

## Gene Screening of 23 Japanese Families with Complete Thyroxine-Binding Globulin Deficiency: Identification of a Nucleotide Deletion at Codon 352 as a Common Cause

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**Abstract.** Thyroxine-binding globulin (TBG) is a major thyroid hormone transport protein in human serum. Its complete deficiency (TBG-CD) is one of inherited TBG abnormalities that transmit on X-chromosome. We previously reported a nucleotide deletion at codon 352 of the TBG gene (TBG-CDJ) in Japanese families with TBG-CD. To determine the prevalence of this mutation in Japanese with TBG-CD, 23 affected subjects (19 males and 4 females) belonging to unrelated families living in 4 major islands of Japan were analyzed with regard to the mutation at codon 352. Their genomic DNAs were amplified by the polymerase chain reaction with allele specific primers. Nineteen male and four female subjects were shown to have the mutation as hemizygotes and heterozygotes, respectively. It is concluded that TBG-CDJ may be a common cause of TBG-CD in Japanese and might have appeared in the ancestors of the Japanese after the human race divergence.

**Key words:** Thyroxine-binding globulin, TBG-CDJ, Gene screening, Allele specific amplification.  
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**THYROXINE**-binding globulin (TBG) is a 54 kD glycoprotein which consists of a 395 amino acid core polypeptide with 4 oligosaccharide chains [1, 2]. It is synthesized in the liver and serves as a major transport protein for thyroid hormones in human serum. It binds 70% of thyroxine ( $T_4$ ) and triiodothyronine ( $T_3$ ) in human serum [2–5]. A decrease in, or absence of, serum TBG is usually accompanied by abnormally low serum total  $T_4$  and  $T_3$  concentrations while free  $T_4$  and free  $T_3$  values are unaltered, if the thyroid status remains euthyroid. Partial TBG deficiency is defined as a low TBG level in serum, while complete TBG deficiency (TBG-CD) is characterized by TBG in serum which is undetectable even with a sensitive

assay. Several kinds of hereditary defects in the TBG molecule have been characterized [6–13]. They are inherited in X-linked fashion [5, 14, 15]. Therefore, defective TBG phenotypes are fully expressed in males. Meanwhile, females carrying both normal and TBG-CD alleles are usually phenotyped as partial TBG deficiency, and may not be recognized as TBG deficient because the extent of the decrease in TBG tends to be mild.

The TBG gene is located on the long arm of the X-chromosome [16] and has been shown to be a member of the serpin (serine protease inhibitor) superfamily [1, 17]. Mutations in the coding regions of the TBG gene have been reported in some of the TBG variants [18–29]. As for TBG-CD, replacement of Leu227 by Pro was detected in three of six French Canadian pedigrees [19]. Single nucleotide deletion at codon 165 was also reported to lead to TBG-CD in a family with an English background [21]. We have described a nucleotide deletion at codon 352 of the genomic

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TBG gene (TBG-CDJ) in Japanese subjects. A nucleotide deletion at codon 352 resulted in 22 amino acid truncation and further 22 amino acid divergence at its carboxy-terminus [22].

We have shown the existence of nucleotide deletion in six families by utilizing primer directed mutagenesis in the polymerase chain reaction (PCR)[22]. Primer directed mutagenesis with selective enzyme digestion is a useful method to use in detecting a known mutation. However, it is time consuming to digest PCR products. Recently, an easier and more direct approach, namely allele specific amplification, was established [30]. In this report, we carried out a screening program with this method to examine the prevalence of this particular mutation in 23 Japanese pedigrees with TBG-CD. A strong linkage between single nucleotide deletion at codon 352 and TBG-CD in Japanese was demonstrated.

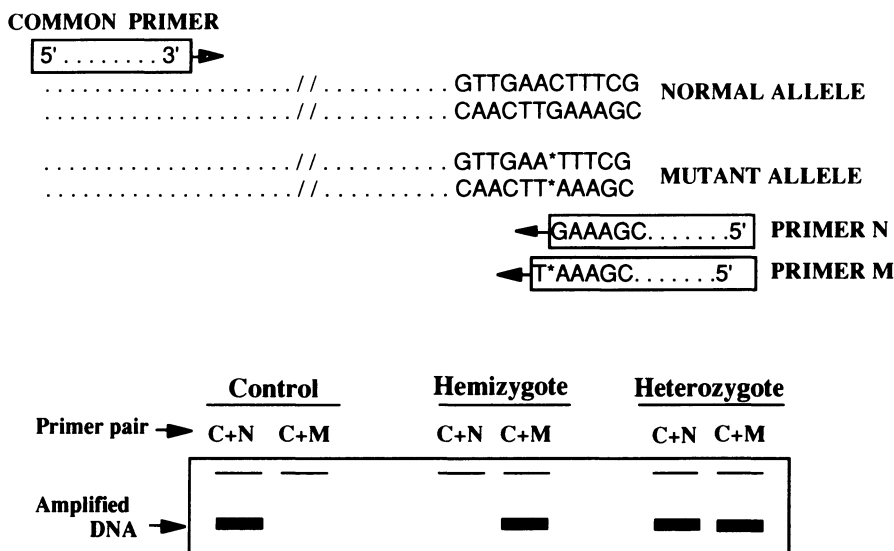
### Materials and Methods

Twenty-three families suspected of including TBG-CD subjects were analyzed in this study. In 19 of 23 families, there was an affected male

subject available for study. Female subjects were chosen from the remaining 4 families.

Serum total T<sub>4</sub>, total T<sub>3</sub> and TSH concentrations were determined with commercial RIA kits. Serum TBG concentrations in candidates were determined with a highly sensitive TBG enzyme immunoassay (EIA) kindly provided by Amano Pharmaceuticals Co., Nagoya, Japan. The detection limit of this kit was 0.1 mg/L. The diagnosis of TBG-CD was defined as undetectable TBG in serum with this assay.

Genomic DNA was extracted from peripheral white blood cells as described previously [18]. Oligonucleotide primers specific for normal (primer N) and mutant (primer M) TBG genes were chemically synthesized with a DNA synthesizer (Model 381 A, Applied Biosystems, Foster city, CA). Both primers were complementary to the sense strand of the TBG gene and were identical with each other except for the nucleotide at the 3' end (G for primer N and T for primer M, respectively). Common primer (C) was also synthesized as complementary to the antisense strand. The sequence and location of each primer are as follows: N: (3446) 5'-TTTTCAGGCTGATCCGAAG-3' (3427), M: (3446) 5'-TTTTCAGGCTGATCCGAAAT-3' (3426), and C: (3015) 5'-ATCCT-



**Fig. 1.** The strategy of screening for the mutation with allele specific amplification. In the upper part of the figure, the relationships between primer pairs and TBG genes are shown. Asterisks in the mutant allele show a nucleotide deletion recognized in TBG-CDJ. Primer N and primer M were complementary to the sense strands of the normal and mutant TBG alleles, respectively. The primer pair of C and N amplifies the normal TBG allele, and the primer pair of C and M amplifies the mutant one. The relationships between amplified bands and genotypes are shown in the lower part of the figure.

TGACCTTTATGTCCC-3' (3034), where numbers in parentheses represent the positions of the first and last nucleotides from the translation initiation codon (ATG) in the genomic TBG gene sequence (Fig. 1).

PCR was carried out as described elsewhere [25] with some modifications. Briefly, the reaction was carried out in a final volume of 100  $\mu$ l with 50 pmol of each primer. One  $\mu$ g of genomic DNA from white blood cells was used as a template. Initial denaturation for 240 sec at 94°C was followed by 30 cycles of denaturing for 90 sec at 94°C, annealing for 150 sec at 56°C, and primer extension for 210 sec at 72°C, with final extension for 15 min at 72°C. The PCR products were electrophoresed on a 1.5% agarose gel, stained with ethidium bromide, and visualized by UV light. PhiX-174 phage DNA digested with Hae III (Toyobo, Tokyo, Japan) was used as a molecular

weight marker. The relationships between the amplified DNA fragments and various genotypes are shown on Fig. 1. The specificity of the primers has been confirmed and reported elsewhere [31].

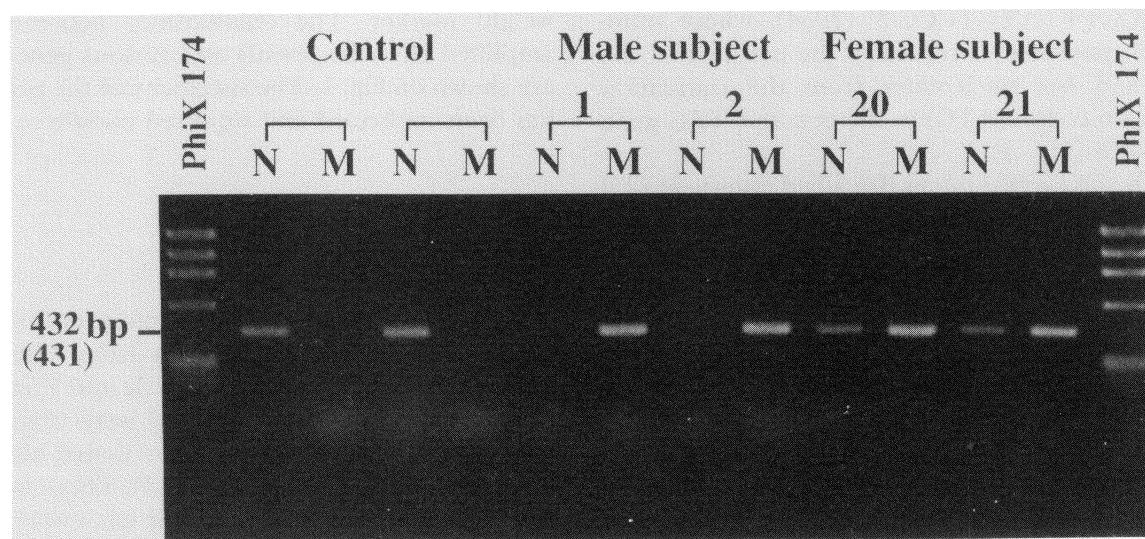
## Results

In response to our requests of many institutions in Japan to send blood samples from TBG deficient subjects, a total of 23 (19 male and 4 female) samples from unrelated families were obtained. Their residences were widely distributed all over the main Japanese islands (Table 1). There was no consanguinity among them. Their laboratory data are summarized on Table 1. Three males (Nos. 2, 7 and 9) and one female (No. 21) treated with *l*-thyroxine had undetectable serum TSH and low

**Table 1.** Serum TT<sub>4</sub>, TT<sub>3</sub>, TSH and TBG concentrations and native places

|                  | TT <sub>4</sub><br>(nmol/L) | TT <sub>3</sub><br>(nmol/L) | TSH<br>(mU/L)      | TBG<br>(mg/L) | RESIDENCE |
|------------------|-----------------------------|-----------------------------|--------------------|---------------|-----------|
| <b>Males</b>     |                             |                             |                    |               |           |
| 1 <sup>c)</sup>  | 43.8                        | 0.92                        | 2.7                | <0.1          | Honshu    |
| 2 <sup>c)</sup>  | 61.8                        | 0.92                        | <0.1 <sup>a)</sup> | <0.1          | Honshu    |
| 3 <sup>c)</sup>  | 46.3                        | 0.92                        | 1.8                | <0.1          | Kyushu    |
| 4 <sup>c)</sup>  | 41.2                        | 0.92                        | 0.5                | <0.1          | Honshu    |
| 5                | 16.7                        | 0.76                        | 6.0                | <0.1          | Kyushu    |
| 6                | 32.2                        | 0.92                        | 1.0                | <0.1          | Honshu    |
| 7                | 27.0                        | 0.61                        | <0.1 <sup>a)</sup> | <0.1          | Honshu    |
| 8                | 46.3                        | 0.92                        | 0.1                | <0.1          | Honshu    |
| 9                | 28.3                        | 0.31                        | <0.1 <sup>a)</sup> | <0.1          | Honshu    |
| 10               | 29.6                        | 1.04                        | 0.5                | <0.1          | Honshu    |
| 11               | 43.8                        | 0.90                        | 0.6                | <0.1          | Kyushu    |
| 12               | 42.5                        | 0.22                        | 1.7                | <0.1          | Kyushu    |
| 13               | <16.7                       | <0.31                       | 1.8                | <0.1          | Honshu    |
| 14               | 9.3                         | 1.06                        | 1.8                | <0.1          | Shikoku   |
| 15               | 21.9                        | 0.76                        | 1.3                | <0.1          | Shikoku   |
| 16               | 64.4                        | 0.92                        | 2.7                | <0.1          | Honshu    |
| 17               | 46.3                        | 1.21                        | 2.8                | <0.1          | Hokkaido  |
| 18               | 39.9                        | 1.10                        | 1.8                | <0.1          | Honshu    |
| 19               | 30.9                        | 1.09                        | 2.8                | <0.1          | Kyushu    |
| <b>Females</b>   |                             |                             |                    |               |           |
| 20 <sup>c)</sup> | 60.5                        | 1.38                        | 3.0                | 7.8           | Honshu    |
| 21 <sup>c)</sup> | 55.3                        | 1.22                        | <0.1 <sup>a)</sup> | 11.0          | Honshu    |
| 22               | 32.2                        | 0.92                        | 1.8                | 4.4           | Honshu    |
| 23               | 97.8                        | 3.48                        | <0.1 <sup>b)</sup> | 5.0           | Honshu    |
| Normal ranges    | 64.4–167.3                  | 1.07–2.91                   | 0.5–3.5            | 15.6–29.1     |           |

Total T<sub>4</sub>(TT<sub>4</sub>), Total T<sub>3</sub>(TT<sub>3</sub>) and TSH were measured with commercial RIA kits. TBG was determined by means of a highly sensitive EIA as described in Materials and Methods. As the native place of the subjects, the names of the 4 major islands of Japan are indicated under "residence". a) under *l*-thyroxine supplementation therapy for primary hypothyroidism. b) untreated hyperthyroidism. c) previously reported in Ref [22].



**Fig. 2.** Detection of normal and TBG-CDJ mutant allele by means of allele specific amplification. DNA samples from 2 control subjects were amplified only with a primer pair of C and N (lane N). DNAs from affected males (Nos. 1 and 2), hemizygous for TBG-CDJ, were amplified only with a primer pair of C and M (lane M). Affected females (No. 20, 21), heterozygous of normal and mutant alleles, were amplified with both primer pairs (lanes N and M). PhiX 174 digested with Hae III served as a molecular weight marker.

total  $T_4$  and/or total  $T_3$  levels. Also a severe hyperthyroid subject (No. 23) with a suppressed serum TSH level had normal total  $T_4$  and slightly increased total  $T_3$ . Serum TBG levels of all males were below the detection limit (0.1 mg/L) of the EIA, indicating that they were hemizygotes of TBG-CD. Four females were shown to have decreased serum TBG (7.8, 11.0, 4.4 and 5.0 mg/L).

Their genomic DNAs were extracted from the peripheral white blood cells and subjected to polymerase chain reaction with allele specific primers. As shown in Fig. 2, a 431 base pair fragment amplified with the primer pair of C and M was clearly demonstrated at lane M of male subjects. A pair of primers, C and N, gave no bands at 432 base pairs in any of them (lane N in Fig. 2). These results indicate that two male subjects (No. 1, 2) have the nucleotide deletion on their genomic DNAs as hemizygotes. In the other 17 male subjects also, this mutation was detected (data not shown).

In all 4 female subjects, PCR utilizing both primers pairs (C and N, C and M) produced normal and mutant bands (432 and 431 base pairs, respectively). In Fig. 2, the results for two female subjects (No. 20, 21) are shown. Thus, it was indicated that they were heterozygotes for normal and TBG-CD alleles regarding this mutation.

Their decreased TBG was due to this heterozygosity.

Thus the same mutation at codon 352 in the TBG gene was demonstrated in all the 23 TBG-deficient subjects analyzed in this study. In contrast, genomic DNA from an unaffected subject with a normal serum TBG concentration led to the amplification of the DNA fragment only with a C and N primer pair, not with a C and M pair (Fig. 2, control).

## Discussion

Three kinds of mutations have been reported to manifest inherited TBG-CD. Firstly, replacement of Leu227 by Pro was detected in three of six French Canadian pedigrees [19]. Then, single nucleotide deletions at codon 165 in a family with an English background [21] and at codon 352 in Japanese [22] were also shown to lead to this hereditary condition.

As the mutation at codon 352 does not create or eliminate a recognition site for restriction enzymes in the TBG gene, we cannot utilize the standard analysis of restriction fragment length polymorphism. In a previous study we detected the mutation by generating a novel recognition sequence in the

mutant gene by the method of primer directed mutagenesis [22]. In this study, we employed allele specific amplification because of its simplicity. The screening of 23 subjects belonging to unrelated Japanese families with inherited TBG-CD has revealed that all families harbored the single nucleotide deletion at codon 352 without exception. It is concluded that TBG-CDJ may be a common cause of TBG-CD in Japanese. Furthermore it has been detected only in Japanese. Therefore it is postulated that TBG-CDJ might have appeared in the ancestors of the Japanese at an early stage after the divergence of the human races. Because defects in the TBG molecule cause no disabilities or disadvantages in the affected subjects, the mutation should have been well preserved. This could explain the higher frequency of the TBG-CD in Japanese than in Caucasians [32–35].

The mechanism by which this mutation causes the loss of immunoreactivity and biological activity had been evaluated in an expression experiment with mammalian cells [36]. TBG-CDJ was synthesized as a truncated molecule and retained within the rough endoplasmic reticulum, resulting in a complete absence of secretion.

Further study on the prevalence of this muta-

tion in Orientals, especially in Mongoloids, may provide information on the origin of this mutation as well as of the Japanese race itself.

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