A New Point Mutation (A420G) in the Thyroid Hormone Receptor-β Gene of a Patient with Resistance to Thyroid Hormone

Maria C. Villadolid M. D.

First Department of Internal Medicine, Nagasaki University School of Medicine, 1-7-1 Sakamoto, Nagasaki 852, Japan

Resistance to thyroid hormone (RTH) is a syndrome of reduced responsiveness of tissues to thyroid hormone. We present a new point mutation at nucleotide 1544 of the T3 binding region of thyroid hormone receptor-β (TRβ) resulting in a lysine to arginine substitution in codon 420 in a female patient diagnosed with RTH in Nagasaki, Japan. The clinical characteristics of this patient is extremely milder than those reported in other families with RTH, namely goiter, tachycardia, and learning disability. On the other hand, the thyroid function tests are typical of other subjects with RTH and TSH levels were not suppressed even when supraphysiologic doses of T3 (75 µg/day for 7 days) were given. Basal peripheral tissue response parameter levels did not alter even after administration of T3. Thyrotropin-releasing hormone (TRH), 200 µg i.v., caused a rise in TSH, T3, and T4, indicating that the pituitary-thyroid system was intact and the patient's TSH was biologically active.

To further confirm the biological activity of this mutant TRβ, a mutant TRβ gene chimera was constructed in vitro using the Spliced Overlap Extension Method. In vitro translation product of this mutant TRβ gene demonstrated 40% reduction in binding capacity compared to the wild type. It is now known that thyroid hormone resistance presents with a wide spectrum of phenotypes accompanying the presence of point mutations in different areas of the T3 binding domain of the TRβ gene. These data suggest that a subclinical RTH with familial or sporadic cases may also possess a point mutation of TRβ as well as typical RTH.

Key words: Resistance to Thyroid Hormone, Point Mutation, T3 Binding Region, Thyroid Hormone Receptor β

Introduction

The syndrome of resistance to thyroid hormone (RTH) is usually autosomal dominant inheritance and characterized by goiter and high levels of circulating thyroid hormone, with inappropriately normal or slightly elevated TSH (1, 2). Recent advanced techniques in molecular biology have evaluated a number of genetic abnormalities of thyroid hormone receptor (TR) in patient with RTH. These are including point mutations resulting in a single amino acid substitution or early termination, single nucleotide insertions resulting in frame shifts, and addition of two amino acid at the carboxyl-terminus of the receptor molecule, deletion of a single amino acid of TR-β gene (3-5). Although the pattern of inheritance in a hundred with RTH was autosomal dominant except one case (1), the molecular basis for RTH have been reported date have mutation in one allele of the thyroid hormone receptor-β (TRβ) gene on chromosome 3. Thus, manifestation of RTH in heterozygous individuals required the expression of a mutant receptor that inhibit normal TR-β by “dominant negative” interaction and decrease T3 binding (6-8).

RTH have been a significant diversity of the clinical picture of the different kindred (9-11). Clinically, they presented either generalized or dominantly pituitary resistance to thyroid hormone. Studies describing TRβ mutation have noted that most are distributed along the 2 distinct clusters, one in the 5’end (codon 305-351) of and the other in the 3’end (codon 430-461) of the T3 binding region (3-5). The generalized type of RTH have presented the mutation of TRβ in both cluster, on the other hand pituitary type of RTH in only the former cluster (12, 13). In the present study, we report a new point mutation at nucleotide 1544 in the dimerization subdomain of T3 binding region of TRβ, resulting in a lysine to arginine substitution in codon 420 in a patient diagnosed with generalized RTH in Nagasaki. We will discuss the causes of diversity of clinical picture among the patients of RTH patient show the amino acid change of the T3 binding region of TRβ produced by a single point mutation.

Methods

Case Report

The patient is a 16 year-old high school girl who initially
consulted for goiter. She was born fullterm with other growth and developmental parameters being normal. There was no history of other major illnesses or hospitalization. History of hyperactivity, weight loss, speech and learning disability was not elicited. She was within average in general performance at school. There was no history of any major illnesses in the family. On physical examination height was 160 cm which is within 1~2 SD above the mean, and weight was 48 kg. Body proportions were normal. Mean resting pulse was 85 per min and the blood pressure 110/80 mmHg. The thyroid was 1.5 times enlarged with no bruit. There was no exophthalmos, lid lag, muscle weakness, tremor or other obvious signs of hyperthyroidism. There was no organ enlargement. The skin had normal moisture with no erythema or onycholysis. Deep tendon reflexes relaxation phase were not prolonged. Serum cholesterol was within normal range and there was no pituitary tumor visualized by computer tomographic scan of the head.

Basal thyroid function tests, T3 suppression test, TRH test, and other basal tissue metabolic parameters were done including serum ferritin, total cholesterol and sex hormone binding globulin. Thyroid function tests were done for available family members.

Cloning and Sequencing of the TRβ

High molecular weight genomic DNA from patient was isolated from lymphocytes (14). Oligomer primer pairs used to amplify the T3 binding domain of the TRβ gene, Exons 7 and 8, were synthesized according to known protocols (6). Using 1 μg of genomic DNA as template and 100 pmol of each primer pair, a 100 μl reaction mixture underwent 40 cycles of PCR reaction. The first denaturation was set at 94°C for 5 minutes, followed by 39 cycles of 1 minute annealing at 58°C, 1 minute extension at 72°C and 1 minute denaturation at 94°C.

PCR amplified fragments of Exon 7 and 8 of our subjects were fractionated on 2% NuSieve GTG Agarose gel (FMC, Rockland, ME, USA) and purified by Oligomer Kit (Bio 101, Inc, La Jolla, CA, USA) and subcloned into Bluescript SK- (Stratagem, La Jolla, CA, USA). Positive clones were picked up, and sequenced using direct sequence analysis kit (United State Biochemical, Ohio, USA) labeled with 32P (Amersham)., and fractionated on 8% polyacrylamide gel and exposed to autoradiography film.

In Vitro Expression of Normal and Mutant TRβ

We prepared BSK II SK- plasmid vector in which a wild type human c-erb AβcDNA was inserted (pe A12 a gift from Dr. R. M. Evans, Howard Hughes Medical Institute, the Salk Institute for Biological Studies San Diego, CA, USA). The 5′end of the insert containing the initiation codon site was inserted into the T3 promoter ends of pBluescript II SK- and the 3′end of the insert containing the poly A tail into the T3 promoter end of the same plasmid vector (Figure 3). To construct the mutant TRβ chimera, we utilized the Spliced Overlap Extension Method (15) and synthesized the complimentary internal oligonucleotide primers containing the mutation in Exon 8 and amplified this by PCR using the T3 and T3 promoter regions outside the primers and the wild type TRβ receptor cDNA inserted into Bluescript SK+ vector as template to produce the 1.6 Kb chimera receptor. The structures of both wild type and mutant plasmids were confirmed by dyeoxy-nucleotide sequencing. The plasmid containing the mutant or wild type TRβ cDNA was linearized with Hind III and transcribed by T3 RNA polymerase (Stratagene mCAP kit, La Jolla, CA, USA) in the presence of the CAP analog containing 5′7me Gppp 5′G. The mRNA product obtained was used for in vitro translation using a rabbit reticulocyte system with or without labelled methionine according to the protocol (In Vitro Translation kit, Stratagene, La Jolla, CA, USA).

T3 Binding Studies

The T3 binding affinity measurements were done by saturation analysis with 100T3, 2 μl of the in vitro translation product was incubated overnight at 4°C with varying amounts of 100T3 (NEN, 2200 Ci/mmol, 0.01-0.5 nM, New England Nuclear, MA., USA.) in T3 binding buffer (50 mM NaCl, 10% glycerol, 2 mM EDTA, 5 mM DDT, 20 mM Tris-HCl ph 7.6) at a total volume of 100 μl. Nonspecific binding was determined in the presence of a large excess of cold T3 (0.05 μM). Protein bound T3 was separated from free T3 by a 0.45 μm nitrocellulose filter according to the protocol by Inoue et al. (16) and T3 binding was measured in triplicate.

Results

Clinical Studies

The patient demonstrated high levels of circulating thyroid hormones with inappropriate secretion of TSH (Table 1). Circulating antibodies to T1, T2, TSH-binding inhibitory immunoglobulins, antimicrosomal antibodies or antithyroglobulin antibodies were not detected. Thyrotropin-releasing hormone test (TRH), 200 μg i. v., caused a rise in TSH to 32.7 μU/ml at 30 minutes then down to 10.6 μU/ml at 120 minutes, with T3 rising to 20.1 μg per dl, free T3 to 6.7 ng/dl, T4 to 276 ng/dl, and Free T3 to 13.4 pg/ml, indicating that the pituitary-thyroid system was intact and the patient’s TSH was biologically active (Table 2). The sensitivity of the pituitary cells to TRH inspite of elevated serum thyroid hormone levels suggested that the pituitary was not sensitive to
Table 1. T₃ Suppression test

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>TSH</th>
<th>T₄</th>
<th>FT₄</th>
<th>FT₃</th>
<th>Tg</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.9</td>
<td>17.8</td>
<td>4.9</td>
<td>9.8</td>
<td>190</td>
</tr>
<tr>
<td>30</td>
<td>32.7</td>
<td>20.1</td>
<td>6.7</td>
<td>13.4</td>
<td>276</td>
</tr>
<tr>
<td>60</td>
<td>17.6</td>
<td>12.7</td>
<td>8.0</td>
<td>231</td>
<td>227</td>
</tr>
<tr>
<td>90</td>
<td>12.7</td>
<td>10.6</td>
<td>13.0</td>
<td>24.6</td>
<td>227</td>
</tr>
</tbody>
</table>

Basal measurements of TSH, serum thyroid hormones, ferritin, total cholesterol, sex hormone binding globulin were taken. The patient was given 75 µg/day of T₃ for 7 days. Results in this table shows that T₃ given in supraphysiologic doses over a period of 7 days failed to suppress TSH and caused a further rise in serum thyroid hormone levels but no change in basal metabolic parameters (ferritin, total cholesterol and sex hormone binding globulin).

Table 2. TRH test

<table>
<thead>
<tr>
<th>Time (day)</th>
<th>TSH</th>
<th>T₄</th>
<th>FT₄</th>
<th>FT₃</th>
<th>TBG</th>
<th>Ferritin</th>
<th>T. chol</th>
<th>SHBG</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.7</td>
<td>22.5</td>
<td>4.6</td>
<td>8.0</td>
<td>23.1</td>
<td>21.5</td>
<td>193</td>
<td>33</td>
</tr>
<tr>
<td>&lt;T₃ 75µg/day&gt;</td>
<td>9.6</td>
<td>26.1</td>
<td>5.3</td>
<td>13.0</td>
<td>24.6</td>
<td>19.0 (ng/ml)</td>
<td>197 (mg/dl)</td>
<td>34 (nmol/l)</td>
</tr>
<tr>
<td>7</td>
<td>368</td>
<td>254</td>
<td>5.3</td>
<td>13.0</td>
<td>24.6</td>
<td>19.0 (ng/ml)</td>
<td>197 (mg/dl)</td>
<td>34 (nmol/l)</td>
</tr>
</tbody>
</table>

Thyrotropin-releasing hormone (TRH), 200 µg iv, caused a brisk rise in TSH to 32.7 µU/ml at 30 minutes and finally at 10.6 µU/ml at 120 minutes. Serum T₄, T₃, free T₄, and free T₃ rose proportionately indicating that the pituitary-thyroid system was intact and patient's TSH was biologically active.

Sequencing of the T₃ Binding Domain of the TRB Gene

Amplification of Exon 7 (nucleotides 1171-1429) and Exon 8 (nucleotides 1444-1698) produced similar length PCR products of 258 and 254 bp respectively. PCR Products from the patient representing amplified fragments of Exon 7 (nucleotides 1171-1429) and 8 (nucleotides 1441-1692) of the TRB gene were sequenced. Exon 7 of and Exon 8 of the parent's clones did not contain any variant sequence. In Exon 8 of the patient, there was a single base mutation at nucleotide position 1544, with the Codon 420 AAA (lysine) being converted to AGA (arginine) (Figure 2). This mutation was detected in 3 out...
Figure 3. Partial sequence of the antisense strand of Exon 8 (nucleotides 1444-1698) of the TRB gene. The 254 bp fragment of genomic DNA from the patient (Mutant) and parent (Normal) was amplified using Primers B and D and sequenced as described in Materials and Methods. A point mutation was detected in 4 out of 7 separately amplified PCR products that changed Codon 420 for lysine (TTT) to one for arginine (TCT).

7 independent clones of the patient. The mutant clones were derived from separate PCR amplifications. Sequencing of the antisense strands also produced the complementary base mutation (Figure 3).

T₃ binding studies

Wild type receptor was produced by inserting pEA 12 into BSKII SK- plasmid vector and using this as template to amplify with internal oligoprimer pairs containing the single mutation (Figure 4A, B). Normal and mutant cDNAs were utilized to synthesize cRNA and in vitro translated in a rabbit reticulocyte system. The translation products were used to study T₃ binding. Ligand binding assay showed the mutant TR had 40% decreased binding activity compared to the wild-type TR (Figure 5A, B).

Discussion

The patient described here was diagnosed to have generalized RTH when she presented with a mild, nonnodular goiter with no accompanying signs and symptoms of hypermetabolism in spite of having high levels of circulating thyroid hormone. Growth and developmental parameters were within the normal values. Peripheral tissue response parameters to thyroid hormone were normal in the face of persistent elevation of thyroid hormone levels with poor suppressibility of TSH secretion by exogenous T₃. Inconsistent with the usual findings in patients with Graves' disease, however, were the persistently measurable serum levels of TSH which responded as in normal subjects to administered TRH. A pituitary tumor was ruled out. The results of the studies carried out gave strong evidence...
that the patient had a decreased responsiveness to circulating thyroid hormone. Target organ unresponsiveness could include the pituitary, for the negative feedback effect of high circulating thyroid hormones was unusually weak. While the rest of the family failed to show evidence of the same dysfunction, we tried to carry out some basic molecular studies to further investigate this phenomenon.

Thyroid hormone receptor is encoded by the proto-oncogene c-erbA (17). There are multiple isoforms such as TR β1, β2, α1, variant αs, and αs (18). TRs consist of a superfamily of nuclear hormone receptor (19), and have a characteristic domain structure including DNA-binding domain, ligand (T3) binding domain and dimerization domain with TR-axillary protein such as retinoid X receptor (20). Since the majority of the mutations published in the literature concerning RTH have been found around Exon 7 and 8 of the TR β gene, we concentrated our analysis on this area. The TR β gene of this patient was analyzed and we identified a point mutation, A to G, at nucleotide 1544, Codon 20, resulting in an amino acid change, from lysine to arginine. Both of these amino acids are basic, therefore the change from lysine to arginine does not alter the charge and may not tertiary structure. The in vitro transcription/translation product of the mutant receptor is biochemical evidence that this mutant receptor, when produced in vitro, is defective in thyroid hormone binding. This receptor, however, has the ability to bind thyroid hormone approximately 40% less than the wild-type receptor which could partially explain why the patient could compensate and maintain a euthyroidal status inspite of elevated circulating levels of thyroid hormone.

So far the mutations identified in RTH cases have been found in the T3 binding region of the TR β gene, but not in the dimerization domain (21, 22). The area where our mutation lies is located in what is believed to be the dimerization subdomain (23), an area encoding heptad repeats forming a leucine zipper structure which means this area is important for receptor dimerization. Glass (24) synthesized mutations in this region, specifically Codon 423, near our C 420 mutation, and noted that although dimerization activity of the mutant was not abolished, its presence could give rise to dominant negative mutants that maintain dimerization activity but destroy other important functions such as DNA binding, ligand binding or transactivation. This may also explain the lack of symptoms in our patient. For example, the presence of a mutation in this region could form a mutant homodimer, or a wild-type mutant heterodimer, thus decreasing the number of functional wild-type homodimers or decreasing the chances of functional wild-type receptors to compete for transcription factors (25, 26).

Many discrepancies between the clinical picture of patients with thyroid hormone resistance and in vitro and in vivo studies of their receptors have been found (3-5, 9, 10, 27). In this report, we have not excluded the possibility
of other factors and other mutations existing in other parts of her TR β gene and other types of TR receptors, for example TR α. Other studies also note that the degree of thyroid hormone resistance as manifested by the clinical severity or absence of symptoms of RTH does not necessarily correlate with the degree of T3 binding impairment of mutant receptors (26, 27). Individual genetic differences and other factors involved in thyroid hormone action may also contribute to the subject’s phenotype.

Acknowledgements

The author wishes to acknowledge the members of the Endocrine Unit, First Department of Internal Medicine and Department of Cell Physiology, Atomic Disease Institute, Nagasaki University School of Medicine, especially Dr. Akira Ohtsuru and Dr. Naokata Yokoyama, for their expert advice and technical support in accomplishing this work.

References

8) Nagaya T and Jameson JL 1993 Thyroid hormone receptor dimerization is required for dominant negative inhibition by mutations that causes thyroid hormone resistance. J Biol Chem 268 : 17693-17697.
21) O'Donnell AL, Rosen EV, Darling DS, Koenig RJ 1991 Thyroid hormone receptor mutations that interfere with transcriptional activation also interfere with receptor interaction with a nuclear protein. Mol Endocrinol 5 : 94-99.