Note

Sequence Analysis of Thyroid Transcription Factor-1 Gene Reveals Absence of Mutations in Patients with Thyroid Dysgenesis but Presence of Polymorphisms in the 5' Flanking Region and Intron

AKIRA HISHINUMA, TAKEO KURIBAYASHI*, YUMIKO KANNO**, KAZUMICHI ONIGATA***, KANJI NAGASHIMA***, AND TAMIO IEIRI

Department of Clinical Pathology, *Department of Pediatrics, Dokkyo University School of Medicine, Tochigi 321-0293, **Sumitomo Metal Bio-Science Inc., Tokyo 100-0004, and ***Department of Pediatrics, Gunma University School of Medicine, Maebashi, Gunma 371-8511, Japan

Abstract. Congenital hypothyroidism is caused by several mechanisms. The most common cause worldwide is iodine deficiency, but in iodine-sufficient regions thyroid dysgenesis is the most common cause of congenital hypothyroidism. In the present study we analyzed the thyroid transcription factor-1 (TTF-1) gene in patients with congenital hypothyroidism due to thyroid dysgenesis: three patients with athyrosis, five with ectopy, and one with hypoplasia. Genomic DNA was isolated from peripheral leukocytes, and the TTF-1 gene, including a 5' flanking region, two exons and one intron was amplified by polymerase chain reaction (PCR) with 4 pairs of primers. The PCR products were directly sequenced by the Dye Terminator Cycle Sequencing method. We could not find any mutations specific for the thyroid dysgenesis in the 5' flanking region, two exons and one intron in the TTF-1 gene, but two heterozygous nucleotide substitutions were detected in the intron: a G to A transition at nucleotide 469 (G469A) and a C to A transversion at nucleotide 866 (C866A). The same nucleotide changes were detected in some normal subjects. Allelic frequencies of the polymorphisms G469A and C866A were 23% and 10%, respectively. Another normal polymorphism in the 5' flanking region was a G to T transversion at nucleotide -845 from the transcription start site (G-845T). The allelic frequency of the polymorphism G-845T was 28%. We also found 12 polymorphisms in the 5' flanking region, two in the intron and one in the 3' untranslated region. These polymorphisms were detected in 100% chromosomes. These results suggest that congenital hypothyroidism associated with thyroid dysgenesis is unlikely to be caused by mutations in the TTF-1 gene in which, however, were detected normal polymorphisms in the 5' flanking region, intron and 3' untranslated region.

Key words: Thyroid dysgenesis, Thyroid transcription factor-1, Polymorphism, Hypothyroidism


Developmental defects in thyroid organogenesis include the complete absence of the thyroid (athyrosis), ectopy of the thyroid in sublingual to mediastinal locations and hypoplasia in the normal position in the neck, but the underlying molecular mechanisms remain elusive. Much attention has been focused on the thyrotropin receptor (TSHR) gene, but a mutation in the TSHR gene caused thyroid dysgenesis in only one out of 100 patients [1]. In another study no linkage to the TSHR gene was found in patients with familial congenital hypothyroidism [2].
Normally the thyroid glands develop from the ventral wall of the primitive endodermal pharynx between the first and second branchial arch by gestational day 16 in humans, and then migrate caudally to the final position in the neck ventral to the trachea by gestational week 10. Thyroid transcription factor-1 (TTF-1), a homeodomain-containing nuclear transcription protein, is first expressed in the thyroid anlage committed to become the thyroid before expression of the thyroid-specific proteins such as thyroglobulin and thyroperoxidase [3]. It was shown that TTF-1 transcriptionally stimulates expression of these thyroid-specific proteins [4]. The TTF-1 disrupted mouse displayed absence of the thyroid glands as well as defective organogenesis of the lungs, ventral forebrain and pituitary gland [5]. Recently, it was reported by two separate groups that mutations in the coding region of the TTF-1 gene were not detected in European patients with thyroid dysgenesis [6, 7]. In the present study we analyzed the TTF-1 gene including the 5' flanking region, two exons and one intron in Japanese patients with congenital hypothyroidism due to thyroid dysgenesis.

**Patients and Methods**

**Patients**

Nine patients with thyroid dysgenesis aged between 5 months and 29 years have been studied, three with athyrosis, five with ectopy, and one with hypoplasia. The diagnosis was confirmed by echography as well as the measurement of serum concentrations of thyroxine (T4), triiodothyronine (T3) and TSH. 123I scintigraphy was employed for some patients. For example, the patient with hypoplasia was hypothyroid (T4 and T3, under the detection limit; and TSH 880 mIU/ml) and a half-sized thyroid gland was detected in the normal position by echography.

**Screening of mutations by RNase cleavage assay and direct sequencing of PCR products**

Genomic DNA was isolated from EDTA-treated blood with a QIAamp Blood Kit (QIAGEN, Hilden, Germany). The final DNA concentration was 10 to 20 ng/μl. One to five μl of DNA was amplified by polymerase chain reaction (PCR) in a GeneAmp 9600 thermal cycler (Perkin Elmer, Foster City, CA, USA) with four pairs of primers which span the 5' flanking region, two exons and one intron of the TTF-1 gene. The nucleotide sequences are 5'-ATA TTT AGG TGA CAC TAT AGG AGG CAG TCG ATC CCC TAC TCA GC-3' (1F), 5'-AAT AAT ACG ACT CAC TAT AGG ACC CTC CAT GCC CAC TTG CTG GTA-3'(1R), 5'-AAT AAT ACG ACT CAC TAT AGG ACA GCC GCC GCC GAA TCA T-3' (2F), 5'-ATA TTT AGG TGA CAC TAT AGG AGA GAG GTT AGG AGA GGG GGT GTT GA-3' (2R), 5'-AAT AAT ACG ACT CAC TAT AGG ATC AAC ACC CCC TCT CCT AAC CTC TC-3' (3F), 5'-ATA TTT AGG TGA CAC TAT AGG CCT GGC GCT TCA TTT TGT AG-3' (3R), 5'-AAT AAT ACG ACT CAC TAT AGG ATG GTG GCG GTG AGC AAG AAC AT-3' (4F), and 5'-ATA TTT AGG TGA CAC TAT AGG AGG GTT AGA ACG GAG CAG GAC AGC AGG-3' (4R). The locations of the primers in the TTF-1 gene are shown in Fig. 1. Since the GC content of the TTF-1 gene was very high, DNA was denatured at 98 °C. The PCR buffer was optimized with pH 9.5 and 3.5 mM MgCl₂ (HotWax OptiStart Kit, Invitrogen, San Diego, CA, USA). In order to avoid non-specific amplification, nucleotide polymerization was catalyzed at 63 °C by TaKaRa

![Fig. 1. Primer locations in the TTF-1 gene.](image-url)
POLYMORPHISMS IN TTF-1 GENE

Ex Taq polymerase (Takara, Kyoto) reacted with TaqStart Antibody (Clontech, Palo Alto, CA, USA). Since the primers were tagged with T7 and SP6 RNA polymerase sequences at their 5' ends, the PCR products were first used for RNase cleavage assay (Mismatch Detect kit, Ambion, Austin, TX, USA). The PCR products were then purified by Geneclean II (BIO 101, La Jolla, CA, USA) and directly sequenced by a cycle sequencing method (Dye Terminator Cycle Sequencing Ready Reaction Kit, Perkin Elmer) with the primers used in the PCR reaction on an automatic DNA sequencer (Model 377 DNA sequencer, Perkin Elmer). The nucleotide sequences were compared to the sequences reported by Ikeda et al. [8] and Lonigro et al. [9] with DNASIS software (Takara). The heterozygous polymorphisms were confirmed by cloning the PCR products in the pCR2.1 plasmid vector (Original TA cloning kit, Invitrogen, San Diego, CA, USA) and sequencing. The polymorphisms in the intron were studied in 11 controls by direct sequencing of the PCR products generated with an extra set of primers (5'-GCG GGT ACT CGG CGT CTC CT-3' and 5'-GGC TGC CCT CCC TCA GTC TCA-3'). The polymorphisms in the 5' flanking region were analyzed in 11 controls by sequencing the PCR products with the primers 1F and 1R with an additional primer 1aR (5'-CGG GCA CGG ACA GGT CT-3').

**Results and Discussion**

We first screened the TTF-1 gene for mutations by RNase cleavage assay, but we could not find any RNase sensitive sites. Since the RNase cleavage assay only detects 75% of mutations depending on sequences, we further sequenced the PCR products of all the cases.

We did not detect any mutation in the protein coding region of the TTF-1 gene, but several nucleotide substitutions were found in the intron and 5' flanking region. In the intron we detected two heterozygous nucleotide substitutions. A guanine to adenine transition at nucleotide 469 (G469A) was found in two patients with ectopy and one with hypoplasia (Fig. 2A), and a cytosine to adenine transversion at nucleotide 866 (C866A), in one patient with athyrosis (Fig. 2B), but the polymorphism G469A was observed in 23% of 40 chromosomes and the polymorphism C866A, in 10% (Fig. 3).

In the 5' flanking region of the TTF-1 gene we detected a guanine to thymine transition at nucleotide -854 upstream from the translation start site (G-854T) in two patients with ectopy and one with hypoplasia (Fig. 2C). Although the precise extent of the promoter was not yet identified in the human TTF-1 gene in thyroid cells, the 5' flanking region of about 1,000 nucleotides from the translation start site was analyzed. This region is
HISHINUMA et al. reported to be responsible for about two thirds of the promoter activity in MLE-15 lung adenocarcinoma cells [10]. Several transcription factors activate transcription of the TTF-1 gene by binding to this region. For example, HOXB3 binds to two regions of the rat TTF-1 gene (−300 and −170 from the transcription start site) and activates expression of the TTF-1 gene in the thyroid [11], and Oct1 and hepatocyte nuclear factor 3 bind to the same region or nearby regions in the proximal promoter of the human TTF-1 gene and activate the TTF-1 expression in lung epithelial cells [10, 12]. The nucleotide −854 is more upstream from these regions which mediate activation of transcription of the TTF-1 gene. The polymorphism G-854T would not affect expression of the TTF-1 gene because this nucleotide substitution was found in normal controls. The allelic frequency was 28%.

We also found 12 polymorphisms in the 5' flanking region, two in the intron and one in the 3' untranslated region (3'UTR) (Fig. 3). These polymorphisms were detected in 100% chromosomes. Polymorphisms G59C and 60insG in the intron and a polymorphism 40insC in the 3'UTR were also reported by Lonigro et al. [9]. All of the polymorphisms in the 5' flanking region have not been reported. It is not certain if these polymorphisms originate in the racial difference between Orientals and Caucasians or the reported sequence was not correct.

In conclusion, the thyroid dysgenesis is unlikely to be caused by mutations in the TTF-1 gene including the 5' flanking region, two exons, and one intron. This result is associated with an indispensable role of TTF-1 in embryonic development because it was reported that TTF-1 disrupted mice were born dead and defects in several organs, including the thyroid, lung, pituitary and a restrict region of the forebrain, were found [3]. To elucidate the mechanisms of thyroid dysgenesis, the distal promoter of the TTF-1 gene is of interest because the rat TTF-1 gene contains two consensus TTF-1 binding motifs which positively regulate transcription of the TTF-1 gene [13]. Other transcription factors which might be explored include PAX8 [14] and TTF-2 [15].

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References


