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ORIGINAL ARTICLE

Hormonal aggressiveness according to the expression of cellular markers in corticotroph adenomas

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Abstract

Purpose The molecular mechanisms underlying tumor growth in Cushing's disease (CD) still remain a challenge. Moreover, clinical manifestations of CD may vary depending on hormonal activity; however, factors involved in the hormonal aggressiveness of adrenocorticotropic hormone (ACTH)-secreting pituitary tumors have not been fully clarified. We investigated the association between the expression of cellular markers regarding pituitary tumor progression and initial or postoperative hormone levels in patients with CD.

Methods Tumor tissues from 28 corticotroph adenomas (female 26, male 2, mean age 39.21 ± 10.39 years) were subject to immunohistochemical study using the following antibodies: pituitary tumor-transforming gene 1 (PTTG1), cyclin D1, p16, p27, brahma related-gene 1 (Brg1), and Ki-67. We then analyzed the relationship between each cellular marker expression and hormone levels, including 24 h urinary free cortisol (UFC), plasma ACTH, and serum cortisol.

Results PTTG1 and Ki-67 were expressed in 100% and 50% of patients, respectively. However, the levels did not reflect initial hormonal activity. The cyclin D1-negative group showed higher serum cortisol levels compared to the cyclin D1-positive group ($p = 0.01$). The 24 h UFC levels were significantly higher in the p27-negative group than in the p27-positive group ($p = 0.04$), whereas the Brg1-positive group revealed higher serum cortisol levels than in the Brg1-negative group ($p = 0.02$).

Conclusions Although PTTG1 and Ki-67 play an essential role in developing ACTH-secreting tumors, cyclin D1, p27, and Brg1 may be better biomarkers to determine hormonal aggressiveness of the tumor. Further research is needed to understand the influence of cellular markers on hormonal activity in CD.

Keywords Cushing syndrome · ACTH-secreting pituitary adenoma · Hormones · Biomarkers

Introduction

Cushing's disease (CD) is characterized by the overproduction of glucocorticoid (Gc) resulting from adrenocorticotropic hormone (ACTH)-secreting pituitary tumors [1]. The prevalence of CD is rare, with an estimated 2.4 cases per million in the population [2]. Endogenous hypercortisolism leads to an increase in various morbidities, including diabetes mellitus, hypertension, and osteoporosis [3, 4]. In addition, subjects with uncontrolled hypercortisolism have a 4-fold to 5-fold increase overall mortality than the general population [1]. Thus, early diagnosis and appropriate treatment are very important in patients with CD [5]. The first-line therapy in the management of CD is

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surgical removal of the pituitary adenomas [6, 7], and the immediate postoperative remission rates seem to be promising ranging from 65 to 94% [3, 8, 9]. Relatively high recurrence rates, ranging from 20 to 25% on the long-term follow-up, still remain a challenge [10, 11]; however, second-line treatments such as radiation and medical therapy have been used as adjuvant therapies.

Corticotroph adenomas, which account for about 10–15% of all pituitary tumors [12], are composed of functional and silent corticotroph adenomas [13]. Most of the ACTH-secreting pituitary adenomas are microadenomas [3]; however, the clinical manifestation of corticotroph adenoma may vary depending on hormonal activity. Nonetheless, the molecular mechanism underlying tumor growth in CD or factors involved in the aggressiveness of ACTH-secreting pituitary tumors have not fully been clarified. Previous studies suggested that preoperative plasma ACTH [14] and early postoperative serum cortisol levels [3, 15], as well as age at diagnosis and the presence of diabetes or hypertension [4, 16], might be useful clinical predictors for the cure of CD patients, but there is a paucity of data on biomarkers reflecting hormonal activity in ACTH-secreting pituitary tumors.

Here, we investigated whether cellular markers that are linked to cell proliferation and differentiation of pituitary tumors are expressed in corticotroph adenomas. Based on these results, we aimed to evaluate the association between each cellular marker and the initial and follow-up hormone levels in patients with CD.

Patients and methods

Study participants

Twenty-eight patients, 26 women and 2 men, with CD underwent transsphenoidal surgery (TSS) performed by a single neurosurgeon (SH Kim) in Severance hospital from 1996 to 2010. Three patients had already undergone TSS in other hospitals and were referred to our hospital to remove remnants of the pituitary tumors. In all patients, the diagnosis of Cushing's syndrome was confirmed based on clinical features and the findings of biochemical tests, including 24 h urinary free cortisol (UFC) excretion, low-dose and/or high-dose dexamethasone suppression tests (DSTs.), magnetic resonance imaging, and abdominal computed tomography. In this study, hormonal aggressiveness was defined as high hormonal activity, including 24 h UFC, plasma ACTH (AM), and serum cortisol levels (AM). Preoperative and postoperative protocols in patients with CD were the same as described in our previous study [17]. The study was approved by the Institutional Review Board of the Severance Hospital (IRB No. 4-2018-0475).

In all patients, preoperative Gc replacement was not performed. Serum cortisol level was checked every 6 h during the first day, at 48 h and 72 h after TSS. Within 24 h after TSS, immediate therapeutic outcome was determined by postoperative serum cortisol levels. If the serum cortisol concentration is less than 2 μg/dL or if symptoms and signs of adrenal insufficiency are present, Gc. replacement therapy was initiated. If the serum cortisol level is reduced to levels between 2 and 5 μg/dL, the patient was monitored for developing clinical signs of adrenal insufficiency until 72 h after TSS and thereafter, Gc. replacement therapy was started. In case of serum cortisol levels of more than 5 μg/dL, reoperation was considered. The 24 h UFC levels, including non-suppressed serum cortisol and plasma ACTH levels, confirmed initial remission. Except for one subject whose postoperative data were not available, 25 patients (92.6%) achieved remission by surgery, one subject still had tumor remnants even after TSS, and the one remaining patient showed unclear results regarding initial cure due to old medical records. In a total of 27 patients, the 24 h UFC excretion tests, serum cortisol, and plasma ACTH levels were regularly checked during follow-up. Serum cortisol and plasma ACTH levels were regularly assessed at 1 week, 2 weeks, and 3 months after the surgery; then, they were monitored every 6 months. Based on the criteria proposed by Ayala et al. [18], if 24 h UFC level was elevated beyond a reference range (20–90 μg/day, radioimmunoassay) or serum cortisol levels of greater than 2 μg/dL were detected during this process, we conducted overnight or low DSTs, and determined whether remission status was maintained. Hydrocortisone replacement was usually stopped within 6 months in patients without sign or symptoms of hypopituitarism, while hydrocortisone of 10–20 mg daily was maintained in those with sign or symptoms of hypopituitarism. In order to assess the hormonal status more accurately, a combined pituitary function test (CPFT) was performed more than 6 months after TSS; whether to maintain hydrocortisone replacement therapy was determined according to the results of CPFT.

Differential diagnosis of Cushing's disease

To differentiate central origin from ectopic sources of ACTH, high-resolution MRI and inferior petrosal sinus sampling (IPSS) were performed before TSS in all patients. Sella MRI was performed using a 3-T MRI unit (MR Systems Achieva, release 3.1, Philips, The Netherlands). The sella MRI and IPSS protocols were the same as described in our previous research [17]. The standard protocol for sella MRI included T1-weighted and T2-weighted turbo spin echo sequences in the sagittal plane, a T2-weighted turbo spin echo sequence in the axial plane, and high-resolution images in a T2-weighted turbo spin echo

sequence in the coronal plane. Coronal dynamic acquisition with a slice thickness of 2 mm was started simultaneously with intravenous gadolinium injection (0.2 ml/kg), followed by a T1-weighted turbo spin echo sequence in the sagittal and coronal planes. A single neuroradiologist, who was blinded to the findings of the IPSS, reviewed the MRI images. IPSS was successful in all patients and no major complications were detected in any patient. The IPSS results were interpreted according to the criteria described by Oldfield et al. [19]; a central to peripheral ACTH ratio ≥2 at baseline or ≥3 after Corticotropin-releasing hormone (CRH) injection was used to indicate evidence of pituitary ACTH secretion, and an ACTH ratio between the right and left inferior petrosal sinuses ≥1.4 at baseline or after CRH injection was regarded as the existence of lateralization.

Immunohistochemical staining and interpretation

We performed immunohistochemical staining to evaluate whether each cellular marker was expressed in ACTH-secreting pituitary tumors. Tumor tissues obtained from 28 corticotroph adenomas were available. The immunohistochemical staining protocols were the same as described in our previous study [20]. Sectioned slides were deparaffinized three times in xylene for 20 min each and rehydrated using a graded alcohol solution. Antigen retrieval was conducted in 10 mM citrate buffer at pH 6.0 for 10 min in a microwave. Slides were allowed to cool to room temperature and sequentially rinsed three times in PBS and 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, and Tween 20 (0.025%; TBS-T) for 2 min each. Endogenous peroxidase activity was quenched by incubation in peroxidase-blocking reagent (code S2001; Dako Cytomation, Carpinteria, CA). Each incubation step was carried out at room temperature, followed by three sequential washes of TBS-T for 5 min each. Sections were incubated in primary antibody diluted in 10% serum (goat serum, Jackson ImmunoResearch Laboratories Inc., West Groove, PA; rabbit and horse serum, Vector Laboratories Inc., Burlingame, CA). The secondary antibody was diluted in antibody diluent (Dako Cytomation) and incubated with a biotinylated secondary antibody for 30 min, peroxidase-labeled streptavidin for 20 min (LSAB-2; Dako Cytomation), and diaminobenzidine chromogen substrate (Dako Cytomation) for 5 min. Slides were counterstained with hematoxylin, dehydrated in a graded alcohol solution, and mounted.

The following primary antibodies were used to detect the expression associated with cellular proliferation and differentiation in the current study: rabbit antipituitary tumor-transforming gene 1 (anti-PTTG1, 18-0278; Invitrogen, San Diego, CA), rabbit polyclonal cyclin D1 (sc-718; Santa Cruz Biotechnology Inc.), mouse monoclonal p16 (sc-56330; Santa Cruz Biotechnology Inc.), mouse monoclonal p27 (sc-1641; Santa Cruz Biotechnology Inc.), rabbit polyclonal brahma related-gene 1 (Brg1, sc-10768; Santa Cruz Biotechnology Inc.), and mouse monoclonal Ki-67 (clone MIB-1; Dako). The results were interpreted by a single pathologist (MK Lee), who scored on a scale of 0 to 3 according to percentage positivity, which means the number of cells staining positive (grade 0, 0%; grade 1+, less than 10%; grade 2+, 10–49%; grade 3+, more than 50%). Moreover, Ki-67 labeling index (LI) was estimated from mean numbers of cells positive for Ki-67 immunostaining per 1000 tumor cells in the highly positive area [21]. The data are expressed as percentages.

Statistical analysis

Data analyses were performed using IBM SPSS version 23.0 for Windows (SPSS Inc., Chicago, IL, USA) and SAS 9.4 version (SAS Inc., Cary, NC, USA). Due to the small number of patients, data were analyzed by nonparametric tests. Continuous variables are presented as median, minimum and maximum. Comparisons between groups were conducted using the Mann–Whitney *U* test, Kruskal–Wallis test, and Fisher's exact test. The Wilcoxon rank sum test was performed to compare hormone levels between cellular marker-positive and negative groups. The Wilcoxon signed-rank test was used to compare hormone levels between baseline and each follow-up time point according to the expression of cellular markers. Two-sided *p* values less than 0.05 were considered statistically significant.

Results

Mean age was 39.21 ± 10.39 years (range: 18–60 years), and our subjects showed a female predominance with a male to female ratio of 1:13. Moreover, the average follow-up period was 88.0 ± 45.4 months. A total of 27 patients who had available postoperative data were followed for a minimum of 10 months and a maximum of 230 months; among them, 22 (81.5%) were monitored for longer than 5 years.

Differences in initial hormone levels according to the expression of cellular markers in patients with CD

As shown in Table 1, Fig. 1, and Supplemental Table 1, PTTG1 was over-expressed in all patients (1 + –3 +); however, there were no significant differences in hormonal levels, including 24 h UFC, plasma ACTH, and serum cortisol, according to the degree of PTTG1 expression. Cyclin D1 was variously expressed in corticotroph adenomas; 13 patients (46.4%) showed positive staining for

Table 1 Differences in initial hormone levels according the expression of each cellular marker in patients with Cushing's disease

Expression of cellular markers	24 h UFC (μg/day)	*p* value	Plasma ACTH (pg/mL)	*p* value	Serum cortisol (μg/dL)	*p* value
PTTG1						
1 + ~2 + ($n = 8$)	448.2 (99.7–2739.3)	0.89	97.4 (27.4–289.1)	0.82	30.4 (18.1–48.1)	0.19
3 + ($n = 20$)	443.9 (28.5–6275.0)		71.3 (14.6–325.8)		25.8 (8.8–71.6)	
CD1						
Negative ($n = 15$)	429.8 (44.4–6275.0)	0.71	99.0 (14.6–325.8)	0.21	27.6 (13.8–71.6)	0.01
Positive ($n = 13$)	480.6 (28.5–1480.6)		53.0 (27.4–289.1)		19.9 (8.8–48.1)	
p16						
Negative ($n = 9$)	534.0 (99.7–6275.0)	0.49	95.7 (27.4–325.8)	0.20	26.9 (18.1–71.6)	0.33
Positive ($n = 19$)	407.7 (28.5–2739.3)		74.6 (14.6–295.9)		26.7 (8.8–48.1)	
p27						
Negative ($n = 7$)	763.1 (379.0–6275.0)	0.04	99.0 (40.0–325.8)	0.26	29.5 (19.9–71.6)	0.09
Positive ($n = 20$)	350.3 (28.5–1520.6)		80.3 (14.6–295.9)		24.4 (8.8–48.1)	
Brg1						
Negative ($n = 13$)	443.9 (28.5–1480.6)	0.37	56.6 (14.6–116.2)	0.05	18.1 (8.8–48.1)	0.02
Positive ($n = 15$)	473.6 (161.2–6275.0)		114.8 (27.4–325.8)		27.5 (19.9–71.6)	

UFC urine free cortisol, *ACTH* adrenocorticotropic hormone, *PTTG1* antipituitary tumor-transforming gene 1, *CD1* cyclin D1, *Brg1* brahma related-gene 1

cyclin D1 (1 + –3 +), and the remaining 15 subjects were categorized as the cyclin D1-negative staining group. In addition, serum cortisol levels in the cyclin D1-negative group were significantly higher compared to the cyclin D1-positive group ($p = 0.01$), although no statistical difference was observed in plasma ACTH levels between the two groups. Furthermore, Ki-67 was expressed in 50% of patients; however, Ki-67 LI did not reflect hormonal levels of corticotroph adenomas (data not shown). The majority (20, 71.4%) of the tumors were categorized as microadenoma; among them, 7 cases were invisible tumors on the sella MRI (Supplemental Table 1). In addition, total of 21 tumors (75%) were categorized as Hardy type 1; Hardy type 3 and 4 accounted for only 10.7% of cases.

Representative images for Ki-67, p16, p27, and Brg1 expression are presented in Fig. 2. Expression of p16 was absent in 9 patients (32.1%); however, the presence of p16 expression did not influence hormone levels. Meanwhile, p27 staining was properly done in 27 tumors (96.4%); the positivity of p27 staining was present in 20 patients (71.4%). The 24 h UFC levels were significantly higher in the p27-negative group than in the p27-positive group ($p = 0.04$). In the case of Brg1, 15 patients (53.6%) had positive staining. Even though the Brg1-positive group tended to show higher 24 h UFC and plasma ACTH levels compared to the Brg1-negative group, only serum cortisol level was significantly different between the two groups ($p = 0.02$). As shown in Supplemental Table 2, the number of patients with Hardy type 2 or greater and macroadenomas showed a tendency to increase in the Brg1-positive group compared to the Brg1-negative group, although the statistical significance was not definite.

Differences in follow-up hormone levels after surgery according to the expression of cellular markers in patients with CD

To analyze the effects of cellular marker expression on changes in ACTH-secreting pituitary tumor hormonal activity, we used each difference between the values of hormone at baseline and those within 3 months after TSS because several studies found that early postoperative hormone levels, such as basal serum cortisol and plasma ACTH levels, can predict remission after surgery for CD patients [3, 15, 22, 23]. Most of the postoperative hormonal changes were not associated with the expression of cellular markers, except for PTTG1 and p16. In the case of PTTG1, the degree of reduction in 24 h UFC levels measured at 1 week after the surgery was significantly greater in the high-grade group (3+) than in the low-grade group (1 + –2 + , $p = 0.037$, Supplemental Table 3). Moreover, compared to baseline plasma ACTH levels, there were significant differences in the change in plasma ACTH levels measured at 24 h after TSS between p16-positive and p16-negative groups ($p = 0.035$, Supplemental Table 4). Supplemental Fig. 1 shows changes in postoperative plasma ACTH levels according to each time point and the expression of cyclin D, p27, and Brg1, respectively.

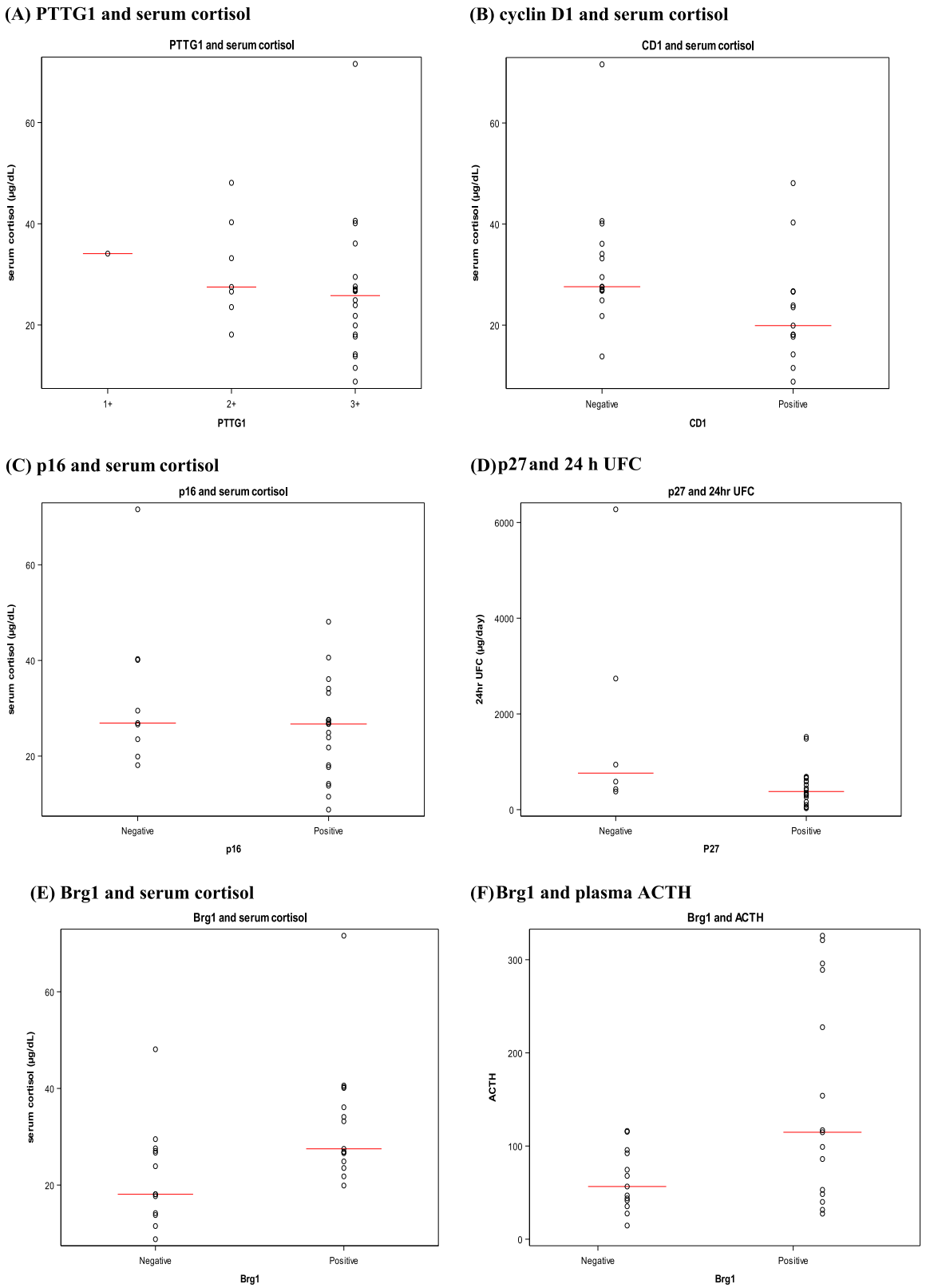
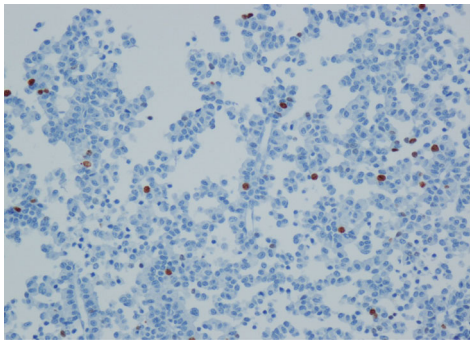
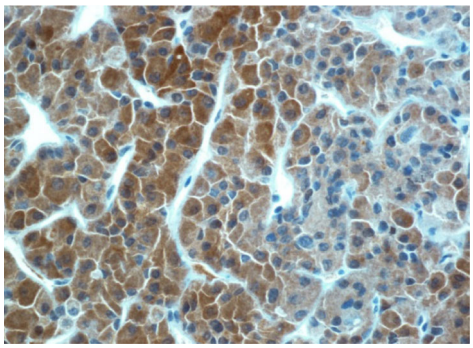


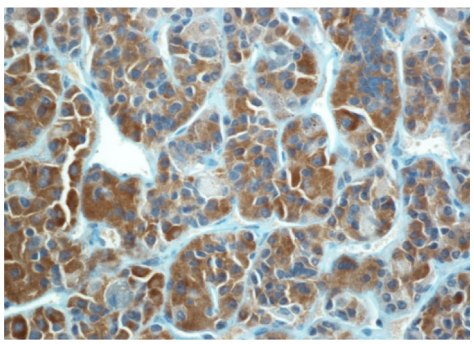
Fig. 1 Distribution of hormonal levels according to the expression of each cellular marker in patients with Cushing's disease. **a**, **b**, and **c** show the distribution of serum cortisol levels according to PTTG1, cyclin D1, and p16, respectively. **d** The pattern of 24 h UFC levels according to the presence of p27. **e** and **f** show serum cortisol and plasma ACTH levels according to the expression of Brg1. Among them, **b**, **d**, and **e** showed significant results. The red line indicates the median value of each hormone level. UFC urine free cortisol, ACTH adrenocorticotropic hormone, PTTG-1 antipituitary tumor-transforming gene 1, CD1 cyclin D1, Brg1 brahma related-gene 1



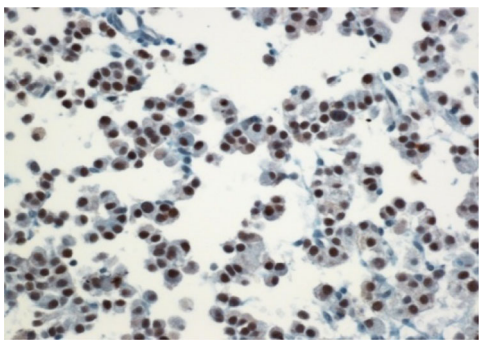
(A) Ki-67, X200 magnification.



(B) p16, X400 magnification.



(C) p27, X400 magnification



(D) Brg1, X400 magnification

Fig. 2 Immunohistochemical staining patterns for Ki-67, p16, p27, and Brg1 in corticotroph adenomas. **a**, **b**, **c**, and **d** show Ki-67 (×200 magnification), p16, p27, and Brg1 expression patterns (×400 magnification) in corticotroph adenomas, respectively. Ki-67 nuclear expression, p16 cytoplamic and/or nuclear expression, p27 cytoplasmic expression, Brg1 nuclear expression. Brg1 brahma related-gene 1

Immunohistochemical staining characteristics in patients with CD who showed recurrence

Surgical remission was achieved in 25 patients (92.6%); among them, tumor recurrence occurred in only three patients. Table 2 summarizes the immunohistochemical staining findings and preoperative hormone levels in CD patients with recurrence. All patients were female and their median age was about 28 years (range: 19–50 years), which was younger than those without recurrence (median: 40 years of age, range: 18–60 years), although there was no significant difference in age according to the presence of recurrence. In particular, p16 was expressed in all patients who showed recurrence. Moreover, all cases were categorized as Hardy type 1; only 1 case showed macroadenoma with elevated hormone levels beyond a reference range.

Discussion

Hypercortisolism is associated with increased risk for a variety of co-morbidities, including diabetes mellitus, dyslipidemia, arterial hypertension, sarcopenia, and osteoporosis, contributing to increased mortality in CD patients [4, 24]. Accordingly, Harvey Cushing reported a 4.6-year median survival [25]; the major causes causing mortality in Cushing's syndrome were cardiovascular events (40%) and infection (12%) [26], rather than tumor size or invasiveness itself. CD is usually caused by benign and small pituitary corticotroph adenomas [27] that are sometimes invisible on MRI; nevertheless, none of the diagnostic tests are 100% reliable [28]. That's why we should pay attention to the hormonal aggressiveness in CD patients. Moreover, a previous study reported that 25% of CD subjects who achieved remission after TSS showed recurrence during a mean follow-up of 45 months [10]. Because it is not certain whether remission can entirely normalize these morbidities or survival rates, long-term follow-up is required even after successful tumor surgery [7]. Nonetheless, no existing biomarker has sufficient accuracy for predicting the outcome of CD patients when used alone. Furthermore, until now, there have been various approaches to explore several oncogenes or loss of tumor-suppressor genes involved in the development of ACTH-secreting tumors [13, 29]; however, it is not known how they affect hormonal aggressiveness closely related to clinical manifestation in subjects with CD. Therefore, the current study was designed to investigate the effect of cellular marker expression regarding tumor progression on initial or follow-up hormone levels in patients with CD.

A variety of factors, including genetic events, hormone, and growth factors, have been implicated as etiologies for

Table 2 Comparison of corticotroph adenoma immunohistochemical staining and preoperative hormone levels or tumor invasiveness in CD patients who showed recurrence

Patient No.	Age	Sex	Ki-67 Li	PTTG1	CD1	p16	p27	Brg1	24 h UFC (μg/day)	Plasma ACTH (pg/mL)	Serum cortisol (μg/dL)	Tumor size	Hardy type
1	50	F	0	3+	1+	2+M	3+	0	28.5	74.6	11.5	Micro	1
16	19	F	2.52	3+M-S	2+	2+M	3+	0	57.8	47.0	17.7	Micro	1
18	28	F	3.76	2+	0	2+W	0	3+W	379.0	40.0	33.2	Macro	1

Ki-67 LI Ki-67 labeling index, *PTTG1* antipituitary tumor-transforming gene 1, *CD1* cyclin D1, *Brg1* brahma related-gene 1, *UFC* urine free cortisol, *ACTH* adrenocorticotropic hormone, *Micro* microadenoma, *Macro* macroadenoma *grade 0*, 0%, *grade 1+* less than 10%, *grade 2+* 10–49%, *grade 3+* greater than 50%, *W* weakly positive, *M* moderate, *S* severe

the development of pituitary tumors [30, 31] as they seem to interact with one another initiating cell transformation and promoting tumor proliferation [12]. In addition, impaired feedback inhibition might be one of the underlying mechanisms for developing pituitary adenomas [12]. Corticotroph adenomas, which produce excess ACTH, are also known to be resistant to Gc negative feedback [32], and the loss of Gc feedback has been thought to be an early event in tumorigenesis [27].

PTTG, a securin responsible for separation of chromatin during mitosis, might also be involved in pituitary tumorigenesis as a proto-oncogene [29, 33]. Even though many studies showed that PTTG is generally correlated with the invasiveness of pituitary adenomas [29], the relationship between PTTG expression and aggressiveness of pituitary tumors is still debated [34]. Meanwhile, increased expression of Ki-67, an index for cellular proliferation [33], was reported to be better for predicting recurrence than PTTG in pituitary adenomas [35]. On the other hand, according to Scheithauer et al., the association between Ki-67 expression and tumor recurrence was not found [36]. Similarly, one study also reported that Ki-67 was not related to ACTH-secreting pituitary tumor recurrence status [33]. In this study, expression of PTTG1 was detected in 100% of corticotroph adenomas, whereas Ki-67 expression was present in only 50%; contrary to our expectations, we observed that both PTTG1 and Ki-67 LI were not linked to initial hormone levels of ACTH-secreting tumors. Although PTTG1 expression appeared to be associated with the reduction of 24 h UFC levels measured at 1 week after surgery, we do not yet know the clinical significance of this finding due to the small sample size.

It is well known that alterations in the expression of cell cycle regulators such as cyclins and/or cyclin-dependent kinases have been implicated in pituitary tumorigenesis [29, 37]; about 80% of pituitary adenomas in humans revealed changes in at least one cell cycle regulator [38]. Particularly, cyclin D1, a protein encoded for by the bcl-1 or PRAD1-CCND1 gene, together with CDK4 and CDK6, is known to be responsible for S phase transition of the cell cycle during the G1 phase, thereby initiating oncogenesis [39]. According to Jordan et al., cyclin D1 overexpression was frequent in non-functioning and aggressive pituitary tumors, whereas it was not frequently expressed in corticotroph adenomas compared to normal pituitary tissue [39]. Furthermore, one study reported a significant downregulation of cyclin D1 expression in ACTH-secreting pituitary adenomas [40]. The current study also showed only about half of the tumors had positive staining for cyclin D1. In this study, it is noteworthy that downregulation of cyclin D1 in human ACTH-secreting tumors may reflect hormonal activity, such as serum cortisol levels, suggesting the potential of cyclin D1 as a biomarker for identifying hormonal aggressiveness in patients with CD. Reisman D et al. demonstrated that Gc appears to directly inhibit cell proliferation by virtue of its ability to suppress the expression of cyclin D in lymphoid cells, regardless of cell cycle progression [41]. Therefore, further study is needed to investigate the exact role of downregulated cyclin D1 expression in developing hormonal aggressiveness of CD.

p16 is one of the CDK inhibitors that inhibits CDK4; it functions as a tumor suppressor through preventing phosphorylation and the following inactivation of retinoblastoma-associated protein (Rb) [29]. If p16 silencing occurs, Rb1 can be phosphorylated, which enables cell cycle progression through activated E2F transcription factor [42]. Meanwhile, epigenetic silencing of the CDK inhibitor p27 has also been regarded as another mechanism of tumorigenesis in subjects with CD [12]; one study demonstrated the relationship between a lack of p27/kip1 expression and malignant corticotroph adenomas [43]. Especially, decreased p27 expression has been reported to cause upregulation of cyclin E expression in ACTH-secreting tumors [39, 44]. Moreover, Roussel-Gervais et al. found that $p27^{Kip1}$ loss-of-function and cyclin E upregulation may cooperatively be involved in pituitary tumorigenesis [45]. Nonetheless, the expression of cyclin E in ACTH-secreting tumors appears to be controversial. Tani et al. showed that increased expression of $p16^{INK4a}$ with the concomitant downregulated cyclin E1, as well as cyclin D1 might contribute to the small size of corticotroph adenomas. Gc excess might directly lead to the upregulation of CDK

inhibitor 2A ($p16^{INK4a}$), although the significant correlation between $p16^{INK4a}$ expression and serum cortisol level was not observed [40], which is in part consistent with our findings.

In this study, we also detected the expression of both p16 and p27 in corticotroph adenomas (67.9% and 74.1%, respectively); however, there was significant difference in 24 h UFC levels according to the presence of p27 expression ($p = 0.04$), but not p16. Additionally, even though the statistical significance was not definite, the remaining hormone levels such as plasma ACTH and serum cortisol also had a tendency to increase in the p27-negative group, suggesting the possibility that p27 can reflect initial hormonal aggressiveness in patients with CD. Moreover, there was statistically significant differences in reduction of plasma ACTH levels measured at 24 h after TSS according to the presence of p16 ($p = 0.035$); however, it was difficult to determine how p16 influences follow-up hormone levels due to the small sample size of the current study. Additionally, all cases with recurrence were categorized as Hardy type 1; among them, only 1 case showed macroadenoma with elevated hormone levels beyond a reference range, indicating that tumor size and invasiveness or preoperative hormone levels cannot fully explain the recurrence of corticotroph adenomas. However, it is noteworthy that increased expression of p16 was identified in all of the CD patients who showed recurrence. Recently, senescence of the pituitary has been proposed as a significant mechanism in pituitary tumorigenesis [46, 47]; one study demonstrated that pituitary adenomas showed decreased p16 and increased PTTG, whereas normal pituitary exhibited exactly the opposite pattern [46]. Hence, further investigations using a larger sample size are needed to clarify the influence of p16 expression on tumor aggressiveness in subjects with CD.

Brg1, which is one of the ATPase subunits of mammalian SWI/SNF2 enzymes, has been regarded as a tumor suppressor [48]. In contrast, recent studies have shown that Brg1 overexpression is associated with cell proliferation and cancer progression in various tumor types, including melanoma, glioma, pancreatic cancer, and others [48]. Brg1 may be partly involved in the process of Gc repression of the *POMC* gene [32], and the loss of nuclear Brg1 might lead to Gc resistance and disrupted control of the cell cycle through derepression of cyclin E expression in corticotroph cells [45]. Moreover, Brg1 loss of function is correlated with a loss of $p27^{Kip1}$ in corticotrophinomas [45]. In the current study, Brg1 expression was identified in 53.6% of CD patients, similar to previous results [27]. Intriguingly, serum cortisol level was significantly higher in the Brg1-positive group relative to the Brg1-negative group. Furthermore, 24 h UFC and plasma ACTH levels had a tendency to increase in the Brg1-positive group, although no significant differences in these hormone levels between the two groups were revealed. We speculated that increased expression of Brg1 is linked to more aggressive clinical features in ACTH-secreting tumors, suggesting the possibility that Brg1 may play an essential role as a tumor driver, unlike the traditional role of Brg1 in CD.

To our knowledge, this is the first clinical study to investigate how the expression of cellular markers of cell proliferation and tumor progression in corticotroph adenomas can affect hormonal aggressiveness in patients with CD. Moreover, a single pathologist interpreted the immunohistochemical stained slides to reduce inter-individual variation. Additionally, patients with CD are usually treated and followed according to same protocol; therefore, both baseline and follow-up hormone levels could be measured and investigated at the same time points. However, our study also has some limitations. First, the sample size of the current study was small. Moreover, nonparametric statistics have inevitable limitations because the comparison was performed based on the rank of variables without using mean values, which could be why we did not find associations between each marker and postoperative hormone activity. Second, we conducted this study using a single measurement of hormone levels. Thirdly, we did not confirm the association between the expression of these cellular markers and hormone levels in the control group. Fourthly, we did not have enough medical records such as pathologic diagnosis of the tumors due to the long-term follow-up duration. Finally, our findings itself cannot show how these cellular markers affect hormonal levels in CD patients.

In summary, although PTTG1 and Ki-67 play essential roles in developing ACTH-secreting tumors, cyclin D1, p27, and Brg1 may be better biomarkers to determine initial hormonal aggressiveness of corticotroph adenomas; their baseline hormone levels were associated with decreased expression of cyclin D1 and p27 and increased expression of Brg1. Our findings also suggest the possibility that these markers may help provide important insights into potential new therapeutic targets for CD. Therefore, a larger study with long-term follow-up will be needed to understand the role of cellular markers in the progression or recurrence of corticotroph adenomas.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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