Spiking expression of μ-crystallin mRNA during treatment with methimazole in patients with Graves' hyperthyroidism.

Satoru Suzuki, Masahiro Takei, Shin-ichi Nishio, Hidefumi Inaba, Ai Sato, Masanori Yamazaki, Ken Shinomiya, Kiyoshi Hashizume

Department of Aging Medicine and Geriatrics, Institute on Aging and Adaptation, Shinshu University, Graduate School of Medicine, 3-1-1, Asahi, Matsumoto, 390-8621, JAPAN,

running title Thyroid hormone regulates CRYM expression.

Correspondence should be addressed to Satoru Suzuki, M.D., Ph.D., Department of Aging Medicine and Geriatrics, Institute on Aging and Adaptation, Shinshu University, Graduate School of Medicine, 3-1-1, Asahi, Matsumoto, Nagano, 390-8621, Japan

TEL: +81-263-37-2686

FAX: +81-263-37-2710 E-MAIL: soutaro@hsp.md.shinshu-u.ac.jp

Disclosure statement: The authors have nothing to disclose.

Abstract

Context: μ-crystallin is an NADPH-dependent cytosolic T3-binding protein. A knockout study in mice showed that μ-crystallin has a physiological function as a reservoir of T3 in the cytoplasm *in vivo*. Patients with nonsyndromic deafness were reported to have point mutations in the μ-crystallin gene. The expression of μ-crystallin is regulated by multiple factors.

Objective: The present study was performed to determine whether thyroid function is related to the expression of μ-crystallin mRNA in peripheral mononuclear cells.

Design and setting: We examined 23 normal healthy male and female subjects and 15 patients with Graves' disease.

Methods: μ-crystallin protein expression was determined immunohistochemically in peripheral mononuclear cells. The expression of μ-crystallin mRNA was assessed by reverse transcription of total RNA from peripheral mononuclear cells followed by quantitative PCR.

Results: μ-crystallin protein was detected in peripheral mononuclear cells. The mRNA expression was negatively correlated with age in normal female subjects. The values in female subjects were significantly higher than those in males. The values were positively correlated with serum TSH concentration. The values in the thyrotoxic patients with Graves' disease were lower than those in healthy subjects. A transient increase in μ-crystallin expression was observed within 14–42 days after the initial treatment with anti-thyroid medication.

Conclusions: Thyroid hormone inversely relates to the expression of μ-crystallin mRNA in euthyroid mononuclear cells. Abrupt suppression of thyroid function leads to overexpression of μ-crystallin mRNA in thyrotoxic mononuclear cells. Thyroid hormone-regulated μ-crystallin expression may control thyroid hormone action *via* the intracytoplasmic T_3 capacity.

key words: CRYM, thyroid hormone, thyroid hormone binding protein, crystallin, hyperthyroidism

Introduction

3,5,3'-Triiodo-L-thyronine (T3) and thyroxine (T4) are secreted from the thyroid gland as active thyroid hormone [1]. In peripheral target cells, these two forms of iodothyronine enter the cytoplasm across the cell membrane [2], where they are subsequently transferred to the nucleus. 3,5,3'-Triiodo-L-thyronine-binding protein (μ-crystallin; CRYM) is present in the cytoplasm and is known to bind T3 with high affinity in the presence of NADPH [3]. This protein plays a role in binding T3 and may transport T3 from the cytoplasm to the nucleus [4]. Clinically, two patients with nonsyndromic deafness have been reported to have point mutations in the μ-crystallin gene [5], and the T3-binding properties of CRYM protein may affect the clinical symptoms of deafness [6]. The expression of CRYM is regulated by numerous factors, including T3 [7]. In the rat liver, the treatment with T3 up-regulates the maximal binding capacity of NADPH-dependent T3 binding [8]. It has also been reported that CRYM mRNA level increases after treatment with T3 in human retinal WER1 cells [9].

To clarify whether the expression of CRYM is controlled by thyroid hormone *in vivo*, especially in humans, we measured the levels of CRYM mRNA in human peripheral blood mononuclear cells (PBMCs). We measured the amount of the CRYM mRNA in healthy male and female Japanese volunteers and patients with Graves' disease, and assessed the relationship between CRYM mRNA expression and thyroid hormone function. We monitored changes in the CRYM mRNA level for at least 6 weeks in patients with Graves' disease.

Materials and Methods

Experimental subjects

This study was approved by the Ethical Committee of Shinshu University, and all participants gave their written, informed consent. The subjects consisted of 23 healthy volunteers aged 25–49 yr and 15 patients with Graves' disease aged 23–65 yr attending the Department of Aging Medicine and Geriatrics, Shinshu University Hospital. All Graves' patients are newly diagnosed cases in the study.

Study Protocol

Blood samples to obtain total RNA were collected from subjects into PAXgene tubes (PreAnalytiX, Zurich, Switzerland). Serum for measurement of thyroid hormone was simultaneously obtained into silicone-coated tubes. Exclusion criteria for the present study were diabetes and inflammation. All Graves' patients received 15 or 20 mg of methimazole as initial treatment after the first blood samples were obtained. Steady amounts of methimazole were administered for a further 42 to 56 days. Samples were obtained at least three times during the period of treatment.

Measurement of thyroid hormones and TSH

Serum free T3, free T4, and TSH were measured with Chemilumi ACS-T3, T4 (Bayer Med, Tokyo, Japan) and Architect TSH (Abbott Japan, Tokyo, Japan).

RNA extraction

Total RNA was extracted from samples of 2.5 ml of blood using PAXgene Blood RNA tubes and a PAXgene Blood RNA kit (Qiagen Inc., Valencia, CA) according to the manufacturer's protocol. RNA integrity was assessed by electrophoresis on 1% agarose gels. RNA concentrations were determined by monitoring A_{260}, and purity was assessed by determining the $A_{260/280}$ as an indicator of the RNA to DNA ratio.

Quantitative RT-PCR

Aliquots of 50 ng of the extracted RNA were reverse transcribed in 20-μl reaction volumes using a Quantitect RNA kit (Qiagen Inc.) according to the manufacturer's protocol. CRYM mRNA expression levels were analyzed using an ABI Prism 7900 sequence detection system (PerkinElmer Applied Biosystems, Tokyo, Japan), which employs TaqMan chemistry for highly accurate quantification of mRNA levels. Reactions were performed in a volume of 50 μl on 96-well plates in buffer containing TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and aliquots of 2 μl of reverse transcribed cDNA template. The amount of 18S ribosomal RNA was measured using commercially available probes in accordance with the manufacturer's protocol (Applied Biosystems). Reactions were as follows: 50°C for 2 min, 95°C for 10 min, and then 50 cycles of 95°C for 15 s and 60°C for 1 min. Oligonucleotide primers and the TaqMan probe for human CRYM were as follows: forward, CAGAGCCCATTTTGTTTGGTGAAT, reverse TCATCATCCAGTTCTCTCCAGTCA, and probe FAM ATCAATGCTGTTGGAGCCAG NFQ. Samples were run in triplicate and normalized to a standard curve of serial dilutions of reverse transcribed RNA obtained from the EBV-transformed lymphoblasts of a healthy 30-year-old woman [10]. Briefly, a standard curve of serial dilutions of reverse transcribed RNA obtained from the EBV-transformed lymphoblasts was performed in each 96-well assay. The quantification was performed by crossing-point inter- and extrapolation into the standard curve by using ABI Prism 7900 sequence detection system software. Relative units represent the ratio of concentration between the CRYM mRNA and 18S ribosomal RNA as a housekeeping mRNA.

Immunostaining

Venous blood (10 ml) was drawn from healthy volunteers and collected in tubes containing EDTA. PBMCs were isolated by gradient centrifugation separation (Lymphoprep; Axis-Shield, Oslo, Norway). The cells were taken to make cytospin preparations, which were fixed and permeabilized using InterPrep permeabilization reagent (Beckman Coulter, Fullerton, CA). The construction and characterization of CRYM–specific antibody were described previously [11]. After blocking with 1% bovine serum albumin in phosphate-buffered saline (PBS), sections were reacted with CRYM-specific primary antibody at room temperature. For competition studies, sections were reacted with CRYM-specific antibody preabsorbed with the immunizing peptide as described previously [11]. After washing with PBS, sections were incubated with TRITC-labeled mouse anti-rabbit IgG (Zymed, San Francisco, CA) for 2 h at room temperature. After washing with PBS, the sections were mounted with Slow-Fade (Molecular Probes, Eugene, OR, USA).

Statistical analysis

Data are presented as means±SD. StatView Version 4.1 (Abacus, Berkeley, CA) was used for statistical analyses. Differences between two groups were assessed by the nonparametric Mann-Whitney *U* test for variables that were not normally distributed. In the normal subject group, Spearman nonparametric correlation coefficients were used to examine relationships between variables, and best-fit lines were obtained by linear regression using the least-squares method. $P<0.05$ was considered statistically significant.

Results

CRYM protein is expressed in the cytoplasm of PBMCs

We initially assessed the expression of CRYM protein in PBMCs. Immunohistochemically, TRITC-labeled second antibody recognized the CRYM polyclonal antibody in the cytoplasm of all mononuclear cells, as shown in Fig. 1a. The heterogeneous expression was observed in the cytoplasmic region of some mononuclear cells. The CRYM-specific antibody preabsorbed with the immunized peptide did not react in the cytoplasm of the cells (Fig 1b).

Gender differences in normal subjects

The level of expression of CRYM mRNA was lower in patients with Graves' disease than in normal controls. Table 1 summarizes the age, thyroid functions, and CRYM mRNA expression of the normal subjects and the Graves' disease patients before administration of methimazole. There were no gender differences in either age or thyroid function between the two groups. Both serum free T3 and T4 were higher in Graves' patients than in normal subjects. CRYM mRNA expression in the normal women was significantly higher than that in normal men, although there were no significant differences in CRYM mRNA expression between men and women in the Graves' patients. The level of CRYM mRNA expression of the Graves' patients was significantly lower than that of the normal subjects in either male or female.

Age-dependent inverse relation in female but not in male subjects

Figure 2a shows that the relationship between age and CRYM mRNA expression. There was a significant inverse correlation between age and the expression level of CRYM mRNA in the normal women (P=0.0451). In male subjects, an inverse correlation was observed but it was not statistically significant (P=0.729).

Thyroid function and the CRYM mRNA are negatively correlated

To clarify the relationship between thyroid function and CRYM mRNA expression, we compared the serum concentrations of free T3, free T4, TSH, and CRYM mRNA expression. As shown in Fig. 3 b-d, there was a significant positive relation between TSH and CRYM mRNA expression (P=0.0365). Although other parameters for thyroid function were not significantly related to CRYM mRNA expression, there were inverse correlations between either free T3 or free T4 and CRYM mRNA expression (*vs*. free T3 P=0.791, *vs*. free T4 P=0.269).

Spiking expression was observed during treatment

In the patients' group, there was no correlation between either free T3 or free T4 and CRYM mRNA expression at the time of the initial measurement

(*vs*. free T3 *P*=0.338, *vs*. free T4 *P*=0.405). After the initial measurement of thyroid function and CRYM mRNA expression, all patients received 15 or 20 mg methimazole per day. With the exception of case 5, a transient increase in expression of CRYM mRNA was observed within 56 days in all cases (Fig. 3). The concentrations of free T3 and free T4 decreased during the period of treatment with this agent. In case 5, the value was lower on day 70 than on day 42 after starting the anti-thyroid medication (data not shown). As shown in Fig. 4, transient increases of 1.25 to 8.18–fold in the level of expression were recognized during the treatment in all cases studied with the exception of case 5.

Discussion

In this study, we demonstrated that CRYM protein is expressed in human peripheral mononuclear cells. The expression is not homogeneously distributed in cytoplasm of some cells. As an electron microscopic study demonstrated the expression of CRYM protein in mitochondria and nucleus, CRYM protein may be present not only in cytoplasm but also in other organelles [12]. Examination of mRNA expression in 73 different human tissues showed that CRYM mRNA is expressed at high levels in brain and heart, while the level of expression is low in peripheral leukocytes [11]. As the expressions of thyroid hormone nuclear receptors are abundant in target tissues, such as brain, heart, liver and kidney, many articles demonstrate thyroid hormone action using the classical target tissues. Clinically, the effects of thyroid hormone on the hematopoietic and immune systems are not well documented. However, nuclear thyroid hormone receptor knockout studies demonstrated that thyroid hormone stimulates the maturation of hematopoietic cells and modifies immunological responses *in vivo* [13][14]. These observations indicate that thyroid hormone affects physiological function of peripheral mononuclear cells through CRYM as well as its nuclear receptors.

CRYM mRNA expression was higher in females than in males, and the level of expression was negatively correlated with age in females. This may be related to the potency of estrogen action. It has been reported that CRYM mRNA expression is reduced in ovarian follicles 6 h after follicle-stimulating hormone treatment relative to that in untreated cells of rodents [15]. In this context, the expression of thyroxine-binding protein is also positively regulated by estrogen [16]. These data suggest that estrogen may contribute to the predominant expression of thyroid hormone-binding proteins, including CRYM, in female subjects.

Free T3 and free T4 are not associated with CRYM mRNA expression. In contrast, serum concentration of TSH is positively related with the expression of CRYM mRNA. The peripheral action of thyroid hormone may reflect TSH concentration more precisely than free T3 or free T4 concentration. These observations suggest that CRYM mRNA expression is inversely related to the thyroid hormone action in normal human peripheral cells. The level of mRNA expression was significantly lower in Graves' patients than in normal subjects, suggesting that CRYM mRNA expression may be negatively regulated by thyroid hormone. One the contrary, it is reported that CRYM mRNA level increases after 48-hour incubation with T3 in human retinal WER1 cells [9]. Currently, we do not

have any clear interpretations for up- and down regulation of CRYM expression by thyroid hormone. As the incubation time for T3 in WER1 cells is obviously shorter than the time for thyrotoxicosis in Graves' patients, it may be possible that acute effect of T3 is different from chronic effect on CRYM mRNA expression. We note that CRYM mRNA expression may be affected by the autoimmune process in PBMCs of untreated Graves' disease, as Graves' disease is developed by the abnormal response in the autoimmune system [17].

Thyroid hormone concentrations decreased linearly until 56 days after the initial treatment with anti-thyroid medication, while the expression of CRYM mRNA increased transiently from day 14 to 42 after starting the medication. After the increase, the expression was suppressed until day 56 except in case 5. More data points may clarify the more precise regulation, although this study was performed based on the patients' compliance. These spiking expression patterns suggest that abrupt depletion of thyroid hormone from tissues may stimulate the increase in level of CRYM mRNA expression. Currently, it is not clear why the CRYM mRNA expression did not continue to increase during the period when the serum concentration of thyroid hormone was decreasing. Although the numbers of each cell type in PBMCs are not significantly altered during the treatment with methimazole (data not shown), autoimmune responses, which may be affected by Graves' disease itself or methimazole, may reflect the temporal expression of CRYM in PBMCs. While, some as yet unidentified factor(s) may suppress the expression after the transient increase to stabilize the level of the CRYM mRNA expression.

CRYM has been reported to regulate T3-mediated transactivation through the T3 content in cytoplasm of the living cells [4]. Knockout study showed that CRYM stores T3 and delayed T3 clearance from the tissues *in vivo* [18]. It might also be possible that alterations of the redox state and NADPH / NADP+ ratios in mononuclear cells might be altered in hyperthyroidism or after antithyroid drug treatment. These alterations also affect free thyroid hormone concentration in cytoplasm. The results of the present study demonstrated that T3 inversely relates to CRYM mRNA expression. These observations imply that peripheral thyroid hormone action may be regulated, at least in part, through the intracellular CRYM-dependent T3 capacity, which is controlled by thyroid hormone itself at the cellular level.

In conclusion, the level of CRYM mRNA expression was negatively correlated with thyroid hormone in human mononuclear cells. Gender differences were detected in the CRYM mRNA expression in normal subjects. Abrupt suppression of thyroid hormone function stimulated overexpression of CRYM mRNA. These observations suggest that inverse expression of CRYM mRNA by thyroid hormone may affect the thyroid hormone action through regulation of intracellular T3 concentration. The precise regulation of CRYM expression remains to be elucidated.

References

1. Larsen PR, Davies TF, Schlumberger M-J, Hay ID. Thyroid physiology and diagnostic evaluation of patients with thyroid disorders. In: Kronenberg HM, Melmed S, Polonsky KS, Larsen PR (eds). Williams textbook of endocrinology 11ed. Philadelphia: Saunders, 2008: 299-332

2. Visser WE, Friesema EC, Jansen J, Visser TJ. Thyroid hormone transport in and out of cells. Trends Endocrinol Metab 2008; 19: 50-56

3. Suzuki S, Mori J-I, Kobayashi M, Inagaki T, Komatsu A, Yamashita K, Takeda T, Miyamoto T, Ichikawa K, Hashizume K. Presence of functional domains in NADPH-dependent cytosolic 3,5,3'-triiodo-L-thyronine (T3)-binding protein (p38CTBP) molecule: analysis with deletion mutants. Horm Metab Res 2003; 35: 577-582

4. Mori J-I, Suzuki S, Kobayashi M, Inagaki T, Komatsu A, Takeda T, Miyamoto T, Ichikawa K, Hashizume K. Nicotinamide adenine dinucleotide phosphate-dependent cytosolic T3 binding protein as a regulator for T3-mediated transactivation. Endocrinology 2002; 143: 1538-1544

5. Abe S, Katagiri T, Saito-Hisaminato A, Usami S, Inoue Y, Tsunoda T, Nakamura Y. Identification of CRYM as a candidate responsible for nonsyndromic deafness, through cDNA microarray analysis of human cochlear and vestibular tissues. Am J Hum Genet 2003; 72: 73-82.

6. Oshima A, Suzuki S, Takumi Y, Hashizume K, Abe S, Usami S. CRYM mutations cause deafness through thyroid hormone binding properties in the fibrocytes of the cochlea. J Med Genet 2006; 43: e25.

7. Suzuki S, Mori J-I, Hashizume K. μ-crystallin, a NADPH-dependent T3-binding protein in cytosol. Trends Endocrinol Metab 2007; 18: 286-289

8. Nishii Y, Hashizume K, Ichikawa K, Takeda T, Kobayashi M, Nagasawa T, Katai M, Kobayashi H, Sakurai A. Induction of cytosolic triiodothyronine (T3) binding protein (CTBP) by T3 in primary cultured rat hepatocytes. Endocr J 1993; 40: 399-404

9. Yan L, Li F, Ding-Geng C, Samir SD. Identification of novel retinal target genes of thyroid hormone in the human WER1 cells by expression microarray analysis. Vision Res 2007; 47: 2314-2326

10. Komatsu A, Suzuki S, Inagaki T, Yamashita K, Hashizume K. A kindred with cockayne syndrome caused by multiple splicing variants of the CSA gene. Am J Med Genet 2004; 128A: 67-71

11. Suzuki S, Mori J, Kobayashi M, Inagaki T, Inaba H, Komatsu A, Yamashita K, Takeda T, Miyamoto T, Ichikawa K, Hashizume K. Cell-specific expression of NADPH-dependent cytosolic 3,5,3'-triiodo-L-thyronine-binding protein (p38CTBP). Eur J Endocrinol 2003; 148: 259-268

12. Usami S, Takumi Y, Suzuki N, Oguchi T, Oshima A, Suzuki H, Kitoh R, Abe S, Sasaki A, Matsubara A The localization of proteins encoded by CRYM, KIAA1199, UBA52, COL9A3, and COL9A1, genes highly expressed in the cochlea. Neuroscience 2008; 154: 22-28

13. Arpin C, Pihlgren M, Fraichard A, Aubert D, Samarut J, Chassande O, Marvel J. Effects of T3Rα1 and T3Rα2 gene deletion on T and B lymphocyte development. J Immunol 2000; 164: 152-160

14. Kendrick TS, Payne CJ, Epis MR, Schneider JR, Leedman PJ, Klinken SP, Ingley E. Erythroid defects in TRα -/- mice. Blood 2008; 111: 3245-3248
15. Ko C, Grieshaber NA, Ji I, Ji TH. Follicle-stimulating hormone suppresses cytosolic 3,5,3'-triiodothyronine-binding protein messenger ribonucleic acid expression in rat granulose cells. Endocrinology 2003; 144: 2360-2367
16. Bartalena L. Recent achievements in studies on thyroid hormone-binding proteins. Endocr Rev 1990; 11: 47-64
17. Liu C, Papewalis C, Domberg J, Scherbaum WA, Schott M Chemokines and autoimmune thyroid diseases. Horm Metab Res 2008; 40: 361-368
18. Suzuki S, Suzuki N, Mori J-I, Oshima A, Usami S, Hashizume K. μ-crystallin as an intracellular 3,5,3'-triiodothyronine holder *in vivo*. Mol Endocrinol 2007; 21: 885-894

Figure Legends

Fig. 1. CRYM protein is expressed in human peripheral mononuclear cells.
a. Cytospin preparation of human mononuclear cells was incubated with anti-CRYM antibody, followed by staining with TRITC-antibody. b. Cytospin preparation of human mononuclear cells was incubated with anti-CRYM antibody preabsorbed with the immunizing peptide as described previously [11]. After washing with phosphate-buffered saline, the section was stained with TRITC-antibody as a negative control.

Fig. 2. Relationship between age (a), free T3 (b), free T4 (c), TSH (d) and CRYM expression in normal male and female subjects combined.
a. Dotted and bold lines indicate best-fit lines of male and female subjects, respectively. Lines were obtained by linear regression using the least-squares method. b-d. Bold lines indicate best-fit lines of normal subjects.

Fig. 3. Serum concentrations of free T3, free T4, and CRYM expression during treatment with anti-thyroid medication in all cases.
After obtaining samples for measurement of thyroid hormones and CRYM expression, methimazole at a dose of 15 or 20 mg/day was started in all cases. Cases 1 to 10 were female, while cases 11 to 15 were male.

Fig. 4. Time course of changes in CRYM expression during the initial 42 to 56 days of treatment with methimazole.
The value at the beginning of methimazole treatment was given an arbitrary value of 1. Subsequent values were normalized relative to that obtained at the beginning of methimazole administration in each case. All of the data are superimposed on the same graph. Cases 1 to 10 were female, and cases 11 to 15 were male.

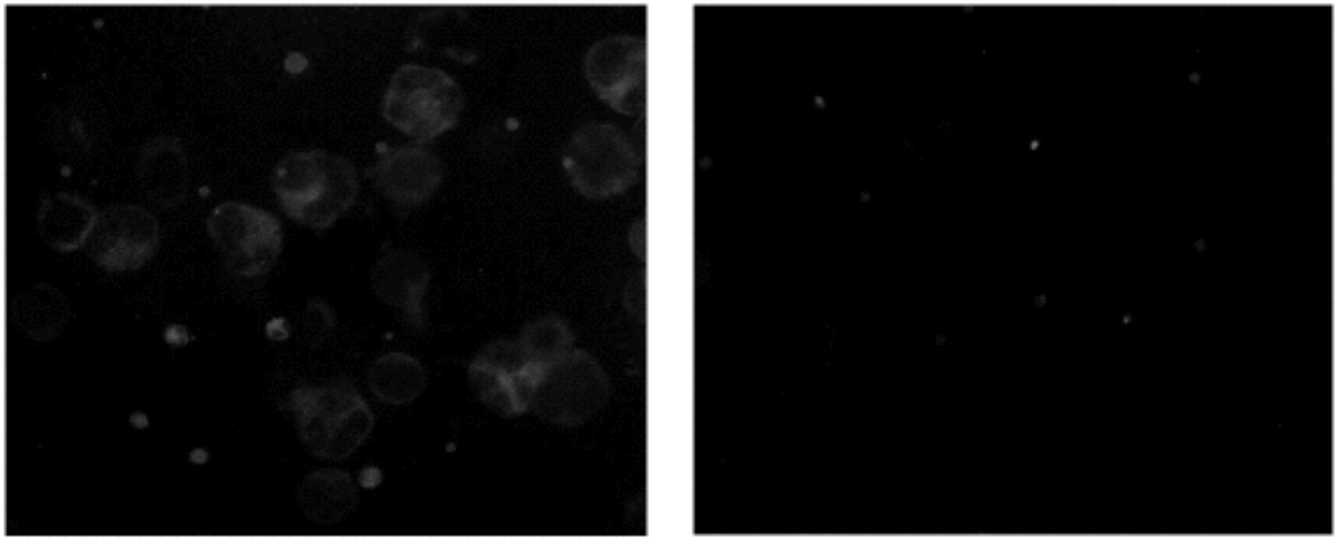
Table 1. Clinical characteristics of healthy subjects and patients with Graves' disease.

	Normal subjects		*P* value	Graves' patients[a)]		*P* value	*P* value
	male n=12	female n=13	male *vs*. female	male n=5	female n=10	male *vs*. female	Normal control *vs*. Graves' patients
Age (yr)	36.8±11.1	36.3±10.3	0.913	43.6±14.0	43±12.7	0.9025	0.100
Free T3 (pg/ml)	3.28±0.21	3.04±0.36	0.480	15.01±9.33	10.3±5.32	0.327	<0.001
Free T4 (ng/dl)	1.26±0.14	1.23±0.14	0.572	4.84±2.80	2.97±1.05	0.270	<0.001
TSH (μIU/ml)	1.05±0.30	1.57±1.01	0.550	n.d.	n.d.		
CRYM (A.U.)	0.31±0.09	0.54±0.25	0.019	0.20±0.042	0.15±0.037	0.126	male; 0.045, female; <0.001

a) Values obtained from samples prior to starting the medication.

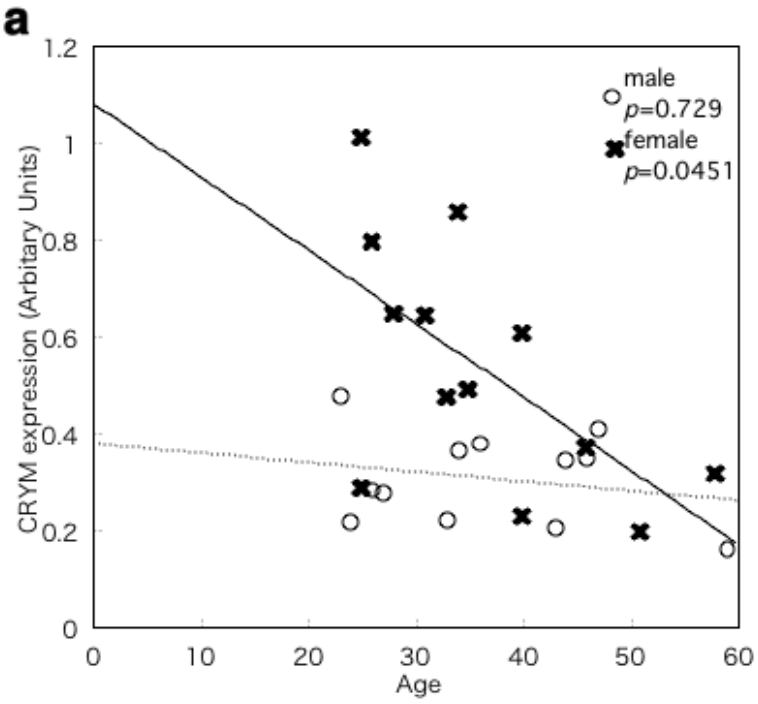
A.U. arbitrary units

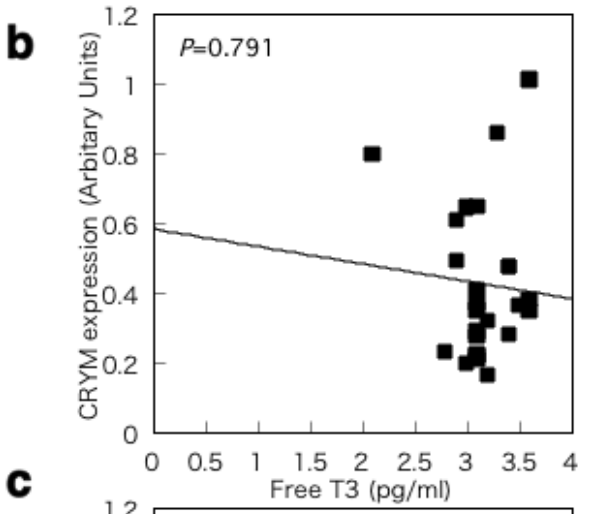
n.d. not detectable.

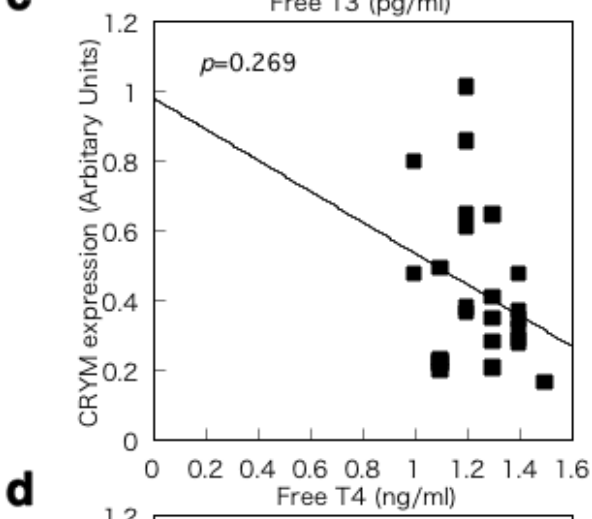


a b

Fig 1


a
CRYM expression (Arbitary Units)
Age
male
p=0.729
female
p=0.0451


b
P=0.791
CRYM expression (Arbitary Units)
Free T3 (pg/ml)


c
p=0.269
CRYM expression (Arbitary Units)
Free T4 (ng/ml)

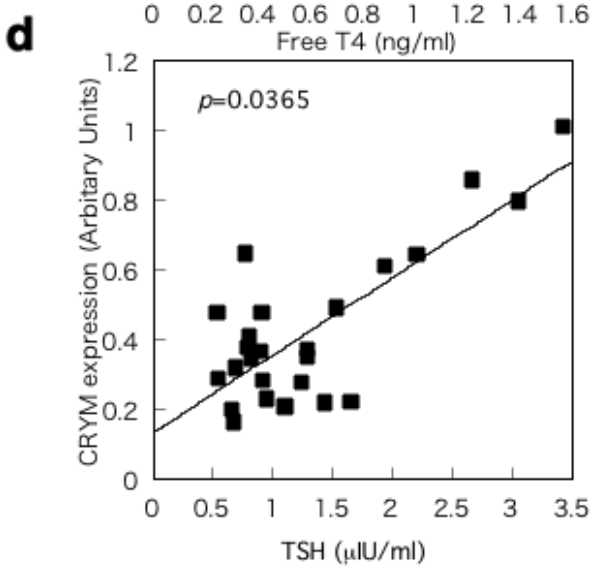

d
p=0.0365
CRYM expression (Arbitary Units)
TSH (μIU/ml)

Fig 2

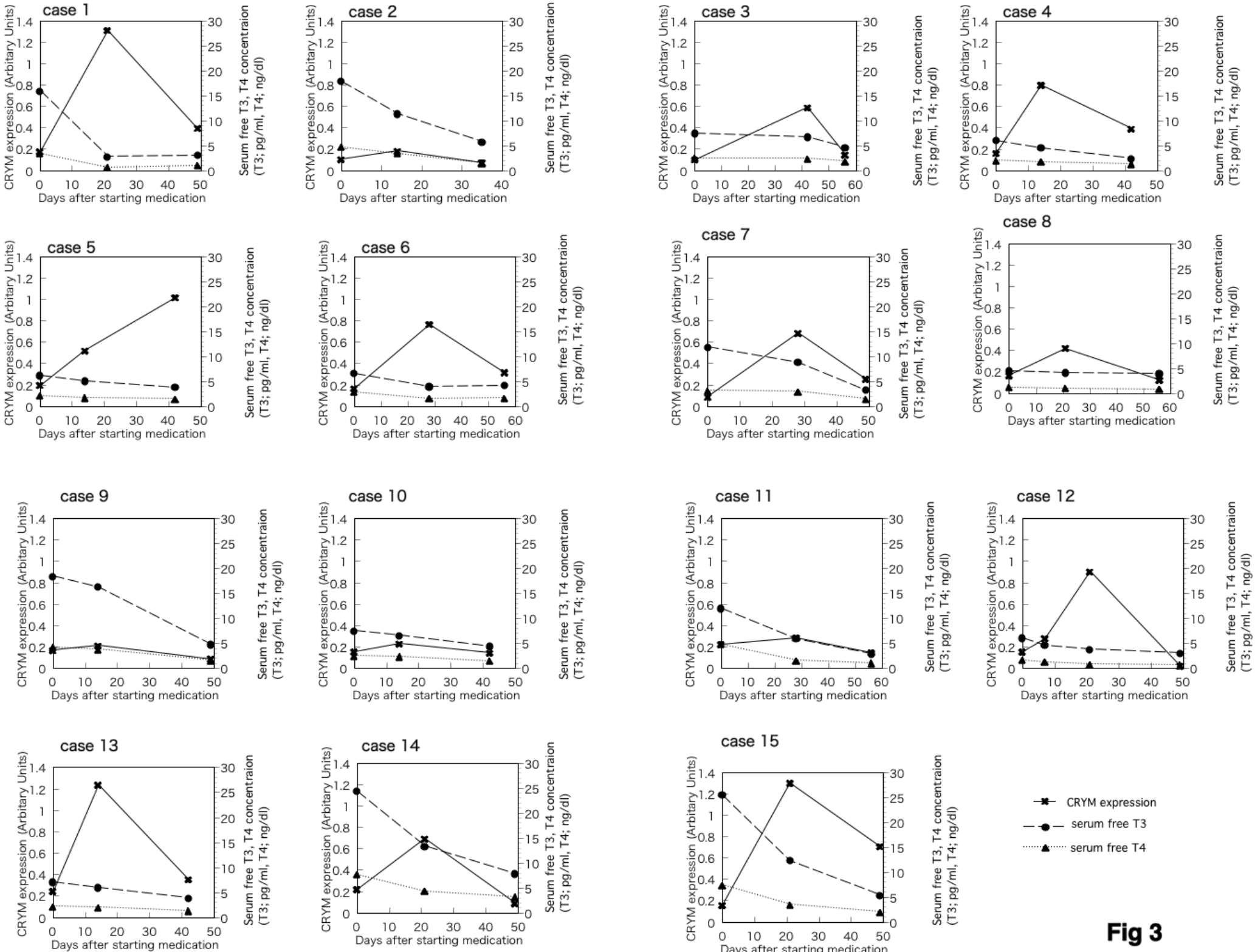

case 1
case 2
case 3
case 4
case 5
case 6
case 7
case 8
case 9
case 10
case 11
case 12
case 13
case 14
case 15
CRYM expression (Arbitary Units)
Serum free T3, T4 concentraion (T3; pg/ml, T4; ng/dl)
Days after starting medication
CRYM expression
serum free T3
serum free T4

Fig 3

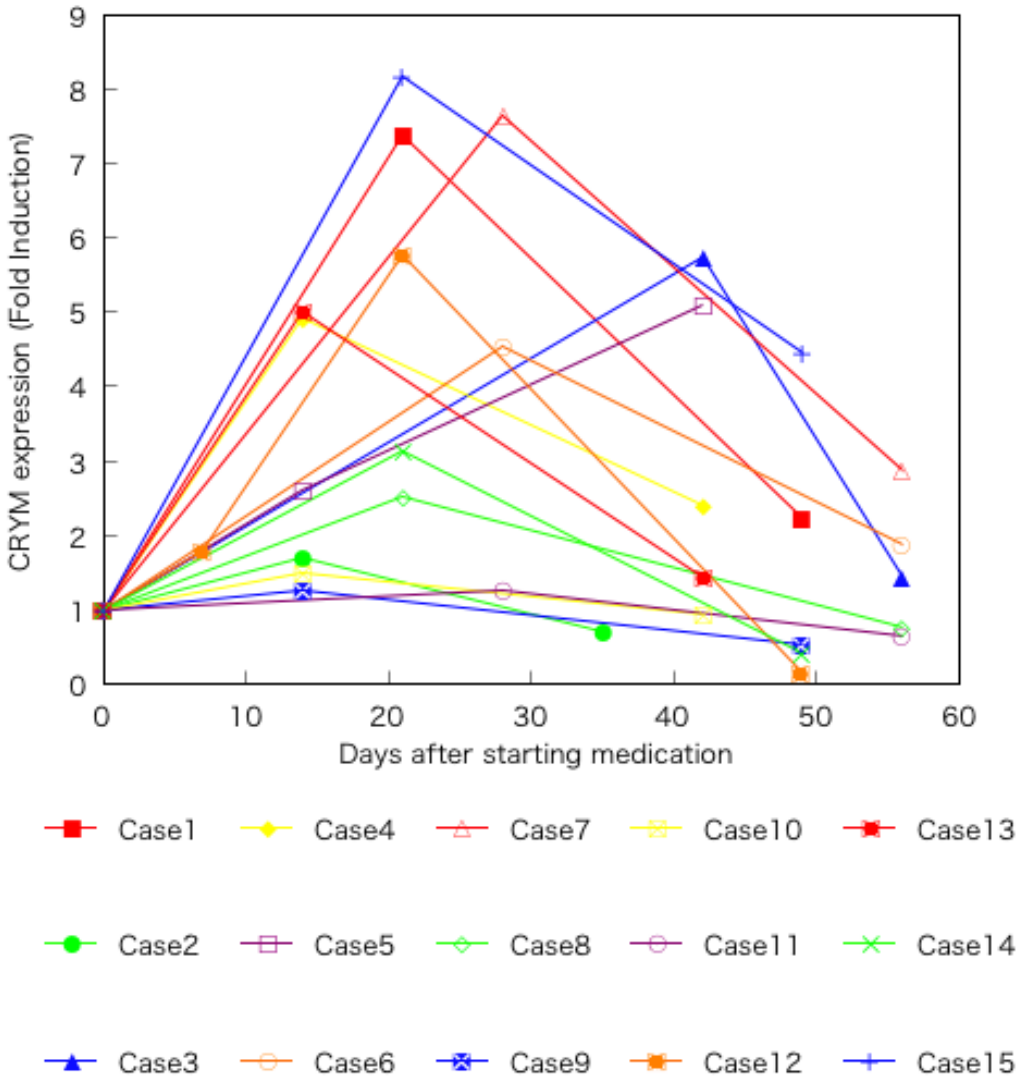

CRYM expression (Fold Induction)
Days after starting medication
0
1
2
3
4
5
6
7
8
9
0
10
20
30
40
50
60
Case1
Case4
Case7
Case10
Case13
Case2
Case5
Case8
Case11
Case14
Case3
Case6
Case9
Case12
Case15

Fig 4