [2]. The gene structure and the amino acid sequence have a very high homology with members of serine protease inhibitors (serpin) superfamily [3]. The TBG gene is located on the long arm of the X-chromosome (Xq21–q22) [4] and is composed of five exons [2, 3]. The non-coding first exon is named exon 0 [2]. TBG defects lead to three different phenotypes according to serum TBG concentrations in affected hemizygous males: complete TBG deficiency (TBG-CD), partial TBG deficiency (TBG-PD), and TBG excess (TBG-E) [5]. These defects show a diagenic transmission, according to the X chromosome localization of the gene.

Consequently, TBG defects are clinically fully expressed in hemizygous males and rarely in Turner syndrome or homozygous females, whereas in heterozygous females TBG deficiency is usually partial. Due to dosage compensation, normally achieved through the random inactivation of one of the two X chromosomes, heterozygous females usually show intermediate TBG levels between affected and unaffected males [6]. However, on occasion inactivation may be selective for one of the alleles resulting in a phenotype undistinguishable from that in affected males [7]. TBG defects do not lead to major metabolic alterations in affected individuals, and the diagnosis of TBG-CD is suspected in clinically euthyroid subjects with low total serum thyroid hormone concentrations and normal TSH levels.

Since the first description of the complete sequence of human TBG gene in 1986 [3], 24 variants have been associated with TBG defects [8–28]. Either nonsense or missense mutations, randomly distributed throughout the TBG gene, have been reported in association with the typical biochemical alterations. In particular, nonsense variants lead to non-functional truncated proteins, while missense mutations alter the ternary structure of the protein and its processing in the Golgi, thus leading to abnormal properties and/or concentration of TBG variants [29]. On the contrary, gene duplications or triplications have been shown to be the cause of TBG-E [30].

From data of neonatal screening for congenital hypothyroidism, based on total T4 determination, the incidence of TBG-CD is extremely variable, being higher in Japanese populations (1:1,200–1:1,900 newborn/year) [26] than in Caucasian (1:5,000–1:15,000 newborn/year) [31]. The variability is likely due to types of mutations and their ethnic preponderance. For example, the complete deficiency, TBG-CDJ, is common in Japanese [32, 33], while the frequency of partial deficiency, TBG-A, in Australians is 40% [9]. Furthermore, the prevalence of TBG deficiency is underestimated by the inability to detect the defects in heterozygous female and those with TBG-PD.

In the present paper, two unrelated cases of TBG-CD harboring novel mutations in the TBG gene are reported.

## Materials and methods

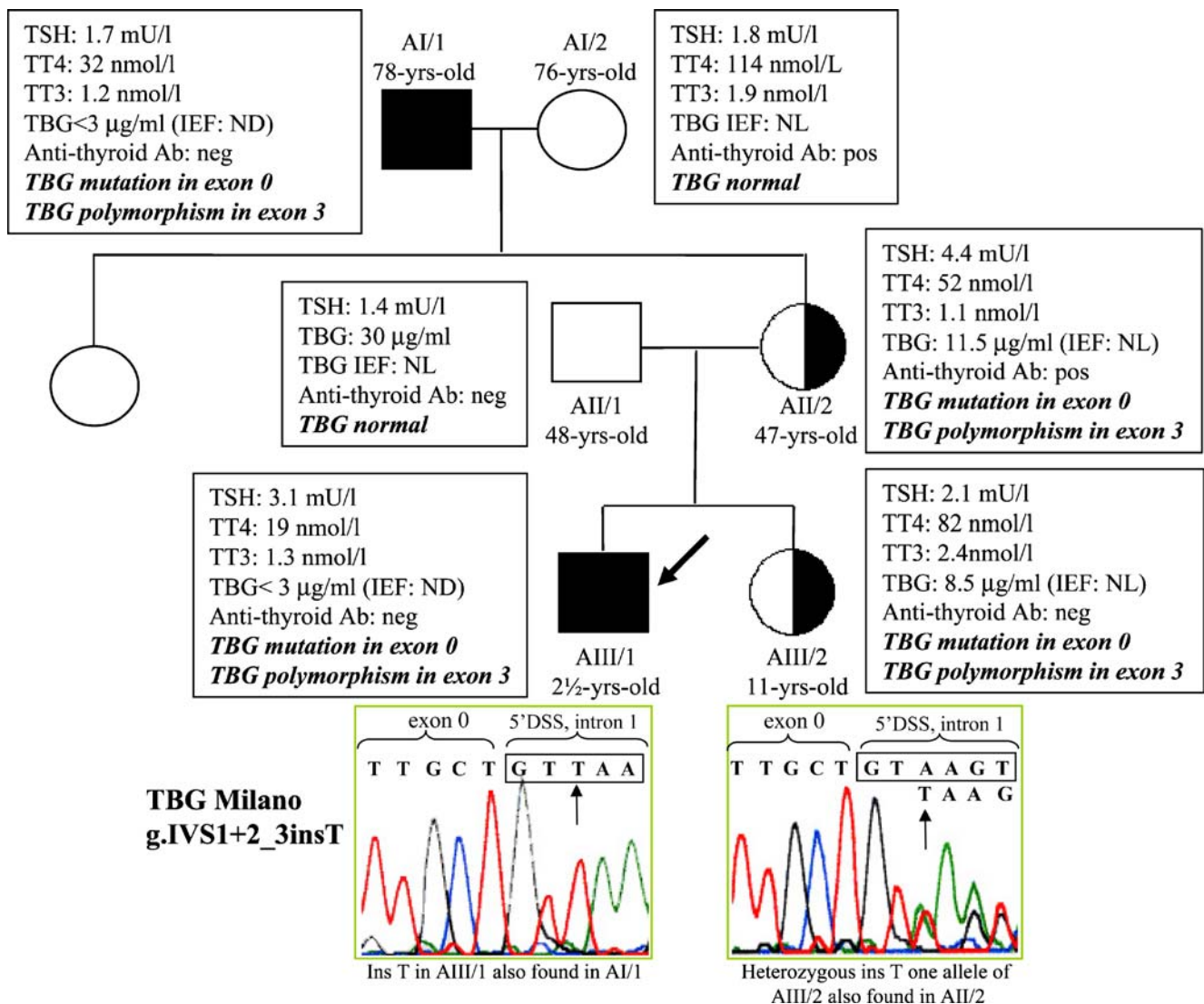
Seven patients from two unrelated families and from non-consanguineous parents, referred to us with a diagnosis of TBG deficiency, were studied.

*Family A* comprises six members. The proband (AIII/1) is an Italian boy, positive at neonatal screening for congenital hypothyroidism with a normal TSH of 5 mU/l but low total T4 (TT4) of 19 nmol/l. Re-evaluation confirmed the low levels of TT4 as well as total T3 (TT3) with normal TSH levels. The ultrasound examination showed a normal thyroid gland and thyroid scintigraphy revealed a normal uptake. L-T4 therapy was started and withdrawn at the age of 2 years and 6 months when he came to our attention and was studied together with his family members (Fig. 1). The previous L-T4 therapy did not result in any side effects. Indeed, the child showed a normal growth and an appropriate neuro-psychological development.

*Proband B* is an adopted 3-year-old Russian boy, and no information about his parents is available. The suspicion of TBG deficiency was raised by the finding of low total thyroid hormone concentrations and normal TSH levels, during routine examinations. These data were confirmed on a follow-up examination, and TBG was not detectable (Fig. 2).

### Thyroid function testing

TBG concentrations were measured by conventional radioimmunoassay able to detect values above 3 µg/ml. Total absence of TBG (TBG-CD) was confirmed by isoelectric focusing (IEF), able to detect as little as 5 µg TBG/l [34] (Fig. 3). Immunoreactive TSH was measured using ultrasensitive third generation methods [AutoDelfia® sUltra (Wallac, Turku, Finland): 0.002 mU/l]. TT3 and TT4 were measured with enzyme immunoassay kit (Beckman Analytcat S.p.A.). Direct dialysis was used to measure free T4



**Fig. 1** Family A: clinical and molecular features of the family affected with TBG complete deficiency (TBG-CD). Hemizygous members for the TBG mutation are indicated as *black symbols*, the heterozygous women are indicated as *half-darkened symbols*, and the unaffected members as *white symbols*. The proband is indicated with an *arrow*. Biochemical data were obtained in the proband (AIII/1) after 6 months of levothyroxine (L-T<sub>4</sub>) withdrawal at 2 years and a half of age, and in mother (AII/2) and maternal grandmother (AI/2).

Electropherograms correspond to the TBG-Milano mutant (g.IVS1+2\_3insT) identified in the proband and found also in the maternal grandfather and the same mutation in one allele of the mother and also found in the sister. Normal values: thyrotropin (TSH): 0.26–4.2 mU/l, total T<sub>4</sub> (TT<sub>4</sub>): 64–154 nmol/l, total T<sub>3</sub> (TT<sub>3</sub>): 1.2–3.0 nmol/l, thyroxine-binding globulin (TBG): 13–32 μg/ml, TBG IEF (isoelectric focusing, see also Fig. 3): *NL* within normal limit, *ND* not detectable

levels. Anti-thyroglobulin and anti-peroxidase antibodies were determined using a Liaison Kit (Byk-Sangtec Diagnostica, Dietzenbach-Germany).

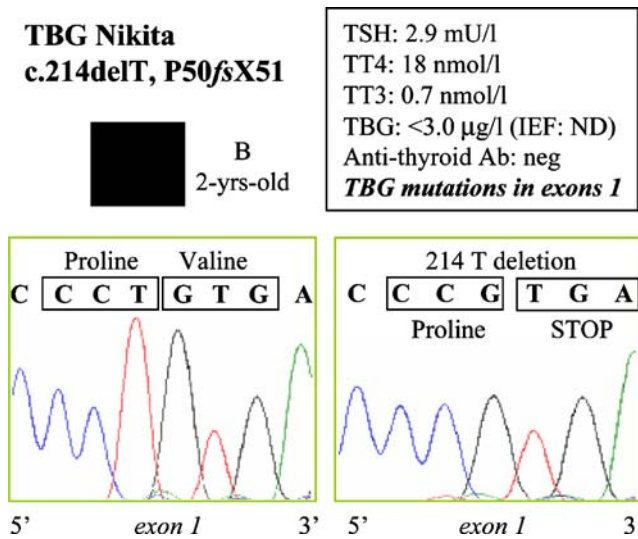
#### Genomic DNA analysis

DNA was extracted from peripheral blood leukocytes by a standard method. All five TBG exons were amplified using intronic oligonucleotide primers, as previously described [21]. PCR products were directly sequenced. Briefly, an aliquot of 3–10 ng/100 bp of purified DNA and 3.2 pmol of

either the forward or reverse primer were used in standard cycle sequencing reactions with ABI PRISM Big Dye terminators and run on an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, CA, USA). Both exonic sequences and intronic boundaries were sequenced.

#### Restriction endonuclease analysis of the mutation in family A

The mutation found in family A creates a restriction endonuclease site for the enzyme MseI. To confirm the



**Fig. 2** Proband B: clinical and molecular findings of the proband with TBG complete deficiency (TBG-CD) (*black symbol*). The electropherograms correspond to the TBG-Nikita mutation (c.214delT, P50fsX51) identified in the proband and a wild-type sequence obtained from a healthy control. Normal values: thyrotropin (TSH): 0.26–4.2 mU/l, total T4 (TT4): 64–154 nmol/l, total T3 (TT3): 1.2–3.0 nmol/l, thyroxine-binding globulin (TBG): 13–32 µg/ml, TBG IEF (isoelectric focusing, see also Fig. 3): *ND* not detectable

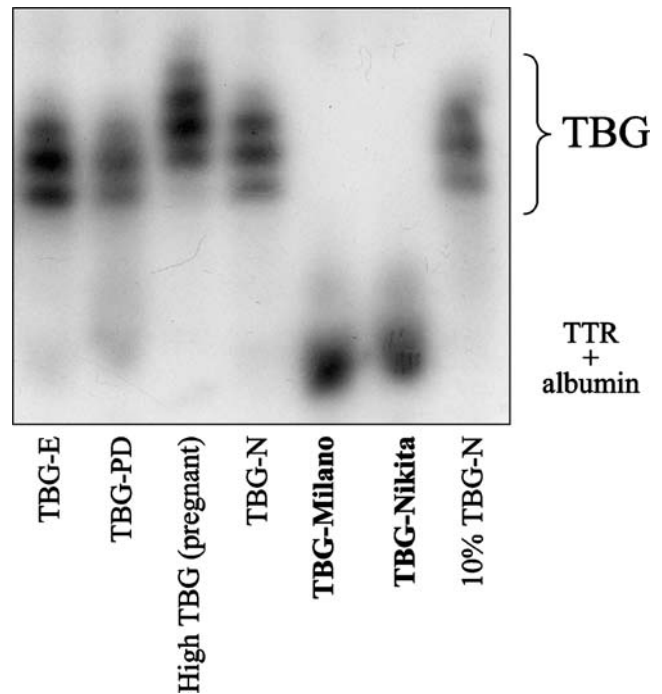
presence of this mutation in all family members affected with TBG defect, PCR products were digested with MseI and electrophoresed on 3% agarose gel.

**Results**

**Biochemical evaluation**

In family A, in addition to the proband, low levels of total thyroid hormones with normal TSH concentrations and absent serum TBG levels were recorded in the maternal grandfather (AI/1). The mother and the sister of the proband (AII/2 and AIII/2) showed total thyroid hormones straddling the low–normal range and only slightly reduced TBG levels, consistent with the X-linked transmission of the defect. The father and the maternal grandmother had normal TSH and TBG values (Fig. 1). Thyroid autoantibodies were negative in all family members except for AI/2 and AII/2. These two individuals had also Hashimoto’s thyroiditis, confirmed by thyroid ultrasonographic pattern and elevated TSH levels during the follow up. L-T4 therapy was thus introduced.

Free T4 levels, measured by equilibrium dialysis, were normal in all affected individuals of family A and in proband B. They were 2.3, 2.7, and 2.1 ng/dl for subjects A/I, A/III, and B, respectively (normal range 0.8–2.7).



**Fig. 3** Analysis of TBG by isoelectric focusing (IEF). Tracer amount of <sup>125</sup>I-T4 were added to serum before submission to IEF and radioautography. TBG is microheterogeneous due to differences in sialic acid content and shows three major bands between pH 4.35 and 4.50. Note the absence of bands in the region of TBG in serum from subjects AIII/1 and B, labeled TBG-Milano and TBG-Nikita, respectively. In these samples, all <sup>125</sup>I-T4 activity is confined to transthyretin (TTR) and albumin. Control sera are from a subject with TBG excess (TBG-E); partial TBG deficiency (TBG-PD); high TBG concentration due to pregnancy showing anodal shift due to increased sialic acid content [45]; normal TBG (TBG-N) is shown undiluted and tenfold diluted. The method can detect as little as 0.3% of TBG-N

**Mutational analysis**

A novel germline TBG mutation was found in family A and was named TBG-Milano. It is a T insertion at 5’ donor splice site of exon 0, between nucleotides 2 and 3 at the beginning of intron 1 (g.IVS1+2\_3insT). It was found in the proband (AIII/1) and in the maternal grandfather (AI/1) (Fig. 1). The mother AII/2 and the sister AIII/2 were found to be heterozygous for the same mutation. These results were confirmed by restriction analysis in all family members (data not shown). TBG-Milano clearly segregates with the TBG-CD phenotype in males of family A, indicating its key role in the pathogenesis of the defect. Exonic and intronic recognition sequences, as well as the consensus sequences in the immediate vicinity of the exon-intron boundaries, have an established role for splice site selection [35, 36]. A consensus value (CV) can be calculated by comparing the mutated nucleotide sequence (CVM) with the wild-type consensus sequence (CVN) [37]. The downstream splice donor site, both normal and with the mutant-T insertion, were thus analyzed using the primate

nucleotide weight table proposed by Shapiro and Senapaty [37]. The calculated CVN of 0.8 was 0.5 for CVM corresponding sequence TBG-Milano.

Affected members with TBG-CD of family A also share a polymorphism in exon 3 (L283F). At position 283, a G to T transition (c.2690G>T), leading to a leucine to phenylalanine substitution, is present with hemizygous or heterozygous pattern according to the gender. This missense substitution leads to a well-known polymorphism (TBG-Poly, Fig. 4) whose frequency has been studied in different populations. It is found in 16% of French Canadian men [10] and Australian aborigines [9], in about 20% of the Japanese population [33] and up to 31% of the Han Chinese population [27]. In contrast, the TBG gene sequence was normal in the two unaffected members of family A (AI/2, AII/1), lacking either the splice site mutation or the polymorphic variant L283F (Fig. 1).

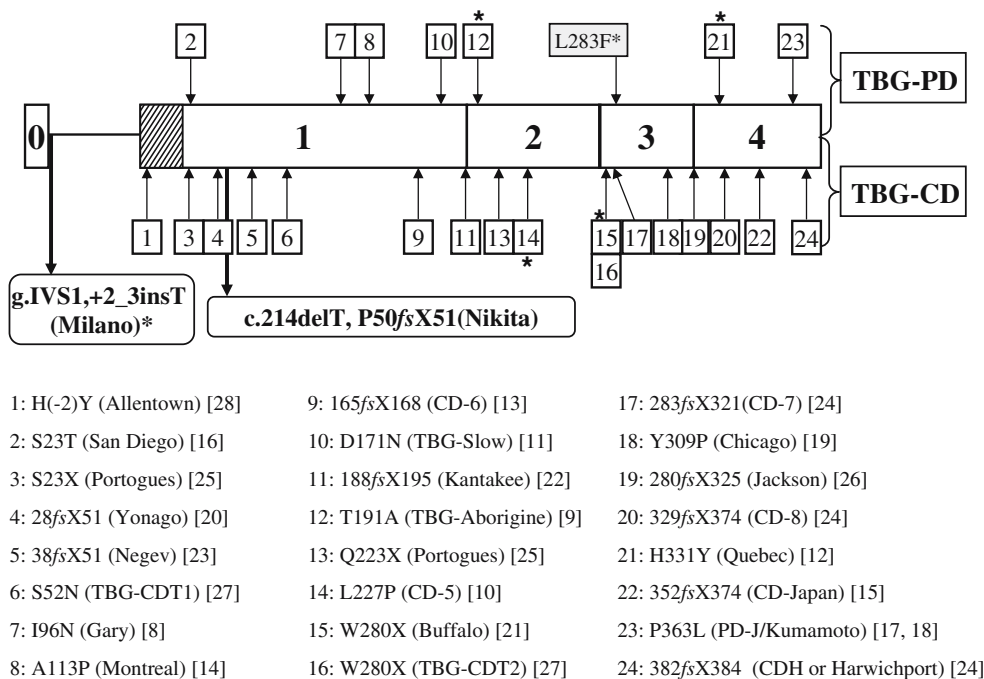
In case B, a single base-pair deletion at nucleotide 214 of exon 1 was found (Fig. 2). This T deletion leads to a frameshift at codon 50 with the creation of a premature stop at position 51 (c.214delT, P50fsX51). No family members were available for investigations, as the proband was adopted.

**Discussion**

The present study reports two novel mutations in the TBG gene. They are both associated with a phenotype of complete protein deficiency (TBG-CD) in male family members. The first variant is a T insertion at the beginning of intron 1 between nucleotide 2 and 3 (g.IVS1+2\_3insT), while the second is a T deletion in exon 1 leading to a truncated protein (c.214delT, P50fsX51).

Both mutations fail to produce functional TBG molecules as it is not detectable by either immunologic or binding methods. In particular, no TBG binding activity could be shown by IEF, a method capable of detecting as little as 0.3% of the average normal TBG concentration in the serum [34].

To date, 26 different mutations have been reported in the TBG gene, including the two novel mutations herein described (Fig. 4). Missense and nonsense mutations in the coding exons or in donor or acceptor splice sites have been described, without clustering in “hot regions”. Only nine mutations are transitions in CpG dinucleotides, that are frequent sites of point mutations in several other genes [38], while no mutations in GC-rich regions (defined as ≥4 consecutive Gs or Cs) have been recorded. It is worth noting that the majority of complete TBG defects are



**Fig. 4** All TBG mutations reported in the literature. According to the recent nomenclature recommendations, the A of the ATG of the initiator Met codon is denoted as +1 [46]. However, due to the presence of a 20-amino acid signal peptide cleaved at the time of protein synthesis and corresponding to the beginning of exon 1, the starting A of the ATG in exon 1 as has been numbered as -20 in all published papers. Mutations reported in the upper part of the figure are associated with TBG-PD,

while mutations on the bottom associate with TBG-CD. The only known polymorphism (L283F) is indicated in grey with an asterisk. Accordingly, an asterisk has been added when a mutation has been described as associated to the polymorphism. At the bottom, all mutations are reported with their proper name and reference number. Black arrows indicate the two novel mutations reported in the present study

associated with nonsense mutations generating precocious stop codon or with missense mutations associated with the presence of the polymorphism L283F (TBG-Poly) (Fig. 4). Only two mutations occurring in a noncoding region have been previously associated with TBG-CD (TBG-Kantakee and TBG-Jackson). TBG-Kantakee [22], an intronic mutation at the 3' acceptor splice site of intron 2, causes a frameshift and an early termination signal. The mutation in TBG-Jackson [26], located at the 5' donor splice site of intron 4, causes an alteration of the normal mRNA splicing process leading to exon 3 skipping, and consequently to a nonsense sequence from codon 280 to codon 325. In contrast, partial TBG defects are typically due to single-missense mutations [39].

As far as the TBG-Milano is concerned, it clearly segregates with the TBG-CD phenotype. Moreover, the analysis performed by means of the primate nucleotide weight table proposed by Shapiro and Senapathy [37] indicated a lower translation efficiency for this mutant. It is logical to hypothesize that the frameshift created by the sequence variation in the 5' donor splice site of exon 0 (TBG-Milano) could alter the correct initiation of the transcription process, or, alternatively, lead to an unstable mRNA. In particular, it is worth noting that in the 5' UTR (containing exon 0), the *cis*-acting transcriptional regulatory elements including TATA box, CAAT box and a hepatocyte nuclear factor 1 binding motif are located in the region upstream exon 0. Moreover, the signal peptide, which is crucial to SRP (signal recognition particle) recognition and to the following targeting to the ER (endoplasmic reticulum), maps in the region downstream exon 0 [2, 40]. It is tempting to speculate the present mutation lying in the 5' donor splice site of exon 0 could lead to alterations both in the upstream regulatory elements and in the downstream signal peptide resulting in its complete absence in the serum.

Interestingly, as already reported for other TBG defects (Fig. 4), also the present mutation co-segregates with the L283P polymorphism, which is also frequently found in normal population and does not alter the molecular structure of TBG. Both mutation and variant are located on the same allele as they were both transmitted, on the X-chromosome, from the maternal grandfather to the niece through the mother.

TBG-Nikita leads to a short protein of 50 residues. Two other TBG mutations leading to premature stop codons at the residue 51 have been described [20, 23]. In all cases, the associated phenotype is that of TBG-CD.

Recently, a complete analysis of relationship among known serpins with a detailed interacting genomic, functional, and structural information has been published [41]. In the phylogeny of serpin superfamily, both TBG and CBG (cortisol-binding globulin), fall in the clade identified with the name of "antitrypsin-like" based on the high percentage

of homology. In particular, the reactive site-residues specific for this class, Met-Ser of  $\alpha$ 1-antitrypsin, and Leu-Ser of  $\alpha$ 1-antichymotrypsina, are predicted to correspond to Leu352 and Ser353 residues of TBG sequence [3].

The present mutations produce no protein molecule or one truncated at amino acid 50. The latter will lack the hinge region (P15-P9), which provides mobility essential for the conformational change of the reactive center loop needed to control ligand release [42]. Moreover, it will have no specific T4-binding (Phe249) and glycosylation (Leu246) sites that are highly conserved in most of the serpins [43].

As expected, the thyroid function tests were consistent with the X-linked transmission of the defect. In particular, female members harboring the TBG-Milano showed a partial reduction in TBG concentration with normal TSH values, reduced total thyroid hormone concentrations and TBG levels in the lower limit of the normal range. On the contrary, affected male members, harboring either TBG-Milano or TBG-Nikita, showed complete TBG deficiency with normal TSH values and levels of total thyroid hormones well below the lower limit of normal.

In conclusion, we report two novel mutations of the TBG gene causing a complete TBG deficiency. The first mutation lies at the 5' donor splice site of exon 0 affecting splicing, while the second is a single nucleotide deletion leading to premature stop codon. The carboxyl terminus of the protein was previously demonstrated to be essential for the transport of the protein from the rough endoplasmic reticulum to the Golgi apparatus [44]. It is, thus, expected that both the TBG-Milano and the TBG-Nikita will have similar defect in intracellular transport and secretion. This would explain the lack of detectable TBG in the serum of individuals harboring the present mutations.

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