Differential gene expression profiles of POMC-related enzymes, transcription factors and receptors between non-pituitary and pituitary ACTH-secreting tumors

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Abstract. The differential gene expression of proopiomelanocortin (POMC)-related processing enzymes, transcription factors, and receptors responsible for ACTH secretion between non-pituitary and pituitary ACTH-secreting tumors remains obscure. This study was attempted to determine the gene expression profiles of transcription factors (Tpit, NeuroD1 and IKZF1), proprotein convertase (PC) 1/3 and PC2, and several key receptors linked to ACTH secretion, including corticotrophin releasing hormone receptor (CRHR1), vasopressin receptor 1b (V1bR), somatostatin receptor (SSTR) subtype-2, -5 and dopamine receptor type 2 (D2R) in non-pituitary and pituitary ACTH-secreting tumors. Surgical tissue specimens from carcinoid tumors causing ectopic ACTH syndrome (EAS: n=4) and pituitary tumors causing Cushing’s disease (CD: n=13), were subjected to real-time RT-PCR for measurements of each mRNA levels. POMC and CRHR1 mRNA levels in CD were far greater than those in EAS, whereas IKZF1, PC2, SSTR-2 and -5 mRNA levels in EAS were significantly greater than those in CD. NeuroD1, Tpit, PC1/3, V1bR and D2R mRNA levels were comparable between EAS and CD. In conclusion, differential gene expression profiles revealed more abundant mRNA expression in EAS than in CD of 1) IKZF1 with its potential implication of cell differentiation and hormone secretion, 2) PC2 with its possible enhanced processing activity of mature ACTH, and 3) SSTR-2 and -5 with their potential therapeutic application of more selective agonists in EAS patients.

Key words: Gene expression, Ectopic ACTH syndrome, Transcription factor, Somatostatin receptor subtype, Proprotein convertase

ECTOPIC secretion of ACTH from non-pituitary tumors, referred to ectopic ACTH syndrome (EAS), accounts for about 10-20% of Cushing’s syndrome [1]. A series of transcription factors that participate both in the differentiation of corticotroph lineage and in POMC gene expression have been identified [2-5]. Although the POMC promoter region is unaltered in pituitary ACTH-secreting tumors [6], the molecular basis of ectopic ACTH production and POMC gene transcription in non-pituitary tumors remains obscure. Since somatostatin receptors (SSTR) and dopamine receptors have been identified in non-pituitary tumors causing EAS, somatostatin analogues (SSA) and dopamine (DA) agonists have been used as an effective therapy to block ACTH secretion in some EAS patients [7]. However, the differential expression profiles of the genes related to POMC transcription factors, processing enzymes and various key receptors linked to ACTH secretion between non-pituitary and pituitary ACTH-secreting tumors have not been characterized yet. Therefore, the present study was designed to elucidate whether the genes of interest are differentially expressed between non-pituitary and pituitary tumors by a reverse transcriptase-PCR (RT-PCR) technique.

Patients and Methods

Patients

Tumor samples from 4 consecutive EAS patients (2006-2010) and 13 randomly-selected CD patients were collected during surgery at the Tokyo Medical and
Dental University (TMDU) and Toranomon Hospital, respectively. The protocol was approved by the ethical committees of each institute. Informed consent was obtained from each patient before surgery. Dynamic endocrine tests were performed according to the clinical guideline for the diagnosis of CD proposed by the working group of the Ministry of Health, Labour and Welfare of Japan [8]. Thirteen patients underwent transsphenoidal surgery for the removal of pituitary adenomas at Toranomon Hospital, and tissue specimens were used as control: Three patients (Cases 1, 2, 3) and one patients (Case 4) diagnosed as EAS were treated and followed-up at TMDU Hospital and Ome Municipal General Hospital, respectively; EAS was differentiated from CD on the basis of negative pituitary tumor on MRI in combination with the following dynamic endocrine tests; no response to CRH stimulation test or no suppression by overnight high-dose (8mg) dexamethasone suppression test (HDDST), and negative ACTH gradient during inferior petrosal sinus (IPS)/ cavernous sinus (CS) sampling. Desmopressin stimulation test and octreotide (OCT) suppression test were performed in 3 and 4 cases, respectively. Somatostatin receptor scintigraphy (SRS) were performed in 3 cases. Plasma ACTH and serum cortisol levels were measured by an immunoradiometric assay (Mitsubishi IRMA kit, Mitsubishi Kagaku Medience, Tokyo, Japan), and an enzyme immunoassay (EIA: TOSOH, Tokyo, Japan), respectively. Presence of ACTH immunoreactivity was demonstrated in all 4 cases by immunohistochemical study.

Clinical characteristics of 4 EAS patients studied are shown in Table 1. The mean age of 4 EAS patients was 61.8 ± 10.0 year-old and they were all females. The tumor histology was bronchial carcinoid tumors (3) and thymic carcinoid tumor (1), with mean tumor size of 13.8 ± 3.2 mm. All 4 cases presented with Cushingoid features (moon face, central obesity, easy bruising, hirsutism), hypertension, diabetes mellitus/ impaired glucose tolerance (IGT), and two (Cases 1, 3) with skin pigmentation. Endocrine studies showed elevated plasma ACTH levels (143.4 ± 28.3 pg/mL) and serum cortisol levels (40.3 ± 3.3 μg/dL). Neither suppression after overnight HDDST nor positive response to CRH stimulation were noted in 3 of 3 cases (100%) and 3 of 4 cases (75%), respectively, while positive response to DDAVP stimulation and suppression after OCT were 2 of 3 cases (67%) and 2 of 4 cases (50%), respectively. Two of 3 cases (67%) showed positive results on SRS. The mean age of 13 CD patients (2 male and 11 female) was 38.9 ± 4.2 year-old, with mean tumor size of 11.1 ± 2.2 mm. Both plasma ACTH levels (164.6 ± 29.2 pg/mL) and serum cortisol levels (32.5 ± 4.5 μg/dL) were elevated.

Quantification of mRNA

Total RNA was extracted from the surgical tumor specimens. Five μg of total RNA were reverse transcribed. The transcripts of the following genes were quantified with Chromo4™-based (Bio-Rad Laboratories) real-time RT-PCR using fluorescent SYBR green technology essentially as described [9]. The PCR primers were synthesized by Griner bio-one (Tokyo, Japan) and their sequences are shown in Table 2. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the endogenous internal control to compare the relative expression of the following genes: proopiomelanocortin (POMC), proopiomelanocortin convertase (PC)-1/3 and -2, T-box 19 (Tpit), neurogenetic differentiation 1 (NeuroD1), and IKAROS family zinc finger 1 (IKZF1), corticotrophin releasing hormone receptor 1 (CRHR1), arginine vasopressin receptor 1 b (V1bR), somatostatin receptor (SSTR)-2, -5, and dopamine receptor type 2 (D2R). The relative expression of mRNA are expressed as median and interquartile range.

Statistical analysis

Data are expressed as means ± s.e.m. Differences between groups were examined for statistical significance with Mann-Whitney test, and P value less than 0.05 were considered statistically significant.

Results

Gene expression profile of POMC, PC-1/3 and -2 in tumors from EAS and CD patients is shown in Fig. 1. POMC mRNA levels in EAS were far less than those in CD (P < 0.01). PC1/3 mRNA levels did not show any significant difference between EAS and CD, whereas PC2 mRNA levels in EAS were markedly (32-fold) greater than those in CD (P < 0.01).

Gene expression profile of the POMC-related transcription factors (Tpit, NeuroD1, IKZF1) in tumors from EAS and CD patients is shown in Fig. 2. IKZF1 mRNA levels in EAS were significantly (65-fold) greater than those in CD (P < 0.05), whereas neither Tpit nor NeuroD1 mRNAs showed any significant differences between EAS and CD.

As shown in Fig. 3, CRHR1 mRNA levels in CD were markedly (35-fold) greater than those in EAS (P < 0.01),

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Despite the lower POMC mRNA expression in EAS than in CD, plasma ACTH and serum cortisol levels in our EAS patients were comparable to those in CD patients. It should be noted that two EAS patients (Cases 1, 2) treated with preoperative octreotide therapy showed lower POMC mRNA expression than the other two (Cases 3, 4) (Fig. 1, Table 1). It has been recently reported that octreotide up-regulated BMP-Smad signaling to suppress POMC mRNA expression in AtT-20 cells [10]. Thus, it is reasonable to speculate that the apparent discrepancy between POMC gene expression and circulating ACTH levels in our EAS and CD patients may be partly accounted for by the effects of the preoperative octreotide therapy.

PC1/3 is a cleaving enzyme responsible for the

whereas V1bR mRNA levels did not differ between EAS and CD. Both SSTR-2 and -5 mRNA levels were significantly (P < 0.05) greater in EAS than those in CD by about 5- and 7-fold, respectively, whereas D2R mRNA levels did not differ between EAS and CD.

**Discussion**

In this study, we clearly showed differential expression profiles of several key genes related to transcription (Tpit, NeuroD1, IKZF1), POMC processing (PC-1/3, -2), and receptors linked to ACTH secretion (CRHR1, V1bR, SSTR-2, -5) between non-pituitary ACTH-secreting tumors causing EAS and pituitary ACTH-secreting tumors causing CD. Despite the lower POMC mRNA expression in EAS than in CD, plasma ACTH and serum cortisol levels in our EAS patients were comparable to those in CD patients. It should be noted that two EAS patients (Cases 1, 2) treated with preoperative octreotide therapy showed lower POMC mRNA expression than the other two (Cases 3, 4) (Fig. 1, Table 1). It has been recently reported that octreotide up-regulated BMP-Smad signaling to suppress POMC mRNA expression in AtT-20 cells [10]. Thus, it is reasonable to speculate that the apparent discrepancy between POMC gene expression and circulating ACTH levels in our EAS and CD patients may be partly accounted for by the effects of the preoperative octreotide therapy.

PC1/3 is a cleaving enzyme responsible for the

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**Table 1** Clinical characteristics of 4 ectopic ACTH syndrome patients studied

<table>
<thead>
<tr>
<th>Case #</th>
<th>Age/gender</th>
<th>Cushingoid feature</th>
<th>Skin pigmentation</th>
<th>ACTH (pg/mL)</th>
<th>Cortisol (μg/dL)</th>
<th>Dynamic endocrine test</th>
<th>Preoperative therapy</th>
<th>Histopathological diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>75 F (+)</td>
<td>(+)</td>
<td>158</td>
<td>31.6</td>
<td>(+)</td>
<td>CRH (+) DDAVP (+) HDDST (8mg) (+) OCT (+)</td>
<td>Met (0.75 g/day) Oct (200 μg/day)</td>
<td>Bronchial carcinoid tumor</td>
</tr>
<tr>
<td>2</td>
<td>71 F (+)</td>
<td>(-)</td>
<td>142</td>
<td>43.2</td>
<td>(-)</td>
<td>ND (-) (+) (+) (+) (+)</td>
<td>Met (1 g/day) Oct (LAR20 mg/day)</td>
<td>Bronchial carcinoid tumor</td>
</tr>
<tr>
<td>3</td>
<td>69 F (+)</td>
<td>(+)</td>
<td>205</td>
<td>47.0</td>
<td>(-)</td>
<td>(-) (-) (-) (-) (-) (+)</td>
<td>Met (1 g/day) DEX (0.5 mg/day)</td>
<td>Bronchial carcinoid tumor</td>
</tr>
<tr>
<td>4</td>
<td>32 F (+)</td>
<td>(-)</td>
<td>68.7</td>
<td>39.2</td>
<td>(-)</td>
<td>ND (-) ND (+) (+)</td>
<td>Met (2 g/day)</td>
<td>Thymic carcinoid tumor</td>
</tr>
</tbody>
</table>

CRH: corticotropin releasing hormone, DDAVP: desmopressin, HDDST: high-dose dexamethasone suppression test, OCT: octreotide, SRS: somatostatin receptor scintigraphy, Met: metyrapone, LAR: Sandostatin LAR®, DEX: dexamethasone, ND: not determined

**Table 2** PCR primers used for RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer sequences</th>
<th>Reverse primer sequences</th>
<th>PCR product size (bp)</th>
</tr>
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<tr>
<td>POMC</td>
<td>AGACAGGCCACTTGCTGGATT</td>
<td>GGCTCTGCAAGAAAGCAACA</td>
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<tr>
<td>PC1/3</td>
<td>CGCTGACCTGCAACAATGACT</td>
<td>CAGCAACAAGGCTGCTGCACT</td>
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<tr>
<td>PC2</td>
<td>CTTGCAAAGGCCAAGAGAGAAG</td>
<td>TTTCGGTCAATCCTCTCGTG</td>
<td>101</td>
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<tr>
<td>Tpit</td>
<td>GCCAGCATGTCATCTTTTCCT</td>
<td>CTGCTTGACACTCTCATCT</td>
<td>60</td>
</tr>
<tr>
<td>NeuroD1</td>
<td>CTGCTCAGAGCTACTAAACAA</td>
<td>GTTTCAGCTTGGAGGACCTT</td>
<td>105</td>
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<tr>
<td>IKZF1</td>
<td>CTTCCGGGGCAGACTGTA</td>
<td>TCTCTCTGATCCTATCTGCCA</td>
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</tr>
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<tr>
<td>V1bR</td>
<td>CAAGATCCGAACAGTGAAAGTG</td>
<td>CATAGAGATGGAAAGCACA</td>
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<tr>
<td>SSTR2</td>
<td>AGACCAAGCAGCTAAGGATT</td>
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<tr>
<td>SSTR5</td>
<td>CGCCGTCTTCCATCTACTA</td>
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<tr>
<td>D2R</td>
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<td>113</td>
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<tr>
<td>GAPDH</td>
<td>GCTGAGAACGGGAAGCTCTTGT</td>
<td>TCCCATGTTGGAAGACGCA</td>
<td>136</td>
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</table>

Abbreviations used: POMC, proopiomelanocortin; PC, proprotein convertase; Tpit, T-box 19; NeuroD1, neurogenetic differentiation 1; IKZF1, IKAROS family zinc finger 1; CRHR1, corticotropin releasing hormone receptor 1; V1bR, arginine vasopressin receptor 1b; SSTR, somatostatin receptor; D2R, dopamine receptor type 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase
Furthermore, 2 of 4 our EAS patients (Cases 1, 3) with skin pigmentation showed higher expression of PC2 mRNA than the others (Cases 2, 4), suggesting the possible involvement of α-MSH in the development of skin pigmentation. Our data are consistent with those of previous study showing more predominant production of α-MSH and CLIP by non-pituitary tumors causing EAS resulting from the enhanced PC2 expression by the tumors [13].

PC1/3 mRNA expression comparable between EAS and CD as demonstrated in this study, also lend credence to the contention that non-pituitary tumors are capable of processing POMC as actively as pituitary tumors to secrete ACTH.

**Fig. 1** Gene expression of POMC and proprotein convertase (PC)-1/3 and -2 in non-pituitary and pituitary ACTH-secreting tumors. Data are expressed as fold increase. Each case is represented by the following symbols: ● (Case 1), ▲ (Case 2), ■ (Case 3), ○ (Case 4). The box represents the median and intraquartile range (25th to 75th percentile) and whiskers represent the farthest points that are not outliers. A dot represents more than 3/2 times the interquartile range from the end of a box. **P < 0.01 between the groups. Abbreviation used: EAS, ectopic ACTH syndrome; CD, Cushing’s disease.

**Fig. 2** Gene expression of transcription factors in non-pituitary and pituitary ACTH-secreting tumors. Data are plotted as in Fig. 1. Each case is represented by the same symbols as in Fig. 1: ● (Case 1), ▲ (Case 2), ■ (Case 3), ○ (Case 4). * P < 0.05 between the groups. Abbreviation used: Tpit, T-box 19; NeuroD1, neurogenic differentiation 1; IKZF1, IKAROS family zinc finger 1.

Post-translational processing of POMC into mature ACTH(1-39) in corticotrophs of the pituitary, whereas PC2 is responsible for the proteolytic processing of ACTH(1-39) into α-melanocyte stimulating hormone (α-MSH) and its C-terminal fragment, termed corticotrophin-like intermediate lobe peptide (CLIP), in the intermediate lobe, but not the anterior lobe of the pituitary. We have previously shown that PC1/3 mRNA is more abundantly expressed than PC2 mRNA in pituitary ACTH-secreting tumors causing CD [11], and both PC1/3 and PC2 are expressed in neuroendocrine tumors (NET) including carcinoid tumors [12]. The present study clearly showed that PC2 mRNA expression in the carcinoid tumors causing EAS was far greater than that in the pituitary adenomas causing CD. Furthermore, 2 of 4 our EAS patients (Cases 1, 3) with skin pigmentation showed higher expression of PC2 mRNA than the others (Cases 2, 4), suggesting the possible involvement of α-MSH in the development of skin pigmentation. Our data are consistent with those of previous study showing more predominant production of α-MSH and CLIP by non-pituitary tumors causing EAS resulting from the enhanced PC2 expression by the tumors [13]. PC1/3 mRNA expression comparable between EAS and CD as demonstrated in this study, also lend credence to the contention that non-pituitary tumors are capable of processing POMC as actively as pituitary tumors to secrete ACTH.

Tpit, a member of transcription factors of the T-box family [14], and NeuroD1, a basic helix-loop-helix
transcription factor in synergy with other transcription factor Ptx1 [2], play pivotal roles in the differentiation of corticotroph lineage and transcription of POMC gene. The present study, however, revealed that there were no differences of Tpit or NeuroD1 expression between non-pituitary and pituitary ACTH-secreting tumors. Thus, both Tpit and NeuroD1 are required to achieve corticotroph differentiation in non-pituitary tumors in the same manner as in pituitary tumors. However, Tpit and NeuroD1 expressions have been shown in ACTH-negative carcinoid tumors [15], suggesting that yet-unspecified transcription factors other than Tpit and NeuroD1 are needed to induce transcription of pituitary-like POMC mRNA in EAS.

The present study demonstrated for the first time that IKZF1 gene expression was far greater in EAS than that in CD. Ikaros gene, a transcription factor that binds to regulatory sequences of genes expressed in lymphoid cells [16, 17], contains seven exons yielding eight isoforms by alternative splicing. In particular, IKZF1 expressed in the normal human pituitary and neoplastic tissues, has been shown to bind and enhance activity of the low-density lipoprotein receptor (LDL-R) and POMC promoter [18, 19]. In fact, stable transfection of IKZF1 in AtT20 cells resulted in enhanced endogenous POMC expression and ACTH secretion in conditioned culture media [19]. As previously explained, the discrepancy between POMC and IKZF1 mRNA levels in our EAS patients may be accounted for the treatment with octreotide before operation. In contrast, it has been demonstrated that IKZF1-transfected cells have enhanced LDL endocytosis with abundant endoplasmic reticulum, large Golgi complexes, and prominent secretory granule formation caused by incorporation of robust cholesterol [18]. These findings expand repertoire of Ikaros actions to include regulation of the cholesterol uptake metabolic pathway as well as growth and differentiation of pituitary cell. Therefore, it is also possible to speculate that membrane trafficking functions may be more active and efficient in non-pituitary ACTH-secreting tumors than in pituitary ACTH-secreting tumors. If that is the case, therapeutic implications of IKZF1-reducing drugs and/or lipid-modifying drugs for inhibition of ACTH secretion and
tumor growth could be speculated [20].

CRHRI and V1bR, two specific receptors for potent ACTH secretagogues, have been shown to be overexpressed in pituitary ACTH-secreting tumors causing CD [11]. Although CRH stimulation test has a great sensitivity (94%) in CD patients, about 5-30% of EAS patients respond to CRH stimulation [21-23]. On the other hand, desmopressin stimulation test had a 40% false-positive response in EAS patients [24]. In fact, false-positive results were observed in 1 of 4 cases after CRH stimulation as recently reported [25] as well as in 2 of 3 cases after desmopressin stimulation in this study. The present study showed that CRHRI mRNA expression in EAS was lower than CD as expected, and that V1bR mRNA expression was comparable between EAS and CD. Thus, desmopressin test appears less useful than CRH test for differentiation of EAS from CD. In fact, a robust response of ACTH to DDAVP stimulation in a patient with thymic carcinoid tumor (Case 4) is consistent with the highest V1bR levels among all 4 EAS cases. Given a very small number of our EAS patients of carcinoid tumors, the differential gene expression between bronchial and thymic carcinoid tumor remains unknown.

It has been known that NET causing EAS, such as bronchial carcinoid tumors, often express functional SSTR-2 and SSTR-5 [26], and that corticotroph tumors express multiple SSTR subtypes [27]. EAS patients (36–57%) have positive results on SRS [28-30] due to higher binding affinity of octreotide to SSTR-2 and SSTR-5. In fact, 2 cases who showed suppression of ACTH by octreotide in our study were SRS-positive [28]. The present results with more abundant expression of both SSTR-2 and SSTR-5 mRNA in EAS than CD are consistent with the more effective medical treatment of octreotide in EAS patients than in CD patients.

Although DA agonists (bromocriptine, cabergoline) used for medical treatment in CD patients have proven to be effective in some patients [31], few studies have been studied for the potential use of DA compounds in EAS patients; one DA-responsive case with the abundant D2R expression in the tumor [32], and another case with synergistic effect by lanreotide and cabergolin [33]. The comparable D2R mRNA expression between EAS and CD in this study, along with its lower expression in CD than non-functioning pituitary tumors in our previous study [34], suggests the limited use of DA agonists for medical treatment in EAS as well as CD.

In conclusion, our study demonstrated that ectopic ACTH-secreting tumors have more abundant gene expression of IKZF1 with potential implication in differentiation and secretory activities, and of SSTR-2 and -5 with their therapeutic potential for medical treatment in EAS.

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