Thyroglobulin gene mutations producing defective intracellular transport of thyroglobulin are associated with increased thyroidal type 2 iodothyronine deiodinase activity

Short title: High thyroidal D2 activity with Tg mutation (37 characters)

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Abstract

Context: Most patients with defective synthesis and/or secretion of thyroglobulin (Tg) present relatively high serum free T3 (FT3) concentrations with disproportionately low free T4 (FT4) resulting in a high FT3/FT4 ratio. The mechanism of this change in FT3/FT4 ratio remains unknown.

Objective: We hypothesize that increased type 2 iodothyronine deiodinase (D2) activity in the thyroid gland may explain the higher FT3/FT4 ratio which is frequently observed in patients with abnormal Tg synthesis.

Design: We recently identified a compound heterozygous patient (Patient A) with a Tg G2356R mutation and one previously described (C1245R) that is known to cause a defect in intracellular transport of Tg. In the current study, after determining the abnormality caused by G2356R, we measured D2 activity as well as its mRNA level in the thyroid gland. We also measured the thyroidal D2 activity in 3 patients with Tg transport defect and in normal thyroid tissue.

Results: Morphological and biochemical analysis of the thyroid gland from Patient A, complemented by a pulse chase experiment revealed that G2356R produces a defect in intracellular Tg transport. D2 activity but not type1 deiodinase in thyroid glands of patients with abnormal Tg transport was significantly higher than in normal thyroid glands, whereas D2 mRNA level in Patient A was comparable with that in normal thyroid glands. Furthermore, there was a positive correlation between D2 activity and FT3/FT4 ratios.

Conclusion: Increased thyroidal D2 activity in the thyroid gland is responsible for the

higher FT3/FT4 ratios in patients with defective intracellular Tg transport.

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Conclusion: Increased thyroidal D2 activity in the thyroid gland is responsible for the

higher FT3/FT4 ratios in patients with defective intracellular Tg transport.

Introduction

Thyroid dyshormonogenesis due to mutations in the thyroglobulin (Tg) gene is relatively rare with an estimated prevalence of 1 in 100,000 newborns (1). Inherited in an autosomal recessive manner, the majority of patients have large goiters of elastic and soft consistency (2). Several mutations in the Tg gene have been reported in man [reviewed in (3, 4)]. These includes truncated Tg molecules due to premature termination of translation (5, 6), alternative splicing resulting in the deletion in a segment of the Tg molecule (7), or a simple amino acid substitution (a missense mutation) (8). In Japanese families, several different types of missense mutations have been reported (9).

We recently presented the clinical findings in a congenital goiter (Patient A) caused by two missense mutations (C1245R and G2356R) in each allele of Tg gene (10). The replacement of cysteine 1245 produces retention of the Tg molecule within the rough endoplasmic reticulum (RER) (8). However, the consequences resulting from the G2356R remained unknown. We now report immunohistochemical and electron microscopic studies on thyroid tissue obtained from Patient A. In addition, we evaluated by pulse-chase experiments whether G2356R causes retention of the mutant Tg within a cell.

Although the degree of thyroid dysfunction varies considerably among patients with defective Tg synthesis, patients usually have a relatively high serum free T3 (FT3) concentration with disproportionately low free T4 (FT4) level. The maintenance of relatively high FT3 levels prevent profound tissue hypothyroidism except in brain and pituitary which are dependent of T4 supply (11, 12), resulting in the neurological- and intellectual defects in some of cases (2). However, the mechanism by which higher FT3 levels are maintained relative to those of FT4 remains unknown. As in the majority of cases, Patient A also had a high serum FT3 concentration with relatively a low FT4 level. Since type 2 iodothyronine deiodinase (D2) is expressed in the human thyroid gland and is

postulated to play an important role as a source of plasma T3 (13), we determined D2 activity in thyroid tissue obtained from Patient A as well as 2 other patients with a homozygous C1245R mutation. We found that it was increased in all three patients with Tg defect as compared to normal thyroid tissues obtained from patients with thyroid tumors. The results suggest that in patient with Tg transport abnormality, increased thyroidal D2 activity is possibly responsible for the higher serum T3 concentration relative to that of T4.

Case presentation

Patient A is a 38-year-old man who presented with sleep dyspnea due to a large goiter that was found at birth. When he was 3 years old, methimazole (MMI) was given and the prescription was continued until 8 years of age, resulting in lethargy. At another medical center MMI was stopped and levothyroxine (LT4) was instituted. Thereafter, he continued to take LT4. The dose was 100 μg/day when he was first seen by us. He had a recent gain of 20 kg since he stopped exercising and complained of dyspnea when supine due to neck compression. His sister had a goiter since birth but the nonconsanguineous parents had no goiter nor thyroid function abnormalities.

The patient's height was 178 cm and weight 104 kg. Blood pressure was 132/92 mm Hg, and the pulse 96 beats per minute. The thyroid was markedly enlarged, weighing approximately 300 g, diffuse, of soft texture and nontender. A prominent vascular bruit was audible. There were no other physical findings except for a fine finger tremor. Blood count and electrolytes were normal and urinalysis was positive for protein and sugar. The postprandial blood glucose was 368 mg/dl and HbA1c was 7.0%, indicating that the patient had diabetes mellitus. Serum total cholesterol was 152 mg/dl, triglyceride 333 mg/dl, and

HDL-cholesterol 31 mg/dl. Changes in serum TSH, FT4 and FT3 during LT4 administration (100μg/day) and after the cessation of LT4 are shown in Table 1. Serum concentration of Tg was undetectable and antibodies against the Tg, thyroid peroxidase, and TSH-receptor were all negative. The thyroidal uptake of ^{123}I was 93% at 3 h and did not decline following perchlorate administration. Dyspnea in the supine position worsened. He could not tolerate an attempt to further suppress serum TSH by increase in the LT4 dose because of tachycardia and tremor. Therefore, a total thyroidectomy was performed.

Materials and Methods

1. Subjects

Thyroid tissue was obtained from Patient A, as well as from 2 patients with homozygous C1245R mutation of the Tg gene and one patient homozygous for C1977S (8). Normal tissues were obtained from surgically resected glands of euthyroid patients with non-functioning thyroid adenomas (3 cases) or carcinomas (2 cases). In the experiment of endoglycosidase H (Endo H) digestion, the thyroid gland obtained from a patient with Graves' disease was used as control. FT4 and FT3 were measured before surgery. The analyses of the Tg genes of patients was approved by the Ethics Committees at Nagoya University School of Medicine and Dokkyo University School of Medicine. Subjects gave written informed consent for participation in this study. Members of the family of Patient A declined to participate in the study.

2. Sequencing and haplotype analysis of the Tg gene

Total RNA extracted from the thyroid gland of Patient A was used to prepare Tg cDNA by reverse transcription coupled PCR (RT-PCR) and was directly sequenced. The RT-PCR conditions and the sequencing primers have been previously described (14).

For haplotype analysis, three cDNA fragments encompassing nucleotide positions from

3240 to 4931, from 4090 to 5892, and from 5442 to 7343 were amplified by RT-PCR. Products were ligated to pCR2.1 plasmid (Invitrogen Corp., Carsbad, CA) and HB101 E. Coli were transformed by the obtained plasmids. Then, the amplified Tg cDNA fragments were sequenced.

3. Tissue preparations and morphological analysis

The thyroid glands obtained at the surgery were quickly frozen in liquid nitrogen and stored at -80 C until used for RNA extraction and the determination for D2 and type 1 deiodinase (D1) activities.

Tg immunohistochemistry was performed using HISTOFINE^{TM} SAB-PO (R) kit (Nichirei Co., Tokyo, Japan) according to a protocol supplied by the manufacturer. In this kit rabbit polyclonal antibody to human Tg (code: 422691) was included. For electron microscopy, thyroid tissues were fixed in 2% glutaraldehyde, postfixed in osmium-tetrachloride and dehydrated. Tissue blocks were embedded in epon and cut into ultra thin sections. After double staining with uranylacetate and lead-citrate, sections were examined by an electron microscope (JEM-2000X; JOEL, Akishima, Japan).

4. Endo H digestion

Approximately 20 mg of thyroid tissue was homogenized in 100 μL Tris buffer (10 mmol/L; pH8.0) that contained a cocktail of protease inhibitors (Complete Protease Inhibitor Cocktail Set, Roche, Mannheim, Germany). The homogenate was centrifuged at 18,000xg twice, for 30 min each time, and the supernatant was used as a thyroid tissue extract. Tg contents in the homogenate was measured using a RIA kit (Eiken Chemical C., Tokyo, Japan) and an aliquot of the thyroid extract containing 2 μg Tg was digested with 0.3 mU/L Endo H (Roche), then, analyzed by 4-15% gradient SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (8).

5. Expression of human Tgs in HEK293 cells and a pulse-chase experiment

An expression plasmid containing the full-length Tg gene was constructed as follows. RNA was extracted from surgically isolated thyroid tissues using the RNeasy Mini kit (Qiagen, Valencia, CA). Three segments of Tg cDNA, the nucleotide position at 16 to 3032, 2986 to 5717 and 5719 to 8323 (named as fragment A, B and C, respectively), were amplified by reverse transcription-polymerase chain reaction (RT-PCR). The PCR amplifications were performed using Expand High-Fidelity kit (Roche, Mannheim, Germany). The primers used were as follows: for the fragment A, forward 5'-AGGAAGGGCCAGGAAAAT-3' and reverse 5'-AAATCTAGAAAAGCGGCGTCTCTGATA-3'; for the fragment B, forward 5'-CTGGCGGCTCAGTCTACCTTA-3' and reverse 5'-AAATCTAGAATCTATGCTAGCTGACAGAAAGAGTGCTCCT-3'; for the fragment C, forward 5'-TCAGCTAGCAGAGATAACAGAGAGTGCATCC-3' and reverse 5'-AATTCTAGAGGAGCTCAAGGGCTGGTC-3'. For cloning purposes, the 5718th nucleotide of Tg cDNA was changed from cytosine to adenine, which does not alter the amino acid residue concerned. The amplified fragment A was cloned into pcDNA3.1/V5/His-TOPO plasmid (Eukaryotic TOPO TA Cloning Kit, Invitrogen, Carlsbad, CA) and then ligated to the fragment B at Bfr I and Xba I sites. This plasmid was ligated again to the fragment C at Nhe I and Xba I sites to obtain the full length Tg cDNA.

To introduce the G2356R mutation, a guanine was replaced by an adenosine at nucleotide at 7123 from the translation start site by PCR-based mutagenesis using the human wild type Tg cDNA as a template. The mutation was confirmed by sequencing. The cDNA fragment containing the G2356R mutation was digested with NdeI and EcoRI and used to replace the corresponding fragment of wild type human Tg-pcDNA3.1/V5-His-TOPO. The cDNA encoding C1245R mutant human Tg was also subcloned into pcDNA3.1/V5-His-TOPO (Invitrogen Corp.) (8). All plasmids were purified

using QIAGEN Plasmid kit (Qiagen, Valencia, CA) prior to transfection.

HEK293 cells were grown in 60-mm diameter plastic dishes and transfections were performed by adding 3.3 μg of plasmid DNA/dish by the calcium-phosphate method (15). Forty eight hours after transfection, cells were labeled with ^{35}S-methionine [EXPRE^{35}S^{35}S 5.6 mega Becqurel (MBq)/dish, SA: 43.5 tera Bq/mmol; PerkinElmer Life Sciences Inc. Boston, MA] for 3 h. Then, excess of unlabeled methionine was added to each dish to stop further incorporation of labeled methionine into proteins and the labeled proteins were chased for 0, 3, 6 and 24 h. After the chase, medium was collected and cells were lysed in 1 ml Buffer A (16). Samples of medium and cell lysates were kept frozen at -20 C until used for immunoprecipitation.

Immunoprecipitation of Tg using 0.5 μl of rabbit polyclonal anti-human Tg antibody (NeoMarkers, Fremont, CA) was performed as described previously (17) followed by 5-15% gradient SDS-PAGE. For control, 0.5 ml non-immune rabbit serum was added to medium and cell lysate obtained from 0 h chase of wild type Tg.

7. Measurement of D2 and D1 activities in the thyroid tissue

D2 activity in the microsomal fraction of thyroid tissue was measured as previously described (18). Thyroidal D1 was also measured with [^{125}I]-reverse T3 as a substrate as described previously (19).

8. Northern blot analysis

Total RNA was extracted by the method of Chomczynski and Sachi (20). To determine the expression levels of D2 and D1 mRNAs, we employed cRNA probes labeled with [α-^{32}P] UTP. Details of this procedure were described elsewhere (18). After hybridization, autoradiography was performed by exposing the membranes to X-ray film (Kodak XAR-2,

Eastman Kodak Co., Rochester, NY) for 24 h. The amounts of D2 and D1 mRNAs were measured by densitometry using NIH Image (version 1.61) and expressed in arbitrary units after correction for the amount of 28S ribosomal RNA (rRNA).

9. Statistical analysis

Statistical differences were evaluated by the Student's *t* test. Correlation between D2 activity and FT3/FT4 ration was evaluated by the Pearson's correlation coefficient test. *P* values less than 0.05 were determined as significant difference.

Results

1. Two mutations, C1245R and G2356R, found in each allele of the Tg gene in Patient A

By sequence analysis of Tg cDNA, we found two nucleotide substitutions. One is a nucleotide replacement from thymine to cytosine at position 3790, resulting in the substitution of the normal cysteine with an arginine at codon 1245 (C1245R). This mutation was previously reported to be located on codon 1263 (8) because the signal peptide was formerly included in the codon numbering of the Tg molecule. The other mutation was a substitution of the normal guanine with an adenine at position of 7123. This substitution replaces the normal glycine with an arginine at codon 2356 (G2356R). We screened 103 random subjects as for these two nucleic acid substitutions found in Patient A and found none. Thus, the two substitutions found in Patient A are not polymorphisms.

Since we could not obtain DNA samples from the parents of Patient A, we performed haplotype analysis to determine whether two mutations found in Patient A was located on different Tg alleles. The result indicates that this patient is a compound heterozygous for Tg gene (Fig. 1).

2. Morphological and biochemical analysis of the thyroid of Patient A, which confirms that the mutant Tgs of Patient A are retained in the rough endoplasmic reticulum (RER)

The thyroid gland of Patient A, removed by surgery, was diffusely enlarged (380 g in weight) with several scattered cystic lesions, having the gross appearance of an adenomatous goiter. As reported (10), an occult papillary carcinoma was found. Microscopically most of the follicular lumens were dilated to variable degrees (data not shown). Immunohistochemistry demonstrated the presence of Tg in follicular cells, but none in the follicular lumen (Fig. 2A). Electron microscopy showed dilated RER within the follicular cell. These findings are consistent with those observed in a case of defective

intracellular transport of Tg (Fig. 2B) (14).

As shown in Fig. 2C, the Tgs of Patient A were sensitive to Endo H as was that of a patient with homozygous C1245R mutation whereas the Tg of a normal subject was resistant. These results indicate that both forms of mutant Tgs are retained within the RER and are not transported to the Golgi apparatus.

3. Demonstration by the pulse chase experiment that the G2356R mutation caused defective intracellular transport of Tg

To demonstrate whether the G2356R mutation causes a defect in intracellular transport of Tg as does C1245R, we expressed in HEK293 cells the mutant Tgs, G2356R and C1245R, separately as well as the wild-type Tg. As shown in Fig. 3, normal Tg was detected within the cell up to 6 h of the chase but little remained after 24 h. Tg accumulated in the medium during 24 h of the chase, indicating that most of the wild-type Tg was secreted to the medium 24 h just after its synthesis.

Both mutant Tgs (C1245R and G2356R) were detected in cell lysates as a band of 330 kDa molecular mass. The time course of intensity of the Tg bands within cells were similar to that of the wild-type Tg. However, in contrast to the normal Tg, the mutant Tgs were not secreted into medium at any time during the chase. These results demonstrate that the novel mutant Tg, G2356R, as well as the previously described C1245R mutant, were retained and decayed within cells.

4. Increased D2 activity in thyroid tissues of patients with defective Tg transport

In order to explore the mechanism(s) by which patients with defective Tg transport have higher serum T3 levels relative to those of T4, D2 activity was measured in the thyroid tissues obtained from Patient A, 2 patients with homozygous C1245R mutation and

one patient with homozygous C1977S mutation. Control tissues were obtained from 5 normal thyroid glands surgically removed for non-functioning thyroid adenomas (3 cases) or carcinomas (2 cases). D2 activity in thyroid glands of patients with defective Tg transport was 2553±479 fmol/mg microsomal protein/h (mean±SE, n=4) whereas the mean activity in normal thyroid tissues was 933±392 fmol/mg microsomal protein/h. Thus, D2 activity in the thyroids with defective Tg transport was significantly higher ($p<0.05$) than normal (Fig. 4A). Among the normal thyroid tissues, we found that one from a patient with benign adenoma showed high D2 activity that overlapped with that of patients with defective Tg transport. Interestingly, this patient with thyroid adenoma also had a high serum T3 level relative to that of T4, resulting in high FT3/FT4 ratio (Fig. 4B). We therefore correlated the FT3/FT4 ratio with the corresponding D2 activity in the thyroid tissues and found a positive correlation (r=0.839, $p<0.01$, Fig. 4B). On the other hand, thyroidal D1 activity in patients with Tg defect was 1194±525 pmol/mg microsomal protein/min (mean±SE, n=4) and that in controls was 249±70 pmol/mg microsomal protein/min. There was no significant difference in thyroidal D1 activity between the patients and controls ($p=0.083$) nor was the correlation between thyroidal D1 activity and FT3/FT4 ratio (r=0.371, $p=0.365$).

We, furthermore, examined whether increased thyroidal D2 activity in patient with defective Tg transport is associated with the increase in D2 mRNA. As shown in Fig. 4C, D2 mRNA level in the thyroid gland of Patient A was not different from that in normal thyroid tissues. Also D1 mRNA level was not increased in the thyroid gland of Patient A (data not shown). Therefore, the increased D2 activity in the thyroid glands with defective Tg transport is not due to a pretranslational changes of D2.

Discussion

In the present study, we identified two missense mutations of the Tg gene

patients with defective Tg transport as compared with that in normal thyroid tissues. This increase in D2 activity is likely due to posttranslational control because D2 mRNA was not increased in the thyroid gland of Patient A. Since the disproportional increases in serum T3 levels are not limited in patients with defective Tg transport but also observed among the patients with congenital goiter due to mutations in the Tg gene (2), it is highly likely that the abnormality of Tg synthesis and/or transport is associated with increased D2 activity in the thyroid gland, resulting in high serum free T3 levels relative to the T4 concentration.

D2 has a very short half-life (<1 h) as compared to D1 (>12 h) (23). Thus, the posttranslational control of D2 seems to be important for the control of its activity. Furthermore, T3 and T4 can exert suppressive effects on D2 activity by pre- and posttranslational mechanisms, respectively, *in vivo* (24). This may explain why D2 activity in the human thyroid glands is suppressed disproportionately to the high levels in its mRNA because T4 must exist in very high concentration in the normal thyroid tissue (23). On the other hand, it is highly possible that T4 production is impaired in cases of defective Tg synthesis and/or transport. Actually, serum T4 levels are low in most cases of defective Tg synthesis (2) as well as in our cases with defective Tg transport (10). Therefore, the lower T4 contents in their thyroid glands may explain the mechanism by which thyroidal D2 activity is much increased in patients with defective intracellular transport of Tg.

Another possible mechanism of the increased D2 activity in thyroid glands with defective Tg transport is impaired proteolysis of D2 by ubiquitination. D2 is ubiquitinated and the D2-ubiquitin conjugates (Ub-D2) are taken up by a proteasome, and then rapidly degraded in cells (25). For this process, two ubiquitin-conjugating enzymes, namely Ubc6p and Ubc7p, are required (26). On the other hand, a recent study showed that the expression of molecular chaperones is increased in the thyroid gland with abnormal Tg due to unfolded protein responses (UPR) (27). In fact, a number of molecular chaperones were

demonstrated in thyroid tissues obtained from patient A and a patient homozygous for C1245R (Fig. 2C). It is well known that both Ubc6p and Ubc7p are involved in UPR {reviewed in (28)}. We thus assumed that a majority of Ubc6p and Ubc7p are recruited in UPR in patients with defective Tg transport and become deficient for D2 degradation, resulting in increased D2 activity.

In summary, we demonstrate that the novel Tg G2356R mutation found in Patient A produces defective intracellular transport of Tg by expressing the mutant Tg in HEK293 cells and performing pulse-chase experiment. In thyroid glands of patients with such defective intracellular transport of Tg, D2 activity was significantly increased and correlated with serum FT3/FT4 ratio. Therefore, we suggest that the increased thyroidal D2 activity accounts for a relatively high serum FT3 level with a disproportionately low FT4 level, which is frequently observed in patients with defective intracellular transport of Tg.

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Legends of figures

Fig 1: Haplotype analysis of the Tg gene of Patient A

Three overlapping cDNA fragments were amplified by RT-PCR. Primers used in PCR reactions are indicated by arrows. After ligation into the pCR2.1 plasmid, clones were sequenced. Allele 1 contains a mutation (guanine to adenosine) at position of 7123 (in a square) that yields G2356R Tg whereas Allele 2 includes a mutation (thymine to cytosine) at position of 3790 (in a square) that yields C1245R.

Fig. 2: Tg in Patient A is not secreted in the follicular lumen and retained in rough endoplasmic reticulum (RER)

Tg immunohistochemistry and electron microscopy of the thyroid gland of Patient A are shown in (A) and (B), respectively. Note that Tg immunostaining was found only in the follicular cell whereas it was conspicuously absent in the follicular lumen and that extremely dilated vesicles of RER are present in the follicular cells.

(C) Digestion of thyroid tissue extracts by Endoglycosidase H (Endo H): Thyroid tissues were obtained from a patient homozygous for Tg C1245R, Patient A and a patient with Graves' disease that did not contain a mutation in Tg cDNA (Normal). An aliquot of the thyroid extract containing 2 μg of Tg was digested with Endo H (0.3 mU/L) and subjected to 4-15% gradient SDS-PAGE using the Phast System (Pharmacia, Uppsala, Sweden). Undigested Tg or Tg resistant to Endo H migrated as a 330 kDa band, whereas Endo H sensitive Tg was smaller in size. In the thyroid glands of a patient homozygous for Tg C1245R and Patient A, proteins of smaller in size than the Tg (either resistant or sensitive to Tg) were present. These presumably represent molecular chaperones. MW: the molecular weight marker

Fig. 3: Pulse chase experiment on Tg synthesized by HEK293 cells

Tgs with C1245R and G2356R mutation and the wild-type Tg were individually expressed in HEK 293 cells and labeled with [^{35}S]-methionine for 3 h. Then, the labeled Tg was chased for 1, 6, or 24 h. After the harvest, the Tg in the medium and in the cell lysates was immunoprecipitated with anti-Tg antibody and analyzed by 5-15% gradient SDS-PAGE. As a molecular weight marker (MW), a [methyl-^{14}C]-methylated Protein Molecular Weight Marker (PerkinElmer Life Sciences) was used. Fluorography was performed using Amplify^{TM} (Amersham International plc, Amersham, UK) and signals were analyzed by Fuji Bioimage Analyzer (BAS 5000; Fuji Photo Film, Tokyo, Japan).

In the cell lysates (Cell, upper panel) all three Tgs were detected (open arrow) during the chase of at least 6 h. On the other hand, in the medium (Medium, lower panel), only the wild-type Tg (open arrow) was detected. Tgs with either C1245R or G2356R mutations were not detected at any time of the chase, indicating that these mutant Tgs were not secreted into the medium.

NIS: non immune serum

Fig. 4: D2 activity is increased at a posttranslational level in the thyroid glands of patients with defective intracellular transport of Tg and a positive correlation between the thyroidal D2 activity and serum FT3/T4 ratio

(A) Thyroidal D2 activities were measured in 4 patients with defective intracellular transport of Tg (2 patients with homozygous C1245R mutation, one with homozygous C1977S mutation and Patient A). For control, D2 activity was also measured in normal thyroid tissues obtained from 3 patients with non-functioning thyroid adenomas and 2

patients with carcinomas. Results are expressed as mean±SE fmol/mg microsomal protein/h.

(B) A positive correlation was observed between FT3/FT4 ratio and thyroidal D2 activity. Closed circles indicate patients with defective intracellular Tg transport whereas open circles indicate controls. One patient homozygous for Tg C1245R is not included because data of serum FT4 and FT3 were not available.

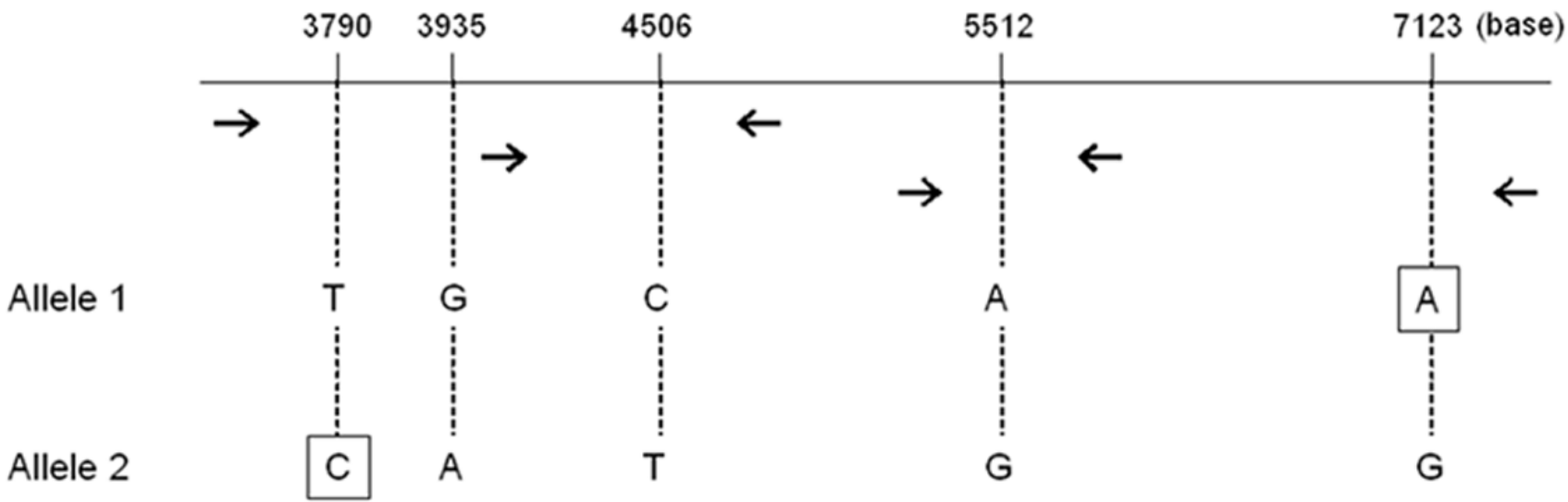
(C) Northern blot analysis of thyroidal D2 mRNA

D2 mRNA in the thyroid gland of Patient A is compared to that in normal thyroid tissues. An autoradiograph is shown on the left panel. The radioactivities of bands were quantitated by densitometry. The amount of D2 mRNA was corrected for the densities of 28S rRNA and expressed in an arbitrary unit (right panel).

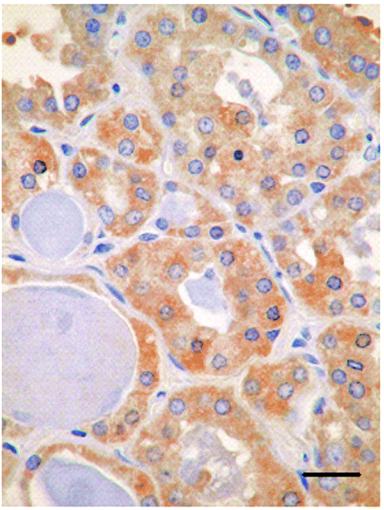
			TSH (μU/ml)	FT4 (pM)	FT3 (pM)	FT3/FT4
Patient A	Before operation	On LT4 (100 μg/day)	1.18	15.2	7.79	0.513
		2W after stopping LT4	1.67	11.6	6.65	0.573
		4W after stopping LT4	3.28	8.6	6.19	0.72
	After operation	On LT4 (200μg/day)	2.36	23.8	5.45	0.229
Patient B			1.6	7.2	4.92	0.66
Patient C			1.7	12.3	6.61	0.537
Reference range			0.75-4.67	9.1-19.6	3.67-6.21	

Table 1. Changes in serum TSH, FT4 and FT3 in Patient A during LT4 therapy, after stopping LT4 before thyroidectomy as well as those after the operation

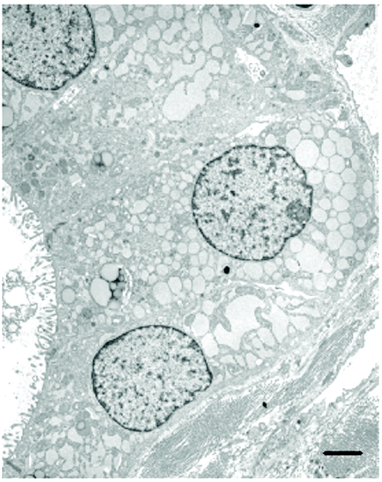
The values in Patient B and C before the operation without LT4 administration are also shown.


3790
3935
4506
5512
7123 (base)
Allele 1
T
G
C
A
A
Allele 2
C
A
T
G
G

A

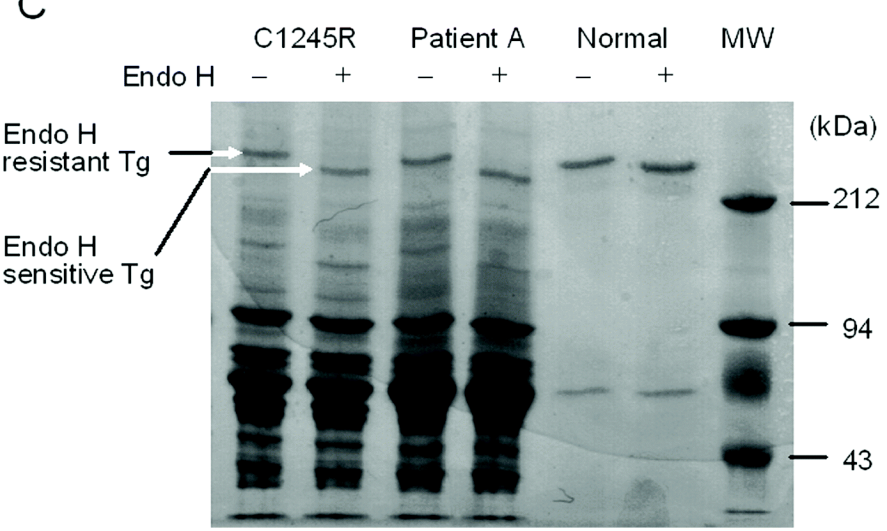


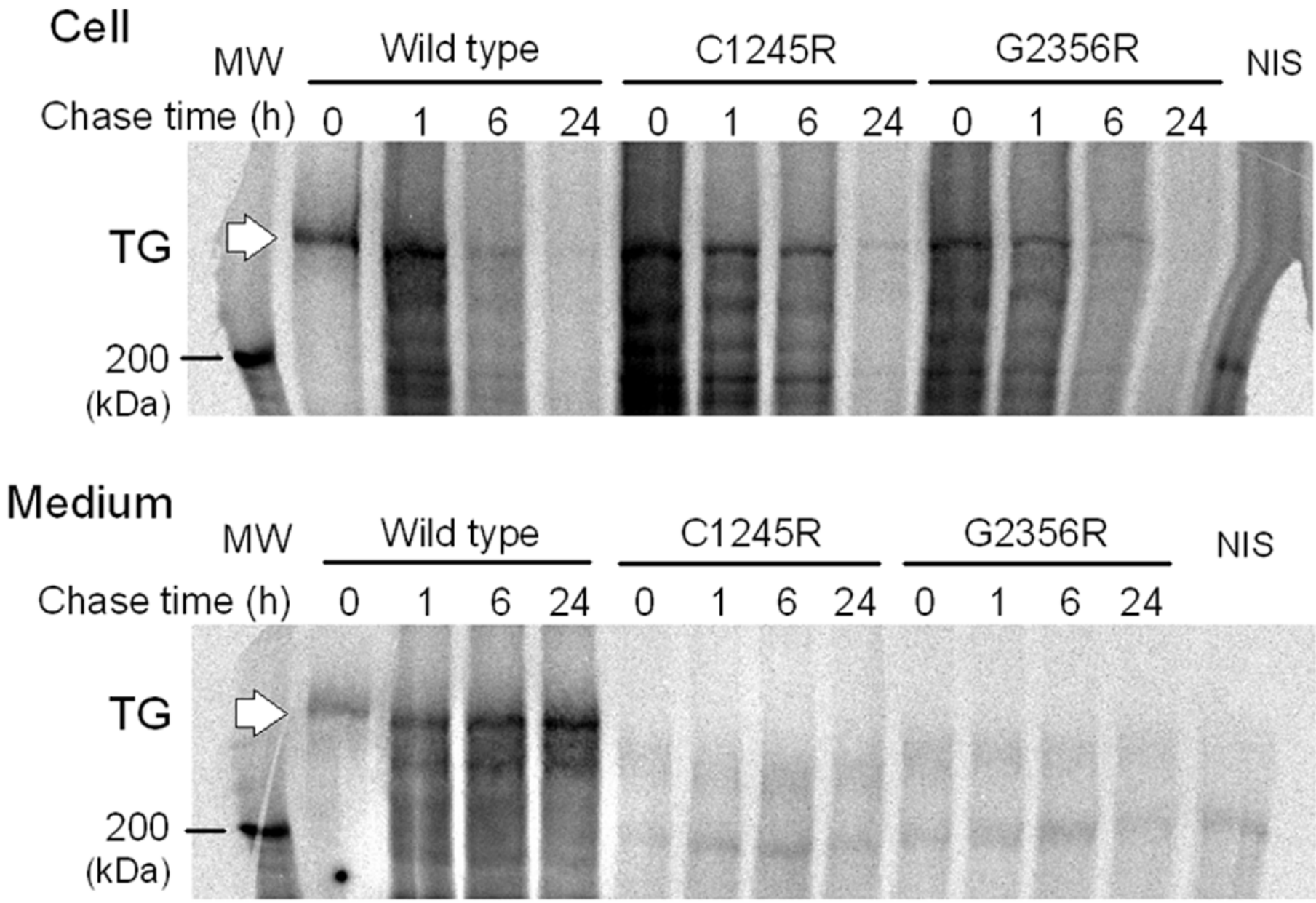
B

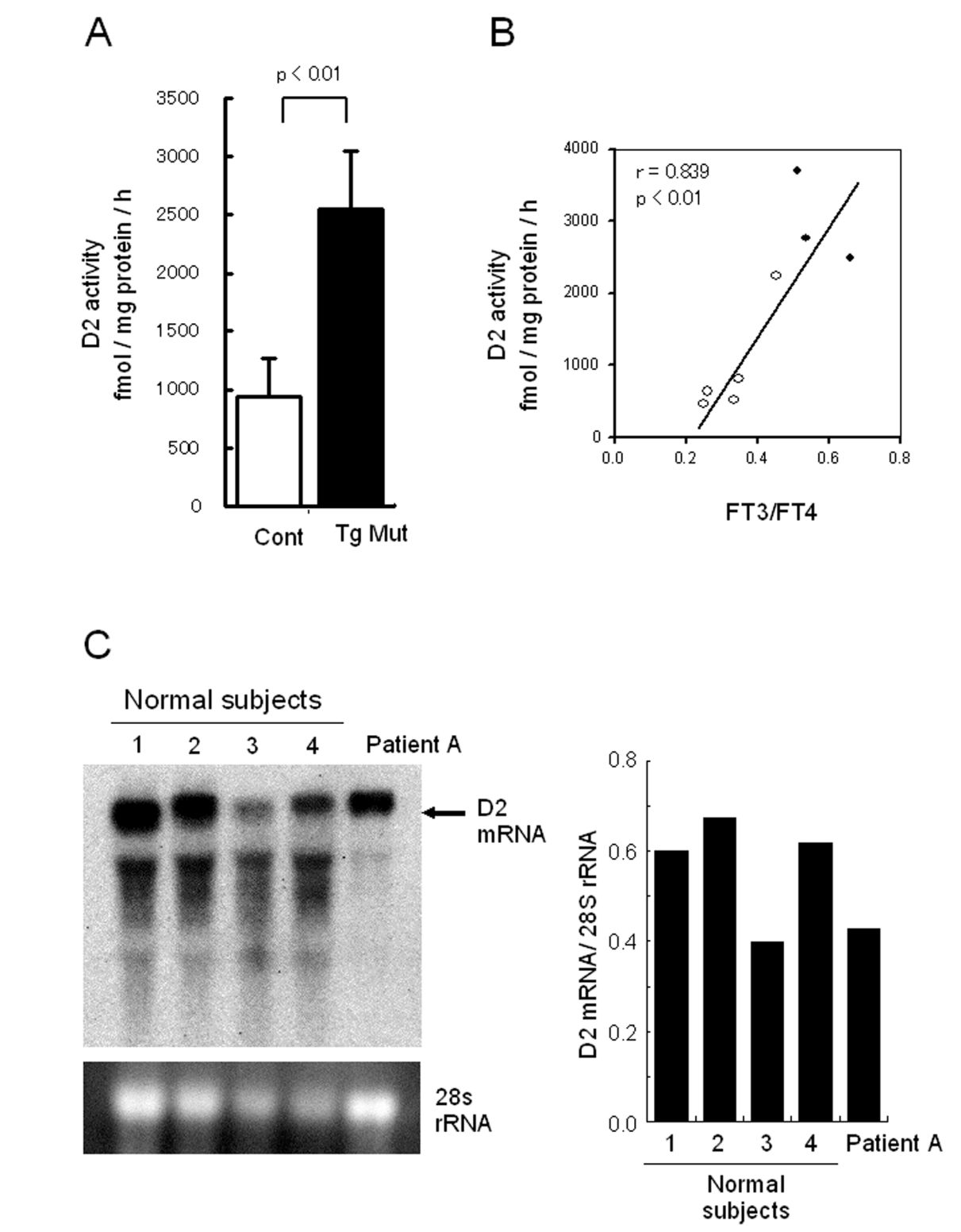


A) Scale bar = 100 µm
B) Scale bar = 2 µm

C




Cell
MW
Wild type
C1245R
G2356R
NIS
Chase time (h)
0
1
6
24
0
1
6
24
0
1
6
24
TG
200
(kDa)
Medium
MW
Wild type
C1245R
G2356R
NIS
Chase time (h)
0
1
6
24
0
1
6
24
0
1
6
24
TG
200
(kDa)


A
p < 0.01
3500
3000
2500
2000
1500
1000
500
0
D2 activity
fmol / mg protein / h
Cont
Tg Mut
B
4000
3000
2000
1000
0
r = 0.839
p < 0.01
D2 activity
fmol / mg protein / h
0.0
0.2
0.4
0.6
0.8
FT3/FT4
C
Normal subjects
1
2
3
4
Patient A
D2
mRNA
28s
rRNA
0.8
0.6
0.4
0.2
0.0
D2 mRNA/ 28S rRNA
1
2
3
4
Patient A
Normal
subjects