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
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 ± 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 µg octreotide (Sandostatin®, Sandoz, Switzerland) or 2.5 mg bromocriptin (Parodel®, Novartis, Switzerland) was administrated 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 µg 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.
Gene expressions in somatotroph adenomas 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 Spearmann’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>
<thead>
<tr>
<th>Table 1</th>
<th>PCR primers for real-time RT-PCR</th>
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<tbody>
<tr>
<td>Genes</td>
<td>probes sequences</td>
</tr>
<tr>
<td>SSTR2 #47</td>
<td>Forward: attgcagcggaaaagea</td>
</tr>
<tr>
<td></td>
<td>Reverse: eagccagecagagateta</td>
</tr>
<tr>
<td>SSTR5 #55</td>
<td>Forward: gcegggctaacctctt</td>
</tr>
<tr>
<td></td>
<td>Reverse: gaaccttgtggagctggό</td>
</tr>
<tr>
<td>D2R #17</td>
<td>Forward: aaacagggcagaggtgtg</td>
</tr>
<tr>
<td></td>
<td>Reverse: egtaacctgcctctcteg</td>
</tr>
<tr>
<td>GAPDH #60</td>
<td>Forward: gcetctgtcctctggtc</td>
</tr>
<tr>
<td></td>
<td>Reverse: agcacaatcegtgtgact</td>
</tr>
</tbody>
</table>

**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 ± 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 Spearmann’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).

<table>
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<tr>
<th>Table 2</th>
<th>Clinical, endocrinological and neuroimaging characteristics of DG-type and SG-type adenomas</th>
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<tbody>
<tr>
<td></td>
<td>DG ((N^a))</td>
</tr>
<tr>
<td>Age</td>
<td>48.6±2.0 (44)</td>
</tr>
<tr>
<td>Gender</td>
<td>0.51</td>
</tr>
<tr>
<td>Male</td>
<td>52.3% (23)</td>
</tr>
<tr>
<td>Female</td>
<td>47.7% (21)</td>
</tr>
<tr>
<td>Tumor size</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Macroadenoma</td>
<td>71.1% (27)</td>
</tr>
<tr>
<td>Microadenoma</td>
<td>28.9% (11)</td>
</tr>
<tr>
<td>Cavernous sinus invasion</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>+</td>
<td>34.1% (14)</td>
</tr>
<tr>
<td>-</td>
<td>65.8% (27)</td>
</tr>
<tr>
<td>Basal GH (ng/mL)</td>
<td>55.5±20.2 (44)</td>
</tr>
<tr>
<td>IGF-1 (ng/mL)</td>
<td>830.4±41.9 (44)</td>
</tr>
<tr>
<td>%GH suppression by octreotide</td>
<td>87.9±3.3 (29)</td>
</tr>
<tr>
<td>%GH suppression by bromocriptin</td>
<td>58.2±5.0 (27)</td>
</tr>
</tbody>
</table>

a, available data
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 ($r = -0.32$, $P = 0.31$).
Gene expressions in somatotroph adenomas

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-

![Image](image_url)

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 (○).

![Image](image_url)

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

Table 3  SSTR2 level as evaluated by immunohistochemical study

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Protein level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(−) 1+ 2+ 3+</td>
</tr>
<tr>
<td>DG (18)</td>
<td>2 5 11 0</td>
</tr>
<tr>
<td>SG (16)</td>
<td>14 2 0 0</td>
</tr>
</tbody>
</table>

The percentage of cells staining sstr2A was evalutaed as follows: (−) negative staining, (+) <20%, (2+) 20-50%, (3+) >50%.
DG: densely granulated somatotroph adenoma, SG: sparsely granulated somatotroph adenoma
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 effec-
tive than SSAs in lowering GH and IGF-I in treatment
of somatotropinomas, DAs can be orally administered
at lower costs than SSAs. It has been reported that the
suppression of GH/PRL by D2R agonists was corre-
lated to $D_2R$ gene expression in lactotroph and soma-
totroph tumors in vitro [20, 21]. In the present study,
however, no significant differences were detected in
responsiveness to bromocriptin between the two sub-
types, 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 desen-
sitization of SSTR2 and D2R and octreotide respon-
siveness on SG-type adenomas.

In conclusion, the present study strongly suggests
that SSTR2 expression is involved in determine dif-
ference in responsiveness to octreotide between the
two distinct subtypes (DG- and SG-) of “pure” soma-
totroph adenomas. Further studies are required to clar-
ify the whole picture of molecular mechanism underly-
ing the clinical differences between DG- and SG-type
adenomas.

**Disclosure**

**Declaration of interest**

The authors declare that there is no conflict of inter-
est that could be perceived as prejudicing the impartial-
ity of the research reported.

**Funding**

This research did not receive any specific grant from
any funding agency in the public, commercial or not-
for-profit sector.

**Acknowledgements**

We thank Dr. T. Tateno for his cooperation in this study.
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