

Function of cell-cycle regulators in predicting silent pituitary adenoma progression following surgical resection

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Abstract. The present study investigated the use of cell-cycle regulators for predicting the progression of silent pituitary adenoma (SPA) following surgical resection, via immunohistochemical analysis of tumor samples obtained by surgical resection. The medical records of patients diagnosed with SPA between January 2000 and December 2013 in the Samsung Changwon Hospital, Sungkyunkwan University School of Medicine (Changwon, South Korea) were reviewed. Immunohistochemical staining was performed on sections of the archived, paraffin-embedded tissues obtained by surgery, with all tissues stained for cell-cycle regulatory proteins p16, p15, p21, cyclin-dependent kinase (CDK)4, CDK6, retinoblastoma protein (pRb) and cyclin D1, as well as E3 ubiquitin-protein ligase mib1 (MIB-1) antigen and p53. The primary end-point was to investigate the expression of cell-cycle regulatory proteins in SPA. The secondary end-point was to estimate the progression-free survival of patients with SPA following surgical resection and to identify its association with the expression of cell-cycle regulatory proteins. Of the 127 SPA samples, 44 (34.6%) were from patients with progression during a mean follow-up period of 62.4 months (range, 24.2-118.9 months). Immunohistochemical overexpression was identified in 61 samples (48.0%) for p16, 38 samples (29.9%) for p15, 19 samples (15.0%) for p21, 49 samples (38.6%) for CDK4, 17 samples (13.4%) for CDK6, 57 samples (44.9%) for pRb and in 65 samples (51.2%) for cyclin D1. Multivariate analysis revealed that null cell adenoma [95% confidence interval (CI), 0.276-0.808], somatotroph SPAs (95% CI, 1.296-3.121), corticotroph SPAs (95% CI, 1.811-4.078), pluripotent SPAs (95% CI, 2.264-5.194), decreased expression of p16 (95% CI,

2.724-5.588), overexpression of pRb (95% CI, 2.557-5.333), cyclin D1 (95% CI, 1.894-4.122) and MIB-1 (95% CI, 1.561-4.133), increased mitotic index (95% CI, 1.228-4.079), increased p53 expression (95% CI, 1.307-4.065) and invasion into the cavernous sinus (95% CI, 3.842-7.502) predicted SPA progression following resection. The results of the present study suggested that specific cell-cycle regulators, including p16, cyclin D1 and pRb, were associated with SPA progression.

Introduction

Pituitary adenomas (PAs) are typically benign and slow-growing tumors that arise in and consist of adenohypophyseal cells (1). PAs are the third most common type of neoplasm in the central nervous system, the most common type of lesion in the sellar region, and represent between 10 and 15% of intracranial tumors (1). Depending on the hormonal secretion of PAs, they are classified as functioning or non-functioning (silent) (2). Silent PAs (SPAs) can either lack secretion of a sufficient level of hormonal product to increase the serum concentration (totally silent), or can secrete hormonal products that do not cause clinical symptoms or signs that are usual for that hormone (clinically silent) (2). In a cross-sectional study of the inhabitants of Banbury (UK), the prevalence of SPA was 77.6 cases per 100,000 individuals and of these, 28% exhibited totally silent adenomas (3). In a retrospective study of a Finnish population covering 16 years, 37% of 164 new cases of PA were classified as SPA, which is equivalent to an incidence rate of ~3 novel clinical SPAs per 100,000 individuals over 2 years (4). Therapeutic strategies for SPAs vary according to the neurological symptoms and the clinical manifestations caused by excessive adenohypophyseal hormone (5). Observation without treatment may be an option for patients without neurological symptoms. Transsphenoidal surgery is the only treatment with a high likelihood of rapidly alleviating symptoms. Radiation therapy may be used to prevent regrowth of residual adenomas following resection (5). Finally, pharmacological treatment is available for a number of subtypes of SPA, including dopamine agonists for lactotroph adenomas and somatostatin analogs for somatotroph adenomas (5).

The totally silent subgroup of PAs exhibits biological behaviors that are different from the clinically silent

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subgroup (6). Null cell adenomas and oncocytomas are assumed to be slow-growing tumors, whereas a number of clinical SPAs, particularly silent corticotroph adenomas and somatotroph adenomas, grow more rapidly, are more prone to be associated with apoplexy or invasiveness and exhibit a higher recurrence rate (7-11). Thus, the application of a reasonably established therapeutic strategy may occur if the surgical specimens are predictive of recurrence. Due to this fact, previous studies have investigated the histological indices of proliferative potential in resected tumors, including bromodeoxyuridine, proliferating cell nuclear antigen and Ki-67 cell cycle-specific nuclear antigen, to determine the association between histological invasiveness and PA recurrence (12-15). According to a previous analysis of human PAs, there are two major molecular alterations to cell cycle regulation during pituitary tumorigenesis; one is the disruption of cyclin-dependent kinase (CDK) regulation and CDK inhibition suppression (16), another is derived from damage to the retinoblastoma protein (pRb) signaling pathway (17). In spite of the effect of cell-cycle deregulation during pituitary tumorigenesis, the prognostic significance of alterations in the expression of cell-cycle regulators remains unknown in SPAs.

In the present study, the immunohistochemical expression of cell-cycle regulators (CDK4, CDK6, p16, p15, p21 and cyclin D1) and the pRb signaling pathway was investigated in SPA samples to determine the prognostic value for SPA progression following surgical resection. In addition, factors associated with progression-free survival in patients with SPA were characterized.

Patients and methods

Patients. The present study protocol was approved by the Institutional Review Board of Samsung Changwon Hospital (Changwon, South Korea), and all patients or families provided written informed consent. A retrospective case study and clinical review was conducted of 312 PAs treated surgically between January 2000 and December 2013. All patients underwent surgery and a tumor sample was obtained for diagnosis. Inclusion criteria were: i) No clinical manifestation of excessive pituitary hormone; and ii) serum pituitary hormone <1.5 times the upper normal limit [8 ng/ml for growth hormone (GH); 23.0 ng/ml for prolactin (PRL), 21 mIU/ml for follicle-stimulating hormone (FSH), 95 mIU/ml for luteinizing hormone (LH), 4.2 μ IU/ml in thyroid-stimulating hormone (TSH) and 60 pg/ml for adrenocorticotrophic hormone (ACTH)]. Patients undergoing or with a history of medical treatment for functioning PA, including dopamine agonist or somatostatin treatment, were excluded from the present study. Surgical indications were as follows: i) Presence of focal neurological symptoms, including visual field defect and extraocular muscle palsy; ii) altered mentation due to pituitary apoplexy; iii) tumor expansion causing headache and/or hydrocephalus; and iv) patient's requirement to relieve a fear of possessing a growing tumor, even one with a small size.

Biochemical analysis was performed for serum adeno-hypophysial hormones and pituitary function assessed by basal and dynamic testing. According to the protocol of our institute (18), preoperative serum concentrations of ACTH, TSH, LH, FSH, GH, PRL, cortisol, free T4, estradiol and testosterone

were measured. Due to the lack of clinical markers associated with hormonal hypersecretion of ACTH or GH (e.g., hypercortisolism and acromegalic features), screening and dynamic diagnostic tests for Cushing's syndrome or acromegaly were not performed. Preoperative evaluations of adrenal function, including insulin tolerance tests (ITT) and rapid ACTH stimulation test, were not routinely performed as perioperative glucocorticosteroid replacement was routinely conducted during transsphenoidal surgery of pituitary adenomas at Samsung Changwon Hospital. Postoperative evaluations of pituitary function, such as thyroid function tests, and determinations of LH, FSH, estradiol and testosterone concentrations were conducted in all patients. Tests for the evaluation of postoperative adrenal insufficiency (e.g., ITT or rapid ACTH stimulation tests) were performed for all the patients who exhibited decreased levels of ACTH or cortisol compared to the preoperative results.

Central hypothyroidism was defined as a low free T4 level in the presence of an inappropriate TSH level. Central hypogonadism was diagnosed in the presence of a low estradiol or testosterone level and an inappropriately normal or low gonadotrophin level. Concomitant hyperprolactinemia was defined as a PRL level above the normal range and not consistent with the diagnosis of PRL-secreting adenoma. Hormone values were interpreted according to the normal ranges observed in our laboratory. Postoperative serum pituitary hormone level was regularly checked at every 6 month at least 2 years.

Neuroradiological results of SPAs. SPAs were categorized using the Knosp classification, which determines parasellar growth of SPAs on the basis of coronal sections of pre- and gadolinium-enhanced magnetic resonance imaging (MRI) (19). Knosp grade 0-I lesions were enclosed adenomas and Knosp grade II-IV lesions were invasive adenoma (19).

All patients underwent preoperative dynamic sellar MRI. Postoperative sellar MRI was performed immediately after surgery to evaluate any residual mass, and the again at 3-month or 6-month intervals within the first 2 years. If tumor-related symptoms were suspected or hormonal alterations developed, sellar MRI was performed immediately. Surgical extent [gross total resection (GTR) and subtotal resection (STR)] was estimated using intraoperative results and by the immediate postoperative MRI, typically within 72 h after surgery.

Tumor progression was defined as the development of a new tumor in patients with a completely resected tumor or evidence of a new growth of an incompletely resected tumor on serial postoperative MRI. Radiological reviews were conducted by two independent neuroradiologists who were blinded to patient information to classify the SPAs according to the radiological scheme and to determine the tumor progression.

Immunohistochemistry. All SPA specimens were immunohistochemically examined for adeno-hypophysial hormones [GH (cat. no. NB500-364; dilution 1:1,000; Novus Biologicals, LLC, Littleton, CO, USA), PRL (cat. no. LS-C50522-0.25; dilution 1:500; LifeSpan BioSciences, Inc., Seattle, WA, USA) FSH (cat. no. GTX41060; dilution 1:500; GeneTex, Inc., Irvine, CA, USA), LH (cat. no. LH-101AP; dilution 1:2,000; FabGennix International, Inc., Frisco, TX, USA), TSH (cat. no. LS-C194634-500; dilution 1:200; LifeSpan BioSciences,

Table I. Results of ROC curve analysis of cell-cycle regulatory proteins and proliferative markers, and determination of the threshold values.

A, Cell cycle regulatory proteins					
Protein	Proportion of IHC staining nuclei ^a	AUC in ROC curve	Threshold value, %	Sensitivity, %	Specificity, %
p16	6.43±3.51	0.62	7	58.2	80.2
p15	9.39±5.28	0.64	10	53.1	78.3
p21	10.20±7.28	0.72	12	62.4	70.6
CDK4	18.35±12.29	0.70	20	66.7	79.3
CDK6	33.21±18.08	0.77	35	73.2	75.6
pRb	22.43±13.58	0.72	25	61.4	76.8
Cyclin D1	4.52±3.18	0.78	5	69.1	76.4
B, Proliferative markers					
Marker	Proportion of IHC staining nuclei ^a	AUC in ROC curve	Threshold value, %	Sensitivity, %	Specificity, %
MIB-1	1.76±0.91	0.70	2	62.1	77.0
Mitotic index	1.67±0.81	0.75	2	68.8	70.7
p53	2.56±1.32	0.67	3	64.4	67.3

^aMean ± standard deviation. AUC, area under curve; CDK, cyclin-dependent kinase; IHC, immunohistochemical; pRb, retinoblastoma protein; MIB-1, E3 ubiquitin-protein ligase mib1; ROC, receiver operating characteristic.

Inc.) and ACTH (cat. no. GTX89560; dilution 1:50; GeneTex, Inc.), cell-cycle regulatory proteins (p16, p15, p21, CDK4, CDK6, pRb and cyclin D1) and proliferative markers [E3 ubiquitin-protein ligase mib1 (MIB-1) antigen, mitosis and p53].

Immunohistochemical analysis with a labeled streptavidin-biotin method was performed as described previously by Shim *et al* (20). Monoclonal or polyclonal primary antibodies against cell cycle regulators and proliferative markers were p16 (cat. no. abx018127; dilution 1:100; Abexa Ltd., Cambridge, MA, USA), p15 (cat. no. ab53034, dilution 1:100; Abcam, Cambridge, MA, USA), p21 (cat. no. LS-C136937-100; dilution 1:100; LifeSpan BioSciences, Inc.), CDK4 (cat. no. LS-C330939-50; dilution 1:100; LifeSpan BioSciences, Inc.), CDK6 (cat. no. LS-C26471-100; dilution 1:100; LifeSpan BioSciences, Inc.), pRb (cat. no. LS-C51764-50; dilution 1:75; LifeSpan BioSciences, Inc.), cyclin D1 (cat. no. GTX54957; dilution 1:100; GeneTex, Inc.), MIB-1 antigen (cat. no. 43095; dilution 1:100; Signalway Antibody LLC, College Park, MD, USA) and p53 (cat. no. 2526S; dilution 1:100; Cell Signaling Technology, Inc., Danvers, MA, USA). Negative controls omitted the primary antibody and normal pituitary gland sections from autopsy specimens from 3 cadavers were used as positive controls for ACTH, GH, PRL, TSH, LH, FSH, p16, p15, p21, CDK4, CDK6, pRb and cyclin D1. Under high-power magnification (x400), 10 fields were selected in regions with the greatest concentrations of immunopositive nuclei. Each field had between 700 and 1,000 cells, similar to the tumor specimen. Areas of necrosis, normal adenohypophysial cells and endothelial cells were excluded from evaluation.

Immunoreactivity of proteins and markers was determined as a proportion of immunopositive cells from 1,000 cells, determined by manual counting. Mitosis was counted after hematoxylin and eosin staining, and the mitotic index defined as the number of mitotic cells/10 high-power fields. All slides were separately reviewed by two neuropathologists who were blinded to the clinical and radiological information. SPAs were divided into 6 categories according to immunohistochemical staining for adenohypophysial cells: Null cell adenoma, gonadotrophic SPA, somatotrophic SPA, corticotrophic SPA, lactotrophic SPA and pluripotent SPA. This was determined using the protocol of a previous study (5).

Immunoreactivity of cell-cycle regulators and proliferative markers was used to determine whether the markers affected SPA progression. Receiver operating characteristic (ROC) curve analysis was performed using SPSS software version 12.0 (SPSS Inc., Chicago, IL, USA) for immunoreactivity of the cell-cycle regulatory proteins and proliferative markers to predict the likelihood of progression (21). The area under the ROC curve was used to determine the optimal threshold of the mean proportion of immunopositive cells from 1,000 cells. Sensitivity was calculated as the true positive rate (number of true positives divided by the sum of the number of true positives and number of false negatives), specificity as the true negative rate (number of true negatives divided by the sum of the number of true negatives and number of false positives) and accuracy as the sum of the number of true positives and true negatives, divided by the total number of positives and negatives. True positives mean that the immunoreactivity proportion above the threshold value has an influence on the

long time to progression (TTP), and true negatives mean that the immunoreactivity proportion below the threshold value has an influence on the short TTP. The threshold of immunoreactivity with the greatest sensitivity and specificity was determined, and using sensitivity-specificity analysis, a threshold point at which sensitivity and specificity crossed that associated with recurrence was determined for each marker (Table I). On the basis of the threshold value for immunoreactivity for each protein and marker, sequential association analysis for SPA progression was performed.

Statistical analysis. Differences between subgroups were analyzed using Student's t-test for normally distributed, continuous values and the Mann-Whitney test for abnormally distributed, continuous values. χ^2 -tests were used to analyze categorical variables. Variables significantly associated with SPA progression by univariate analyses were used in multivariate analyses. In addition, several variables of interest that were associated with tumor progression in the literature were used in multivariate analysis. The impact of variables on progression was evaluated by comparing progression-free survival curves using a log-rank test. In multivariate analysis, the Cox proportional hazard regression model was used to assess the independent effects of specific factors on the progression rate of SPAs and to define hazard ratios (HRs) for significant covariates. $P < 0.05$ was considered to indicate a statistically significant difference. SPSS software version 12.0 (SPSS Inc.) was used for statistical analysis.

Results

Clinical characteristics of patients with SPAs. According to the review of medical records, during the study period, 312 patients underwent surgical resection for newly diagnosed PAs and 127 (40.7%) were diagnosed with SPA. All patients were treated by an endoscopic endonasal trans-sphenoidal approach. The mean patient age was 48.9 years (range, 24.2–85.6 years), with 71 males and 56 females. The most common presenting symptom was headache (35.4%) (Table II).

Radiographically, the mean maximal diameter of SPAs was 31.5 mm (range, 8.0–65.5 mm); 33.7 mm (range 10.0–65.5 mm) in patients with focal neurological symptoms, including visual field defect or extraocular muscle palsy, and 29.8 mm (8.0–52.3 mm) in those without focal neurological symptoms ($P = 0.458$, Student's t-test). GTR was performed for 82 SPAs (64.5%); 35 SPAs (62.5%) in patients with focal neurological symptoms and 47 SPAs (66.2%) in those without focal neurological symptoms ($P = 0.525$, χ^2 -test). Therefore, among patients with focal neurological symptoms, 35 patients underwent GTR, and among those without focal neurological symptoms, 24 patients underwent STR ($P = 0.172$, χ^2 -test). The size of SPA and major clinical features were not significantly influenced by the immunoreactivity of cell cycle regulators (Table III).

No patients underwent adjuvant radiotherapy without evidence of recurrence or regrowth during follow-up. In 45 patients who had residual tumors following subtotal resection, marked improvement of clinical symptoms was observed. During follow-up with regular examination of serum pituitary hormone and clinical symptoms, patients did not experience worsening symptoms or increased serum pituitary hormone.

Table II. Clinical characteristics of patients with silent pituitary adenoma.

Variable	Value
Total patients, n	127
Mean age (range), years	48.9 (24.2–85.6)
Male:female ratio	71:56
Presenting symptom, n (%)	
Headache	45 (35.4)
Visual field defect	43 (33.9)
Extraocular muscle palsy	13 (10.2)
Altered mentation	7 (5.5)
None (incidental detection)	19 (15.0)
Cell type, n (%)	
Null cell	29 (22.8)
Gonadotroph	41 (32.3)
Somatotroph	19 (15.0)
Corticotroph	16 (12.6)
Lactotroph	15 (11.8)
Pluripotent	7 (5.5)
Knosp classification, n (%)	
0-I (enclosed adenoma)	76 (59.8)
II-IV (invasive adenoma)	51 (40.2)
Mean maximum diameter (range), mm	31.5 (8.0–6.5)
Extent of surgical resection, n (%)	
Gross total resection	82 (64.6)
Subtotal resection	45 (35.4)

Immunohistochemical staining. Adenohypophysial cells were immunohistochemically stained, which revealed gonadotrophic cells in 41 samples (32.2%), somatotrophic cells in 19 samples (15.0%), corticotrophic cells in 16 samples (12.6%), lactotrophic cells in 15 samples and ≥ 2 adenohypophysial cells in 7 samples (5.5%) (Table II).

According to the ROC curve analysis, immunohistochemical overexpression above the threshold value was determined as follows: 7% in p16, 10% in p15, 12% in p21, 20% in CDK4, 35% in CDK6, 25% in pRb, 5% in cyclin D1, 2% in MIB-1 and 3% in p53. Cell-cycle regulatory protein analysis demonstrated that p16 was immunohistochemically overexpressed above the threshold value in 61 samples (48.0%), p15 in 38 samples (29.9%), p21 in 19 samples (15.0%), CDK4 in 49 samples (38.6%), CDK6 in 17 samples (13.4%), pRb in 57 samples (44.9%) and cyclin D1 in 65 samples (51.2%) (Fig. 1). Immunohistochemical overexpression of the proliferative marker MIB-1 was identified in 54 samples (42.5%), mitotic index increased in 51 samples (40.2%) and immunohistochemical overexpression of p53 was observed in 39 samples (30.7%). Additionally, there was no statistical difference in the immunoreactivity for cell cycle regulators and proliferative markers according to the cell types (Table IV).

Progression of SPAs following surgery. All patients were followed for ≥ 24 months and the mean follow-up duration was 60.9 months (range, 25.3–137.4 months). During follow-up,

Table III. Size of silent pituitary adenomas and the presence of focal neurological symptoms, including visual field defects and extraocular muscle palsy, according to the results of immunohistochemical staining for cell-cycle regulators.

Protein	IHC staining	Mean maximal diameter ($\pm$ SD), mm	P-value ^a	Patients with focal neurological symptoms, n (%)	P-value ^b
p16	Overexpression	28.9 ($\pm$ 10.2)	0.162	25 (41.0)	0.118
	Decreased expression	33.9 ($\pm$ 11.0)		31 (47.0)	
p15	Overexpression	30.7 ($\pm$ 9.8)	0.758	16 (42.1)	0.843
	Decreased expression	31.8 ($\pm$ 10.5)		40 (44.9)	
p21	Overexpression	28.4 ($\pm$ 8.7)	0.097	9 (47.4)	0.251
	Decreased expression	32.0 ($\pm$ 10.3)		47 (43.5)	
CDK4	Overexpression	33.8 ($\pm$ 11.5)	0.254	22 (44.9)	0.913
	Decreased expression	30.1 ($\pm$ 8.8)		34 (43.6)	
CDK6	Overexpression	32.6 ($\pm$ 9.6)	0.682	7 (41.2)	0.681
	Decreased expression	31.3 ($\pm$ 7.9)		49 (44.5)	
pRb	Overexpression	33.4 ($\pm$ 10.4)	0.206	27 (47.4)	0.104
	Decreased expression	30.0 ($\pm$ 11.2)		29 (41.4)	
Cyclin D1	Overexpression	32.2 ($\pm$ 12.1)	0.341	30 (46.2)	0.237
	Decreased expression	30.8 ($\pm$ 9.6)		26 (41.9)	

^aStudent's t-test, ^b χ^2 -test. CDK, cyclin-dependent kinase; IHC, immunohistochemical; pRb, retinoblastoma protein; SD, standard deviation.

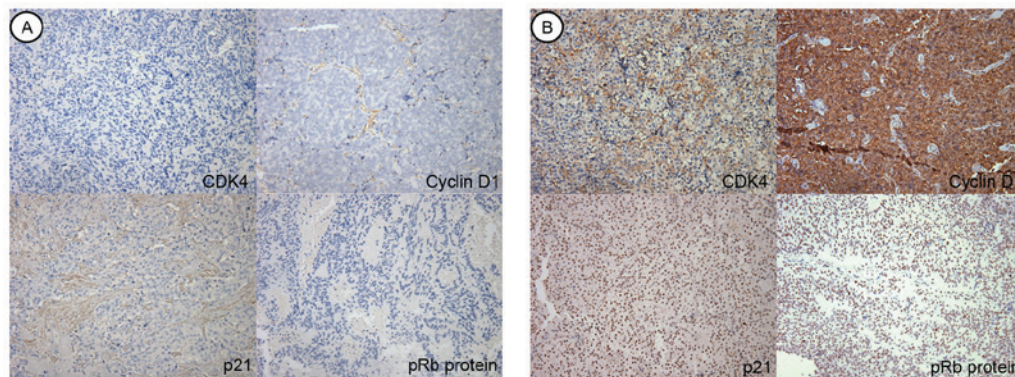


Figure 1. Results of immunohistochemical staining for cell cycle regulatory proteins (magnification, x10). (A) immunohistochemical understaining of CDK4 (<20%), cyclin D1 (<5%), p21 (<12%) and pRb (<25%). (B) Immunohistochemical overstaining of CDK4 ($\geq$ 20%), cyclin D1 ($\geq$ 5%), p21 ($\geq$ 12%) and pRb ($\geq$ 25%). CDK, cyclin-dependent kinase; pRb, retinoblastoma protein.

44 patients (34.6%) experienced progression following surgical resection and the mean TTP following surgery was 36.6 months (range, 5.6-89.4 months). By cell type, progression occurred for 6/29 (20.7%) null cell adenomas, 10/41 (24.4%) gonadotrophic SPAs, 9/19 (47.4%) somatotrophic SPAs, 8/16 (50.0%) corticotrophic SPAs, 6/15 (40.0%) lactotrophic SPAs and 5/7 (71.4%) pluripotent SPAs.

Immunohistochemical overexpression and decreased staining are associated with mean TPP. In the cell-cycle regulatory proteins, the mean TTP was increased in patients with immunohistochemical overexpression compared with that in patients with decreased expression ($P=0.002$). The mean TTP was also shorter in patients with immunohistochemical overexpression, compared with those with decreased expression, for pRb ($P=0.009$) and cyclin D1 ($P=0.028$) (Table V). In the

proliferative markers, the mean TTP was decreased in patients with immunohistochemical overexpression, compared with those with immunohistochemically decreased expression, for MIB-1 antigen ($P=0.014$), mitotic index ($P=0.031$) and p53 ($P=0.047$; Table V).

Univariate analysis of predisposing factors for SPA progression. Univariate analysis revealed that progression occurred significantly more often with somatotrophic SPAs ($P=0.040$), corticotrophic SPAs ($P=0.013$) and pluripotent SPAs ($P<0.001$) compared with null cell adenomas (Table VI). In terms of cell-cycle regulatory proteins, patients with SPA samples that were immunohistochemically overexpressed for p16 exhibited significantly decreased progression rates compared with patients with decreased expression ($P<0.001$). In addition, patients with SPA samples that were

Table IV. Distribution of immunohistochemically overexpressed cell cycle regulators and proliferative markers in silent pituitary adenomas according to the cell types (n=127).

A, Cell cycle regulators

Protein	Null cell adenoma (n=29)	Gonadotroph adenoma (n=41)	Somatotroph adenoma (n=19)	Corticotroph adenoma (n=16)	Lactotroph adenoma (n=15)	Pluripotent adenoma (n=7)	P-value ^a
p16	15 (51.7)	20 (48.8)	9 (47.4)	7 (43.8)	7 (46.7)	3 (42.9)	0.816
p15	10 (34.5)	12 (29.3)	5 (26.3)	4 (25.0)	5 (33.3)	2 (28.6)	0.774
p21	5 (17.2)	6 (14.6)	2 (10.5)	2 (12.5)	3 (20.0)	1 (14.3)	0.635
CDK4	11 (37.9)	16 (39.0)	8 (42.1)	6 (37.5)	5 (33.3)	3 (42.9)	0.592
CDK6	4 (13.8)	5 (12.3)	3 (15.8)	2 (12.5)	2 (13.3)	1 (14.3)	0.872
pRb	12 (41.4)	18 (43.9)	9 (47.4)	8 (50.0)	7 (46.7)	3 (42.9)	0.526
Cyclin D1	15 (51.7)	21 (51.2)	10 (52.6)	9 (56.3)	7 (46.7)	3 (42.9)	0.603

B, Proliferative markers

Protein	Null cell adenoma (n=29)	Gonadotroph adenoma (n=41)	Somatotroph adenoma (n=19)	Corticotroph adenoma (n=16)	Lactotroph adenoma (n=15)	Pluripotent adenoma (n=7)	P-value ^a
MIB-1	11 (37.9)	16 (39.0)	10 (52.6)	9 (56.3)	5 (33.3)	3 (42.9)	0.087
Mitotic index	10 (34.5)	15 (36.6)	10 (52.6)	8 (50.0)	6 (40.0)	2 (28.6)	0.069
p53	7 (24.1)	12 (29.3)	7 (36.8)	6 (37.5)	5 (33.3)	2 (28.6)	0.381

^a χ^2 test. CDK, cyclin-dependent kinase; pRb, retinoblastoma protein; MIB-1, E3 ubiquitin-protein ligase mib1.

immunohistochemically overexpressed for pRb (P=0.008) and cyclin D1 (P=0.038) exhibited significantly increased progression rates compared with that for patients with immunohistochemically decreased expression. Patients with SPA samples that were immunohistochemically overexpressed for proliferative markers exhibited significantly increased progression rates compared with those for patients with immunohistochemically decreased expression; MIB-1 (P=0.010), mitotic index (P=0.026) and p53 (P=0.037; Table VI).

Regarding radiological factors, patients with invasive SPAs exhibited an increased progression rate compared with that of patients with enclosed SPAs (P<0.001). Furthermore, patients with SPAs $\geq$ 25 mm exhibited an increased progression rate compared with that of patients with SPAs <25 mm (P=0.042). The extent of surgical resection for SPA was not associated with progression rate (P=0.683).

Multivariate analysis of predisposing factors for SPA progression. Multivariate analysis using the Cox proportional hazard regression model for SPA progression following surgical resection demonstrated that factors independently associated with progression were null cell adenoma [hazard ratio (HR), 0.553; 95% confidence interval (CI), 0.266-0.841], somatotrophic SPAs (HR, 2.197; 95% CI, 1.298-3.096), corticotrophic SPAs (HR, 2.951; 95% CI, 1.820-4.082), pluripotent SPAs (HR, 3.694; 95% CI, 2.227-5.161), SPA samples with immunohistochemically decreased expression for p16 (HR, 4.170; 95% CI, 2.812-5.522), SPA samples overexpressing pRb (HR, 3.918; 95% CI, 2.532-5.304), SPA samples overexpressing

cyclin D1 (HR, 3.022; 95% CI, 1.898-4.146), SPA samples overexpressing MIB-1 (HR, 2.835; 95% CI, 1.549-4.121), SPA samples overexpressing p53 (HR, 2.484; 95% CI, 1.267-3.701), increased mitotic index (HR, 2.673; 95% CI, 1.235-4.111) and Knosp grades II-IV (HR, 5.677; 95% CI, 3.840-7.514) (Table VII). The Kaplan-Meier survival curve analysis and log-rank test revealed the same results (Fig. 2).

Discussion

The present study had a similar proportion of SPAs to PAs and surgical outcome as previous studies (2,5). The composition of SPA subtypes (null cell adenoma, and gonadotroph, lactotroph, somatotroph, corticotroph and pluripotent SPAs) was similar to other studies (2,5). Categorizing SPAs into clinical and true SPAs is controversial; in the present study, SPAs were defined according to the description by Mayson and Snyder (2,5). In the systemic review and meta-analysis in 2012 by Chen *et al* (22), the rate of STR was between 21.4 and 79.4% following transsphenoidal surgery of SPA, and between 12 and 46% of patients with SPA exhibited tumor regrowth between 42 and 112 months after surgery. The surgical outcomes demonstrated by Chen *et al* (22) were similar compared with the results of the present study.

According to a previous study, the primary treatment of SPA is surgical resection focused on relieving the mass effect, restoring pituitary function and validating a tissue diagnosis (23). As the majority of the SPAs are macroadenoma at diagnosis, the traditional indication of surgery for SPA is the tumor expansion associated with focal neurological symptoms.

Table V. Time to progression and progression rate according to the result of immunohistochemical staining for the cell-cycle regulators and proliferative markers, and cell types.

A, Cell cycle regulators

Protein	Threshold value, %	Sample with immunohistochemical overexpression		Sample with immunohistochemically decreased expression		P-value
		Progression, n (%)	TTP, months ^a	Progression, n (%)	TTP, months ^a	
p16	7	11/61 (18.0)	43.7±17.2	33/66 (50.0)	31.3±14.0	0.002 ^b
p15	10	11/38 (28.9)	38.2±6.8	33/89 (37.1)	32.8±15.3	0.337
p21	12	7/19 (36.8)	37.9±14.5	37/108 (34.3)	33.4±15.8	0.795
CDK4	20	19/49 (38.8)	35.2±3.8	25/78 (32.1)	37.3±15.0	0.718
CDK6	35	6/17 (35.3)	35.5±14.7	38/110 (34.5)	37.0±13.8	0.854
pRb	25	29/57 (50.9)	31.3±12.6	15/70 (21.4)	43.1±15.9	0.009 ^b
Cyclin D1	5	28/65 (43.1)	32.4±13.5	16/62 (25.8)	41.2±16.2	0.028 ^b

B, Proliferative markers

Protein	Threshold value, %	Sample with immunohistochemical overexpression		Sample with immunohistochemically decreased expression		P-value
		Progression, n (%)	TTP, months ^a	Progression, n (%)	TTP, months ^a	
MIB-1	2	25/54 (46.3)	32.2±12.1	19/73 (26.0)	41.9±15.5	0.014 ^b
Mitotic index	2	22/51 (43.1)	33.0±14.6	22/76 (28.9)	39.1±14.3	0.031 ^b
p53	3	16/39 (41.0)	33.7±10.2	28/88 (31.8)	38.8±15.2	0.047 ^b

^aMean ± standard deviation. ^bP<0.05, Mann-Whitney U test. CDK, cyclin-dependent kinase; pRb, retinoblastoma protein; MIB-1, E3 ubiquitin-protein ligase mib1; SD, standard deviation; TTP, time to progression.

Although patients with SPA in the present study underwent surgical resection following the aforementioned indications, the results of surgery for endocrine-inactive adenomas are not as clearly defined as those of surgery for functioning adenomas, as there is no clear criterion for a cure (5).

Expression levels for p16 and pRb in pituitary adenomas were similar to those identified in the study by Kirsch *et al* (23), which observed positive staining >25% for p16 in 57% of samples and for pRb in 48% of pituitary adenomas by immunohistochemical analysis. However, in the present study, the rate of positive immunoreactivity >25% was relatively low; for p16 in 13.2% of SPAs and for pRb in 28.7% of SPAs. This discrepancy may be due to the following reasons: i) Kirsch's analysis included all types of pituitary adenomas, including functioning and non-functioning pituitary adenomas; ii) there are the variations in the immunohistochemical analysis due to concentration and dilution of antibodies used; and iii) there is inter-observer variation in the subjective interpretations for immunohistochemical analysis.

The present study demonstrated that the cell-cycle regulatory proteins p16, pRb and cyclin D1, estimated using immunohistochemical staining, were associated with SPA progression following surgical resection. It was identified that pRb is involved in SPA progression. The prognostic ability

of cell-cycle regulatory proteins and proliferative markers has been identified in renal cell carcinoma (24), gallbladder malignancies (17), gastric adenocarcinoma (25) and breast cancer (26). Therefore, if these cell cycle regulators influence the tumor size and the onset of neurological symptoms, the clinical outcome of SPA may be altered. However, the principal clinical features of SPA were not different, according to the status of immunoreactivity of cell cycle regulators, which may explain why there is no effect on tumor progression from the association between cell cycle regulators and clinical features.

In the cell cycle, mitotic stimulation leads cyclin D1 to activate CDK4 and CDK6 during early G₁ phase, which partially phosphorylates pRb. Cyclin D1 typically binds to CDK during G₁ and is required to bypass the cell cycle restriction point into S phase (27), which leads to cell cycle progression and tumor cell proliferation. In addition, pRb is active and hypophosphorylated in quiescent cells, and inactive and hyperphosphorylated at the G₁-S transition. Therefore, the level of phosphorylated pRb indicates cell cycle progression into the S phase (27). Attenuated cyclin D1 and pRb expression in SPA may explain the increased SPA progression observed in the present study. CDK inhibitors, including p16, oppose CDK activation. During early G₁ phase, p16 binds to CDK4 and 6, and prevents cyclin D activation (27). Thus, increased expression

Table VI. Univariate analysis of factors predicting progression of silent pituitary adenomas (n=127).

Factor	Progression rate, %	HR	95% CI	P-value
Age	-	1.318	0.898-1.738	0.714
Cell type				
Null cell	20.7	1.000		
Gonadotroph adenoma	24.4	1.155	0.564-1.647	0.853
Somatotroph adenoma	47.4	4.372	2.325-6.419	0.040 ^a
Corticotroph adenoma	50.0	4.876	2.517-7.235	0.013 ^a
Lactotroph adenoma	40.0	2.174	0.958-3.336	0.052
Pluripotent adenoma	71.4	8.676	5.083-12.269	<0.001 ^b
Immunohistochemical overexpression of cell-cycle regulator				
p16	18.0	0.314	0.109-0.519	<0.001 ^b
p15	28.9	0.793	0.569-1.017	0.124
p21	36.8	1.503	0.759-2.247	0.526
CDK4	38.8	1.732	0.785-2.679	0.433
CDK6	35.3	1.386	0.661-2.011	0.802
pRb	50.9	6.285	3.088-9.482	0.008 ^a
Cyclin D1	43.1	4.394	1.364-7.424	0.038 ^a
Immunohistochemical overexpression of proliferative marker				
MIB-1	46.3	4.249	2.518-5.979	0.010 ^a
Mitotic index	43.1	3.782	1.843-5.721	0.026 ^a
p53	41.0	3.558	1.447-5.669	0.037 ^a
Knosp classification				
0-I	21.1	1.000		
II-IV	54.9	8.840	4.882-12.798	<0.001 ^b
Maximal diameter of Tumor	-	2.857	1.421-4.293	0.042 ^a
Extent of surgical resection				
Gross total resection	32.9	1.000		
Subtotal resection	37.8	1.358	0.790-1.926	0.683

^aP<0.05, ^bP<0.001. For immunohistochemically overexpressed cell cycle regulators and proliferative markers, the TTP of an immunohistochemically normally expressed sample was used as the standard. CDK, cyclin-dependent kinase; CI, confidence interval; MIB-1, E3 ubiquitin-protein ligase mib1; pRb, retinoblastoma protein; TTP, time to progression; HR, hazard ratio.

of p16 may prevent proliferation and SPA, which would explain the results of the present study. Although null cell adenomas are assumed to be slow-growing tumors, a number of types of silent adenoma, in particular silent corticotroph adenomas and somatotroph adenomas, grow more rapidly and are more likely to be associated with apoplexy or invasiveness, and to exhibit increased rates of progression (9,11). The results of the present study demonstrated that SPAs that were somatotroph, corticotroph or pluripotent were associated with progression. Cavernous sinus invasion, which is more frequent for SPAs that are somatotroph, corticotroph or pluripotent, makes total resection difficult and may influence progression rates.

The present study had a number of limitations. First, immunohistochemical study is well-known to have different outcomes depending on the different professionals performing the analysis; therefore, this subjective interpretation should be complemented by other objective analyses, including molecular

functional study, using several quantitative methods. Secondly, the SPA classification presents limitations. Although SPAs may be separated into true and clinical SPAs, using immunohistochemical and electron-microscope appearance (28), electron microscopic studies were not performed in the present study. Therefore, it cannot be guaranteed that the present study identified definitive classifications of SPA subtypes. Thirdly, not all cell-cycle regulatory proteins were examined. In the present study, a portion of the cell-cycle control system, specifically the G₁ to S transition, was focused on, which may have biased the interpretation of the results and neglected a number of interactions between regulatory proteins. The fourth concern is that the present study does not reflect modern molecular and biological interpretations of SPAs that are increasing our understanding of the pathobiology of SPAs.

The results of the present study suggested that expression of a number of cell-cycle regulators, including p16, pRb

Table VII. Multivariate analysis of factors predicting progression of silent pituitary adenomas (n=127).

Factor	HR	95% CI	P-value
Age ^a	1.524	0.882-2.166	0.294
Null cell vs. other cell type	0.553	0.266-0.841	0.002 ^b
Gonadotroph adenoma vs. other cell type	0.851	0.682-1.021	0.068
Somatotroph adenoma vs. other cell type	2.197	1.298-3.096	0.046 ^b
Corticotroph adenoma vs. other cell type	2.951	1.820-4.082	0.023 ^b
Lactotroph adenoma vs. other cell type	1.342	0.876-1.808	0.381
Pluripotent adenoma vs. other cell type	3.694	2.227-5.161	0.011 ^b
IHC staining for p16 (<7 vs. ≥7%)	4.170	2.812-5.522	0.001 ^b
IHC staining for p15 (<10 vs. ≥10%)	2.071	0.938-3.204	0.062
IHC staining for pRb (≥25 vs. <25%)	3.918	2.532-5.304	0.004 ^b
IHC staining for Cyclin D1 (≥5 vs. <5%)	3.022	1.898-4.146	0.013 ^b
MIB-1 (≥2 vs. <2%)	2.835	1.549-4.121	0.031 ^b
Mitotic index (≥2 vs. <2%)	2.673	1.235-4.111	0.020 ^b
IHC staining for p53 (≥3 vs. <3%)	2.484	1.267-3.701	0.029 ^b
Knosp classification (II-IV vs. 0-I)	5.677	3.840-7.514	<0.001 ^c
Maximal diameter of tumors ^a	2.016	0.913-3.119	0.118
Surgical extent (gross total resection vs. subtotal resection)	1.253	0.704-1.802	0.586

^aMultivariate analysis was performed for the factors as a form of continuous variable. ^bP<0.05; ^cP<0.001. HR, hazard interval; CI, confidence interval; IHC, immunohistochemical; pRb, retinoblastoma protein; MIB-1, E3 ubiquitin-protein ligase mib1.

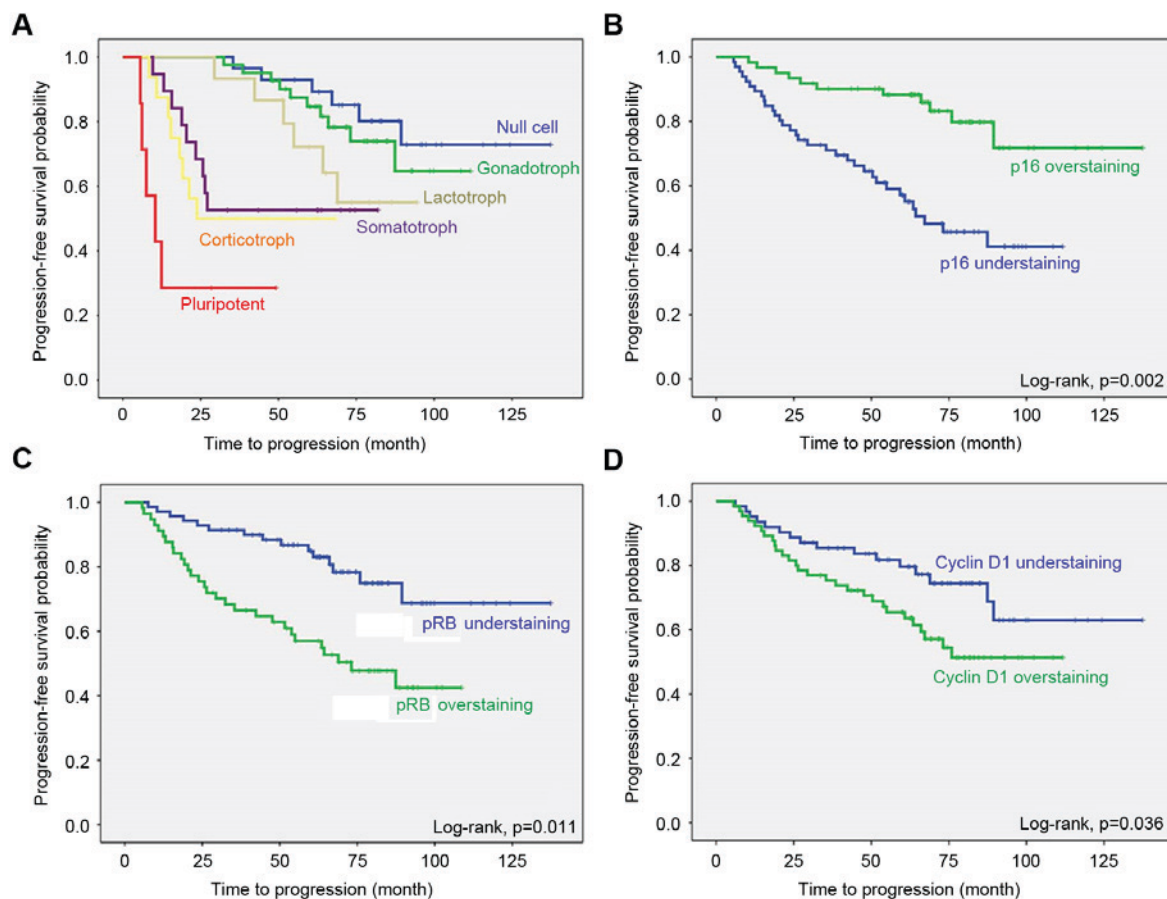


Figure 2. Progression-free survival curve for patients with silent pituitary adenomas. (A) Cells from silent pituitary adenomas. (B) Immunohistochemical understaining (<7%) vs. overstaining (≥7%) for p16. (C) Immunohistochemical understaining (<25%) vs. overstaining (≥25%) for pRb. (D) Immunohistochemical understaining (<5%) vs. overstaining (≥5%) for cyclin D1. pRb, retinoblastoma protein

and cyclin D1, were associated with SPA progression. The results were validated using known proliferative markers of MIB-1, mitotic index and p53, which were also significantly associated with SPA progression. Additional studies using systemically developed molecular biology techniques are required to characterize the pathobiological process of SPA progression.

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