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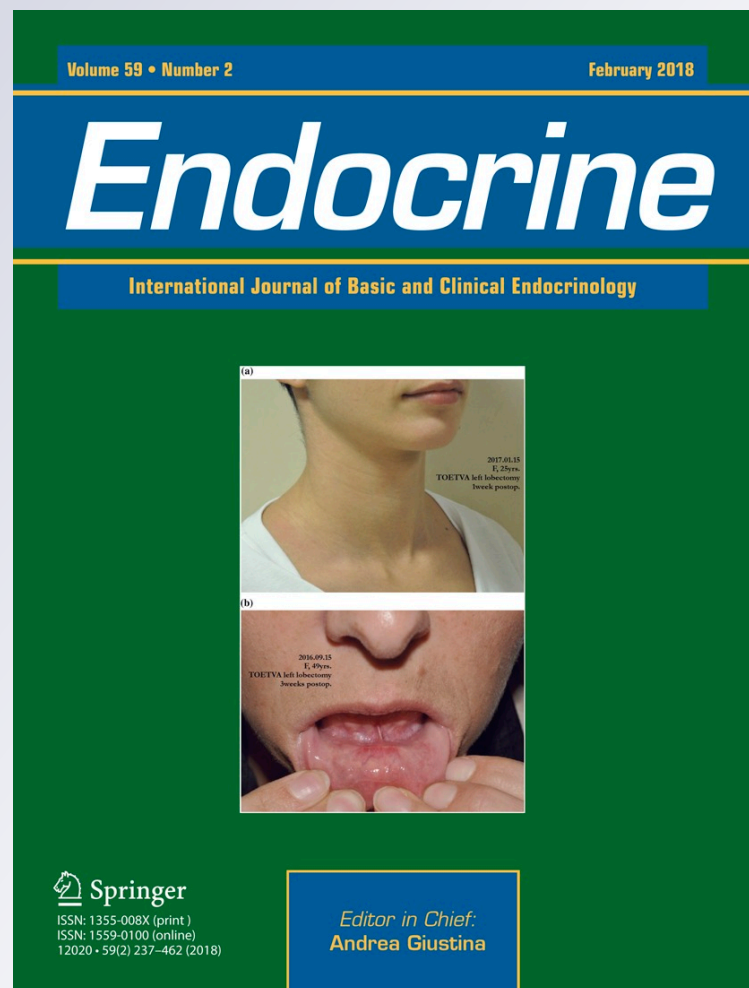
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Effects of growth hormone on thyroid function are mediated by type 2 iodothyronine deiodinase in humans

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Abstract

Purpose Growth hormone (GH) therapy in adults alters thyroid function, and acromegaly often involves thyroid disease. The present study aimed to elucidate roles and mechanisms of GH in regulating thyroid function.

Methods We performed two retrospective observational studies, which focused on consecutive patients with severe adult GH deficiency who received recombinant human GH (rhGH) therapy ($n=20$) and consecutive patients with acromegaly who underwent transsphenoidal surgery (TSS) ($n=25$). In both studies, serum free triiodothyronine (fT3), free thyroxine (fT4), and fT3/fT4 ratio were examined before and after the interventions. We subsequently administered GH to four human cell lines (HepG2, TSA201, MCF7, and HTC/C3) in vitro, and examined changes in mRNA levels of iodothyronine deiodinases (D1, D2, and D3).

Results Median serum fT3 level significantly increased after rhGH therapy from 2.38 to 2.78 pg/mL ($p < 0.001$), and fT4 decreased from 1.115 to 1.065 ng/dL ($p = 0.081$). TSS significantly decreased median serum fT3 from 3.03 to

2.53 pg/mL ($p < 0.001$), and increased fT4 from 1.230 to 1.370 ng/dL ($p < 0.001$). In vitro, GH significantly increased D2 expression at the mRNA level in HTC/C3 cells ($p < 0.01$), as well as D2 protein and its activity.

Conclusions GH increased serum fT3 level and decreased serum fT4 level in humans. Our results suggest that its mechanism involves D2 upregulation. Considering this GH effect on thyroid hormone metabolism, data on thyroid function could be useful in the management of GH deficiency and acromegaly.

Keywords Deiodinase · Growth hormone · Thyroid hormone · Acromegaly · Adult growth hormone deficiency

Introduction

Growth hormone (GH) has pleiotropic effects that promote growth and regulate metabolic activity [1]. Excessive secretion of GH causes acromegaly leading to various clinical features, such as thickening of soft tissue, altered facial appearance, diabetes, dyslipidemia, and hypertension. Thyroid comorbidities in patients with acromegaly frequently occur; goiter is present in 55% [2] and thyroid cancer in 10.6% [3]. In a study on the relationship between acromegaly and thyroid function, Geelhoed-Duijvestijn et al. reported that serum triiodothyronine (T3) transiently decreases after transsphenoidal surgery (TSS) [4].

In adults, GH deficiency leads to a decrease in lean body mass and reduced quality of life [5], while in children it causes growth retardation. Several studies have demonstrated the effects of GH replacement therapy on thyroid function. GH therapy increases serum T3 or free T3 (fT3) [6–9] and decreases serum thyroxine (T4) or free T4 (fT4)

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[6, 8–11]. Conversely, some studies have reported that GH therapy does not significantly change serum T3 or fT3 [10–12], or serum T4 or fT4 [7, 12]. Regardless, previous reports on both acromegaly and adult GH deficiency have suggested that GH has some effects on the regulation of thyroid function.

The regulation of thyroid function consists of three steps. The first step involves the hypothalamus-pituitary-thyroid axis. In particular, thyroid-stimulating hormone (TSH) stimulates the thyroid gland, increases the secretion of T3 and T4, and maintains cell integrity [13]. The second step involves the conversion of T4 to T3 in peripheral tissue, which is mediated by iodothyronine deiodinases. Iodothyronine deiodinases are classified as three types. Type 1 iodothyronine deiodinase (D1) converts T4 to both T3 and reverse T3, type 2 iodothyronine deiodinase (D2) converts T4 to T3, and type 3 iodothyronine deiodinase (D3) converts T4 to reverse T3. The third step involves the modulation of target gene expression by thyroid hormone receptors.

The mechanism of GH for altering thyroid function has not been clarified. Based on the above, if GH regulates TSH, then both T3 and T4 will increase or decrease. Likewise, if GH regulates thyroid hormone receptor, then serum T3 and T4 levels might not change. Thus, we focused on iodothyronine deiodinases: the upregulation of D1 or D2 can increase T3 and decrease T4. Previous reports have shown that D1 activity is increased by GH in the liver of rainbow trout [14], and is decreased in the liver of chicken after hypophysectomy leading to GH deficiency [15]. Nevertheless, an investigation in humans is necessary, because the distribution of deiodinases differs between humans and other species. In particular, D2 is highly expressed in human thyroid glands [16], but very low expression is found in rat thyroid glands [17].

The aim of the present study was to elucidate the roles and mechanisms of GH in the regulation of human thyroid function. Our first hypothesis was that GH increased serum fT3 levels and decreased serum fT4 levels in clinical settings, and this was tested by retrospective observational studies of patients with either acromegaly or adult GH deficiency. Our second hypothesis was that GH upregulated D1 or D2, and was investigated in vitro using human cell lines. We verified these hypotheses by integrating the results.

Materials and methods

Clinical studies

Patients

Two retrospective observational studies were performed using the medical records of patients in the Department of

Diabetes, Endocrinology and Nutrition of Kyoto University Hospital. The first study focused on adult GH deficiency. We selected consecutive adult patients with severe GH deficiency who started recombinant human GH (rhGH) replacement therapy between August 2008 and July 2015. Severe GH deficiency was confirmed principally via peak serum GH levels ≤ 9 ng/mL in a GH-releasing peptide-2 (GHRP-2) test [18, 19], according to guidelines of the Japan Endocrine Society. Serum fT3, fT4, and TSH levels and fT3/fT4 ratio were compared between baseline and 3 months after rhGH therapy. Exclusion criteria were lack of data on thyroid function after 3 months, the need for modification of levothyroxine (LT4) dose, or concomitant primary hypothyroidism or hyperthyroidism during the observational period.

The second study focused on acromegaly. We selected consecutive patients with acromegaly whose endocrinological profiles were examined during hospitalization before and after TSS. A diagnosis of acromegaly was confirmed by a lack of suppression of nadir serum GH < 1 ng/mL in a 75-g oral glucose tolerance test (OGTT) [20]. Serum fT3, fT4, and TSH levels and fT3/fT4 ratio were compared between baseline and after TSS. Exclusion criteria were lack of data on thyroid function or concomitant primary hypothyroidism or hyperthyroidism.

Assays

Serum fT3, fT4, and TSH were measured by electrochemiluminescent immunoassay (Elecys-FT3 II, Elecys-FT4, Elecys-TSH, Roche Diagnostics, Mannheim, Germany); the reference ranges were 2.33–4.00 pg/mL, 0.880–1.620 ng/dL, and 0.500–5.000 μ IU/mL, respectively. Serum GH was measured by enzyme immunoassay (ST AIA-PACK HGH, Tosoh, Tokyo, Japan), and serum insulin-like growth factor 1 (IGF-1) with immunoradiometric assay (IGF-1 IRMA Daiichi, Fujirebio, Tokyo, Japan). SD scores of IGF-1 were obtained from Japanese reference intervals, as previously reported [21].

In vitro studies

In the following sections, we briefly described materials and methods regarding in vitro studies. See supplementary materials and methods for detailed information.

Cell culture

We used the following four cell lines: HepG2, derived from human hepatoblastoma, TSA201, a derivative of human embryonic kidney 293 cells, MCF7, derived from human breast carcinoma, and HTC/C3, derived from human thyroid undifferentiated carcinoma [22].

RNA isolation, Reverse transcription polymerase chain reactions (RT-PCR), and quantitative RT-PCR

We administered 100 ng/mL rhGH (Sigma-Aldrich, St. Louis, MO) to the media and extracted total RNA from cells after incubation for 4 h. RT-PCR were performed to verify *GHR* (the gene coding GH receptor) expression, and quantitative RT-PCR reactions were to verify gene expressions of three deiodinases (*DIO1* codes D1, *DIO2* codes D2, and *DIO3* codes D3, respectively), using *GAPDH* or *PPIA* as endogenous controls (Supplementary Table 1).

Protein extraction and western blotting

We administered 100 ng/mL rhGH to the media and extracted whole cell protein after incubation for 48 h. We performed western blotting analysis using the following primary antibodies: D2 (ab77779; abcam, Cambridge, UK), D3 (NBP1-05767; Novus Biologicals, Littleton, CO), and β -actin (4967; Cell Signaling Technology, Danvers, MA).

T3 generation assay

We administered 100 ng/mL rhGH to the media and then 1 μ M LT4 with and without 1 mM propylthiouracil (PTU) (Nacalai Tesque, Kyoto, Japan), known as a D1 inhibitor, after incubation for 24 h. We collected the cultured media after further incubation for 24 h and measured their T3 concentration.

DIO2 reporter assay

We administered 100 ng/mL rhGH to the media, and simultaneously performed transient transfections including *DIO2* reporter constructs using the Lipofectamine LTX reagent (Thermo Fisher Scientific, Waltham, MA). After incubation for 48 h, we harvested cells and determined luciferase activity using the Dual-Luciferase Reporter Assay System (Promega).

Statistical analysis

Data from the clinical studies were expressed as medians (interquartile range). Comparisons of parameters between two groups were analyzed by Mann-Whitney U test. Correlation was analyzed by calculating Spearman's rank correlation coefficient. Data from in vitro studies were expressed as means $\pm$ standard error of the mean (SEM). Statistical analysis was performed using paired *t*-test. JMP pro version 11.2.0 (SAS institute Inc., Cary, NC) was used for all statistical analyzes. A *p*-value of < 0.05 was considered statistically significant.

Table 1 Baseline characteristics of patients in the adult GH deficiency study

<i>n</i>	20
Age (years)	41 (23–59)
Sex (male/female)	10/10
Body mass index (kg/m ²)	23.1 (20.4–32.0)
fT3 (pg/mL)	2.38 (1.99–2.72)
fT4 (ng/dL)	1.115 (0.964–1.185)
TSH (μ IU/mL)	0.834 (0.074–2.635)
IGF-1 (ng/mL)	95 (60–124)
IGF-1 SD score	−3.2 (−4.7 to −1.4)
GHRP-2 stimulating test (ng/mL)	
Basal GH <i>n</i> = 19	< 0.07 (< 0.07–0.22)
Peak GH <i>n</i> = 19	1.08 (0.18–3.51)
Etiology (<i>n</i>)	
Non-functioning adenoma	7
Craniopharyngioma	5
Undetermined tumor	2
Germinoma	1
Prolactinoma	1
Rathke's cleft cyst	1
Pituitary apoplexy	1
Sheehan's syndrome	1
Pituitary stalk interruption	1
Past treatment (<i>n</i>)	
Surgery	14
Radiotherapy	9
None	6
Replacement therapy (<i>n</i>)	
Hydrocortisone	16
Sex steroids	14
Desmopressin	6
Levothyroxine	17
Dose per body weight (μ g/kg)	0.76 (0.43–1.16)

Data are presented as medians (interquartile range)

N number of patients, *GH* growth hormone, *fT3* free triiodothyronine, *fT4* free thyroxine, *TSH* thyroid-stimulating hormone, *IGF-1* insulin-like growth factor 1, *GHRP-2* GH-releasing peptide-2

Results

Adult GH deficiency study

We collected the medical records of 25 consecutive patients with severe adult GH deficiency, but excluded five patients because of lack of data on thyroid function after 3 months. None of the patients required modification of their levothyroxine doses during 3 months, and none had concomitant primary hypothyroidism or hyperthyroidism. We ultimately analyzed 20 patients.

The baseline characteristics of the patients in the adult GH deficiency study are shown in Table 1, and overall data are shown in Supplementary Table 2. Data of individual patients are shown in Supplementary Fig. 1A. The median serum fT3 level was 2.38 (1.99–2.72) pg/mL, the fT4 level was 1.115 (0.964–1.185) ng/dL, and the TSH level was 0.834 (0.074–2.635) μ IU/mL. Surgical treatment or radiotherapy had been performed at least 3 years before rhGH therapy. A number of patients had received replacement therapy for hypopituitarism, but the doses of hydrocortisone ($n = 16$), sex steroids ($n = 14$), desmopressin ($n = 6$), and levothyroxine ($n = 17$; median dose per body weight: 0.76 (0.43–1.16) μ g/kg) had not been modified for 3 months.

We verified a severe deficit in GH secretion and augmentation of GH effects by rhGH therapy. The median serum IGF-1 level was 95 (60–124) ng/mL, IGF-1 SD score was -3.2 (-4.7 to -1.4), and peak serum GH level in the GHRP-2 stimulating test was 1.08 (0.18–3.51) ng/mL. The median dose of rhGH at 3 months was 0.20 (0.15–0.30) mg/day, and the median serum IGF-1 level significantly increased from 95 (60–124) ng/mL to 179 (110–279) ng/mL ($p < 0.001$), and from -3.2 (-4.7 to -1.4) to -0.7 (-1.8 to 1.8) in the SD score ($p < 0.001$).

Changes in the parameters after 3 months of rhGH therapy are shown in Fig. 1a. The median serum fT3 level significantly increased from 2.38 (1.99–2.72) to 2.78 (2.43–2.91) pg/mL ($p < 0.001$). The fT4 level tended to decrease from 1.115 (0.964–1.185) to 1.065 (0.978–1.160) ng/dL, but this was not significant ($p = 0.081$). The fT3/fT4 ratio significantly increased from 2.16 (1.92–2.35) to 2.51 (2.19–2.78) 10^{-2} pg/ng ($p < 0.001$). The TSH level significantly decreased from 0.834 (0.074–2.635) to 0.624 (0.039–2.320) μ IU/mL ($p = 0.029$). At baseline, serum IGF-1 level positively correlated with fT3/fT4 ratio ($p = 0.466$, $p = 0.038$). Furthermore, changes in serum IGF-1 level positively correlated with changes in fT3/fT4 ratio ($p = 0.463$, $p = 0.040$) (Fig. 1b).

When comparing the two genders, the median serum IGF-1 level at baseline tended to be lower and its changes were significantly smaller in females ($p = 0.080$ and $p = 0.002$, respectively). Likewise, changes in the median serum fT3 level were significantly smaller and changes in the fT3/fT4 ratio tended to be smaller in females ($p = 0.040$ and $p = 0.080$, respectively) (Supplementary Table 2). All three patients with isolated GH deficiency and two of three patients without levothyroxine replacement showed increases in fT3/fT4 ratio likewise the whole cohort.

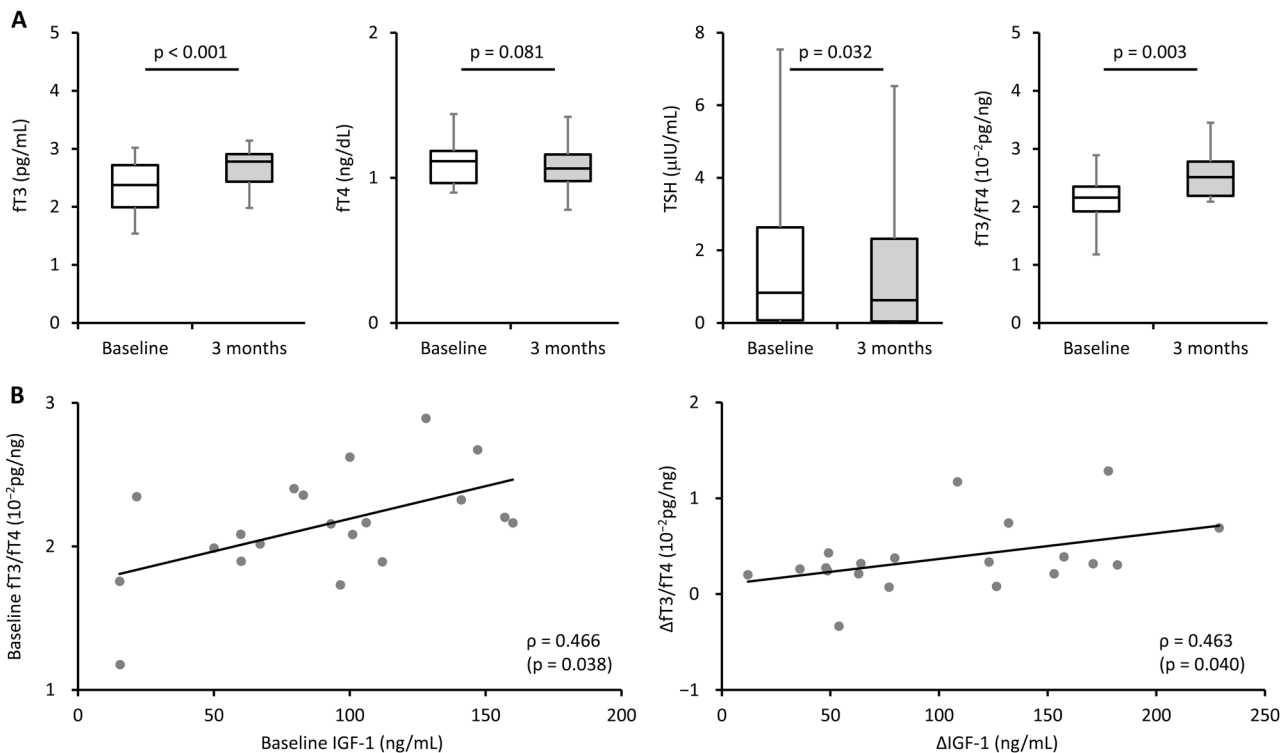


Fig. 1 **a** Box-plot of thyroid function at baseline and 3 months after recombinant human growth hormone (rhGH) replacement therapy in 20 patients with adult GH deficiency. Each box represents the interquartile range. The horizontal line in each box represents the median. The ends of the vertical lines represent the minimum and maximum

values. **b** Linear regression of insulin-like growth factor 1 (IGF-1) and free triiodothyronine /free thyroxine (fT3/fT4) at baseline, and that of changes in IGF-1 (Δ IGF-1) and fT3/fT4 ratio (Δ fT3/fT4) between baseline and 3 months after rhGH therapy. p , Spearman's rank correlation coefficient

We observed variation in serum TSH levels performed a subgroup analysis based on baseline serum TSH levels (Supplementary Fig. 1B); we determined a cut-off value for baseline TSH as 1 $\mu\text{IU/mL}$. Nevertheless, lower TSH group ($n = 11$) still showed significant changes by rhGH treatment in serum TSH levels ($p = 0.005$) and fT3/fT4 ratio ($p < 0.001$), but higher TSH group ($n = 9$) showed no significant changes in either serum TSH levels ($p = 0.150$) or fT3/fT4 ratio ($p = 0.065$). In addition, the degrees of changes in fT3/fT4 ratio were larger in lower TSH group than in higher TSH group ($p < 0.001$).

Acromegaly study

We collected the medical records of 27 consecutive patients with acromegaly, but excluded two patients because of lack of data on thyroid function ($n = 1$) and concomitant primary hyperthyroidism caused by a functional nodule ($n = 1$). We ultimately analyzed 25 patients.

The baseline characteristics of the patients in the acromegaly study are shown in Table 2, and overall data are shown in Supplementary Table 3. In addition, data of individual patients are shown in Supplementary Fig. 1C.

Table 2 Baseline characteristics of patients in the acromegaly study

<i>n</i>	25
Age (years)	52 (38–61)
Sex (male/female)	10/15
Body mass index (kg/m^2)	23.9 (21.4–27.3)
fT3 (pg/mL)	3.01 (2.70–3.24)
fT4 (ng/dL)	1.230 (1.040–1.345)
TSH ($\mu\text{IU/mL}$)	0.771 (0.410–1.380)
IGF-1 (ng/mL)	586 (408–854)
IGF-1 SD score	6.9 (5.1–9.0)
Nadir GH in 75-g OGTT (ng/mL)	10.24 (4.43–16.80)
Pituitary tumor size (mm)	11.0 (7.8–17.2)
Thyroid size (mm)	
Isthmus thickness	2.2 (1.7–3.1)
Right lobe transverse diameter	21.5 (19.6–24.5)
Right lobe thickness	14.9 (12.8–18.3)
Left lobe transverse diameter	20.4 (18.5–22.3)
Left lobe thickness	13.9 (11.1–16.2)
Comorbid thyroid diseases (<i>n</i>)	
None	21
Papillary carcinoma	2
Adenomatous goiter	1
Undetermined tumor	1

Data are presented as medians (interquartile range)

n number of patients, *fT3* free triiodothyronine, *fT4* free thyroxine, *TSH* thyroid-stimulating hormone, *IGF-1* insulin-like growth factor 1, *OGTT* oral glucose tolerance test

Their thyroid function values were within the reference ranges: The median serum fT3 level was 3.01 (2.70–3.24) pg/mL , the fT4 level was 1.230 (1.040–1.345) ng/dL , and the TSH level was 0.771 (0.410–1.380) $\mu\text{IU/mL}$. The thyroid gland was measured by ultrasonography, and 22 patients had a diagnosis of goiter, defined as thickness of the isthmus >4 mm, transverse diameter >20 mm, or thickness of either lobe >15 mm. These criteria are generally used in Japan to diagnose goiter (no reference). Comorbid thyroid diseases were uncommon: papillary carcinoma was found ($n = 2$), adenomatous goiter ($n = 1$), and an undetermined tumor ($n = 1$).

We verified excessive secretion of GH before TSS and attenuation of the GH effect after TSS. Before TSS, the median serum IGF-1 level was 586 (408–854) ng/mL , the IGF-1 SD score was 6.9 (5.1–9.0), and the nadir serum GH level in 75-g OGTT was 10.24 (4.43–16.80) ng/mL . After TSS, the patients were examined at 16th (13th–19th) post-operative day. The median nadir serum GH in 75-g OGTT significantly decreased to 0.88 (0.37–1.67) ng/mL ($p < 0.001$), and the IGF-1 level significantly decreased to 292 (221–354) ng/mL ($p < 0.001$), and 2.8 (1.9–3.9) in the SD score ($p < 0.001$).

Changes in the parameters after TSS are shown in Fig. 2a. The median serum fT3 level significantly decreased from 3.01 (2.70–3.24) to 2.52 (2.15–2.83) pg/mL ($p < 0.001$), and the fT4 level significantly increased from 1.230 (1.040–1.345) to 1.370 (1.205–1.590) ng/dL ($p < 0.001$). The fT3/fT4 ratio significantly decreased from 2.54 (2.17–2.95) to 1.81 (1.52–2.11) 10^{-2} pg/ng ($p < 0.001$). The TSH level significantly increased from 0.771 (0.410–1.380) to 1.230 (0.869–2.070) $\mu\text{IU/mL}$ ($p = 0.025$). At baseline, serum IGF-1 level positively correlated with fT3/fT4 ratio ($\rho = 0.472$, $p = 0.017$). Furthermore, changes in serum IGF-1 level positively correlated with changes in fT3/fT4 ratio ($\rho = 0.609$, $p = 0.001$) (Fig. 2b).

When comparing the two genders, the median serum IGF-1 level at baseline tended to be lower and its changes tended to be smaller in females ($p = 0.053$ and $p = 0.097$, respectively). Likewise, changes in the median serum fT3 level was significantly smaller and changes in the fT3/fT4 ratio tended to be smaller in females ($p = 0.014$ and $p = 0.080$, respectively) (Supplementary Table 3). Even though three patients were withdrawn from β -blockers, the median change in heart rate was -9 (-15 to 6) and positively correlated with the change in fT3/fT4 ratio ($\rho = 0.475$, $p = 0.017$).

Changes in deiodinase expression by GH administration in vitro

To begin with, we examined *GHR* mRNA expression of each cell line in GH-free media. HepG2 cells showed no

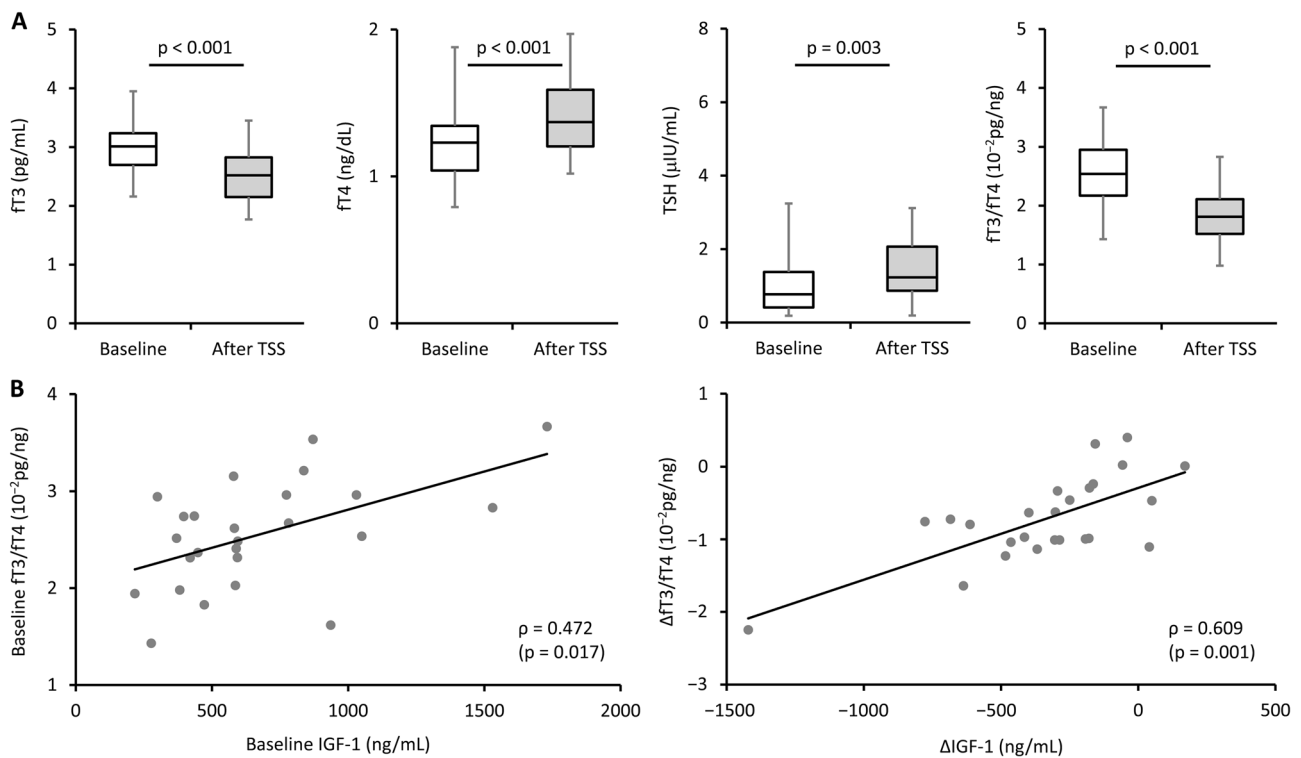


Fig. 2 a Box-plot of thyroid function at baseline and after transphenoidal surgery (TSS) in 25 patients with acromegaly. Each box represents the interquartile range. The horizontal line in each box represents the median. The ends of the vertical lines represent the

minimum and maximum values. **b** Linear regression of IGF-1 and fT3/fT4 ratio at baseline, and that of ΔIGF-1 and ΔfT3/fT4 between baseline and after TSS. ρ, Spearman's rank correlation coefficient

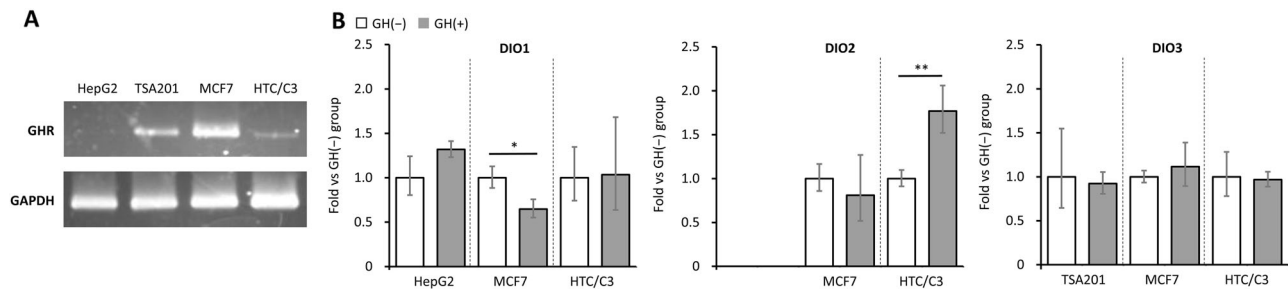


Fig. 3 a Reverse transcription polymerase chain reactions (RT-PCR) analysis of *GHR* mRNA expression in each cell using *GAPDH* as an endogenous control. **b** Changes in gene expressions of deiodinases following GH administration examined using quantitative RT-PCR. GH(-) indicates no rhGH supplementation, and GH(+) indicates 100

ng/mL supplementation. The mRNA levels of deiodinases were normalized to those of *GAPDH* and represented as fold-change versus GH (-) group (means ± standard error of the mean (SEM)). **p* < 0.05; ***p* < 0.01

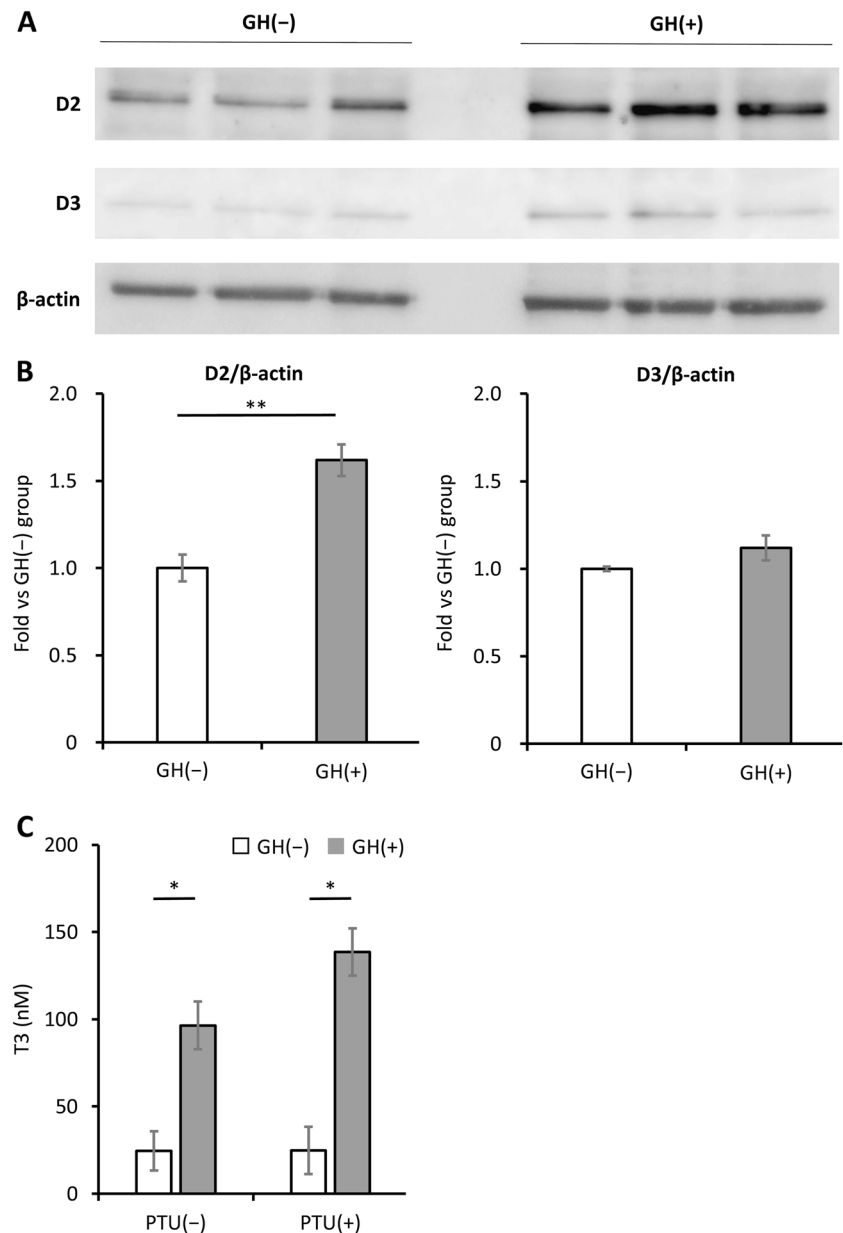
GHR mRNA expression, but TSA201, MCF7, and HTC/C3 cells expressed *GHR* mRNA (Fig. 3a).

We subsequently examined whether gene expression of each of the three iodothyronine deiodinases is changed by supplementing the media containing 1 nM 3,3',5-triiodo-L-thyronine (LT3) with 100 ng/mL rhGH (Fig. 3b). We could not evaluate the mRNA levels of *DIO1* in TSA201 cells, *DIO2* in HepG2 and TSA201 cells, and *DIO3* in HepG2 cells, because their quantitative RT-PCR reactions were unstable or missing values were observed. The mRNA

levels of *DIO1* showed no substantial change in HepG2 and HTC/C3 cells, but significantly decreased in MCF7 cells (*p* < 0.05). *DIO2* did not change in MCF7 cells, but increased significantly in HTC/C3 cells (*p* < 0.01). *DIO3* showed no change in TSA201, MCF7, and HTC/C3 cells.

We performed additional examinations using HTC/C3 cells to confirm relevance of D2 increase by GH. The results of western blotting showed that the protein levels of D2 were significantly increased by GH (*p* < 0.01), while those of D3 were not changed (Fig. 4a, b). Specificity of the used

Fig. 4 a Western blotting analysis of HTC/C3 cells for type 2 iodothyronine deiodinase (D2), type 3 iodothyronine deiodinase (D3), and β -actin. GH(–) indicates no rhGH supplementation, and GH(+) indicates 100 ng/mL supplementation. The D2 bands were detected at a molecular weight of 35,000, D3 at 22,000 and β -actin at 45,000. **b** Relative protein levels of D2 and D3 in western blotting analysis. The protein levels of D2 and D3 were normalized to those of β -actin and represented as fold-change versus GH(–) group (means $\pm$ SEM). $**p < 0.01$. **c** Examination of changes in T3 generated from T4 in cultured media. PTU(–) indicates no propylthiouracil supplementation and PTU(+) indicates 1 mM supplementation. $*p < 0.05$



antibody against D2 was verified using 3xFLAG-tagged mutant D2 protein (Supplementary Fig. 2A). In addition, we measured generated T3 in the cultured media to determine a change in deiodinase activity. As a result, GH significantly increased generated T3 in the media both with and without PTU (Fig. 4c). Selective inhibition of D1 activity by PTU was validated using FLAG-tagged mutant D1 protein (Supplementary Fig. 2B).

DIO2 mRNA increase by GH was also observed in LT3-free media, which was verified using multiple endogenous controls ($p < 0.05$ in *GAPDH* and $p < 0.01$ in *PPIA*) (Fig. 5a). Moreover, *DIO2* mRNA was significantly increased by GH even in 100 nM LT3 ($p < 0.05$) (Fig. 5b). GH tended to increase *DIO2* mRNA in a dose dependent manner (Fig. 5c). In order to investigate mechanisms of GH for

increasing *DIO2* mRNA expression and determine *DIO2* promoter domains responsible for GH action, we subsequently performed *DIO2* reporter assay using HTC/C3 cells. We prepared a series of reporter constructs containing various lengths of *DIO2* promoter sequences, but observed no significant changes of the reporter activity by GH in all constructs used (Fig. 5d). Meanwhile, IGF-1 showed no significant change of *DIO2* mRNA as well as *DIO2* reporter activity (Fig. 5e, f).

Discussion

The present study demonstrated how GH alters thyroid function in humans by examining the effects both in clinical

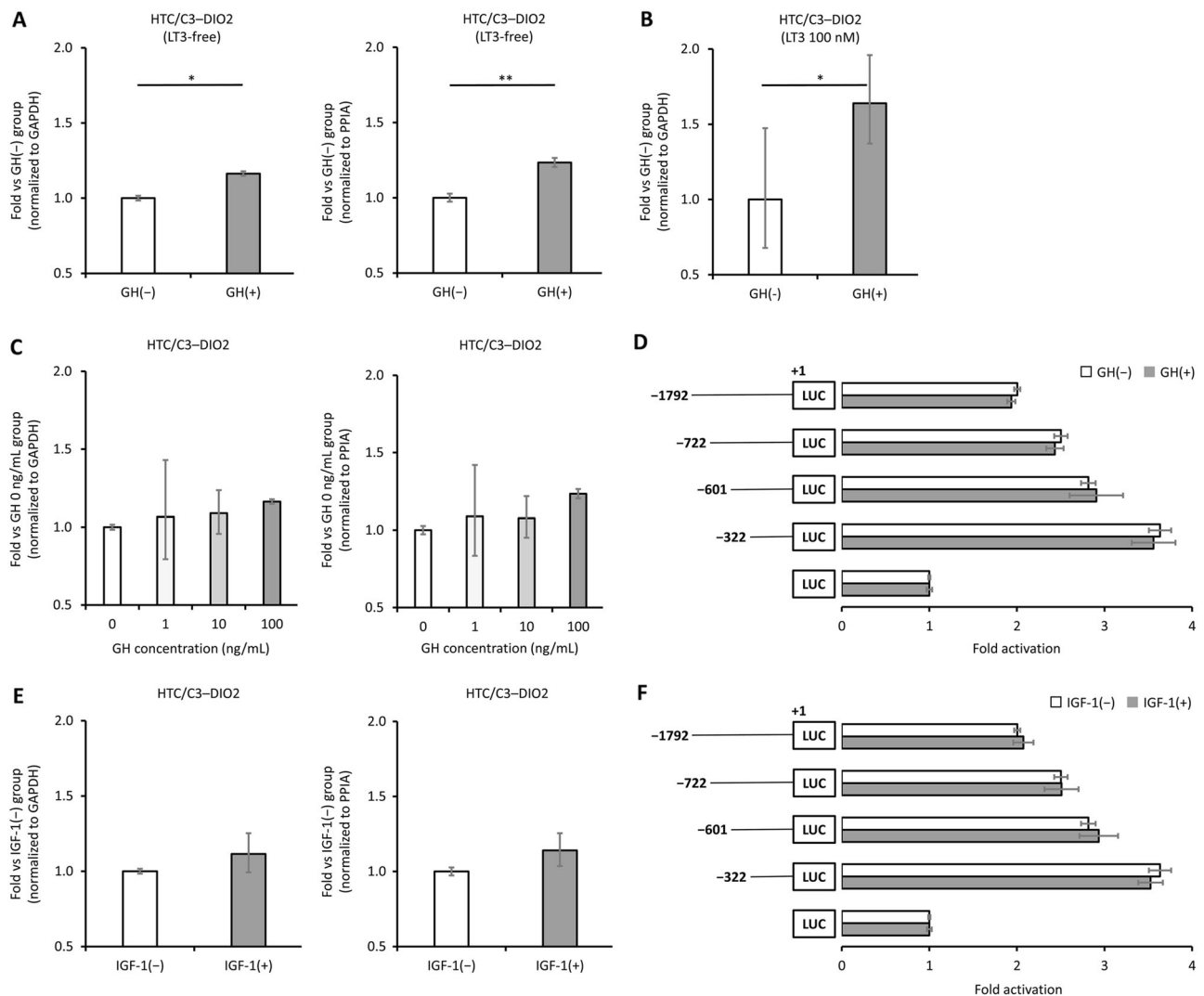


Fig. 5 **a b** Changes in *DIO2* mRNA following rhGH administration examined with HTC/C3 cells in 3,3',5-triiodo-L-thyronine (LT3)-free media or in 100 nM LT3 media using quantitative RT-PCR. GH(-) indicates no rhGH supplementation, and GH(+) indicates 100 ng/mL supplementation. The mRNA levels of *DIO2* were normalized to those of *GAPDH* or *PPIA* and represented as fold-change versus GH(-) group (means $\pm$ SEM). * $p < 0.05$; ** $p < 0.01$. **c** Examination of changes in *DIO2* mRNA according to various concentrations of GH administration (0, 1, 10, and 100 ng/mL). The mRNA levels of *DIO2* were normalized to those of *GAPDH* or *PPIA*, and represented as fold-change versus GH 0 ng/mL group (means $\pm$ SEM). *DIO2* mRNA tended to increase in a dose dependent manner, but this change was not statistically significant. **d** *DIO2* reporter assay with and without rhGH using a series of 5'-deletion mutant constructs of the human *DIO2* promoter fused to the luciferase gene (-1792, -722, -601, and

-322/+1 hDIO2-Luc, the translational start site was set at +1). Fold activation means Firefly luciferase activity /Renilla luciferase activity ratio normalized to that of promoterless pGL 4.10 without rhGH supplementation. No significant changes between GH(-) and GH(+) groups were detected. **e** Changes in *DIO2* mRNA following IGF-1 administration examined with HTC/C3 cells. IGF-1(-) indicates no IGF-1 supplementation, and IGF-1(+) indicates 100 ng/mL supplementation. The mRNA levels of *DIO2* were normalized to those of *GAPDH* or *PPIA*, and represented as fold-change versus IGF-1(-) group (means $\pm$ SEM). No significant changes were detected. **f** *DIO2* reporter assay with and without IGF-1. Fold activation means Firefly luciferase activity/Renilla luciferase activity ratio normalized to that of promoterless pGL 4.10 without IGF-1 supplementation. No significant changes between IGF-1(-) and IGF-1(+) groups were detected

settings and in vitro. rhGH therapy in patients with severe adult GH deficiency was a model showing an increase in GH, which subsequently increased serum FT3, tended to decrease FT4, and decreased TSH. In contrast, surgical intervention for patients with acromegaly, which was a model of a decrease in GH, decreased serum FT3, increased FT4, and increased TSH.

To examine these effects more accurately, we designed two-way studies, and tried to minimize the bias that can arise in retrospective studies by analyzing similar patients who received each intervention. We excluded patients with concomitant primary thyroid dysfunction in both studies, and confirmed that no patients required modification of levothyroxine dose for hypopituitarism in the adult GH

deficiency study. In addition, we limited our investigation to severe cases of adult GH deficiency because we suspected that the discrepancies between some previous reports [6–12] depended on the severity of GH deficiency.

Our results in the clinical studies suggest that GH plays a role in increasing T3 and decreasing T4 in the circulation. We also confirmed that GH increased the fT3/fT4 ratio and its level was correlated with IGF-1, a surrogate marker of GH action. This point is important for interpreting our in vitro results that GH increased D2 expression, because Kanou et al. reported that the fT3/fT4 ratio positively correlated with thyroidal D2 activity in humans [23].

GH decreased the serum TSH level as previously reported [7], but increased the fT3 level, possibly because of negative feedback involving circulating T3 or local upregulation of D2 in the hypothalamus and pituitary gland [24]. The result of subgroup analysis in the adult GH deficiency study suggests that low serum TSH level is a predictive factor for observed changes in thyroid function. In other words, patients with low TSH might be susceptible to cause unmasked central hypothyroidism by rhGH treatment [10]. Whether levothyroxine replacement affects the results of our studies could not be determined because the number of patients were insufficient, but Jorgensen et al. compared patients with and without levothyroxine replacement and obtained similar results in both groups [6].

Gender differences in IGF-1 response to GH were observed in both studies as previously reported; males are more responsive to rhGH treatment than females [25–27]. In addition, we verified that the degrees of changes in the fT3 level and the fT3/fT4 ratio by GH were smaller in females. This suggests that responsiveness of thyroid function to GH, as well as that of IGF-1, might be related to sex hormones.

Subsequently, we performed in vitro studies to elucidate the mechanisms. Our study was characterized as follows: (1) Each cell line originated from human tissue. (2) The iodothyronine deiodinases were analyzed in multiple cell lines. (3) We used the HTC/C3 cell line established from thyroid tissue [22].

The key result was that GH increased *DIO2* mRNA in HTC/C3 cells. This increase was verified in 1 nM LT3 as well as in 100 nM LT3 as the concentration for saturation of the thyroid hormone receptor [28], suggesting that the regulation was independent of T3 or the downstream thyroid hormone receptor. To make sure, we verified that GH increased *DIO2* mRNA even in LT3-free media, considering the possibility that 1 nM LT3 is sufficient for saturating thyroid hormone receptor. We also confirmed that protein levels of D2 and its activity were increased by GH, which corresponds to the results of *DIO2* mRNA.

Moreover, GH did not affect *DIO2* mRNA in MCF7 cells. Considering that GH receptor was expressed in both

HTC/C3 and MCF7 cells, we suspected that other factors were involved. Thyroid specific transcription factors, such as thyroid transcription factor-1, which stimulates the *DIO2* promoter in humans, but not in rats [17], are the likely candidates, because HTC/C3 originates from human thyroid tissue.

To elucidate mechanisms of D2 upregulation by GH, we performed *DIO2* reporter assay. We tried to identify *DIO2* promoter domains responsible for increasing mRNA via GH action, but GH did not affect the reporter activity unexpectedly. This indicates that D2 upregulation by GH is not associated with direct transcriptional activation. Epigenetic regulation might be involved because changes in chromatin at target promoters by GH were previously reported [29]. Furthermore, our results excluded IGF-1 involvement in *DIO2* mRNA expression, although it is well known that GH increases IGF-1. The mechanisms need to be confirmed by further investigations.

Meanwhile, *DIO1* mRNA expression in MCF7 cells was decreased. The results of the clinical studies indicated that GH increased T3 and decreased T4, but D1 downregulation could cause conflicting changes. In addition, HepG2 and HTC/C3 cells showed no change in *DIO1* mRNA, and D1 inhibition by PTU did not change the amount of generated T3 in HTC/C3 cells. Furthermore, we determined that *DIO3* mRNA in all cell lines used and D3 protein in HTC/C3 cells did not change. These findings suggested that D1 and D3 had no relevance in the present study.

Importantly, the results of the clinical studies were supported by the results of our in vitro studies. If GH upregulates D2 in the thyroid glands, more T3 and less T4 will be secreted into the circulation, serum T3 or fT3 level will increase, and serum T4 or fT4 will decrease, as in the case of Graves' disease [16]. In addition, Maia et al. reported that D2 was the major source of plasma T3 in humans [30]. Although rhGH treatment might unmask central hypothyroidism [10], the observed D2 upregulation suggests that thyroid hormone action in peripheral tissue augments in fact. This hypothesis is supported by the result of heart rate in the acromegaly study because the heart is a major target organ of thyroid hormone action.

The previous reports suggested that GH alters the activity of D1 [14, 15]; however, the present study indicated that GH regulates D2. This discrepancy might be because iodothyronine deiodinases are distributed differently in different species; D2 is highly expressed in human thyroid glands [16], but slightly in rat thyroid glands [17]. Since our study examined the effects in humans, it is unsurprising that our results differed from animal studies conducted on rainbow trout or chickens [14, 15].

We expect that our results provide several clinical suggestions as follows. (1) In acromegaly, mild thyrotoxicosis might be improved by an intervention such as TSS. (2) GH

therapy might be more effective for patients with adult GH deficiency who present low serum fT3 levels because GH has potential for increasing fT3. This hypothesis is worth examining because predictors of the clinical response to GH replacement therapy are not well established [31–35]. (3) Children show higher serum fT3 levels than adults [36], which might be explained by the fact they have higher serum GH and IGF-1 levels [21].

The limitations of the present study are listed as follows: the clinical studies were performed retrospectively, so we could not completely exclude bias. Reverse T3 and thyroxine-binding globulin were not measured in all patients, and thyroglobulin was measured in few patients. The results of the in vitro investigation were obtained from cell lines of limited organs, at a certain concentration of hormone, and following a specific time course. We could not examine deiodinases in vivo because it is ethically hard to obtain samples of human tissues including the thyroid gland before and after interventions.

In conclusion, GH increased serum fT3 and decreased serum fT4 and TSH in clinical settings, which might be caused by increased D2 expression as we have shown in the thyroid cell line, HTC/C3. The results of our clinical and in vitro studies corresponded; D2 upregulation increased serum fT3 and decreased fT4. Data on thyroid function could be useful in the management of GH deficiency and acromegaly.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The studies were approved by the Institutional Review Board and Ethics Committee of the Kyoto University Graduate School of Medicine, and were conducted in accordance with the principles of the Declaration of Helsinki.

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