Polymorphism of the polyalanine tract of thyroid transcription factor-2 gene in patients with thyroid dysgenesis

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Abstract

Objective: One of the thyroid-specific transcription factors, thyroid transcription factor-2 (TTF-2), performs a crucial role in the development of the thyroid gland. We performed genetic analysis of the TITF2 gene (encoding TTF-2) in patients with thyroid dysgenesis.

Methods: By direct sequencing of the PCR products of TITF2, we screened the genomic DNA from 46 patients with thyroid dysgenesis (five had agenesis, six had hypoplasia, 15 had ectopy, and 20 were undetermined). We also studied the transcriptional activities of TITF2 by co-expressing the luciferase gene directed by the human thyroglobulin gene promoter.

Results: Human TITF2 consists of a forkhead domain, a polyalanine tract, and unique C-terminal residues. In one of the patients with an ectopic sublingual thyroid, we found a polyalanine tract of 11 alanine residues on one chromosome instead of the 14 alanine residues found in normal controls. In one patient with hypoplasia, the polyalanine tract consisted of 12 heterozygous alanine residues. The reduced polyalanine tracts were not detected in 101 normal individuals. However, the expression study showed that the transcriptional activities of TITF2 with reduced polyalanine-tract lengths were equal to that of TITF2 with an unreduced polyalanine tract.

Conclusion: These results suggest that the polymorphism of the polyalanine tract of TITF2 is not a frequent cause of developmental defects of the human thyroid gland.

Introduction

Thyroid transcription factor-2 (TTF-2) was initially identified to regulate expression of the thyroid-specific genes, thyroglobulin and thyroperoxidase, coordinately with thyroid transcription factor-1 (TTF-1) and paired box homeotic gene 8 (PAX8) (1). TTF-2 is temporarily expressed in the developing thyroid, which is consistent with a role in control of the morphogenesis of the thyroid gland (2). TITF2-null mutant mice exhibited either a sublingual or a completely absent thyroid gland as well as a cleft palate (3). Cloning of the human TITF2 gene showed that human TITF2 was identical to forkhead transcription factor FKHR15 (4).

Thyroid dysgenesis is the main cause of congenital primary hypothyroidism, which affects 1 in 4000 newborns. Developmental defects of the thyroid gland range from agenesis and ectopy to hypoplasia. In most cases of thyroid dysgenesis, genetic abnormalities are unknown, although a few cases, caused by mutations of the thyrotropin receptor (5–7) and PAX8 (8), or deletion of TITF1 (encoding TTF-1) (9, 10), have been reported. Only one case of thyroid dysgenesis associated with a cleft palate and choanal atresia was caused by a missense mutation of TITF2 (11).

In this work, we studied TITF2 in patients with thyroid dysgenesis. TITF2 consists of a single exon that codes for a forkhead domain, a polyalanine tract, and unique C-terminal residues. Initially, the polyalanine tract was reported to consist of 19 residues (4). Subsequently, the length of the major alanine stretch was shown to be 14 residues (12). We detected reduced polyalanine residues in two patients with thyroid dysgenesis, and we performed a functional analysis.

Materials and methods

TITF2 gene analysis

Genomic DNA was isolated from 200 μl peripheral blood, obtained from each of 46 patients with thyroid dysgenesis (five with agenesis, six with hypoplasia, 15 with ectopy, and 20 that were undetermined), using a
QAmp Blood Kit (Qiagen, Hilden, Germany). Using the oligo computer software package, version 4 (National Bioscience, Plymouth, MN, USA), PCR primers were designed to amplify the entire TITF2 gene in two segments, avoiding the formation of primer dimers and hairpins as well as low values for the Gibbs’ free energy of 3’ pentamers. The primer sequences are 5’-CGA CGA TCC CCT GAG-3’ (1F), 5’-GCC GGG TAG GTG GAG AGG TC-3’ (1R), 5’-GCT GGG CGG CAT CTA CAA GTT-3’ (2F), and 5’-GCC TGC TCG GTC TTT TCC AC-3’ (2R). The PCR reaction was performed using the Expand High-fidelity PCR System (Roche, Mannheim, Germany) in a 50 μl solution containing 12% dimethylsulfoxide. Forty-five PCR cycles were used, consisting of denaturation at 98 °C for 4 s, primer annealing at 55 °C for 30 s, and primer extension at 72 °C for 60 s. PCR products were electrophoresed in 1% SeaKem GTG agarose gel (FMC Bioproducts, Rockland, ME, USA) then purified with a GeneClean II kit (Bio101, La Jolla, CA, USA). Sequencing of the PCR products was carried out using a cycle sequencing method (Dye Terminator Cycle Sequencing Ready Reaction Kit; Perkin Elmer, Foster City, CA, USA) employing the same primers as those used for the PCR amplification. Subsequent analysis was performed on an ABI 377 Sequencer (Perkin Elmer). To study the allelic frequency of polyalanine-stretch lengths, genomic DNA from 101 normal individuals was amplified with the forward primer 5’-CTT CAA GCG CTC GGA and the reverse primer 5’-TTT GGT ACC TTT TCC-3’ and the reverse primer 5’-ACG CCG CGG GGT AGT AGA CCC TGC-3’. Differences in PCR-product lengths were analyzed on 2% Metaphor agarose gels (FMC Bioproducts). Polymorphisms of polyalanine-stretch lengths were confirmed by direct sequencing of the PCR products. Genomic DNA from family members of the patients with reduced polyalanine-stretch lengths were confirmed by direct sequencing, as described above. TITF2 expression plasmids with mutations in the forhead domain, R72S, or disruption of the polyalanine tract by valine, (Ala)7(Val)(Ala)6, were also obtained after incorrect incorporation of nucleotides by the polymerase. The human thyroglobulin promoter (−340 bp to −21 bp), which includes a TTF-2 binding site (−147 bp to −136 bp), was amplified using the forward primer 5’-TTT GTG ACC TTT TCC CTC ACT GTG GCT TGA-3’ and the reverse primer 5’-AAA AGC GGA AGG AGG AGA AAC CAC TGC-3’ and ligated to a luciferase reporter plasmid (pGL3-Basic Vector; Promega, Madison, WI, USA) after treatment with the restriction enzymes KpnI and NheI. The TITF2 expression plasmids and the luciferase reporter plasmid, along with a pRL-CMV plasmid (Promega) that was used as an internal control, were co-transfected into the VMRC-LCD and G401 cell lines (both of which were obtained from the Human Science Research Resource Bank, Osaka, Japan), using liposome methods (Tfx-10 for VMRC-LCD and Tfx-20 for G401; Promega). Two days after transfection, the luciferase activity was measured with a luminometer (LUMAT LB 9507; EG&G Berthold, Bad Wildbad, Germany) using the Dual-Luciferase Reporter Assay System (Promega). Four independent transfection experiments were performed, each in triplicate, and the results were statistically analyzed by using paired Student’s t-tests. Values of P < 0.01 were judged as significant.

Results
Sequencing of the entire coding region of the TITF2 gene from 46 patients with thyroid dysgenesis revealed polymorphisms in the length of the polyalanine tract. Initially, the polyalanine tract was reported to consist of 19 residues (4), but the most frequent stretch length was 14 residues in 101 normal individuals (Table 1). The missing 5 residues from the 171th alanine to the 175th alanine are shown in Fig. 1. The longer stretch lengths with 16 alanine residues were found less frequently; the allelic frequency was 3% (6/202). In patients with thyroid dysgenesis, short polyalanine-stretch lengths of 11 and 12 residues were found to be heterozygous in two patients (one ectopy patient had (Ala)11/14 and one hypoplasia patient had (Ala)12/14) and also in one patient with (Ala)14/16. These

<table>
<thead>
<tr>
<th>Normal Individuals</th>
<th>Thyroid dysgenesis</th>
</tr>
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<tbody>
<tr>
<td>11/14</td>
<td>0</td>
</tr>
<tr>
<td>12/14</td>
<td>0</td>
</tr>
<tr>
<td>14/14</td>
<td>96</td>
</tr>
<tr>
<td>14/16</td>
<td>4</td>
</tr>
<tr>
<td>16/16</td>
<td>1</td>
</tr>
</tbody>
</table>

The missing 5 residues from the 171th alanine to the 175th alanine are shown in Fig. 1. The longer stretch lengths with 16 alanine residues were found less frequently; the allelic frequency was 3% (6/202). In patients with thyroid dysgenesis, short polyalanine-stretch lengths of 11 and 12 residues were found to be heterozygous in two patients (one ectopy patient had (Ala)11/14 and one hypoplasia patient had (Ala)12/14) and also in one patient with (Ala)14/16.
reduced polyalanine residues of AAA180–182del and AA168,169del (Fig. 1) were not detected in 101 normal individuals. Other single nucleotide polymorphisms were detected in four locations with allelic frequencies from 10% (2/20) to 5% (1/20), based on the sequences of 10 normal individuals (Table 2). These four single nucleotide polymorphisms did not result in changes of amino acids.

The effects of reduced polyalanine-tract length on the transcriptional activity of \textit{TTF-2} were studied by co-transfecting \textit{TTF-2} expression plasmids into cultured cell lines with a luciferase reporter plasmid transcriptionally directed by the human thyroglobulin promoter. A TTF-1-expressing lung cell line, VMRC-LCD, showed increased expression of luciferase by TTF-2, but a TTF-1-non-expressing kidney cell line, G401, did not show increased expression (data not shown). The degree of transcriptional stimulation by TTF-2 with different polyalanine-stretch lengths (11–14 residues) was not statistically different in the VMRC-LCD cells (Fig. 2). TTF-2 with a disrupted polyalanine tract, (Ala)7(Val)(Ala)6, and a mutation in the forkhead domain, R72S, showed reduced stimulation of luciferase expression.

Discussion

Direct sequencing of the entire coding region of \textit{TTF-2} revealed a highly polymorphic polyalanine tract as well as four single nucleotide polymorphisms which did not alter the amino acid residues. The polymorphism of the polyalanine tract was reported previously, the polyalanine-stretch length being from 12 to 17 residues (12). In the 101 normal Japanese subjects, only two alleles with polyalanine-stretch lengths of 14 and 16 residues were found. Reduced polyalanine-tract length was found only in patients with thyroid dysgenesis, 11 residues being found in a patient with a sublingual ectopic thyroid gland, and 12 residues being found in a patient with thyroid hypoplasia. Alteration of polyalanine-stretch lengths has also been found in a homeobox-containing gene, \textit{HOX D13}, consisting of a polyalanine tract with 15 residues (13, 14). In patients with the autosomal dominant disease synpolydactyly, elongated polyalanine tracts of 22–25 residues were reported.

A computer program that predicts protein secondary structure (nnPredict-UCSF; www.cmpharm.ucsf.edu/nomi/nnpredict.html) indicated that the polyalanine tract of TTF-2 constitutes an \(\alpha\)-helical region C-terminal to the forkhead domain (A Hishinuma, unpublished observations). In several transcription factors which repress the transcription of target genes, alanine-rich

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**Table 2** Single nucleotide polymorphisms in the \textit{TTF-2} gene.

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Amino acid</th>
<th>Normal</th>
<th>Dysplasia</th>
</tr>
</thead>
<tbody>
<tr>
<td>387C → T</td>
<td>L129L</td>
<td>0/20</td>
<td>2/92</td>
</tr>
<tr>
<td>519A → C</td>
<td>A173A</td>
<td>0/20</td>
<td>1/92</td>
</tr>
<tr>
<td>723C → T</td>
<td>P241P</td>
<td>2/20</td>
<td>5/92</td>
</tr>
<tr>
<td>834T → C</td>
<td>S278S</td>
<td>1/20</td>
<td>3/92</td>
</tr>
</tbody>
</table>
regions, which form α-helical regions and are responsible for the transcriptional repression, were identified (15–17). However, these transcriptional effector domains mediate both repression and activation, depending on the cell background (17). Activator or repressor regions of these transcription factors are adjacent or overlapping. For example, an alanine-rich region (residues 139–212) of Drosophila EVE-skipped is responsible for transcriptional activation, while the overlapping region (residues 139–227) mediates transcriptional repression (17).

TTF-2 has a dual function in the development of the thyroid gland (2). In the mouse, TTF-2 shows transient expression during the migration of thyroid precursor cells from the invagination of the pharyngeal endoderm to the final destination in front of the trachea. During this period, TTF-2 represses transcriptional activation of the thyroglobulin and thyroperoxidase promoters by TTF-1 and PAX8, respectively. Subsequently, TTF-2 expression is turned off; in the adult thyroid, TTF-2 functions as a transcriptional activator of thyroglobulin (18) and thyroperoxidase (19, 20).

We performed functional analysis of TTF-2 with reduced polyalanine tracts by co-transfecting TITF2 expression plasmids and luciferase reporter plasmids directed by the human thyroglobulin promoter. We found that TTF-2 activated luciferase expression in cells expressing the TITF1 gene. The TITF2 expression plasmid with a mutation (R72S) in the forkhead domain caused reduced expression of the reporter. The transcriptional function of TTF-2 with a missense mutation (A65V) in the forkhead domain was also abolished in patients with thyroid dysgenesis, cleft palate, and choanal atresia (11). However, no difference in the degree of luciferase expression by TITF2 expression plasmids with polyalanine-stretch lengths varying from 11 to 14 residues was found, though disruption of the polyalanine tract by valine, (Ala)7(Val)(Ala)6, did reduce transcriptional activation. Therefore, we have concluded that it is unlikely that polymorphism of the polyalanine tract is responsible for thyroid dysgenesis.

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References
2 Zannini M, Avantaggiato V, Biffali E, Arnone MI, Sato K, Pischetola M et al. TTF-2, a new forkhead protein, shows a temporal expression in the developing thyroid which is consistent with a role in controlling the onset of differentiation. EMBO Journal 1997 16 3185–3197.

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