CLINICAL STUDY

Genes that characterize T₃-predominant Graves' thyroid tissues

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Abstract

Objective: 3,5,3'-Triiodothyronine (T₃)-predominant Graves' disease is characterized by the increasing volume of thyroid goiter resulting in poor prognosis. Although type 1 and type 2 iodothyronine deiodinases (*DIO1* and *DIO2* respectively) are known to be overexpressed in the thyroid tissues of T₃-predominant Graves' disease, the pathogenesis of this disease is still unclear. The aim of our study is to identify genes that characterize T₃-predominant Graves' disease tissue in order to clarify the molecular mechanism of this disease.

Design and methods: mRNAs from two thyroid tissues of both typical T₃-predominant and common-type Graves' disease were analyzed with DNA microarrays with probes for 28 869 genes. Genes identified to be differentially expressed between the two groups were further analyzed in the second and third screenings using 70 Graves' thyroid tissues by real-time quantitative RT-PCR.

Results: Twenty-three candidate genes were selected as being differentially expressed in the first screening with microarrays. Among these, seven genes, leucine-rich repeat neuronal 1 (*LRRN1*), bone morphogenetic protein 8a (*BMP8A*), N-cadherin (*CDH2*), phosphodiesterase 1A (*PDE1A*), creatine kinase mitochondrial 2 (*CKMT2*), integrin beta-3 (*ITGB3*), and protein tyrosine phosphatase non-receptor type 4 (*PTPN4*), were confirmed to be differentially expressed in *DIO1* or *DIO2* over- and underexpressing Graves' tissues.

Conclusions: These genes are related to the characteristics of T₃-predominant Graves' disease, such as high titer level of serum anti-TSH receptor antibody, high free T₃ to free thyroxine ratio, and a large goiter size. They might play a role in the pathogenesis of T₃-predominant Graves' disease.

European Journal of Endocrinology 168 137–144

Introduction

Treatment with antithyroid drugs usually results in normalization of the serum thyroid hormone levels in Graves' disease, but ~12% of patients, with cases called 3,5,3'-triiodothyronine (T₃)-predominant Graves' disease, show elevated serum T₃ level after serum thyroxine (T₄) becomes normal or even low (1). Patients with T₃-predominant Graves' disease have larger thyroid glands and their sizes tend to increase rapidly, resulting in resistance to antithyroid drug treatment (2, 3, 4, 5). The prevalence of T₃-predominant Graves' disease is high in younger patients (6). Thyroid tissues of T₃-predominant Graves' disease have some typical histological features; for example, the tissue is rich in follicular cells while colloid content is low, and the height of their thyroid follicular epithelial cells is greater than that from patients with the common type of Graves' disease (7).

The source of the high serum T₃ level had not been clarified for a long time. Recently, we found that increase in type 1 and type 2 iodothyronine deiodinase

(D1/*DIO1* and D2/*DIO2* respectively) activities may be the cause of the higher serum T₃/T₄ in T₃-predominant Graves' disease (6). However, many other questions related to the pathogenesis of this disease, for example, why T₃-predominant Graves' disease is often observed in young patients or why the thyroid gland shows rapid growth, remain obscure.

In order to clarify the difference between T₃-predominant and common-type Graves' diseases at the molecular level, we analyzed the gene expression profiles in the thyroid tissues from cases of Graves' disease and identified seven genes that characterize T₃-predominant Graves' disease.

Materials and methods

Thyroid tissues

Thyroid tissues from 74 patients with Graves' disease were collected immediately after surgery at Kuma Hospital. These patients were confirmed to have Graves'

Table 1 Primers and probes used in the first, second, and third screenings.

Gene	Primers and probes
<i>DIO1</i>	5'-ACATGGACATCAGAAATCACCA-3' 5'-CAGGTCAAGGGTAAAAGCCA-3'
<i>DIO2</i>	5'-FAM-ATCCTCTACAAGGGTAAATCTGGCCCTT-TAMRA-3' 5'-TCTTCCTGGCTCTCTATGACTC-3' 5'-CGTGAAAGGAGTCAAGTGG-3'
<i>ACTB</i>	5'-FAM-TCCTCACCCAAATTCACCTGTTTGTAGGZ-TAMRA-3' 5'-TGGACATCCGCAAAGACCTG-3' 5'-TTCATGAGGCACACCTAGCC-3' 5'-FAM-CACCACCATGTACCCTGGCATTGCCGACZ-TAMRA-3'

disease by the serum anti-TSH receptor antibody (TRAb) titer level at the first visit. The patients' informed consent was obtained and the study protocol was approved by the Institutional Ethics Committee. A portion of the resected thyroid tissues was frozen in liquid nitrogen for RNA extraction. The rest of the tissues were fixed in 15% buffered formaldehyde (pH 5.8). After fixation, 5 µm sections were prepared from paraffin-embedded blocks, stained with hematoxylin and eosin, and then examined microscopically. Frozen thyroid tissues were homogenized and total RNA was extracted as described previously (8).

Thyroid function tests

Serum concentrations of TSH, free T₄ (FT₄), and free T₃ (FT₃) with reference ranges of 0.3–5.0 µIU/ml, 0.7–1.6 ng/dl, and 0.17–0.37 ng/dl respectively were measured with a chemiluminescent immunoassay (ARCHTECT i2000, Abbott Japan). TRAb titer levels were measured using an ECLusys TRAb (Roche Diagnostic Japan) with a reference range of <1.3 IU/l. The volume of the thyroid gland was measured by ultrasonography as reported previously (9).

RT

Five microliters of each extracted total RNA were reverse transcribed in an RT mixture, which consisted of 10 µl of 5× first-strand buffer (Invitrogen), 10 mmol/l dithiothreitol, 0.5 mmol/l dNTP (Takara, Shiga, Japan), 500 U Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen), 2 U/µl RNase inhibitor (Takara), and 2.5 µmol/l random hexamer (Takara) in a total volume of 50 µl. The RT reaction was performed for 10 min at 25 °C, 50 min at 37 °C, and 15 min at 70 °C.

Real-time quantitative RT-PCR with TaqMan chemistry

The expression levels of *DIO1*, *DIO2*, and *ACTB* mRNA were measured using THUNDERBIRD Probe qPCR Mix

(Toyobo Life Science, Osaka, Japan) and ABI PRISM 7900HT (Applied Biosystems) according to the manufacturers' instructions. One microliter of cDNA, 0.5 µM each primer, and 7 µM TaqMan probe were used in an assay. Primers and probes were purchased from Operon Biotechnologies (Tokyo, Japan) and are listed in Table 1. The conditions of the PCR were as follows: 95 °C for 1 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. A recombinant pGEM T-vector (Promega) containing a partial cDNA was constructed by PCR cloning with the same primer sets as used in the PCR and was used as a standard sample.

Table 2 Primers used in the second and third screenings.

Gene	Primers
<i>LRRN1</i>	5'-TCGTTTTAAACGTGGATATCGG-3' 5'-TTTCCAAGGTACCTTCGCT-3'
<i>CARTPT</i>	5'-CATCTACCGAGATTAGCTAGA-3' 5'-AATGCCTTGTATGGTGTG-3'
<i>NOQ1</i>	5'-TTCTGGAAGTCATCTCATTCC-3' 5'-TAAGAGGCTGTCTCCATT-3'
<i>BMP8A</i>	5'-CACCCACCAAATGACATCAC-3' 5'-GTGCAGCCTTGAATGCATC-3'
<i>CDH2</i>	5'-TGATGAAGAAGGTGGAGGAGA-3' 5'-TGCAGATCGGACCGGATAC-3'
<i>DALP1</i>	5'-GCCTCGAAAATGTTAAGCC-3' 5'-CCTAAAGTCAAAGGGGAAGA-3'
<i>PDE1A</i>	5'-ACATGTGCAGAAGATGAGG-3' 5'-AGTATATCCCCAGCACAAAC-3'
<i>TSPAL18</i>	5'-GCCCTTTAACAGCTTGCTC-3' 5'-CTAAGCATGTGGGTGGTTC-3'
<i>CKMT2</i>	5'-CCTGGAAAACCTAAGACTC-3' 5'-CCAGGTAATTGACTCCATC-3'
<i>C17orf57</i>	5'-AATCAGCAGCTAAGACAAC-3' 5'-TTTCTAGCTGCATTGGAAC-3'
<i>ITGB3</i>	5'-TCACCATCCACGACCGAAA-3' 5'-TTAAGTGCCCCGGTACGTG-3'
<i>PDE7B</i>	5'-GCCAAGTTGGGCAGATCAG-3' 5'-TGCTCAGCTTCTGAGTAG-3'
<i>PDE10A</i>	5'-GAAATGATGGCAAACGTC-3' 5'-CAGGAAGTCTACTTCGTCT-3'
<i>CLIC6</i>	5'-ATTGGGTCTAGCAGATGTC-3' 5'-GCACTAGTAACATCTGGTAGT-3'
<i>PTPN4</i>	5'-ATGATCCAAACACCTAGTC-3' 5'-GTTTTCCAACACATATCCC-3'
<i>RCAN2</i>	5'-GCATTGCTTGTAATACTCTG-3' 5'-ACTACTAAGTCTGTAGTCGT-3'
<i>GOT1</i>	5'-GTTGTCTGAAGGAGCCAAA-3' 5'-TCTTACCACAGTCACCCTAA-3'
<i>MMRN1</i>	5'-ACCCCTGTAAAGTGGGCATGA-3' 5'-GGGCTCTGGAGAAGACATGG-3'
<i>LYVE1</i>	5'-GCTGAGGATACGACAGTACAC-3' 5'-AGCAGTGGTAGAGACCAGA-3'
<i>CCL21</i>	5'-AGCAGGACTGTCCCCTTGA-3' 5'-GGTGGGAAGACAGAACAGCTT-3'
<i>KIAA1324</i>	5'-ATCTATCACAGCCTACGTC-3' 5'-ACCCCAATAAGTCGATCAG-3'
<i>CXCL13</i>	5'-CGGAGGAGAATTAAGTCCTAC-3' 5'-ACATGTGTACATCACCCTTT-3'
<i>FDCSP</i>	5'-GCGAAAAGTAAACAAGAAGG-3' 5'-TGACTAGAGAATGCTGTGT-3'

Table 3 Data of the patients analyzed in the first screening.

Patient	T1	T2	C1	C2
mRNA ^a				
<i>DIO1/ACTB</i>	0.196	0.158	0.0225	0.0261
<i>DIO2/ACTB</i>	0.273	0.413	0.0952	0.0376
Age (years)	47	32	35	34
Gender	Female	Female	Female	Male
TSH (μIU/ml)	<0.003	0.003	1.052	0.373
FT ₄ (ng/dl)	1.72	0.56	0.96	1.10
FT ₃ (ng/dl)	0.726	0.259	0.280	0.302
FT ₃ /FT ₄	0.422	0.462	0.291	0.274
TRAb (U/l)	17.0	97.1	<1.3	<1.3
Thyroid volume (ml)	24.0	74.9	18.56	49.4

^aData are shown as the mean of duplicate assays.

First screening with DNA microarrays

Among the 74 thyroid tissues collected, two tissues (T1 and T2) were selected as representatives of T₃-predominant Graves' tissue according to the following criteria using the method described by Takamatsu *et al.* (7): i) the tissues showed typical morphological features of T₃-predominant Graves' disease, such as being rich in follicular cells while their colloid content was low (relative area of the epithelium element was more than 20%), with their thyroid follicular epithelial cells being tall (mean height of the epithelium was more than 10 μm); ii) not being accompanied by tumor; iii) being accompanied by no or only slight lymphocyte infiltration; and iv) overexpressing both *DIO1* and *DIO2* mRNAs. Two tissues (C1 and C2) were selected as representatives of common-type Graves' tissue according to the following criteria:

i) the tissues showed typical morphological features of common-type Graves' disease, such as being poor in follicular cells while their colloid content was high (relative area of the epithelium element was <10%), with their thyroid follicular epithelial cells being short (mean height of the epithelium was <7 μm); ii) not being accompanied by tumor; iii) being accompanied by no or only slight lymphocyte infiltration; and iv) underexpressing both *DIO1* and *DIO2* mRNAs. Among these patients, T1, T2, and C1 were administered 150 mg potassium iodide, 400 mg propylthiouracil, 100 mg potassium iodide, and 50 mg propylthiouracil respectively at surgery. As for C2, TRAb was negative at surgery; however, she was diagnosed with Graves' disease with elevated TRAb at her first visit and a large goiter due to enlarged follicles was present at surgery. Thus, we regarded her as a case of transient remission of Graves' disease. These tissues were not used in the second and third screenings.

One hundred micrograms of total RNA extracted from each tissue were labeled and analyzed with GeneChip Human Gene 1.0 ST Arrays (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's protocol. Twenty eight thousand eight hundred and sixty nine genes were analyzed. Signals were quantified with Expression Console Software (Affymetrix) and subjected to quantile normalization. SDS (Z-scores) of each gene in four comparisons (C1 vs T1, C2 vs T1, C1 vs T2, and C2 vs T2) were calculated.

Table 4 Genes selected in the first screening with the microarrays.

Gene	Mean of Z-scores in the four comparisons ^a
<i>LRRN1</i>	7.49
<i>CARTPT</i>	5.75
<i>NQO1</i>	4.15
<i>BMP8A</i>	4.02
<i>CDH2</i>	3.93
<i>DAPL1</i>	3.82
<i>PDE1A</i>	3.76
<i>TSPAL18</i>	3.55
<i>CKMT2</i>	3.22
<i>C17orf57</i>	3.14
<i>ITGB3</i>	2.61
<i>PDE7B</i>	2.58
<i>PDE10A</i>	2.50
<i>CLIC6</i>	2.28
<i>PTPN4</i>	2.22
<i>RCAN2</i>	2.09
<i>GOT1</i>	2.06
<i>MMRN1</i>	-2.55
<i>LYVE1</i>	-2.92
<i>CCL21</i>	-3.45
<i>KIAA1324</i>	-3.72
<i>CXCL13</i>	-6.28
<i>FDCSP</i>	-7.44

^aA greater Z-score represents a higher gene expression level in the T₃-predominant Graves' tissues (T1 and T2).

Figure 1 Microscopic images of the tissues analyzed in the first screening with microarray (×100). C1 and C2 were poor in follicular cells while their colloid content was high, and their thyroid follicular epithelial cells were short. T1 and T2 were rich in follicular cells while their colloid content was low, and their thyroid follicular epithelial cells were tall. Slight lymphocytic infiltrations were observed in C2.

Table 5 Data of the patients analyzed in the second screening.

Group (n=4)	<i>DIO1</i> and <i>DIO2</i> high	<i>DIO1</i> and <i>DIO2</i> low	P value
mRNA ^a			
<i>DIO1/ACTB</i>	0.56 ± 0.12	0.06 ± 0.03	<0.05*
<i>DIO2/ACTB</i>	0.88 ± 0.06	0.10 ± 0.01	<0.05*
Age (years)	40.5 ± 7.59	33.5 ± 13.2	0.561
Gender (female/male)	4/0	2/2	0.181
TSH (μIU/ml)	14.7 ± 11.1	0.321 ± 0.450	0.882
FT ₄ (ng/dl)	0.833 ± 0.430	1.42 ± 0.381	0.665
FT ₃ (ng/dl)	0.557 ± 0.628	0.457 ± 0.182	0.471
FT ₃ /FT ₄	0.682 ± 0.385	0.319 ± 0.094	0.0606
TRAb (U/l)	35.8 ± 39.1	19.1 ± 13.0	0.321
Thyroid volume (ml)	163 ± 121	69.3 ± 50.0	0.47

*Statistically significant.

^aData are shown as the mean ± s.d. of duplicate assays.

Second screening

In the second screening, four samples overexpressing both *DIO1* and *DIO2* mRNAs and four samples underexpressing both *DIO1* and *DIO2* mRNAs were analyzed by quantitative RT-PCR with SYBR Green I chemistry. These samples were not used in the third screening. The expression levels of 23 mRNAs were measured using THUNDERBIRD SYBR qPCR Mix (Toyobo Life Science) and ABI PRISM 7900HT according to the manufacturers' instructions. One microliter of cDNA and 0.5 μM of each primer were used in an assay. Primers were purchased from Operon Biotechnologies and are listed in Table 2. The conditions of the PCR were as follows: 95 °C for 1 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Ct values in each tissue sample were calculated. Genes for which the expression levels in all four samples overexpressing *DIO1* and *DIO2* mRNAs were higher or lower than those in all four samples underexpressing *DIO1* and *DIO2* mRNAs were subjected to the third screening.

Third screening

Quantitative RT-PCR analysis described earlier was performed using RNAs from 62 Graves' tissues. A recombinant pGEM T-vector containing a partial cDNA was constructed by PCR cloning with the same primer sets as used in the PCR and was used as a standard sample.

Statistical analysis

Data were analyzed with StatMate Software (Atoms, Tokyo, Japan). Statistical analysis of differences between the groups was carried out using the Mann–Whitney *U* test. A *P* value <0.05 was considered significant.

Results

Data of the patients analyzed in the first screening are shown in Table 3. Morphologies of each tissue are shown in Fig. 1. In the first screening, genes showing

Z-scores > 2 or < −2 in all four comparisons (C1 vs T1, C2 vs T1, C1 vs T2, and C2 vs T2) were selected. After excluding genes with a weak microarray signal, we chose 23 genes listed in Table 4.

These genes were subjected to the second screening by quantitative RT-PCR. Data of the patients analyzed in the second screening are shown in Table 5. Among the 23 genes, nine genes showed a clear difference in expression between *DIO1* and *DIO2* over- and underexpressing groups.

The expression levels of these nine genes were measured in 62 Graves' tissues. First, data were analyzed comparing the highest 20 and lowest 20 samples in terms of *DIO1* mRNA expression. Data of the patients analyzed and gene expressions are summarized in Table 6 and Fig. 2. Significant differences were observed in terms of FT₃ to FT₄ ratio, TRAb, and thyroid volume. Seven genes, leucine-rich repeat neuronal 1 (*LRRN1*), bone morphogenetic protein 8a (*BMP8A*), N-cadherin (*CDH2*), phosphodiesterase 1A (*PDE1A*), creatine kinase mitochondrial 2 (*CKMT2*), integrin beta-3 (*ITGB3*), and protein tyrosine phosphatase non-receptor type 4 (*PTPN4*), were confirmed to show a significant difference in their expression level between *DIO* over- and underexpressing groups. Secondly, data were analyzed comparing the highest 20 and lowest 20 samples in terms of *DIO2* mRNA expression. Data of the patients analyzed and gene expressions are summarized in Table 7 and Fig. 3. Significant differences were observed in terms of FT₃ to FT₄ ratio, TRAb, and thyroid volume. The same set of seven genes that were detected in *DIO1* analysis, *LRRN1*, *BMP8A*, *CDH2*, *PDE1A*, *CKMT2*, *ITGB3*, and *PTPN4*, were confirmed to show a significant difference in their expression level

Table 6 Clinical data and gene expression in the *DIO1* mRNA high and low groups. Data are shown as the mean ± s.d. of duplicate assays.

Group (n=20)	<i>DIO1</i> mRNA high	<i>DIO1</i> mRNA low	P value
Age (years)	41.8 ± 14.0	34.7 ± 12.3	0.116
Gender (female/male)	15/5	17/3	0.596
TSH (μIU/ml)	6.05 ± 10.9	0.735 ± 1.47	<0.05*
FT ₄ (ng/dl)	0.722 ± 0.316	0.954 ± 0.294	<0.05*
FT ₃ (ng/dl)	0.466 ± 0.320	0.323 ± 0.105	<0.05*
FT ₃ /FT ₄	0.631 ± 0.173	0.353 ± 0.092	<0.01*
TRAb (U/l)	89.9 ± 76.2	42.9 ± 44.0	<0.01*
Thyroid volume (ml)	214 ± 122	73.9 ± 54.1	<0.01*
<i>DIO1</i> ^a	59.7 ± 55.3	3.70 ± 2.19	<0.01*
<i>LRRN1</i>	1.71 ± 1.45	0.627 ± 0.955	<0.05*
<i>BMP8A</i>	5.94 ± 3.57	0.714 ± 0.536	<0.01*
<i>CDH2</i>	0.465 ± 0.644	0.0478 ± 0.0577	<0.01*
<i>PDE1A</i>	4.52 ± 4.73	1.63 ± 2.02	<0.05*
<i>CKMT2</i>	0.779 ± 1.02	0.105 ± 0.126	<0.01*
<i>C17orf57</i>	75.6 ± 102	3660 ± 9490	0.14
<i>ITGB3</i>	2.41 ± 1.31	1.51 ± 1.94	<0.05*
<i>CLIC6</i>	0.842 ± 1.63	0.584 ± 1.05	0.19
<i>PTPN4</i>	0.832 ± 0.814	0.180 ± 0.188	<0.01

*Statistically significant.

^aAll gene expression levels are expressed as expression level relative to *ACTB* mRNA × 10².

Figure 2 The third screening using *DIO1* over- and under-expressing Graves' tissues. Data are shown as the mean of duplicate assays. The Y-axis represents the relative expression level to *ACTB* mRNA. H, *DIO1*-overexpressing tissues; L, *DIO1*-underexpressing tissues. **P*<0.05, ***P*<0.01.

between *DIO2* over- and underexpressing groups. One gene, *C17orf57* (*Efcab13*), showed a significant difference; however, its expression increased in *DIO1*- and *DIO2*-overexpressing samples in the first and second

Table 7 Clinical data and gene expression in the *DIO2* mRNA high and low groups. Data are shown as the mean $\pm$ s.d. of duplicate assays.

Group (n=20)	<i>DIO2</i> mRNA high	<i>DIO2</i> mRNA low	<i>P</i> value
Age (years)	42.2 $\pm$ 13.3	33.6 $\pm$ 9.33	<0.05*
Gender (female/male)	16/4	17/3	0.797
TSH (μ U/ml)	5.51 $\pm$ 10.7	0.946 $\pm$ 1.62	0.228
FT ₄ (ng/dl)	0.701 $\pm$ 0.301	0.928 $\pm$ 0.245	<0.05*
FT ₃ (ng/dl)	0.436 $\pm$ 0.307	0.328 $\pm$ 0.107	<0.05*
FT ₃ /FT ₄	0.615 $\pm$ 0.183	0.370 $\pm$ 0.130	<0.01*
TRAb (U/l)	72.9 $\pm$ 51.2	20.1 $\pm$ 19.7	<0.05*
Thyroid volume (ml)	190 $\pm$ 123	85.4 $\pm$ 48.5	<0.01*
<i>DIO2</i> ^a	135.2 $\pm$ 75.1	20.3 $\pm$ 10.3	<0.01*
<i>LRRN1</i>	1.53 $\pm$ 1.45	0.729 $\pm$ 1.03	<0.05*
<i>BMP8A</i>	3.53 $\pm$ 3.16	1.09 $\pm$ 1.23	<0.05*
<i>CDH2</i>	0.440 $\pm$ 0.649	0.0474 $\pm$ 0.054	<0.01*
<i>PDE1A</i>	4.16 $\pm$ 4.57	1.53 $\pm$ 1.95	<0.05*
<i>CKMT2</i>	0.761 $\pm$ 1.02	0.309 $\pm$ 0.880	<0.01*
<i>C17orf57</i>	120 $\pm$ 302	5100 $\pm$ 9450	<0.05*
<i>ITGB3</i>	2.27 $\pm$ 1.10	1.67 $\pm$ 2.18	<0.05*
<i>CLIC6</i>	0.841 $\pm$ 1.63	1.51 $\pm$ 4.19	0.262
<i>PTPN4</i>	0.832 $\pm$ 0.811	0.267 $\pm$ 0.334	<0.01*

*Statistically significant.

^aAll gene expression levels are expressed as expression level relative to *ACTB* mRNA $\times 10^2$.

screenings, whereas it decreased in the third screening. This phenomenon was probably caused by three samples in the third screening having low *DIO2* expression and extremely high *C17orf57* expression. In summary, in Graves' tissues overexpressing *DIO1* or *DIO2*, seven genes, *LRRN1*, *BMP8A*, *CDH2*, *PDE1A*, *CKMT2*, *ITGB3*, and *PTPN4*, were also overexpressed. Among these genes, *CDH2* was the most differentially expressed and its expression level in *DIO1*- and *DIO2*-overexpressing tissues was approximately ten times greater than that in *DIO1*- and *DIO2*-underexpressing tissues. Medication in the patients in these four groups at surgery is summarized in Table 8. In all groups, the majority of the patients administrated methimazole and/or potassium iodide.

Next, data were analyzed comparing the highest 20 and lowest 20 samples in terms of TRAb, FT₃ to FT₄ ratio, and goiter size (Tables 9, 10, and 11). All these seven genes showed a significant increase in expression level in the high TRAb and FT₃ to FT₄ ratio groups and in the large goiter group.

Discussion

In this study, we identified seven genes that were overexpressed in Graves' tissues with high *DIO1* or *DIO2* expression. The most important start point of our study was how to distinguish T₃-predominant Graves' disease

Figure 3 The third screening using *DIO2* over- and under-expressing Graves' tissues. Data are shown as the mean of duplicate assays. The Y-axis represents the expression level relative to *ACTB* mRNA. H, *DIO2*-overexpressing tissues; L, *DIO2*-underexpressing tissues. **P*<0.05, ***P*<0.01.

Table 8 Medication in the patients at surgery.

<i>DIO1</i> mRNA high group (<i>n</i> =20)	
11	Methimazole (16.4 mg) ^a + potassium iodide (72.7 mg)
6	Methimazole (17.5 mg)
1	Methimazole (30 mg) + thyroxine (25 µg)
1	Methimazole (30 mg) + potassium iodide (100 mg) + dexamethasone (4 mg)
1	Propylthiouracil (300 mg) + potassium iodide (100 mg)
<i>DIO1</i> mRNA low group (<i>n</i> =20)	
7	Methimazole (22.6 mg) + potassium iodide (67.9 mg)
4	Potassium iodide (62.5 mg)
2	Methimazole (18.8 mg)
2	Propylthiouracil (50 mg)
1	Methimazole (10 mg) + thyroxine (25 µg)
1	Methimazole (15 mg) + potassium iodide (50 mg) + thyroxine (25 µg)
1	Propylthiouracil (150 mg) + potassium iodide (100 mg)
1	Potassium iodide (100 mg) + prednisolone (10 mg)
1	No drug
<i>DIO2</i> mRNA high group (<i>n</i> =20)	
8	Methimazole (14.3 mg) + potassium iodide (68.7 mg)
7	Methimazole (18.2 mg)
2	Propylthiouracil (350 mg) + potassium iodide (100 mg)
1	Methimazole (30 mg) + thyroxine (25 µg)
1	Methimazole (30 mg) + potassium iodide (100 mg) + dexamethasone (4 mg)
1	Potassium iodide (150 mg)
<i>DIO2</i> mRNA low group (<i>n</i> =20)	
10	Methimazole (19.5 mg) + potassium iodide (87.5 mg)
2	Methimazole (27.5 mg)
2	Propylthiouracil (50 mg)
1	Methimazole (10 mg) + thyroxine (25 µg)
1	Methimazole (10 mg) + potassium iodide (50 mg) + thyroxine (50 µg)
1	Methimazole (30 mg) + potassium iodide (50 mg) + dexamethasone (4 mg)
1	Propylthiouracil (150 mg) + potassium iodide (100 mg)
1	Potassium iodide (50 mg)
1	No drug

^aThe numbers in parentheses represent the mean administered dose of each drug.

from common-type Graves' disease, as the definitive criteria to distinguish T₃-predominant Graves' disease have not been established yet. The main characteristics of T₃-predominant Graves' disease are as follows: patients are young, goiter is large, serum T₃ level is elevated compared with T₄, and TRAb is high. Thyroid tissue of T₃-predominant Graves' disease shows typical morphology and elevated D1 and D2 expression. However, after collecting tissue samples, we found that cases of T₃-predominant Graves' disease that show all these characteristics were quite rare. Thus, we had to decide which factors should be given priority.

As for the morphology, the majority of the tissues have a mixture of T₃-predominant and common-type morphology. Another problem when we tried to use morphology as a criterion was the fact that objective evaluation was quite difficult. Even though Takamatsu *et al.* (7) established a morphological criterion of T₃-predominant Graves' disease, objective differentiation was quite complicated, and as there are at least two features that characterize T₃-predominant Graves' disease, we had to establish some scoring system

for objective differentiation. For the above reasons, we decided not to use morphology to separate T₃-predominant Graves' from the common-type in the second and third screenings in which we had to deal with many cases.

FT₃ to FT₄ ratio was another candidate criterion. However, we found that FT₃ to FT₄ ratio varied during the clinical course, especially after the use of antithyroidal drugs. In Japan, most patients choose drug therapy. Usually, only patients who have some troubles with antithyroidal drugs, for example, they show poor hormone control or side effects, or their goiter is too large, undergo surgery. As a result, patients who undergo an operation tend to be administered various drugs at various doses and periods before the operation, as serum T₃ level should be kept within the normal range at surgery. Thus, it is hard to estimate the effects of such drugs on the FT₃ to FT₄ ratio in each patient.

Considering these facts, we decided to use four factors, morphology, FT₃ to FT₄ ratio, and *DIO1* and *DIO2* mRNA expression to pick up a small number of typical tissues for the first screening. We then used *DIO1* and *DIO2* mRNA expression in the second and third screenings in order to categorize thyroid tissues simply and objectively. However, in this study, patients with a *DIO1*- or *DIO2*-overexpressing thyroid showed typical clinical features of T₃-predominant Graves' disease, such as high FT₃ to FT₄ ratio, high TRAb, and a large goiter size, in the third screening. Furthermore, all seven genes showed a significant increase in their expression level in the high TRAb and FT₃ to FT₄ ratio groups and in the large goiter group. Thus, we could conclude that these genes also characterize T₃-predominant Graves' tissues. However, there are some limitations in this study. First, we have not validated the protein expression of the detected genes. Secondly, almost all patients analyzed received medication at surgery and it was not clear to what extent such medication altered gene expression in the thyroid.

Although the prevalence of T₃-predominant Graves' disease was reported to be high in younger patients in the previous studies (2, 6), the mean age of patients with a *DIO1*- or *DIO2*-overexpressing thyroid was

Table 9 Gene expression in the high and low TRAb groups. Data are shown as the mean ± s.d. of duplicate assays.

Group (<i>n</i> =20)	High TRAb	Low TRAb	<i>P</i> value
TRAb (U/l)	202.2 ± 128.0	5.7 ± 3.6	<0.01*
<i>LRRN1</i> ^a	1.43 ± 1.21	0.71 ± 0.10	<0.01*
<i>BMP8A</i>	3.54 ± 2.93	1.30 ± 2.05	<0.01*
<i>CDH2</i>	0.335 ± 0.493	0.132 ± 0.330	<0.01*
<i>PDE1A</i>	3.29 ± 4.10	1.83 ± 2.33	<0.01*
<i>CKMT2</i>	0.391 ± 0.348	0.625 ± 1.319	<0.01*
<i>ITGB3</i>	2.73 ± 1.08	1.60 ± 2.13	<0.01*
<i>PTPN4</i>	0.562 ± 0.726	0.413 ± 0.568	<0.01*

*Statistically significant.

^aAll gene expression levels are expressed as expression level relative to *ACTB* mRNA × 10².

Table 10 Gene expression in the high and low FT₃ to FT₄ ratio groups. Data are shown as the mean $\pm$ s.d. of duplicate assays.

Group (n=20)	High FT ₃ to FT ₄ ratio	Low FT ₃ to FT ₄ ratio	P value
FT ₃ /FT ₄	0.711 $\pm$ 0.120	0.283 $\pm$ 0.035	<0.01*
<i>LRRN1</i> ^a	1.53 $\pm$ 1.27	0.54 $\pm$ 0.06	<0.01*
<i>BMP8A</i>	3.71 $\pm$ 2.96	1.31 $\pm$ 2.35	<0.01*
<i>CDH2</i>	0.456 $\pm$ 0.645	0.136 $\pm$ 0.340	<0.01*
<i>PDE1A</i>	3.27 $\pm$ 4.44	1.62 $\pm$ 2.35	<0.01*
<i>CKMT2</i>	0.432 $\pm$ 0.402	0.358 $\pm$ 0.879	<0.01*
<i>ITGB3</i>	2.44 $\pm$ 1.09	1.65 $\pm$ 2.10	<0.01*
<i>PTPN4</i>	0.624 $\pm$ 0.757	0.288 $\pm$ 0.322	<0.01*

*Statistically significant.

^aAll gene expression levels are expressed as expression level relative to *ACTB* mRNA $\times 10^2$.

higher than that of those with a *DIO1*- or *DIO2*-underexpressing thyroid. Probably, this was caused by some social factors, as a considerable number of young patients with a small goiter prefer to undergo surgery to achieve early remission before marriage or employment.

LRRN1 regulates the subcellular localization of specific components of signaling or cell-to-cell recognition pathways (10). *BMP8A* plays a role in calcium regulation and bone homeostasis (11). *CDH2* is a classical cadherin from the cadherin superfamily. The encoded protein is a calcium-dependent cell-to-cell adhesion glycoprotein composed of five extracellular cadherin repeats, a transmembrane region, and highly conserved cytoplasmic tail (12). *PDE1A* plays a role in signal transduction by regulating intracellular cyclic nucleotide concentrations through hydrolysis of cAMP and/or cGMP (13). *CKMT2* is responsible for the transfer of high-energy phosphate from mitochondria to the cytosolic carrier, creatine (14). *ITGB3* is a receptor for cytotactin, fibronectin, laminin, matrix metalloproteinase-2, osteopontin, osteomodulin, prothrombin, thrombospondin, vitronectin, and von Willibrand factor (15). *PTPN4* acts at junctions between the membrane and the cytoskeleton (16).

There is little information on the relationship between these genes and thyroid cell function. In a papillary thyroid carcinoma cell line, TPC-1, *ITGB3* expression was found to correlate with *RET*-mediated invasion in a mouse tumor xenograft model, suggesting that *ITGB3* contributes to cell adhesion and migration downstream of *RET* (17). It is notable that four of seven genes, *LRRN1*, *CDH2*, *ITGB3*, and *PTPN4*, are associated with cellular adhesion. Even though their exact functions in thyroid follicular cells are not clear, they might contribute to the morphological changes in the thyroid tissue of T₃-predominant Graves' disease.

The pathogenesis of T₃-predominant Graves' disease is not fully understood. High titer of TRAb followed by intense TSH stimulation has been believed to be the cause of T₃-predominant Graves' disease. In fact, in this study, a significant increase in TRAb was observed in both *DIO1* and *DIO2* mRNA-overexpressing groups and

all seven genes showed increased expression in the TRAb high group. However, this hypothesis does not fully explain high D1 and D2 expression, a high prevalence in young patients and typical morphological changes in T₃-predominant Graves' disease.

Our data showing increased expression of *CDH2* mRNA in the T₃-predominant Graves' tissues may lead to another explanation. Some similarities between thyroid follicular tumors and T₃-predominant Graves' disease should be noted. Follicular tumors often express a high level of *DIO1* or *DIO2* and thyroid autonomous adenomas overexpress not only *DIO1* but also *CDH2* (18, 19, 20). In recent years, a novel hypothesis named fetal cell carcinogenesis, in which thyroid tumor cells are derived from the remnants of fetal thyroid cells, has attracted increasing attention (21, 22). In this hypothesis, three types of fetal thyroid cell are thought to provoke cancer by generating thyroid cancer cells: anaplastic and papillary carcinomas are derived from thyroid stem cells and thyroblasts respectively and follicular tumors are derived from prothyrocytes. An interesting feature that arises from this hypothesis is that not only thyrocytes but also fetal thyroid cells, probably prothyrocytes that are considered to be the origins of follicular thyroid tumors, are likely to remain in the thyroid of the young. Such fetal thyroid cells are not only the origin of follicular tumors but also control the volume of the thyroid gland by proliferating into thyrocytes. If we hypothesize that T₃-predominant Graves' disease is caused by an increased number of differentiated fetal thyroid cells remaining in the young that can be origins of follicular tumors, we can easily answer the following three questions about T₃-predominant Graves' disease: why its prevalence is high in younger patients, why it shows a large goiter, and why its tissue overexpresses D1 and D2, as such fetal thyroid cells overexpress D1 and D2 and possess high proliferative ability.

The existence of immature thyroid cells in the thyroid gland has long been discussed without any definitive evidence. This is partly because no effective markers have been known to identify such fetal thyroid cells. If the above hypothesis is true, the seven genes identified in this study might be used not only in understanding

Table 11 Gene expression in the large and small goiter groups. Data are shown as the mean $\pm$ s.d. of duplicate assays.

Group (n=20)	Large goiter	Small goiter	P value
Thyroid volume (ml)	261.5 $\pm$ 107.3	36.8 $\pm$ 14.6	<0.01*
<i>LRRN1</i> ^a	1.45 $\pm$ 1.23	0.62 $\pm$ 0.93	<0.01*
<i>BMP8A</i>	3.22 $\pm$ 3.02	1.08 $\pm$ 1.78	<0.01*
<i>CDH2</i>	0.456 $\pm$ 0.644	0.122 $\pm$ 0.332	<0.01*
<i>PDE1A</i>	3.49 $\pm$ 4.39	1.75 $\pm$ 2.34	<0.01*
<i>CKMT2</i>	0.420 $\pm$ 0.397	0.617 $\pm$ 1.31	<0.01*
<i>ITGB3</i>	2.28 $\pm$ 1.03	1.59 $\pm$ 1.99	<0.01*
<i>PTPN4</i>	0.652 $\pm$ 0.766	0.396 $\pm$ 0.566	<0.01*

*Statistically significant.

^aAll gene expression levels are expressed as expression level relative to *ACTB* mRNA $\times 10^2$.

the pathological mechanism of T₃-predominant Graves' disease but also in the detection of fetal thyroid cells.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This research was supported by the Ministry of Education, Culture, Sports, Science, and Technology of Japan by a grant-in-aid for scientific research C, 2011–2013, no.23590670; a Research Grant from the Princess Takamatsu Cancer Research Fund 04-23606.

Acknowledgements

The authors thank Dr Mitsuyoshi Hirokawa for his help in the pathological examination.

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Received 11 June 2012

Revised version received 24 October 2012

Accepted 29 October 2012