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Defective Expression of Prohormone Convertase 1/3 in Silent Corticotroph Adenoma

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Abstract. Silent corticotroph adenoma (SCA) is defined as an ACTH-producing pituitary tumor not associated with clinical and endocrine feartures of Cushing's syndrome, but its underlying molecular mechanism(s) remains unknown thus far. We tested the hypothesis that reduced expression of prohormone convertase (PC) 1/3 responsible for proteolytic processing of proopiomelanocortin (POMC) in SCA may lead to production of unprocessed, biologically inactive POMC and/or precursor of ACTH. Among 30 non-functioning pituitary macroadenomas (NFA) examined, we found 6 SCAs by immunohistochemical study using anti-ACTH antibody. Preoperative endocrine and diagnostic image tests did not reveal any differences between SCA and the remaining NFA except for the higher recurrence rate of SCA. While steady-state PC1/3 mRNA levels determined by RT-PCR were almost comparable between SCAs and NFAs, immunohistochemical study showed negative immunostaining for PC1/3 in all 6 SCAs. Our data suggest that defective PC1/3 expression may lead to preferential production of unprocessed, biologically inactive ACTH variants in SCA.

Key words: Silent corticotroph adenoma (SCA), Prohormone convertase (PC), Proopiomelanocortin (POMC), Immunohistochemistry, Reverse transcriptase-polymerase chain reaction (RT-PCR)

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SILENT corticotroph adenoma (SCA) is originally defined as a pituitary adenoma with positive immunoreactivity for ACTH without any signs and symptoms of Cushing's syndrome [1]. Their clinical and endocrine manifestations are poorly understood and the reasons for the lack of Cushingoid features in SCA remain unknown. Although these tumors may recur more frequently postoperatively [1] and behave more aggressively [3, 4], there are conflicting reports as well [2]. Furthermore, studies on the long-term follow-up and preoperative clinical diagnosis of SCAs are very limited [5]. Possible mechanisms for the lack of Cushing's symptoms in SCA thus far proposed include translational or post-translational abnormalities of ACTH [6], impaired secretion of ACTH from tumor cells [7], and preferential secretion of biologically inactive ACTH molecules [8]. Moreover, possible involvement of prohormone convertase (PC) 1/3 responsible for proper processing of proopiomelanocortin (POMC) into mature ACTH(1-39) in SCAs remains unclear.

In the present study, we studied, 1) prevalence of SCA among non-functioning pituitary adenoma (NFA) by immunohistochemical study for ACTH, 2) clinical and endocrine features between SCA and NFA, and 3) expression of PC1/3 by immunohistochemistry and reverse transcriptase-polymerase chain reaction (RT-PCR) in these tumors.

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Patients and Methods

Patients

A total of 30 consecutive patients (17 male and 13 female) with NFA who underwent transsphenoidal surgery had preoperative endocrine tests, including diurnal rhythm of plasma ACTH/cortisol concentrations and/or overnight small-dose (0.5 mg) dexamethasone suppression, and CRH stimulation before and after surgery. Informed consent for the present study was obtained from each patient before operation.

Immunohistochemistry

Resected tumor tissues were subjected to immunohistochemical studies for ACTH by Envision method (DAKO: Carpinteria, CA) using anti-ACTH monoclonal antibody (1 : 200; DAKO) which could not recognize POMC [9], and PC1/3 by avidin-biotin peroxidase complex method using anti-PC1/3 polyclonal antibody (1 : 400; Chemicon Int,. CA) as previously reported [9].

Real-time quantitative RT-PCR

Tissue specimens obtained from 10 pituitary macroadenoma (3 SCA and 7 NFA) after transsphenoidal surgery were immeadiately forzen at –80°C until analysis. Total RNA from tissues were extracted according to the method provided by TRIzol reagent kit (Gibco, BRL) and first-strand cDNA was synthesized by using a first-strand synthesis kit (GE Healthcare UK), following the manufacturer's instruction. PC1/3 mRNA level was quantified with real-time quantitative RT-PCR using fluorescent SYBR green technology (Light-Cycler; Roche Molecular Biochemicals, Mannheim, Germany). PCR primers were synthesized by Greiner bio-one (Tokyo, Japan). The human PC 1/3 primers were: sense strand 5'-CGCTGACCTGCACAATGACT-3' and antisense strand 5'-CAGACAACCAGGTGCT GCAT-3'. The human GAPDH primers were: sense strand 5'-GCTGAGAACGGGAAGCTTGT-3' antisense strand5'-TCTCCATGGTGGTGAAGACG-3' The mRNA levels of the target sequence were normalized by the GAPDH mRNA levels used as an endogenous internal control; the relative level of PC1/3 mRNA to that of GAPDH was calculated.

Results

Clinical and endocrine features

Six out of the 30 cases with NFA (20%) were identified as SCA based on the positive immunostaining for ACTH in the tumor tissues without clinical signs and symptoms of Cushing's syndrome. As shown in Table 1, they were 1 man and 5 women, aged 48.2 ± 13.6-year-old, and there were no significant differences in tumor size or clinical features between SCA and NFA, including visual field disturbance (100% vs 87.5%), impaired glucose tolerance (0% vs 12.5%), obesity (50% vs 25%), and hypertension (33.3% vs 33.3%), except for the recurrence rate (66.7% vs 16.7%, $p<0.05$). Tumor recurrence occurred in four out of 6 SCA patients during mean follow-up period of 6.1 years (4.2–9.2 years) with the mean postoperative period of 3.8 years (0.5–5.0 years), but none of them developed hypercortisolism during the entire observation period. Endocrine studies showed that neither basal ACTH or cortisol concentrations, their suppression after overnight low-dose (0.5 mg) dexamethasone, nor ACTH response to human CRH (100 μg) stimulation differed between SCA and NFA patients.

Immunohistochemical studies

Immunohistochemical study using anti-ACTH antibody revealed the presence of diffuse ACTH immunoreactivity in tumor cells from a patient with overt Cushing's disease (Fig. 1). In contrast, ACTH immunoreactivities were variably distributed in scattered, clustered and diffuse fashion, and stained in moderate to strong degree in individual tumor tissues from 6 SCAs (Fig. 2). The tumor tissue from overt Cushing's disease showed positive immunoreactivity for PC 1/3 (Fig. 1), whereas immunoreactivity for PC1/3 was negative in all 6 SCA tumor tissues (Fig. 2).

Analyses of PC1/3 mRNA expression

Quantification of PC1/3 mRNA by RT-PCR showed steady-state PC1/3 mRNA levels in tumor tissues were almost comparable between SCAs (Cases 1, 2, 4) and NFAs (n = 7) (Fig. 3).

Table 1. Clinical and endocrine characteristics between silent corticotroph adenoma (SCA) and non-functioning pituitary adenoma (NFA)

	SCA	NFA
Clinical		
Number	6	24
Age (year)	48.2 ± 13.6	54.8 ± 16.0
Male/Female	1/5	16/8
Tumor size (mm)	25.0 ± 3.2 × 27.5 ± 5.2	23.3 ± 3.8 × 27.7 ± 5.3
Visual field disturbances	6	21
Impaired glucose tolerance	0	3
Obesity	3	6
Hypertension	2	8
Tumor recurrence	4	4
Endocrine		
Basal ACTH (pg/ml)[1)]	37 ± 23.6	34.5 ± 19.7
Basal cortisol (μg/dl)[1)]	12.5 ± 5.0	12.0 ± 4.3
CRH stimulation test[2)]		
ACTH (fold increase)[3)]	6.0 ± 4.1	3.4 ± 2.9
Cortisol (fold increase)[3)]	2.0 ± 0.7	2.0 ± 1.3
Dexamethasone suppression test[4)]		
ACTH (pg/ml)[1)]	13.0 ± 6.93	7.0 ± 1.67
Cortisol (μg/dl)[1)]	2.04 ± 0.73	1.10 ± 0.38

[1)] 8:00 (clock time)
[2)] Bolus injection of human CRH (100 μg) at 8:00
[3)] Fold-increase over basal levels
[4)] Dexamethasone (0.5 mg) p.o. at 23:00

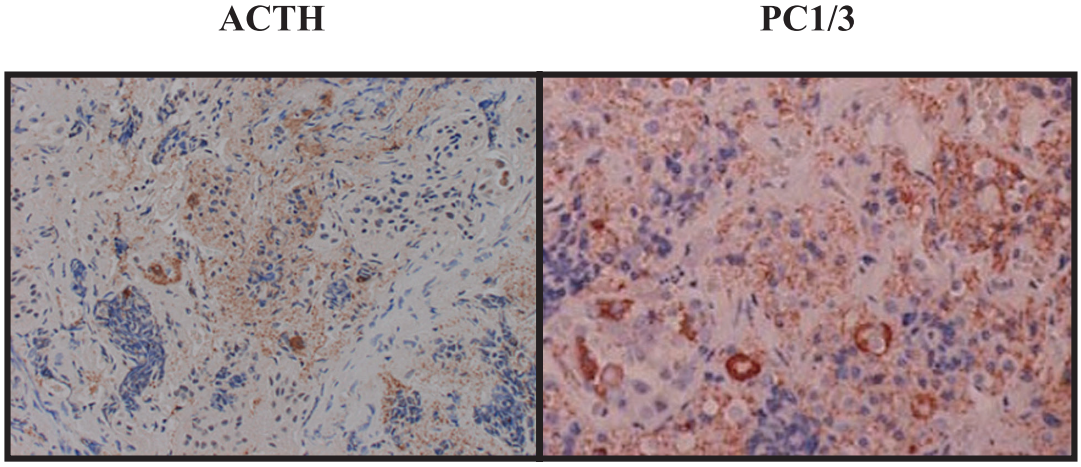

Fig. 1. Immunohistochemistry of ACTH and PC1/3 in corticotroph adenoma causing Cushing's disease. Positive immunostainig for ACTH (left panel) and PC1/3 (right panel) are shown (magnification: ×400).

Discussion

It has been reported that the incidence of SCA among NFA ranged between 6 to 8.1% [1, 10]. The present study showed the higher incidence (20%) of SCA (6 out of 30 NFAs), suggesting that SCA is not so unusual as previously thought. Although SCA patients in our study showed no clinical signs and symptoms of Cushing's syndrome, the metabolic phenotype of Cushing's syndrome, such as hypertension, impaired glucose tolerance and obesity, did not differ between SCAs and NFAs. Furthermore, the present endocrine

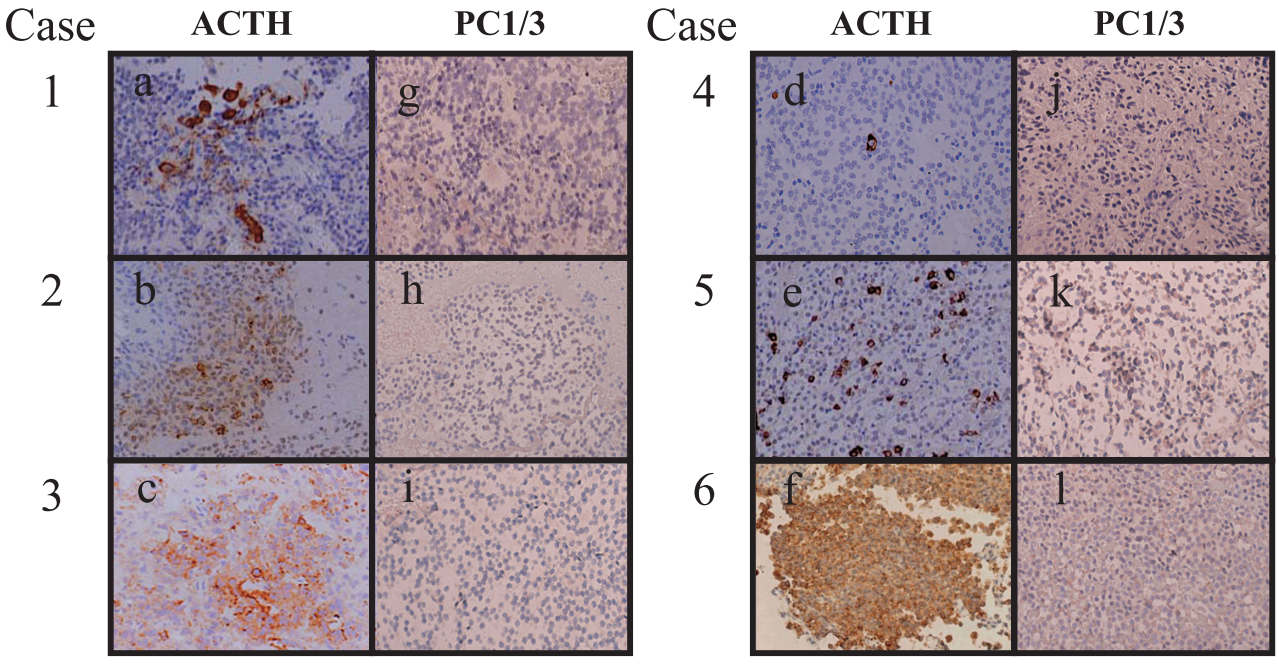

Fig. 2. Immunohistochemistry of ACTH and PC1/3 in silent corticotroph adenomas. Immunohistochemical staining for ACTH (a–f) and PC1/3 (g–l) in tumor tissues from Cases 1–6 are shown (magnification: ×400).

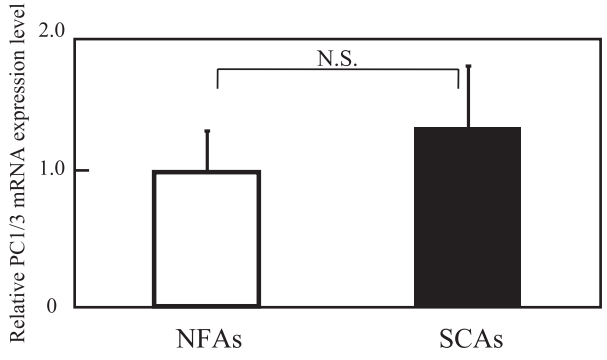

Fig. 3. PC1/3 mRNA expression in silent corticotroph adenoma (SCA) and non-functionig pituitary macroadenoma (NFA) tissues. RT-PCR using fluorescent SYBR green technology was performed using the primers for PC1/3 and GAPDH. The relative levels of PC1/3 mRNA to that of GAPDH are compared between 3 SCAs (closed column: Cases1, 2, 4) and 7NFAs (open column). NS: not significant ($p>0.05$).

data that SCA patients showed normal diurnal rhythm of ACTH/cortisol secretion with suppressibility to low-dose dexamethasone and responsiveness to CRH stimulation, suggest normal regulation of ACTH secretion in SCA. Despite no difference of tumor size by imaging data between SCAs and NFAs, the recurrence rate (66.7%) was higher in SCA patients, suggesting its aggresiveness. These clinical features of SCAs are consistent with those of previous reports [2, 5].

In the present immunohistochemical study, we showed that expression of PC1/3 protein was negative in all 6 SCA tissues in contrast to the positive PC1/3 immunoreactivity in an ACTH-secreting pituitary tumor causing clinically overt Cushing's disease. PC1/3 is responsible for proper processing of POMC into mature ACTH(1-39) in a tissue-specific fashion [11, 12, 13], and highly expressed in ACTH-secreting adenomas [14, 15]. However, there have been few studies on PC1/3 expression in SCA thus far reported; one case with negative PC1/3 expression [16], and another case with positive PC1/3 expression [8].

The present real-time quantitative RT-PCR clearly showed that steady-state PC1/3 mRNA levels were almost comparable between SCAs and NFAs. Our data are compatible with a previous report showing positive expression of PC1/3 mRNA in NFA [17]. Taken together, it is suggested that defective post-transcriptional regulation of PC1/3 is responsible for the discrepancy between mRNA and protein expression in SCA.

Gel exclusion chromatography of plasma samples from some SCA patients showed the presence of two major peaks, one corresponding to authentic ACTH(1-39), and the other high molecular weight (HMW) form,

possibly representing POMC and/or ACTH precursor (unpublished observations). Biological activities of ACTH precursors still remain undetermined; big ACTH is undetectable by *in vivo* bioassay [18], but its biologically activity corresponds to only 3–5% of ACTH(1-39) by *in vitro* assay [19], whereas proACTH has been shown to only 8–33% of ACTH(1-39) by cytochemical assay [20]. Therefore, it is possible to speculate that biologically inactive HMW form of ACTH may be generated from the impaired processing of POMC and/or ACTH precursor by the defective PC1/3 expression in SCA. Thus, the defective PC1/3 expression in SCA may lead to the preferential production of unprocessed, biologically inactive ACTH variants, although its underlying molecular mechanism(s) remains to be determined.

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