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Differential expression of genes related to drug responsiveness between sparsely and densely granulated somatotroph adenomas

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Abstract. There are two main subtypes of GH-producing pituitary adenoma: densely granulated (DG-type) and sparsely granulated (SG-type). Despite the difference in drug responsiveness between the two subtypes, their molecular mechanisms remain unknown. The aim of this study is to evaluate the differential expression of genes related to drug responsiveness between the two subtypes of somatotroph adenoma, and their relationship to the clinical characteristics. Eighty-two acromegaly patients (44 DG-type, 38 SG-type) were studied retrospectively. Clinical characteristics were compared between the two subtypes. Among them, 36 tumor tissue specimens (19 DG-type, 17 SG-type) were available for investigation of the expression of *SSTR2*, *SSTR5* and *D2R* that are reported to be involved in drug responsiveness by real-time RT-PCR. Protein level was evaluated by immunohistochemical study. Patients with SG-type adenomas were younger in age and showed greater GH suppression by octreotide, but not by bromocriptin, and bigger in size and more invasiveness than DG-type adenomas. The mRNA expression of *SSTR2* in DG-type adenomas were greater than those in SG-type adenomas and showed significantly positive correlation with GH suppression by octreotide. There was positive correlation between mRNA and protein levels of *SSTR2*. These data suggested that the differences of responsiveness to octreotide between DG- and SG-type adenomas are based on the expression levels of *SSTR2*.

Key words: Growth hormone-secreting pituitary adenoma, Real-time RT-PCR, *SSTR2*, Drug-responsiveness

ACROMEGALY is caused by excess production of growth hormone (GH) by a GH-secreting pituitary tumor in more than 98% of cases [1]. Although the majority of GH-producing pituitary adenomas are benign, excess amount of long-standing uncontrolled GH and IGF-I increases mortality due to cardiovascular, cerebrovascular and respiratory diseases.

GH-producing adenomas are classified into five subtypes by ultrastructural and histopathological features

based on WHO classification, among which “pure” GH cell adenomas are divided into two distinct subtypes ultrastructurally: densely granulated somatotroph (DG-type) adenomas with large and dense secretory granules in the cytoplasm and sparsely granulated somatotroph (SG-type) adenomas with small and sparse granules.

One of the typical microscopic features of SG-type adenomas is inclusion body called “fibrous body” composed of aggregated cytokeratin filaments. By immunostaining with anti-cytokeratin antibody (CAM5.2), SG-type adenomas show a dot-like distribution pattern in the cells, whereas DG-type adenomas show a perinuclear (ring-like) pattern in the cells.

It has been reported that SG-type adenomas occur more frequently at younger age, are less responsive to

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#Dr. Toshiaki Sano sadly passed away during the completion of this manuscript.

thyrotropin-releasing hormone (TRH) and growth hormone-releasing hormone (GRH), are more resistant to octreotide or bromocriptin, and are larger in size and more invasive than DG-type adenomas [2-4]. Despite the differences of morphological, clinical and hormonal features between these subtypes, the molecular mechanisms underlying these features remain unknown.

To clarify the mechanisms of differences in drug responsiveness between the two subtypes, we investigated the differential expression profiles of genes related to drug responsiveness such as *SSTR2*, *SSTR5* and *D2R* between the two subtypes.

Patients and Methods

Patients

Surgically removed pituitary adenomas from acromegaly patients who admitted to Toranomon Hospital were used. This study was approved by the institutional ethical review committee, and informed consent was obtained from each patient before surgery. All patients were diagnosed as acromegaly based on the clinical manifestation, increased serum GH and IGF-1 levels with lack of serum GH suppression after oral glucose tolerance test, and the presence of pituitary tumors demonstrated by diagnostic magnetic resonance image. "Pure" GH cell adenomas were confirmed by immunohistochemical study after excluded adenomas containing 5% or more prolactin (PRL) positive cells. Pure GH cell adenomas were classified into the two subtypes (DG-type or SG-type) based on cytokeratin distribution pattern in the cytoplasm, as reported [4]. Briefly, the adenoma composed of over 70% perinuclear pattern cells or less than 8% dot pattern cells was categorized as "DG-type." The adenoma composed of over 70% dot pattern cells was categorized as "SG-type." The other adenomas, which did not fall into either of these two categories, were categorized as "intermediate type."

Endocrinological and imaging studies

Basal plasma GH and IGF-1 levels represent the mean $\pm$ SEM of at least three separated samples obtained in the morning after overnight fasting. The size and cavernous sinus invasion of tumors were determined by the neuroimaging. Tumors that were larger than 10 mm in diameter were categorized into macroadenoma, and tumors that were equal to or smaller than 10 mm in diameter were categorized into microadenoma. For

the evaluation of the drug responsiveness, 100 μ g octreotide (Sandostatin[®], Sandoz, Switzerland) or 2.5 mg bromocriptin (Parodel[®], Novartis, Switzerland) was administered subcutaneously or orally; the percentage of GH suppression after administration of octreotide or bromocriptin was calculated.

Analysis of tissue specimens

Tissue specimens obtained during transsphenoidal surgery from each pituitary tumor were used. A portion of the tissue specimens was immediately frozen with RNAlater[®] (Ambion Inc., Austin, TX, USA) and stored at -80°C for quantification of mRNA. The remaining tissue specimens were subjected to histological and immunohistochemical studies.

Quantification of mRNA

Total RNA was extracted from pituitary tumor using QIAzol (Qiagen, Chatsworth, CA) and 5 μ g of total RNA was reverse transcribed with a first-strand cDNA synthesis kit (GE Healthcare, Chalfont, Buckinghamshire, UK), as previously reported [5]. The samples that resulted in lower quality of RNA and/or cDNA were eliminated from the study. Thirty-six samples (19 DG, 17 SG-type adenomas) were used for subsequent evaluation of gene expression. mRNA expression of genes were quantified by TaqMan fluorescence methods using a QuantiTect Probe PCR kit (Qiagen) and a Chromo4[™] Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Japan). Universal Probe Library system (Roche, Nutley, New Jersey, USA) combined with TaqMan technology, which prevents generation of non-specific amplification products, were used to quantify the expression of the following genes: somatostatin receptor subtype 2 and 5 (*SSTR2*, *SSTR5*), Dopamine D2 receptor (*D2R*) and glyceraldehyde-3-phosphate and dehydrogenase (*GAPDH*). After reverse transcription, the reaction mixtures were denatured at 95°C for 3min followed by 40 cycles of PCR at 95°C for 30s, 58°C for 30s, 72°C for 30s. The PCR primers of the genes were synthesized by Griner bio-one (Tokyo, Japan) and their sequences were shown in Table 1. Fluorescence data were quantitatively analyzed by including serial dilutions of control samples in each reaction to produce a standard curve. To compare the relative expression of each gene, *GAPDH* was used as an endogenous internal control and the relative expression of each mRNA to that of *GAPDH* were calculated.

Table 1 PCR primers for real-time RT-PCR

Genes	probes	sequences	PCR product size (bp)
<i>SSTR2</i>	#47	Forward: attgcagcggaaaagcaa Reverse: cagccagcccagagatctta	85
<i>SSTR5</i>	#55	Forward: cgccggcctctactctt Reverse: gaaccttctggaagctctgg	110
<i>D2R</i>	#17	Forward: aacaggcggagagtgtg Reverse: ctgtaccgggtcctctcg	98
<i>GAPDH</i>	#60	Forward: gctctctgctctctctgttc Reverse: acgaccaaaccgttgactc	115

Immunohistochemical study

Immunohistochemical study was performed by the avidin-biotin- peroxidase complex (ABC) technique as reported. The specific antibodies used in this study were a monoclonal antibody to cytokeratin (CAM5.2, Becton-Dickinson, Mountain View, USA), human GH (Dakopatts, Glostrup, Denmark), PRL (Immuno Tech, France) and *SSTR2* (SS-800, Gramsch Lab. Germany). Hematoxylin was used as counterstain. The percentage of cell staining of *SSTR2* was evaluated semi-quantitatively as follows; (–) negative staining, (1+) <20%, (2+) 20-50%, (3+) >50%, respectively. The resulting sections were examined blind, without any prior knowledge of subtype by single observer.

Statistical analyses

Data were shown as mean $\pm$ SEMs. Differences

between groups were examined for statistical significance using Kruskal-Wallis test with Dunn's post hoc test. Their relations between two continuous variables were evaluated using Spearman's rank correlation coefficient. *P* values < 0.05 were considered statistically significant. All statistical analyses were performed using Windows software Prism 5.0 (GraphPad Software, CA, USA).

Results

Patient's profiles

Pure GH cell adenomas resected from 82 patients were categorized 44 (23 males and 21 females, aged 48.6 ± 2.0 years) as DG-type and 38 (17 male and 21 females, aged 40.8 ± 1.8 years) as SG-type. As shown in Table 2, patients with SG-type adenoma were significantly younger in age than DG-type adenoma. There were no significant differences in gender between the two subtypes of adenomas.

The total number of available data on tumor size and cavernous sinus invasion were 74 (38 DG-type, 36 SG-type) and 76 (41 DG-type, 35 SG-type), respectively. Twenty-seven out of 38 DG-type adenomas (71.1%) and 34 out of 36 SG-type adenomas (94.4%) were macroadenomas. Fourteen out of 41 DG-type adenomas (34.1%) and 20 out of 35 SG-type adenomas (57.1%) represented cavernous sinus invasion. These results showed that SG-type adenomas were significantly bigger and more invasive than DG-type adenomas.

Table 2 Clinical, endocrinological and neuroimaging characteristics of DG-type and SG-type adenomas

	DG	(N ^a)	SG	(N ^a)	<i>p</i>
Age	48.6 $\pm$ 2.0	(44)	40.8 $\pm$ 1.8	(38)	< 0.01
Gender					0.51
Male	52.3%	(23)	44.7%	(17)	
Female	47.7%	(21)	55.3%	(21)	
Tumor size					< 0.05
Macroadenoma	71.1%	(27)	94.4%	(34)	
Microadenoma	28.9%	(11)	55.6%	(2)	
Cavernous sinus invasion					< 0.05
+	34.1%	(14)	57.1%	(20)	
-	65.8%	(27)	42.9%	(15)	
Basal GH (ng/mL)	55.5 $\pm$ 20.2	(44)	76.2 $\pm$ 33.6	(38)	0.74
IGF-1 (ng/mL)	830.4 $\pm$ 41.9	(44)	857.8 $\pm$ 59.5	(38)	0.82
%GH suppression by octreotide	87.9 $\pm$ 3.3	(29)	68.2 $\pm$ 3.5	(28)	< 0.0001
%GH suppression by bromocriptin	58.2 $\pm$ 5.0	(27)	53.6 $\pm$ 4.6	(29)	0.54

a, available data

Fig. 1 % GH suppression by medical therapy in two subtypes of GH-producing adenoma

% GH suppression by (a) octreotide (100 µg) and (b) bromocriptin (25 mg) in DG (●) and SG (○) types of adenomas are shown.

*** $p < 0.001$

Fig. 2 Expression profiles of genes related to drug responsiveness

Relative mRNA expression of (a) *SSTR2*, (b) *SSTR5* and (c) *D2R* are shown: DG (●) and SG (○). * $P < 0.05$, ** $P < 0.01$

There were no significant differences of basal GH and IGF-1 levels between the two subtypes. The total number of available data on GH suppression by octreotide and bromocriptin were 57 (29 DG-type, 28 SG-type) and 56 (27 DG-type, 29 SG-type), respectively. GH suppression by octreotide, but not by bromocriptin in DG-type adenoma was significantly greater than that in SG-type adenoma (Table 2, Fig. 1).

Expression profiles of genes related to drug responsiveness

Among tumor specimens from the above 82 patients, 36 samples (19 DG-, 17 SG-type adenomas) were used for subsequent analysis of gene expression. We evaluated the expression of genes related to drug responsiveness including *SSTR2*, *SSTR5* and *D2R* (Fig. 2). *SSTR2* mRNA expression in DG-type adenomas were about

2.6-fold greater than those in SG-type adenomas ($p < 0.05$). Whereas *SSTR5* mRNA expression did not differ between the two subtypes ($p = 0.44$). *D2R* mRNA expression in DG-type adenomas were about 2.0-fold ($p < 0.005$) lower than in SG-type adenomas.

Correlation between gene expression and drug responsiveness

Percentage of GH suppression by octreotide showed a significant positive correlation with *SSTR2* mRNA expression ($r = 0.54$, $P < 0.005$) (Fig. 3) but not with *SSTR5* (data not shown). There was no significant correlation between percentage of GH suppression by bromocriptin and *D2R* mRNA expression ($r = -0.32$, $P = 0.31$).

Immunohistochemistry and protein level

Among the 36 tumor specimens used for gene expression analysis, 34 were available for following immunohistochemical study (18 DG-type, 16 SG-type). Typical staining pattern of cytokeratin and SSTR2 in DG- and SG-type adenomas were shown in Fig. 4. As shown in Table 3, positive immunostaining cells for SSTR2 in DG-type was observed in 16 out of 18 (89%) in DG-type and 2 out of 16 (13%) in SG-type. Based on the immunohistochemical semi-quantitative grading score (Table 3), relative mRNA expression in (2+) group was significantly ($P < 0.05$) greater than that in (-) group (Fig. 5).

Discussion

The present study showed the differences of clinical and hormonal features between DG- and SG-type somatotroph adenomas, and further revealed for the first time the differential expression of genes related to drug responsiveness between the two subtypes.

Clinical characteristics demonstrated in the present study are consistent with the previous reports, showing that SG-type adenomas were more frequent in younger age, larger, more invasive and less responsive to octreotide than DG-type [2, 4], although responsiveness to bromocriptin between the two subtypes were com-

Fig. 3 Correlation between *SSTR2* gene expression and %GH suppression by octreotide
Univariate positive correlation between *SSTR2* mRNA expression and %GH suppression by octreotide are shown: DG (●) and SG (○).

Table 3 *SSTR2* level as evaluated by immunohistochemical study

Subtype (Number of cases)	Protein level			
	(-)	1+	2+	3+
DG (18)	2	5	11	0
SG (16)	14	2	0	0

The percentage of cells staining *sstr2A* was evaluated as follows: (-) negative staining, (+) <20%, (2+) 20-50%, (3+) >50%.

DG: densely granulated somatotroph adenoma, SG: sparsely granulated somatotroph adenoma

Fig. 4 Immunohistochemical analysis in somatotroph adenomas
Typical immunostainings for cytokeratin (CAM5.2) or SSTR2 are shown. The arrows indicated fibrous bodies.

Fig. 5 Correlations of gene expression and protein level of SSTR2 in DG- and SG-type adenomas. Correlation between mRNA expression by real-time quantitative RT-PCR and protein level by immunohistochemical semi-quantitative grading score are shown for SSTR2: DG (●) and SG (○). * $P < 0.05$

parable.

The primary treatment of acromegaly is surgical excision of the somatotroph adenomas by transsphenoidal approach. However, medical treatment of acromegaly with somatostatin analogues (SSA), such as octreotide, is first-line therapy for patients who cannot tolerate surgery or who have residual tumor after surgery. Although octreotide has antisecretory and antiproliferative effects on the somatotroph adenomas, only 50–70% of patients achieve biochemical control by this treatment [6, 7]. The response to octreotide depends on the expression of SSTR2 on the tumor cells, because octreotide binds with higher affinity to SSTR2 among several SSTR subtypes [8–12]. Among five subtypes of SSTR, SSTR2 and SSTR5 are the most dominant isoforms expressed in pituitary adenomas [12–14].

Several studies have shown a positive correlation between GH suppression by octreotide and SSTR2 and/or SSTR5 expression in GH-secreting adenomas [15–19], however to date no studies on differences of SSTR2 and SSTR5 expression between DG- and SG-type adenomas by using quantitative real-time RT-PCR has been reported. The present study revealed for the first time a significant greater SSTR2, but not SSTR5 expression in DG-type adenomas than SG-type adenomas. Furthermore, a positive correlation between the SSTR2 expression and octreotide responsiveness was observed. These results suggest that the different

responsiveness to octreotide between DG- and SG-type adenomas are related to the amount of SSTR2 rather than SSTR5, expressed on the cells of each subtype somatotroph adenomas. Pasireotide (SOM230), a new SSA with higher affinity to SSTR5, than to SSTR2, might be more effective medical treatment for SG-type adenomas than DG-type adenomas.

Although dopamine agonists (DAs) were less effective than SSAs in lowering GH and IGF-I in treatment of somatotrophinomas, DAs can be orally administered at lower costs than SSAs. It has been reported that the suppression of GH/PRL by D2R agonists was correlated to D2R gene expression in lactotroph and somatotroph tumors *in vitro* [20, 21]. In the present study, however, no significant differences were detected in responsiveness to bromocriptin between the two subtypes, despite the greater expression of D2R in SG type than DG-type adenomas. Recently, it was reported that heterodimerization of D2R/SSTR2 was associated with a reciprocal modification of ligand binding and increased internalization of SSTR2 [22, 23]. Thus, it is possible that high expression of D2R promotes desensitization of SSTR2 and D2R and octreotide responsiveness on SG-type adenomas.

In conclusion, the present study strongly suggests that SSTR2 expression is involved in determine difference in responsiveness to octreotide between the two distinct subtypes (DG- and SG-) of “pure” somatotroph adenomas. Further studies are required to clarify the whole picture of molecular mechanism underlying the clinical differences between DG- and SG-type adenomas.

Disclosure

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.

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