

Sequence of a Variant Thyroxine-Binding Globulin (TBG) in a Family with Partial TBG Deficiency in Japanese (TBG-PDJ)

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Abstract. Thyroxine-binding globulin (TBG) is the major transport protein of thyroid hormones in human serum. In this communication, we present a sequence abnormality of the TBG-gene in a Japanese family manifesting partial TBG deficiency (TBG-PDJ). The proband was a male with a reduced concentration of TBG (3.2 µg/ml). Thyroid function tests suggested that the inheritance of this TBG abnormality was X-linked. The TBG exhibited increased heat-lability compared with the common type TBG (TBG-C). The isoelectric focusing pattern of this TBG molecule was indistinguishable from TBG-C. Genomic DNAs from white blood cells of four members of a TBG-PDJ family were subjected to polymerase chain reaction (PCR), and the products were sequenced. The sequencing of the entire coding exons and exon/intron junctions of TBG allele of the proband revealed a single nucleotide substitution: CCT (proline) to CTT (leucine) at amino acid 363 of the TBG-C. The heterozygosity as revealed by the direct sequencing of the PCR product correlated with the TBG concentration in serum. The proline to leucine substitution may cause a change in the TBG tertiary structure and result in decreased heat stability, resulting in decreased TBG levels in the affected subjects.

Key words: Partial TBG deficiency, PCR, Sequencing analysis.

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THYROXINE-binding globulin (TBG) is the major transport protein of thyroid hormones in human serum [1, 2], and consists of a single peptide chain of 395 amino acids [3]. The TBG gene is located on the long arm of the X-chromosome, and most of the TBG abnormalities have been shown to be inherited as X-linked traits [4]. TBG abnormalities can be classified on the basis of serum concentrations, namely excess, reduced or absent TBG. They are suspected when

altered levels of serum total thyroxine are found in euthyroid subjects [5]. Sequencing analysis of the TBG gene in more than 10 families has revealed nucleotide substitution or deletion in TBG exons.

Until now, six types of partial TBG deficiency are characterized based on their concentration in serum and biochemical properties [5]. Sequencing analysis of the variants found in French-Canadian, American black and Australian Aborigines revealed one or two nucleotide substitutions in TBG exons [6–10]. In Japan, no genetic analysis of partial TBG deficiency has been reported. We studied some physico-chemical properties of TBG in a Japanese family manifesting partial TBG deficiency (TBG-PDJ). The affected male has a

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reduced concentration of TBG in serum. This TBG presented heat lability and a normal pattern of isoelectric focusing. To elucidate the molecular nature of this TBG, the entire coding exons and exon/intron junctions of the propositus were sequenced after polymerase chain reaction. A nucleotide substitution was found in the codon for amino acid 363 CTT (leucine) rather than CCT (proline). The same nucleotide substitution was found in his mother as heterozygote. The reduced heat stability due to a single amino acid substitution of the TBG molecule may be the cause of this partial TBG deficiency [13].

Materials and Methods

Subjects

Blood samples were obtained from four members of this partial TBG deficiency family. The pedigree of this family is shown in Fig. 1. The propositus was diagnosed as partial TBG deficiency because of the reduced concentration of TBG in serum. The concentration was determined by means of a highly sensitive EIA provided by Amano Pharmaceutical Co., Nagoya, Japan, as previously described [11]. Serum total T₄ (TT₄), total T₃ (TT₃), free T₄ (FT₄) and TSH were measured with commercial kits.

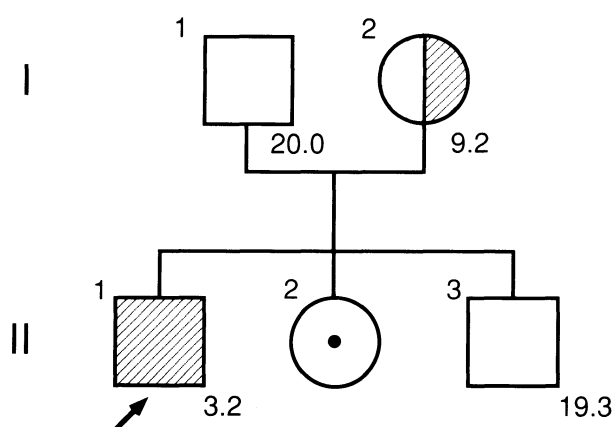


Fig. 1. Pedigree of family with partial TBG deficiency. Subjects are identified by generation (in Roman numerals) and a number to the left and above each symbol. Numbers under each symbol are concentrations of TBG in micrograms per milliliter of serum. Open boxes, unaffected male; hatched box, hemizygous affected male; partially hatched circle, heterozygous affected females; dot, female not studied in detail; arrow, propositus.

Heat lability

The rate of heat denaturation of TBG was determined in individual serum samples by incubating at 42, 48, 54 and 60°C for various periods of time and measuring the residual TBG concentrations by the EIA. Data were expressed as percentages of the TBG concentration before heat exposure [14].

Isoelectric Focusing (IEF)

IEF analysis of TBG was performed as previously described [14]. Five μ l of serum from a patient or normal subject was incubated with [¹²⁵I] thyroxine (Amersham Japan, Tokyo, Japan) and subjected to IEF by means of the Pharmacia Phastsystem (Pharmacia LKB Biotechnology, Sweden).

DNA amplification and sequencing of TBG gene

Genomic DNA was obtained by extraction from white blood cells, as previously described [15]. DNA from the propositus served as a template to amplify the coding regions (Exon 0 through Exon 4) and the adjacent exon/intron junctions of the TBG gene by the polymerase chain reaction (PCR) with Taq DNA polymerase (Perkin-Elmer Cetus Corp., Norwalk, CT), using Parkin-Elmer Cetus Thermal Cycler (model PJ1000), under the conditions and with the oligonucleotide primers described previously [11]. PCR products were isolated, purified and subcloned into the bacteriophage M13mp18 and mp19 by a standard technique. Inserts were then sequenced by the dideoxy chain termination method with α [³⁵S] dCTP (Amersham Japan, Tokyo, Japan) [16]. The strategy of sequencing was described previously [11].

Since single nucleotide substitution was identified in exon 4 in the TBG gene of the propositus, direct sequencing of the PCR product was performed on the DNA templates from four members of this family. The PCR products were alkaline-denatured and subsequently sequenced according to the manufacture's manual (Sequenase Ver. 2.0, United State Biochemical, Cleveland, OH). The internal oligonucleotide TTAGCTTCTAGACCTTCCCAAAGACT or TTCCCTAGAAAGAGAATACTCCTTG was used as the sequencing primer. The samples were analyzed by

electrophoresis on 6% polyacrylamide/6M urea gel, followed by autoradiography with X-AR5 film (Eastman Kodak, Rochester, NY).

Results

Thyroid function tests in four members of a TBG-PDJ family are shown in Table 1. In the proband, the concentration of TBG was the lowest. The heterozygous female had TBG, TT₄, and TT₃ values intermediate to those of the affected male and unaffected family members. All family members were clinically euthyroid and had normal TSH, FT₄ concentrations in serum. As the pedigree in Fig. 1 shows, the inheritance appears to be X-chromosome linked.

When serum samples from a normal control and the proband were heated at 60°C, the rates of TBG disappearance were indistinguishable from each other (Fig. 2). However, when the serum from the proband was incubated at 42, 48 or 54°C, significant disappearance of TBG was noted during 20 min incubation. Note that there was no loss of TBG immunoreactivity in the control serum at these temperatures.

The sequence of the TBG gene of the proband was determined after amplification of the entire coding sequences and exon/intron junctions by PCR and subsequent cloning into M13 bacteriophages. Only one nucleotide substitution was uncovered in exon 4. It was a C- to -T transversion, resulting in the substitution of the normal codon of proline 363 (CCT) by leucine (CTT). This substitution was confirmed by sequencing the two different PCR products. Furthermore, we analyzed exon 4 by direct sequencing of the PCR product from the proband to rule out the

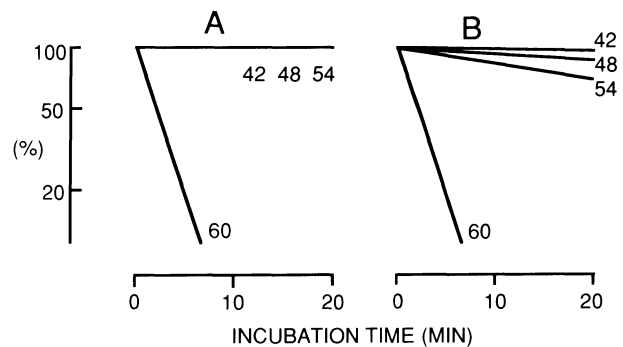


Fig. 2. Rates of TBG denaturation at various temperatures of TBG-PDJ. Serum samples from TBG-C (A) or TBG-PDJ (B) were exposed to 42, 48, 54 and 60°C for 10 or 20 min followed by measurement of TBG by a highly sensitive EIA. Results are expressed as the percentage of the TBG concentration before heat treatment.

possibility that the mutation was derived from PCR error. As shown in Fig. 3-B, the same C- to -T transversion was depicted, confirming his hemizygosity. Sequencing of the products from patients 1-2, revealed C- and T-bands corresponding to the position of C in the common type TBG gene (Fig. 3-C). No nucleotide substitution was detected in two unaffected family members or a normal control.

The substitution of leucine for proline seems not to be associated with a pI change in the TBG-PDJ because they are both neutral amino acids. Indeed, isoelectric focusing of TBG-C and TBG-PDJ were indistinguishable from each other (Fig. 4).

Discussion

A nucleotide substitution in the coding area of the TBG gene was detected by sequencing the

Table 1. Serum thyroid hormones, TSH and TBG concentrations

	TT ₄ (μg/dl)	TT ₃ (ng/dl)	FT ₄ (ng/dl)	TBG (μg/ml)	TSH (μU/ml)
I-1	7.1	105	1.3	20.0	1.80
I-2	4.4	82	1.5	9.2	2.30
II-1	3.6	69	1.3	3.2	2.17
II-3	6.1	132	1.5	19.3	2.09
Normal range	6-13	80-180	0.8-2.2	15-28	0.3-3.2

TT₄, TT₃, FT₄ and TSH were measured with commercial RIA kits. TBG was determined by means of a highly sensitive EIA as described in "Materials and Methods".

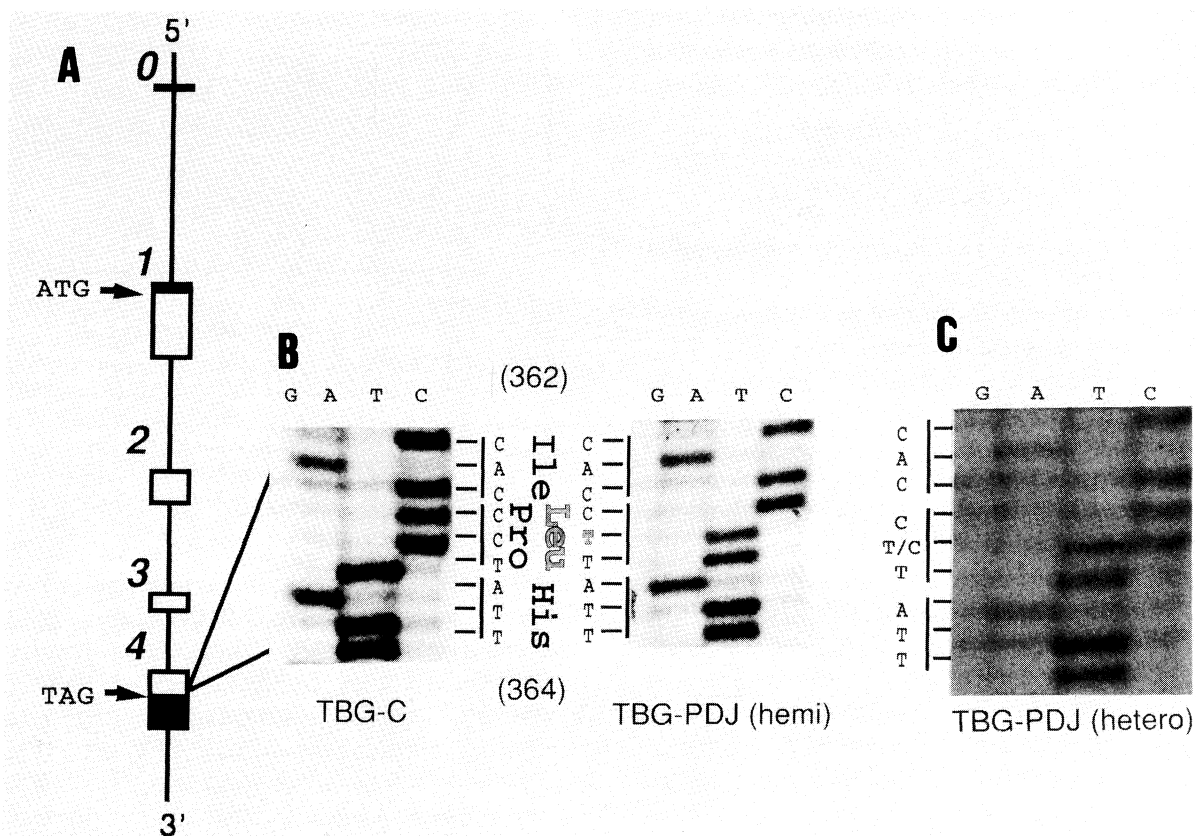


Fig. 3. Direct sequencing of PCR-amplified DNA showing the mutation site in TBG-PDJ. Panel A depicts the organization of the TBG gene. Boxes represent exons, with coding regions in an open box; connecting lines are introns. Start (ATG) and stop (TAG) codons are also indicated. Panel B depicts a part of the sequencing gels of exon 4 from a normal subject and the proband. Amino acid residues numbered consecutively from the N-terminus of the mature protein are shown in brackets. Cytosine in codon 363 of TBG-C is replaced with thymine in TBG-PDJ. The resulting amino acid substitution is depicted. Panel C represents the sequencing gel of the exon 4 from the heterozygous mother (I-2 in Fig. 1).

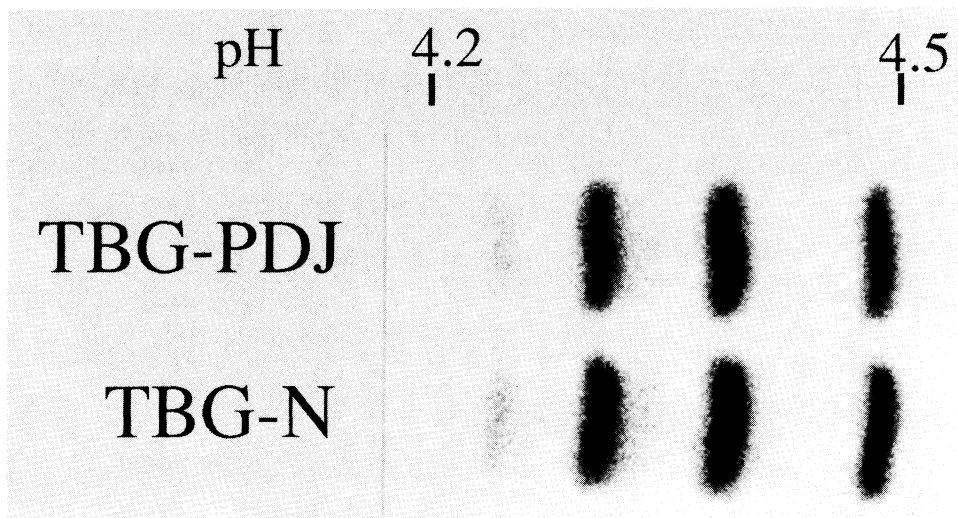


Fig. 4. Isoelectric focusing of serum sample TBG. Serum samples from patient (TBG-PDJ) and normal control (TBG-N) were preincubated with [125 I]thyroxine, then subjected to isoelectric focusing followed by autoradiography.

DNA fragments generated by PCR from a Japanese male with partial TBG deficiency. The segregation of the mutation in the pedigree suggested that this mutation may be responsible for TBG-PDJ. Until now, 6 TBG variants manifesting partial deficiency were reported for various ethnic origins. However, there have been no reports on genetic analysis of Japanese. This is therefore the first report of mutation in the TBG gene in a Japanese family with TBG-PD.

Like other TBG variants, the mutation found in TBG-PDJ was a nucleotide substitution at codon 363, replacing proline with leucine. It is of interest to note that the replacement of leucine by proline was reported in one family with complete TBG deficiency (TBG-CD5) [17]. Since proline is an aromatic amino acid which is thought to cause α -helix breakage, the mutation Pro to Leu or *vice versa* may influence the tertiary structure of the TBG molecule. The change in the tertiary structure may account for the decreased heat stability and the reduced level of immunoreactive TBG in the patient serum. Theoretically, the partial TBG deficiency could be due to alterations in gene expression, abnormal intracellular processing, or

an accelerated rate of degradation due to instability of the variant molecules. The last has been thought to be the main cause of partial TBG deficiency [5]. However, impaired secretion has recently also been shown to contribute to the reduction of TBG in circulation [18, 19]. Further studies, including *in vitro* expression of the mutant TBG, may therefore be required to clarify the mechanism of TBG-PDJ.

Studies of the various physico-chemical characteristics have revealed that some forms of TBG variant manifesting partial TBG deficiency can be conserved in several races, for example TBG-A [14], or prevalent in different areas, for example TBG-S [5]. Thus, the prevalence of TBG-PD having the same mutation should be studied. For complete TBG deficiency, we found only one kind of mutation of the TBG gene, a single nucleotide deletion in exon 4 of the TBG gene, in 18 unrelated Japanese families living in various areas [12]. It is noted that the same mutation was reported in a family with TBG-PD by Shirotani *et al.* in an abstract form [20]. Thus, this mutation could also be prevalent in Japanese, but further investigation is required.

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