Results of Immunohistochemical Staining of Cell-Cycle Regulators: The Prediction of Recurrence of Functioning Pituitary Adenoma

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Key words
- Cell cycle
- Cyclin D1
- p16
- Pituitary adenoma
- RB protein
- Recurrence

Abbreviations and Acronyms
ACTH: Adrenocorticotropic hormone
APA: Anterior pituitary adenoma
CDK: Cyclin-dependent kinase
CDKI: Cyclin-dependent kinase inhibitor
CI: Confidence interval
FPA: Functioning pituitary adenoma
FSH: Follicle-stimulating hormone
GH: Growth hormone
HR: Hazard ratio
ICA: Internal carotid artery
LH: Luteinizing hormone
MRI: Magnetic resonance imaging
pRB: Phosphorylated retinoblastoma
PRL: Prolactin
RB: Retinoblastoma
TSH: Thyroid-stimulating hormone

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OBJECTIVE: **This study was undertaken primarily to investigate the possible prognostic values of several cell-cycle regulators for the prediction of functioning pituitary adenoma (FPA) recurrence after surgical resection by immunohistochemically analyzing tumor samples obtained by surgical resection.**

METHODS: **The medical records of the patients with FPA diagnosed from January 2000 to December 2009 at the Department of Neurosurgery at Samsung Changwon Hospital and Dong-A University Medical Center were selected. Immunohistochemical staining was performed on archived paraffin-embedded tissues obtained by surgical resection for adenohypophysial cells, cell-cycle regulatory proteins (p16, p15, p21, cyclin-dependent kinase [CDK] 4 and 6, phosphorylated retinoblastoma [pRB] protein, and cyclin D1), MIB-1 antigen, and p53.**

RESULTS: **Of the 174 FPAs, 62 (35.6%) recurred during follow-up period (mean duration 62.4 months, range 24.2–118.9 months). Immunohistochemically, overstaining for p16 in 89 samples (51.1%), p15 in 27 samples (15.5%), p21 in 20 samples (11.5%), CDK4 in 54 samples (31.0%), CDK6 in 18 samples (10.3%), pRB protein in 69 samples (39.7%), and cyclin D1 in 87 samples (50.0%). Multivariate analysis using the Cox proportional hazard regression model showed that invasion into cavernous sinus (hazard ratio [HR] of 4.02; $P < 0.001$), immunohistochemical normostaining for p16 (HR of 3.16; $P < 0.001$), immunohistochemical overstaining for pRB protein (HR of 2.45; $P = 0.008$), cyclin D1 (HR of 2.13; $P = 0.029$), MIB-1 antigen (HR of 2.74; $P = 0.002$), and p53 (HR of 2.21; $P = 0.002$), predicted the recurrence of FPA after surgical resection.**

CONCLUSIONS: **Our findings indicate that p16, pRB protein, and cyclin D1 are associated with recurrence FPA after surgical resection.**

INTRODUCTION

Anterior pituitary adenomas (APAs) are benign, epithelial tumors that arise in and consist of adenohypophysial cells (23). APAs are the third most common neoplasm in the central nervous system and the most common lesion in the sella region, representing approximately 10–15% of intracranial tumors on the basis of surgical experience (6, 19, 23). Furthermore, depending on the sections examined, they are noted in 3–24% of unselected autopsies (6, 19). Thus, not surprisingly, the introductions of improved diagnostic techniques have resulted in an increase in the number of APAs discovered (6).

APAs often are slow-growing, expansive tumors, confined to the sellar trucica. However, several APAs are invasive and exhibit more growth and spread into neighboring tissues. Transsphenoidal surgery is the primary option for APAs that require surgical treatment, and a surgical cure can be expected when they are totally removed. However, because of frequent supra- or parasellar extension, surgery infrequently is curative, and tumor remnants regrow in 12–58% of patients within 5 years (45). Even after gross total resection, tumor recurrence is observed after several years in 10–20% of cases (28, 48). Thus, a reasonable organized therapeutic strategy could be instituted if recurrence could be predicted from surgical specimens, and for this reason many authors have investigated histological indices of proliferative potential in resected tumors, such as bromodeoxyuridine (26), proliferating cell nuclear antigen (26), and Ki-67 cell cycle–specific nuclear antigen (29, 56) and attempted to establish the nature of the relationship between histologic invasiveness and tumor recurrence.

Furthermore, recent molecular analyses of human pituitary neoplasias have revealed deregulation of the cell cycle during pituitary tumorigenesis, as indicated by altered cyclin-dependent kinase (CDK) regulation and suppression of the CDK inhibitory mechanism (31). For example, p16 is a member of the protein family that

specifically inhibits cyclin D-dependent kinase, which leads to the suppression of phosphorylation and activation of retinoblastoma (RB) protein (54). In addition, it has been reported that cyclin D1 is often overexpressed in pituitary tumors (22) and exhibits allelic imbalance in some tumor samples (15). Despite of the critical role of cell-cycle deregulation during pituitary tumorigenesis, the prognostic significances of alterations in the expressions of cell cycle regulators, such as CDK4, CDK6, p16, p15, p21, and RB protein, have not been defined.

In this study, by examining tumor samples obtained by surgical resection immunohistochemically, we aimed to determine the prognostic values of several cell-cycle regulators (CDK4, CDK6, p16, p15, p21, phosphorylated RB [pRB] protein, and cyclin D1) in terms of predicting functioning pituitary adenoma [FPA] recurrence after surgical resection. Also, we estimated the immunoreactivity of MIB-1 and p53, which are already known as proliferative makers that can predict the recurrence of pituitary adenomas, and we verified our result. In addition, we examined other factors predisposing patients with FPA to recurrence.

MATERIALS AND METHODS

Patients

The study protocol was approved by the institutional review board of Samsung Changwon Hospital (2011-SCMC-054-000), Dong-A University Medical Center (12-07-004), and all patients or families provided written informed consent. A retrospective case study and clinical review was conducted of the 297 pituitary tumors treated surgically by K.H.K. and Y.Z.K. at Samsung Changwon Hospital and by H.D.K. at Dong-A University Medical Center from January 2000 to December 2009. All patients underwent surgery and provided a tumor sample for diagnosis. Among them, we selected the patients who had an increased level of adenohypophysial hormone in the serum. Patients undergoing or with a history of medical treatment, such as dopamine agonist or somatostatin, for FPA were excluded.

Biochemical analysis was performed for serum adenohypophysial hormones (growth hormone [GH], prolactin [PRL], follicle-stimulating hormone [FSH], luteinizing hormone [LH], thyroid-stimulating hormone [TSH], adrenocorticotropic hormone [ACTH]), and pituitary function was assessed by basal and dynamic testing. Measurements of serum-free thyroid hormones; TSH, PRL, GH, ACTH, LH, and FSH; testosterone or estradiol (in men and premenopausal women); cortisol; and urinary-free cortisol allowed the identification of patients requiring specific replacement therapies. A thorough evaluation of adrenal function was performed to exclude the presence of partial hypoadrenalism, especially in patients with borderline serum and urinary cortisol levels. The preoperative hormone levels in serum were defined to be the greatest values observed before surgery.

Neuroradiologic Findings of FPA

Knosp et al. (27) classified parasellar growth of pituitary adenoma into five grades on the basis of the coronal sections of unenhanced and gadolinium enhanced magnetic resonance image (MRI) scans, with the readily detectable internal carotid artery (ICA) serving as the radiologic landmark. The anatomic, radiologic, and surgical conditions of each grade were considered. Grade 0, I, II, III, and IV are distinguished from each other by medial tangent, the intercarotid line (through the cross-sectional centers), and a lateral tangent on the intra- and supracavernous ICAs. Grade 0 represents the normal condition of the cavernous sinus space. The adenoma does not pass the tangent of medical aspects of supra- and intercavernous ICA. In grade I, the medial tangent is passed, but the extension does not go beyond the intercarotid line, which is the line drawn between the cross-sectional centers of the intra- and supracavernous ICA. Grade II is characterized by the tumor extending beyond the intercarotid line but not extending beyond or tangent to the lateral aspects of the intra- and supracavernous ICA. Grade III is characterized by the tumor extension laterally to the lateral tangent of the intra- and supracavernous ICA. Grade IV is characterized by total encasement of the intracavernous carotid artery. The FPAs with grade 0-III are categorized as enclosed adenoma, and those with grade III-IV are categorized as invasive adenoma.

All the patients underwent preoperative sella MRI. Baseline postoperative sella MRI scans were performed immediately after surgery to evaluate residual mass, and followed 3- or 6-month intervals within the first 2 years. Then, to assess tumor recurrence, serial sella MRI scans were performed at 1- to 1.5-year intervals in asymptomatic patients, but if mass-related symptoms or hormonal alterations developed, a sella MRI scan was performed immediately. Surgical extent (gross total resection or subtotal resection) was estimated by not only surgeon's eye during operation but also MRI, which was performed immediately after surgery, usually within 72 hours after surgery.

Recurrence was defined as the presence of a new tumor in patients with a completely resected tumor, as judged on the first postoperative MRI scan, or evidence of new growth of an incompletely resected tumor on serial postoperative MRI scans versus the immediate postoperative MRI scan. Two neuroradiologists (Y. G. Song and H. Y. Lee) from Samsung Changwon Hospital and one neuroradiologist (S. S. Choi) from Dong-A University Medical Center individually conducted the radiologic review to classify FPAs according to the aforementioned radiological scheme and determined the presence of recurrence.

Immunohistochemical Staining

All tissue specimens were examined for adenohypophysial hormones (GH, PRL, FSH, LH, TSH, and ACTH), cell-cycle regulatory proteins (p16, p15, p21, CDK4, CDK6, pRB protein, and cyclin D1), and proliferative markers (MIB-1 antigen, mitosis, and p53). For this analysis, the labeled streptavidin-biotin method was performed on sections from paraffin-embedded tissues that were used for pathologic diagnoses. Immunohistochemical staining was performed using the DAKO Real EnVision Detection system (Dako, Denmark) on serial 5-ìm tissue sections cut from paraffin-embedded specimens that had been dewaxed and rehydrated through a graded alcohol series. Endogenous peroxidase was blocked by dipping sections in 3% aqueous hydrogen peroxide for 5 minutes, and antigen retrieval was performed with a 13-minute autoclave treatment in 10 mmol/L citrate buffer at pH 6.0. The following monoclonal or polyclonal primary antibodies were used: ACTH (1:50; Neomark, East Topsham, Vermont, USA), GH (1:1000; Neomark), PRL (1:500; Neomark), TSH (1:200; Neomark), FSH (1:500; Neomark), LH (1:2000; Neomark), p16 (1:100; Neomark), p15 (1:100; Dako, Glostrup, Denmark), p21 (1:100; Neomark), CDK4 (1:100; Santa Cruz Biotechnology, Heidelberg, Germany), CDK6 (1:100; Santa

Cruz Biotechnology), pRB (1:75; Life Technologies, Carlsbad, California, USA), cyclin D1 (1:100; Neomark), Bag-1 (1:100; Abnova, Walnut, California, USA), MIB-1 antigen (1:100; Immunotech, Marseille, France), and p53 (1:100 Neomark). Primary antibodies were treated at room temperature for 30 minutes and 1 hours, respectively. After the primary antibody incubation, the sections were incubated with the secondary antibody. After washing samples in phosphate buffer solution for 10 minutes to develop immunoreactions, 3,3′-diaminobenzidine tetrahydrochloride was added as chromogen. Sections were then lightly counterstained with Mayer's hematoxylin and mounted for microscopic examination.

Analysis and Interpretation of Immunoreactivity

Throughout the study, appropriate positive and negative controls were used. Negative controls were obtained by omitting the primary antibody. Sections from normal pituitary gland obtained from autopsy specimens were used as the positive control for ACTH, GH, PRL, TSH, LH, FSH, p16, p15, p21, CDK4 and 6, pRB protein, and cyclin D1. Ten fields were selected in regions with greatest concentrations of immunopositive nuclei and were examined at high power magnification (×400). Each field corresponded to a total number of cells ranging from 700 to 1000, in relation with the cellularity of the tumor specimen. Areas of necrosis, normal adenohypophysial cells, and endothelial cells were excluded from the evaluation. On considering 1000 cells with manual counting, the immunoreactivity of proteins and markers has been described as the percentage of immunopositive cells. Mitosis was counted in the slides, which were stained by hematoxylin and eosin. Mitotic index was defined as the number of mitosis per 10 high-power field. All the slides were reviewed by the same neuropathologist (E.H.L.), who was blinded to patient's clinical and radiologic information. Digital images were captured using a microscope (Model BX41TF; Olympus, Center Valley, Pennsylvania, USA) and digital camera (Model DP70; Olympus).

The purpose of analyzing immunoreactivity of cell-cycle regulators and proliferative marker in this study is to determine whether these markers have an effect on the recurrence of FPA. Therefore, we performed receiver operating characteristic curve analysis of immunoreactivity of the cell-cycle regulatory protein and proliferative markers to predict the likelihood of recurrence (8). We tried to determine the threshold of immunoreactivity with the greatest sensitivity and specificity. Through the sensitivity-specificity analysis, the cut-point (the point that sensitivity and specificity cross) of each marker which was correlated with recurrence was determined (Table 1). Actually, in the autopsy specimen, all the cell-cycle regulatory proteins and proliferative markers were immunohistochemically stained below proportion of the cut-point that we had determined. Therefore, on the basis of the cut-point of immunoreactivity of each protein and marker, sequential correlation analysis with recurrence of FPA was performed.

Classification of FPA

Cells of normal anterior pituitary gland were histologically distinguished from those of pituitary adenoma. The different cell types of the normal anterior gland are grouped in varying combinations into small- or medium-sized nests and cords, each enclosed by a prominent reticulin meshwork to create a prominent alveolar pattern. However, in pituitary adenoma, the alveolar pattern and reticulin architecture of the normal gland is largely effaced in almost cases. Although their borders usually are distinct, adenomas are not truly encapsulated—they have a "pseudocapsule" consisting of compressed adenohypophysial cells and the condensed reticulin fiber network of the adjacent nontumorous anterior lobe. The interface with the surrounding dura was usually discrete. When the effacement was obscure, we confirmed those findings with the reticulin stain, and then included them in this analysis.

On the basis of the serum level of adenohypophysial hormone, FPAs were divided simply into 6 categories: ACTH-secreting adenoma, PRL-secreting adenoma, GH-secreting adenoma, mixed PRL- and GH-secreting adenoma, TSH-secreting adenoma, and gonadotropin-secreting adenoma. Two neuropathologists (E.H.L. and H. W. Lee) reviewed hematoxylin and eosin and immunohistochemical stains, and categorized adenomas as described previously.

Statistical Analysis

Differences between subgroups were analyzed using the Student's t-test for normally distributed continuous values and using the Mann-Whitney test for non-normally distributed continuous values. The χ^2 test was used to analyze categorical variables. The performance of each cell-cycle regulatory protein and proliferative marker as a

Table 1. Results of ROC Curve Analysis and Sensitivity-Specificity Curve of Cell-Cycle Regulatory Protein and Proliferative Markers, and Determining Cut-Point

	Mean Percentage of IHC Staining Nuclei (± SD)	AUC in ROC Curve	Cut-Point, %	Sensitivity, %	Specificity, %
Cell-cycle regulatory protein					
p16	4.62 ± 3.27	0.66	5	53.8	89.7
p15	7.35 ± 5.53	0.68	8	33.3	96.0
p21	8.92 ± 6.43	0.94	10	100.0	76.2
CDK4	22.72 ± 16.42	0.77	25	66.7	82.3
CDK6	34.46 ± 18.35	0.72	40	75.0	81.2
pRB protein	18.24 ± 1.35	0.65	20	61.1	80.4
Cyclin D1	4.76 ± 3.31	0.75	5	66.7	82.0
Proliferative markers					
MIB-1	1.90 ± 0.94	0.79	2	62.8	83.5
Mitotic index	0.86 ± 0.43	0.82	1	72.4	78.2
p53	2.88 ± 1.92	0.85	3	78.4	85.1

ROC, receiver operating characteristic; IHC, immunohistochemical; AUC, area under curve; CDK, cyclin-dependent kinase; pRB, phosphorylated retinoblastoma.

prognostic factor for the recurrence of FPA was investigated by means of receiver operating characteristic curve analysis and sensitivity-specificity analysis. Variables that were found to be significantly associated with recurrence in FPAs by univariate analyses subjected to multivariate analyses. In addition, several variables in which authors were interested and were associated with recurrence of pituitary adenoma in the literatures also were subjected to multivariate analysis. We evaluated the impact on recurrence of variables by comparing the recurrence-free survival curves using the log-rank test. In multivariate analysis, Cox proportional hazard regression model was used to assess the independent effects of specific factors on tumor recurrence rate and to define hazard ratios for significant covariates. Two-sided P values of less than 0.05 were considered statistically significant. SPSS software version 12.0 (SPSS Inc., Chicago, Illinois, USA) was used for the statistical analysis.

RESULTS

Clinical Outcomes

According to our review of medical records, during these periods, 293 patients with pituitary adenoma underwent surgical resection. Among them, 174 patients (59.4%) were diagnosed as FPA. An endoscopic endonasal transsphenoidal approach was used in 89 cases (51.2%), microscopic transsphenoidal approach in 79 cases (45.4%), and craniotomy in 6 cases (3.4%). Mean patient age was 44.8 years (range, 22.6–78.7 years), and there were 102 men and 72 women. Chief complaints at presentation were galactorrhea and amenorrhea in 53 (30.4%), Cushing's disease in 32 (18.4%), visual disturbance in 31 patients (17.8%), headache or dizziness in 26 (14.9%), acromegaly in 21 (12.1%), a decreased libido or impotence in 20 (11.5%), altered mentality in 5 (2.9%), and thyrotoxicosis in 1 patient (0.6%). Three patients (1.7%) had an incidentally found asymptomatic FPA (Table 2).

Gross total removal was performed in 150 cases (86.2%) and subtotal resection in 24 (13.8%). No patients underwent adjuvant radiotherapy without evidence of recurrence or regrowth during follow-up. In the 24 patients who had residual tumors after subtotal resection, significant decrease or normalization of serum adenohypophysial hormone level was found, and clinical symptoms cause by excessive hormone (e.g., galactorrhea and amenorrhea) were also much improved. During follow-up with regular examination of serum adenohypophysial hormone, they did not have any clinical symptoms from excess hormone, nor increase of serum adenohypophysial hormone. Therefore, under the condition of no radiological recurrence, we closely and regularly followed those patients without any medical treatment.

Table 2. Clinical and Radiologic Characteristics of Patients with Functioning Pituitary Adenoma (N = 174)

Variables	Characteristics
Age, mean (range), year	44.8 (22.6–78.7)
Male:female	102:72
Chief complaint	
Galactorrhea and amenorrhea	53 (30.4%)
Cushing disease	32 (18.4%)
Visual symptoms	31 (17.8%)
Headache or dizziness	26 (14.9%)
Acromegaly	21 (12.1%)
Decreased libido or Impotence	20 (11.5%)
Altered mentality	5 (2.9%)
Incidentaloma	3 (1.7%)
Thyrotoxicosis	1 (0.6%)
Clinicopathologic diagnosis	
PRL-secreting adenoma	83 (47.7%)
ACTH-secreting adenoma	36 (20.7%)
GH-secreting adenoma	24 (13.8%)
Mixed PRL- and GH-secreting adenoma	21 (12.1%)
TSH-secreting adenoma	6 (3.4%)
Gn-secreting adenoma	4 (2.3%)
Knosp classification	
0	71 (40.8%)
I	43 (24.7%)
II	35 (20.1%)
III	13 (7.5%)
IV	12 (6.9%)
Maximum diameter	
Mean (range), mm	20.7 (6.5–53.4)

PRL, prolactin; ACTH, aderenocorticotropic hormone; GH, growth hormone; TSH, thyroid-stimulating hormone; Gn, gonadotropin.

Table 3. Result of Immunohistochemical Staining for Adenohypophysial Cells, Cell-Cycle Regulatory Proteins, and Proliferation Markers in the Sample of Functioning Pituitary Adenoma (N = 174)

Variables	Number of Patients (%)
Immunohistochemical staining for adenohypophysial cells	
Corticotroph cell	43 (24.7)
Lactotroph cell	88 (50.6)
Somatotroph cell	41 (24.0)
Thyrotroph cell	21 (14.9)
Gonadotroph cell (LH)	29 (16.7)
Gonadotroph cell (FSH)	33 (19.0)
Immunohistochemical overstaining for cell-cycle regulatory protein	
p16	89 (51.1)
p15	27 (15.5)
p21	20 (11.5)
CDK4	54 (31.0)
CDK6	18 (10.3)
pRB protein	69 (39.7)
Cyclin D1	87 (50.0)
None of the above	22 (12.6)
Immunohistochemical overstaining for proliferative markers	
MIB-1	79 (45.4)
Mitotic index	72 (41.4)
p53	47 (27.0)

LH, luteinizing hormone; FSH, follicule-stimulating hormone; CDK, cyclin-dependent kinase.

Radiographically, there were 71 cases (40.8%) of Knosp grade 0, 43 cases (24.7 %) of grade I, 35 cases (20.1%) of grade II, 13 cases (7.5%) of grade III, and 12 cases (6.9%) of grade IV. Mean maximal diameter of FPAs was 20.7 mm and these ranged from 6.5 to 53.4 mm (Table 2).

Results of Immunohistochemical Staining

In the immunohistochemical staining for adenohypophysial cells, corticotroph cells were stained in 43 samples (24.7%),

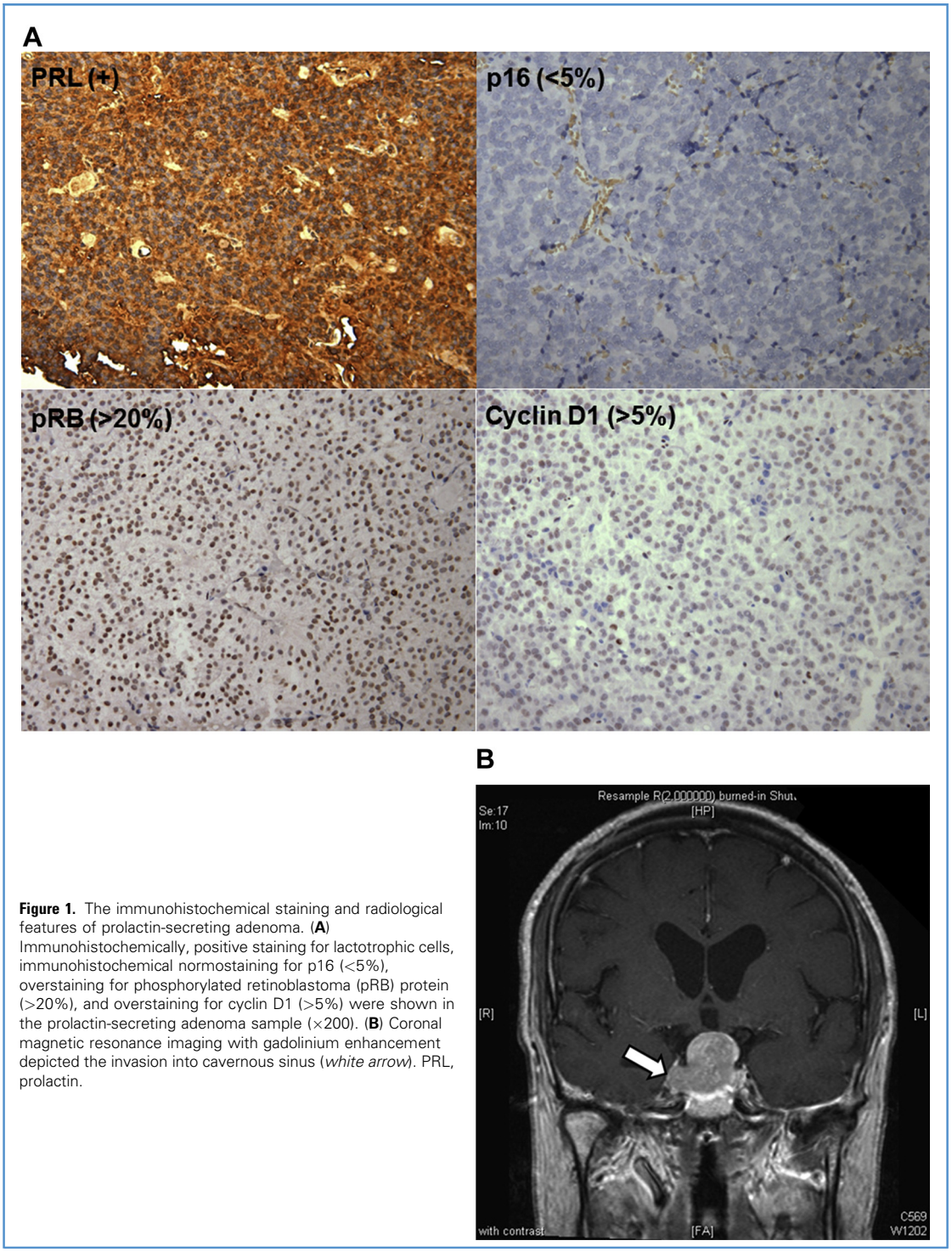

Figure 1. The immunohistochemical staining and radiological features of prolactin-secreting adenoma. (**A**) Immunohistochemically, positive staining for lactotrophic cells, immunohistochemical normostaining for p16 (<5%), overstaining for phosphorylated retinoblastoma (pRB) protein (>20%), and overstaining for cyclin D1 (>5%) were shown in the prolactin-secreting adenoma sample (×200). (**B**) Coronal magnetic resonance imaging with gadolinium enhancement depicted the invasion into cavernous sinus (*white arrow*). PRL, prolactin.

lactotroph cells in 88 samples (50.6%), somatotroph cells in 41 samples (24.0%), thyrotroph cells in 21 samples (14.9%), LH secreting cells in 29 samples (16.7%), and FSH secreting cells in 33 samples (19.0%). Only one adenohypophysial cell was stained in 61 samples (35.1%). However, in 113 samples (64.9%), two or more adenohypophysial cells were immunohistochemically stained: 43 samples (24.7%) had two adenohypophysial cells, eight samples (4.6%) had three cells, five samples (2.9%) had four cells, and two samples (1.1%) had six cells, respectively (Table 3).

In terms of cell-cycle regulatory proteins, p16 was overstained immunohistochemically above cut-point in 89 samples (51.1%), p15 in 27 samples

Table 4. Results of Immunohistochemical Analysis for Cell-Cycle Regulatory Protein And Proliferative Markers According to the Classification of Functioning Pituitary Adenoma (N = 174)

Markers	ACTH-Secreting Adenoma (n = 36)	PRL-Secreting Adenoma (n = 83)	GH-Secreting Adenoma (n = 24)	Mixed PRL- and GH- Secreting Adenoma (n = 21)	TSH-Secreting Adenoma (n = 6)	Gonadotropin- Secreting Adenoma (n = 4)
IHC overstaining for cell-cycle regulatory protein						
p16	8 (22.2%)	59 (71.1%)	11 (45.8%)	6 (28.6%)	5 (83.3%)	0 (0.0%)
p15	4 (11.1%)	15 (18.1%)	3 (12.5%)	2 (10.0%)	2 (33.3%)	0 (0.0%)
p21	3 (8.3%)	11 (10.8%)	2 (8.3%)	3 (14.3%)	1 (16.7%)	0 (0.0%)
CDK4	11 (30.1%)	27 (32.5%)	7 (29.2%)	8 (38.1%)	1 (16.7%)	0 (0.0%)
CDK6	3 (8.3%)	10 (12.0%)	3 (12.5%)	2 (10.0%)	0 (0.0%)	0 (0.0%)
pRB protein	19 (52.8%)	26 (31.3%)	11 (45.8%)	11 (52.4%)	2 (33.3%)	0 (0.0%)
Cyclin D1	24 (66.7%)	30 (36.1%)	16 (66.7%)	15 (71.4%)	2 (33.3%)	0 (0.0%)
IHC overstaining for proliferative markers						
MIB-1	22 (61.1%)	27 (32.5%)	15 (62.5%)	13 (61.9%)	1 (16.7%)	1 (25.0%)
Mitotic index	20 (55.5%)	22 (26.5%)	14 (58.3%)	15 (71.4%)	1 (16.7%)	0 (0.0%)
p53	15 (41.7%)	12 (14.5%)	9 (37.5%)	9 (42.9%)	1 (16.7%)	1 (25.0%)

ACTH, aderenocorticotropic hormone; PRL, prolactin; GH, growth hormone; TSH, thyroid-stimulating hormone; IHC, Immunohistochemical; CDK, cyclin-dependent kinase; pRB, phosphorylated retinoblastoma.

(15.5%), p21 in 20 samples (11.5%), CDK4 in 54 samples (31.0%), CDK6 in 18 samples (10.3%), pRB protein in 69 samples (39.7%), and cyclin D1 in 87 samples (50.0%). In the proliferative markers, immunohistochemical overstaining for MIB-1 was found in 79 samples (45.4%), increased mitotic index in 72 samples (41.4%), and immunohistochemical overstaining for p53 in 47 samples (27.0%; Table 3). Figure 1 illustrates immunohistochemical staining results for cell-cycle regulatory proteins.

Analysis of Immunohistochemical Overstaining According to Classification of FPA

In the results of immunohistochemical staining for cell-cycle regulatory protein, a relatively greater proportion of immunohistochemically overstained protein than average of total samples was found as follows: p16 and p15 in PRL-secreting adenoma (71.1% and 18.1%) and TSH-secreting adenoma (83.3% and 33.3%); p21 in mixed PRL- and GH-secreting adenoma (14.3%) and TSH-secreting adenoma (16.7%); CDK4 in PRL-secreting adenoma (32.5%) and mixed PRL- and GH-secreting adenoma (38.1%); CDK6 in PRL-secreting adenoma (12.0%) and GH-secreting adenoma (12.5%); and pRB protein and cyclin D1 in ACTH-secreting adenoma (52.8% and 66.7%), GH-secreting adenoma (45.8% and 66.7%), and mixed PRL- and GH-secreting adenoma (52.4% and 71.4%) (Table 4). In addition, all the proliferative markers were found to have a relatively greater proportion of immunohistochemical overstaining than average of total samples in ACTH-secreting adenoma, GH-secreting adenoma, and mixed PRL- and GH-secreting adenoma (Table 4).

Analysis of Immunohistochemical Overstaining According to Radiologic Characteristics

The proportion of invasive adenoma was relatively greater than in the samples of immunohistochemically overstaining for p21 (35.0%), CDK4 (18.5%), pRB protein (23.3%), cyclin D1 (23.0%), MIB-1 (25.3%), mitotic index (33.3%), and p53 (59.4%) than average proportion of total samples (14.4%). Among them, χ^2 test showed that adenomas that had immunohistochemical overstaining for p21 ($P < 0.001$), pRB protein ($P = 0.024$), cyclin D1 ($P = 0.002$), MIB-1 ($P = 0.013$), mitotic index ($P < 0.001$), and p53 ($P < 0.001$) were more frequent than other markers with statistical significance (Table 5).

In terms of maximum diameter of FPA, Mann-Whitney U test showed that FPAs of immunohistochemical overstaining for p16 were smaller in size than others ($P = 0.015$), and those of immunohistochemical overstaining for pRB protein ($P < 0.001$), cyclin D1 ($P = 0.020$), MIB-I ($P < 0.001$), and mitotic index ($P < 0.001$) were greater in size than others (Table 5).

Recurrence of FPA

All the patients were followed for 24 months or more, and the mean follow-up duration was 62.4 months (range, 24.2–118.9 months). During follow-up, 62 patients (35.6%) experienced recurrence. Mean duration to recurrence or regrowth after surgery was 24.3 months (range 3.4–80.6 months). In cell-cycle regulatory proteins, the mean time to recurrence was longer in patients with immunohistochemical overstaining than those with immunohistochemical normostaining for p16 ($P < 0.001$), and shorter in patients with immunohistochemical overstaining than those with immunohistochemical normostaining for pRB protein ($P = 0.004$) and cyclin D1 ($P = 0.013$; Figure 2). In proliferative markers, the mean time to recurrence was shorter in patients with immunohistochemical overstaining than those with immunohistochemical normostaining for MIB-1 antigen ($P < 0.001$), mitotic index ($P = 0.032$), and p53 ($P = 0.007$) (Table 6).

Table 5. Results of Immunohistochemical Analysis for Cell-Cycle Regulatory Protein and Proliferative Markers According to the Radiological Characteristics (N = 174)

	Knosp Classification					
	Enclosed Adenoma			Invasive Adenoma		
Markers	0 (n = 71)	I (n = 43)	II (n = 35)	III (n = 13)	IV (n = 12)	Mean Maximum Diameter, mm, ± SD
IHC overstaining for cell-cycle regulatory protein						
p16	36 (40.4%)	39 (43.8%)	9 (10.1%)	3 (3.4%)	2 (2.2%)	18.3 ± 10.2
p15	9 (33.3%)	10 (37.0%)	4 (16.7%)	2 (7.4%)	2 (7.4%)	19.6 ± 11.3
p21	2 (10.0%)	3 (15.0%)	8 (40.0%)	4 (20.0%)	3 (15.0%)	19.2 ± 9.8
CDK4	23 (42.6%)	11 (20.4%)	10 (18.5%)	7 (13.0%)	3 (5.6%)	22.1 ± 10.4
CDK6	9 (50.0%)	4 (22.2%)	3 (16.7%)	1 (5.6%)	1 (5.6%)	21.4 ± 9.2
pRB protein	17 (24.6%)	15 (21.7%)	21 (30.4%)	9 (13.0%)	7 (10.1%)	24.7 ± 11.3
Cyclin D1	18 (20.7%)	21 (24.1%)	28 (32.2%)	10 (11.5%)	10 (11.5%)	23.0 ± 12.2
IHC overstaining for proliferative marker						
MIB-1	11 (13.9%)	15 (19.0%)	33 (41.8%)	11 (13.9%)	9 (11.4%)	25.1 ± 11.7
Mitotic index	11 (15.3%)	12 (16.7%)	25 (34.7%)	12 (16.7%)	12 (16.7%)	24.8 ± 10.6
p53	5 (10.6%)	7 (14.9%)	12 (25.5%)	12 (25.5%)	11 (23.4%)	22.1 ± 12.6
Clinicopathologic diagnosis						
PRL-secreting adenoma	39 (47.0%)	24 (28.9%)	7 (8.4%)	7 (8.4%)	6 (7.2%)	20.9 ± 11.5
ACTH-secreting adenoma	5 (13.9%)	7 (19.4%)	18 (50.0%)	2 (5.6%)	4 (11.1%)	19.3 ± 11.2
GH-secreting adenoma	18 (75.0%)	4 (16.7%)	1 (4.2%)	1 (4.2%)	0 (0.0%)	18.2 ± 12.0
Mixed PRL- and GH-secreting adenoma	7 (33.3%)	4 (19.0%)	5 (23.8%)	3 (14.3%)	2 (9.5%)	24.1 ± 14.3
TSH-secreting adenoma	1 (16.7%)	2 (33.3%)	3 (50.0%)	0 (0.0%)	0 (0.0%)	21.2 ± 12.5
Gn-secreting adenoma	1 (25.0%)	2 (50.0%)	1 (25.0%)	0 (0.0%)	0 (0.0%)	20.3 ± 13.7

IHC, immunohistochemical; CDK, cyclin-dependent kinase; pRB, phosphorylated retinoblastoma; PRL, prolactin; ACTH, aderenocorticotropic hormone; GH, growth hormone; TSH, thyroid-stimulating hormone; Gn, gonadotropin.

Univariate Analysis of Predisposing Factors for the Recurrence of FPA

In clinicopathologic classification of FPA, univariate analysis showed that greater recurrence rate in ACTH-secreting adenoma ($P = 0.038$) and mixed PRL- and GH-secreting adenoma ($P = 0.012$) than other FPA (Table 7).

In terms of cell-cycle regulatory protein, immunohistochemical overstaining for p16 was found less than other cell-cycle regulatory protein in FPAs with recurrence. In addition, immunohistochemical overstaining for pRB protein and cyclin D1 were more common than other cell-cycle regulatory protein. Univariate analysis showed that immunohistochemical overstaining for p16 ($P < 0.001$) were inversely associated with recurrence of FPAs and immunohistochemical overstaining for pRB protein ($P = 0.023$) and cyclin D1 ($P = 0.048$) were directly associated with recurrence of FPA. All the FPA with immunohistochemically overstaining for proliferative marker had greater recurrence rate than average of total FPA patients. Also, univariate analysis showed that immunohistochemical overstaining for MIB-1 ($P = 0.007$), mitotic index ($P = 0.036$), and p53 ($P < 0.001$) were associated with recurrence of FPAs (Table 7).

Regarding radiologic factors, invasive adenomas (52.0%, Knosp grade III-IV) were more common than enclosed adenoma (32.9%, Knosp grade 0-II) in FPAs with recurrences. And the FPAs with maximum diameter ≥25mm (42.4%) were more common than those with maximum diameter <25 mm (31.5%) in FPAs with recurrence. Univariate analysis showed that invasive adenoma ($P < 0.001$) and FPAs with maximum diameter ≥25 mm ($P = 0.033$) were associated with recurrence of FPAs (Table 7).

In addition, FPAs requiring subtotal resection (37.5%) were more common than those requiring gross total resection (35.3%) in recurring FPAs. However, there was no statistically difference in the univariate analysis ($P = 0.579$).

Multivariate Analysis of Predisposing Factors for the Recurrence of FPA

Multivariate analysis using Cox's proportional hazard regression model for recurrence in FPAs after surgical resection showed that the following factors were independently associated with high rate of recurrence: Knosp grade III-IV (hazard ratio [HR] of 4.02, 95% confidence interval [95% CI] 2.69–5.35; $P < 0.001$), immunohistochemical normostaining for

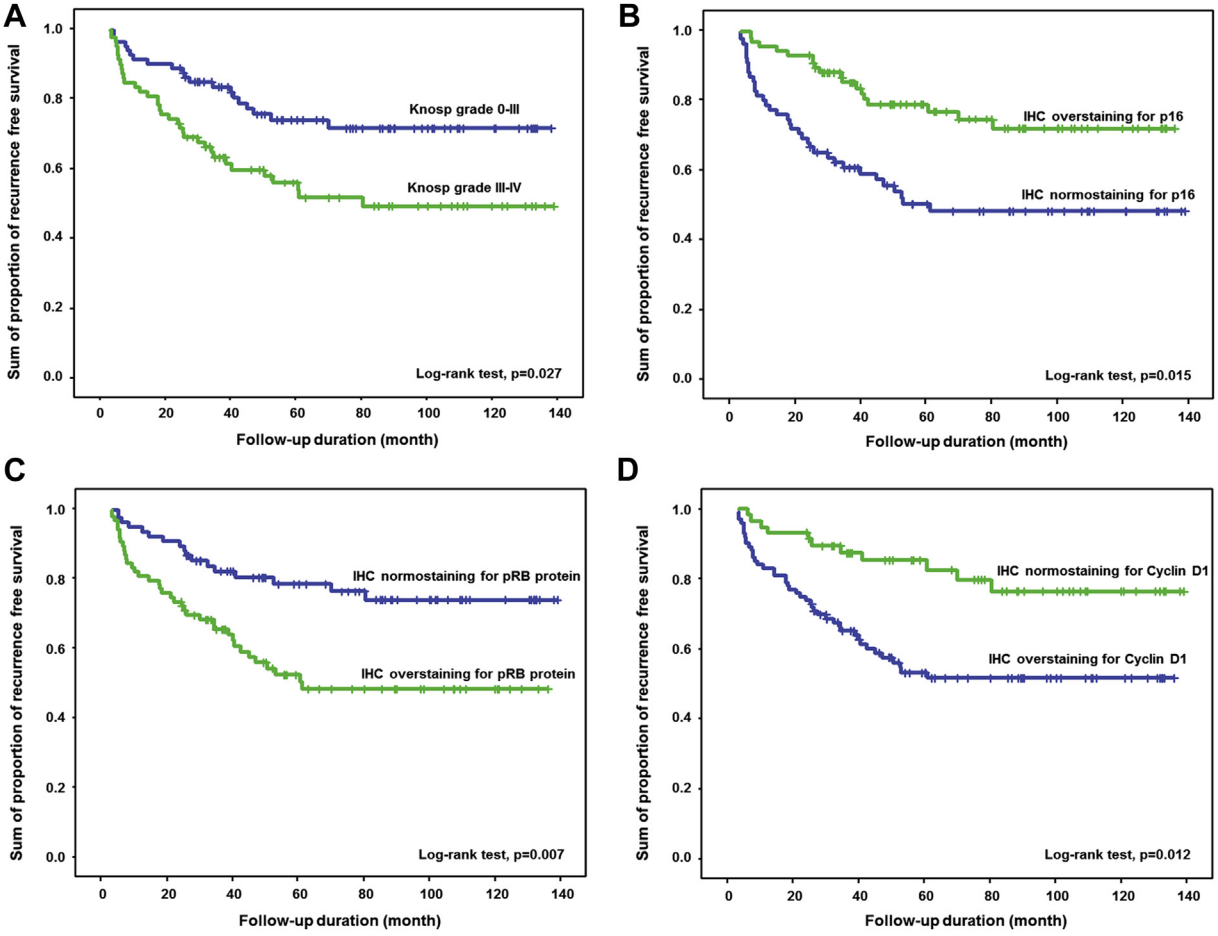

Figure 2. Recurrence-free survival curve for the patients with functioning pituitary adenoma. (**A**) Knosp grade 0-III versus Knosp grade III-IV, (**B**) immunohistochemical (IHC) normostaining (<5%) versus overstaining (≥5%) for p16, (**C**) IHC normostaining (<20%) versus overstaining (≥20%) for phosphorylated retinoblastoma (pRB) protein, and (**D**) immunohistochemical normostaining (<5%) versus overstaining (≥5%) for cyclin D1.

p16 (HR of 3.16, 95% CI 1.70–4.62; $P < 0.001$), immunohistochemical overstaining for pRB protein (HR of 2.45, 95% CI 1.75–3.15; $P = 0.008$), immunohistochemical overstaining for Cyclin D1 (HR of 2.13, 95% CI 1.33–2.93; $P = 0.029$), immunohistochemical overstaining for MIB-1 antigen (HR of 2.74, $P = 0.002$), immunohistochemical overstaining for p53 (HR of 2.21, $P = 0.002$), and mixed PRL- and GH-secreting adenoma (HR of 2.08, $P = 0.032$) (Table 8).

The following factors that were associated with recurrence of FPAs in univariate analysis were not associated with high rate of recurrence in multivariate analysis: size of adenoma and mitotic index. And, other factors which were concerned by authors, such as p15 and p21 were not associated with recurrence of FPA in multivariate analysis (Table 8).

DISCUSSION

In this study, it was found that immunohistochemical staining results for some cell-cycle regulators (p16, pRB protein, and cyclin D1) predict FPA recurrence. During the early G1 phase of the cell cycle, in response to mitotic stimuli, cyclin D activates CDK4 and CDK6, which then partially phosphorylates RB protein. Cyclin D1, one of the G1 cyclins, binds to CDK during the G1 phase of cell cycle and is essentially required for by-passing the cell cycle restriction point into the S phase (34), which enables the cell cycle to progress and tumor cells to proliferate. In addition, RB protein, the product of the RB gene, plays a key role in regulating the cell cycle (30). RB protein exists in an active hypophosphorylated state in quiescent cells and in an inactive hyperphosphorylated state at the G1-S cell cycle transition. Therefore, the level of phosphorylated RB protein is an indicator of cell-cycle progression into S-phase. Thus, attenuated expressions of cyclin D1 and pRB protein in FPA could explain the greater rate of recurrence observed in this study. However, CDK inhibitors, such as p16 (an INK4 family member), oppose CDK activation, and during the early G1 phase of the cell cycle, p16 binds to CDK4 and 6, and prevents cyclin D activation. Thus, increased expression of p16 could prevent the proliferation and recurrence of

Table 6. Time to Recurrence According to the Cell-Cycle Regulatory Protein and Proliferative Markers

	Case with Immunohistochemical Overstaining for Marker		Case with Immunohistochemical Normostaining for Marker		
	No. of Recurrence (%)	Time to Recurrence (Month, Mean ± SD)	No. of Recurrence (%)	Time to Recurrence (Month, Mean ± SD)	Mann-Whitney U test (*P* Value)
Cell-cycle regulatory protein					
p16	16/89 (18.0)	34.1 ± 17.4	46/85 (52.9)	20.9 ± 13.4	<0.001*
p15	7/27 (25.9)	27.4 ± 6.6	55/147 (37.4)	23.9 ± 4.8	0.241
p21	5/20 (25.0)	26.6 ± 5.7	57/154 (37.0)	24.1 ± 3.6	0.427
CDK4	21/54 (38.9)	23.7 ± 4.5	41/120 (34.2)	25.9 ± 15.2	0.758
CDK6	6/18 (33.3)	23.2 ± 14.7	56/156 (35.9)	24.4 ± 3.8	0.805
pRB protein	37/69 (53.6)	19.8 ± 11.4	25/105 (23.8)	31.0 ± 6.3	0.004*
Cyclin D1	42/87 (48.3)	20.2 ± 12.1	20/87 (23.0)	32.9 ± 15.7	0.013*
Proliferative marker					
MIB-1	39/79 (49.4)	19.2 ± 1.0	23/95 (24.2)	32.9 ± 6.2	<0.001*
Mitotic index	34/72 (47.2)	21.1 ± 2.7	28/102 (27.5)	28.2 ± 3.4	0.032*
p53	25/47 (53.2)	17.4 ± 0.2	37/127 (19.1)	29.0 ± 4.5	0.007*

CDK, cyclin-dependent kinase; pRB, phosphorylated retinoblastoma.
*Statistically significant.

pituitary adenoma, which is in accord with our results. The frequencies of the expressions of p16 and pRB protein found in this study are similar to those reported by Kirsch et al. (24), who observed p16 and RB protein positivity in 57% and 48% of pituitary adenomas in the immunohistochemical analysis, respectively.

In terms of RB protein expression, in a study on heterozygote RB gene knockout mice investigators found that animals developed pituitary adenocarcinomas of intermediate lobe origin (21). However, studies on human RB gene loss in pituitary tumors remain controversial. In one study, sustained loss of heterozygosity of the RB1 gene was found in highly invasive or malignant pituitary tumors (38), but this finding was not sustained by a loss of RB protein by immunohistochemical analysis. On the other hand, in another study a lack of RB protein expression in a small proportion of pituitary tumors was found not to be associated with loss of an RB1 intragenic marker (44). Recently, loss of RB protein expression in these tumors was suggested to be caused by methylation in the gene-promoter region (43).

The present study is the first to report that RB protein plays a role in FPA recurrence. In fact, the prognostic role of cell-cycle regulatory protein and proliferative markers has been known in several cancers. The cumulative number of aberrantly expressed cell-cycle and proliferative markers correlated with aggressive pathologic features and inferior oncologic outcomes in patients with renal cell carcinoma (11), gallbladder malignancies (46), gastric adenocarcinoma (25), and breast cancer (14). In addition, there are several studies reporting the role of RB protein in prognosis and progression of malignant brain tumors, such as glioblastoma (3, 12, 16, 52, 53). Among them, Hilton et al. (16) suggested that glioblastoma showing widespread immunohistochemical expression of RB protein have a better prognosis than those without these features, which was opposite to our results. They did not separate RB protein into phosphorylated and unphosphorylated form. Therefore, the data from unphosphorylated RB proteins were intermingled into those from phosphorylated RB proteins, which made an opposite result to ours. As mentioned previously, phosphorylated RB protein is important to transit into S-phase in the cell cycle. Therefore, there is a limitation to interpretation of their result.

In the clinical field, reported recurrent rates vary from one study to another and depend on both the length of follow-up and the number of patients studied. An analysis of some of these studies shows that the absence of residual tumor after surgery, lack of cavernous sinus invasion, and postoperative radiotherapy are invariably associated with lower risks of recurrence (7, 9, 13, 37). In the present study, a recurrence rate of 35.3% (53 of 150) was found for grossly total resected adenomas and 37.5% (9 of 24) for subtotally resected adenomas, which was not significantly different ($P = 0.579$). In fact, this result looks somewhat difficult to understand. However, serum level of adenohypophysial hormone was normalized or much decreased immediately after surgery in all the patients in this study. Even in the patients without normalization of hormone level, clinical symptoms from excessive adenohypophysial hormone also disappeared sequentially. Actually, among 13 patients who did not experience normalization of hormone level after gross total resection, recurrence occurred in 3 patients (23.1%), and among 4 patients without normalization of hormone level after subtotal resection, recurrence occurred in 1 patient (25.0%). This result showed that the presence of postoperative remnant adenoma is not independent predictive

TUMOR

Table 7. Univariate Analysis of Factors Predicting Recurrence of Functioning Pituitary Adenomas After Surgical Resection (N = 174)

	Recurrence, n (%)		Univariate Analysis		
	Yes (n = 62)	No (n = 112)	HR	95% CI	*P*-Value
Clinicopathological classification					
ACTH-secreting adenoma	17 (47.2)	19 (52.8)	1.62*	1.16–2.08	0.038*
PRL-secreting adenoma	21 (25.3)	62 (74.7)	0.72	0.33–1.11	0.243
GH-secreting adenoma	10 (41.7)	14 (58.3)	1.29	0.92–1.66	0.254
Mixed PRL- and GH-secreting adenoma	11 (52.4)	10 (47.6)	1.98*	1.47–2.49	0.012*
TSH-secreting adenoma	2 (33.3)	4 (66.7)	0.90	0.45–1.35	0.846
Gn-secreting adenoma	1 (25.0)	3 (75.0)	0.65	0.22–1.18	0.074
IHC overstaining for cell-cycle regulatory protein					
p16	16 (18.0)	73 (82.0)	0.40*	0.18–0.62	<0.001*
p15	7 (25.9)	20 (74.1)	0.64	0.24–1.04	0.066
p21	5 (25.0)	15 (75.0)	0.61	0.21–1.01	0.052
CDK4	21 (38.9)	33 (61.1)	1.16	0.83–1.49	0.429
CDK6	6 (33.3)	9 (66.7)	0.81	0.36–1.26	0.538
pRB protein	37 (53.6)	32 (46.4)	2.12*	1.63–2.61	0.002*
Cyclin D1	42 (48.3)	45 (51.7)	1.71*	1.39–2.03	0.014*
None of the above	6 (35.3)	11 (64.7)	1.00		
IHC overstaining for proliferative marker					
MIB-1	39 (49.4)	40 (50.6)	1.85*	1.30–2.40	0.007*
Mitotic index	34 (47.2)	38 (52.8)	1.61*	1.21–2.01	0.036*
p53	25 (53.2)	22 (46.8)	2.24*	1.69–2.79	<0.001*
Radiological characteristics					
Knosp classifications 0-II	49 (32.9)	100 (67.1)	1.00		
III-IV	13 (52.0)	12 (48.0)	2.21*	1.52–2.90	<0.001*
Size of adenoma, mm					
<25	34 (31.5)	74 (68.5)	1.00		
≥25	28 (42.4)	38 (57.6)	1.60*	1.18–2.02	0.033*
Surgical extent					
Gross total	53 (35.3)	97 (64.7)	1.00		
Subtotal	9 (37.5)	15 (62.5)	1.10	0.42–1.78	0.579

HR, hazard ratio; CI, confidence interval; ACTH, aderenocorticotropic hormone; PRL, prolactin; GH, growth hormone; TSH, thyroid-stimulating hormone; Gn, gonadotropin; IHC, immunohistochemical; CDK, cyclin-dependent kinase; pRB, phosphorylated retinoblastoma.
*Statistically significant.

factor of recurrence of FPA even in the patients who experienced only decrease of adenohypophysial hormone in serum after surgery.

Similarly, Brochier et al. (2) also reported that the presence of postoperative remnant adenoma is not an independent factor of recurrence in pituitary adenoma. In terms of time to recurrence, among 62 patients with recurrence of FPA, only 4 patients (6.5%) experienced the recurrence of FPA after 5 years of surgery. Almost reccurence occurred within 5 years of surgery even in the subtotally resected FPAs. Also, we examined the time to recurrence according the state of immunohistochemical staining for each markers. The early recurrence within 5 years of surgery was associated with the state of immunohistochemical staining for p16, pRB protein, and cyclin D1. However, the late recurrence 5 years after surgery did not have any tendency of immunohistochemical staining for cell-cycle regulatory proteins.

Cavernous sinus invasion is known to be a strong independent predictive factor of recurrence or regrowth of APA (5, 13). In present study, cavernous sinus invasion was found in 25 FPAs. Interestingly, cavernous sinus invasion was more common for the FPA with immunohistochemical overstaining for p21 (35.0%), CDK4 (18.6%), pRB protein (23.1%), Cyclin D1 (23.0%), MIB-1 antigen (25.3%), mitotic index (33.4%), and p53 (48.9%). Among them, immunohistochemical overstaining for pRB protein, cyclin D1, MIB-1 antigen, mitotic index, and p53 was independently associated with recurrence of FPA. Several investigators reported that they found rates of invasion to be greatest (>50%) in silent subtype 3 adenomas (92%), ACTH adenomas of Nelson's syndrome (56%), silent ACTH adenomas of subtype 2 (75%), as well as male patients (56%) and in PRL-cell adenomas (56%) (43, 44).

Because this study includes only FPA, we could not directly compare our results with theirs. However, among 24 adenomas with cavernous sinus invasion, there were 14 male (58.3%) and 10 female (41.7%) patients in this study. Cavernous sinus invasion also was found more frequently in 15.7% of PRL-secreting adenomas, 16.7% of ACTH-secreting adenomas, and 23.8% of mixed PRL- and GH-secreting adenomas than other adenomas. This study showed that the most common cavernous sinus invasion was found in mixed PRL- and GH-secreting adenoma. This discrepancy between other investigators' results and ours might be originated from undoing categorization of mixed PRL- and GH-secreting adenoma. In addition, invasive status is sometimes difficult to determine, and dural biopsy is not routinely performed during surgery. In addition, microscopic dural invasion has been noted by some pathologists in the vast majority of macroadenoma patients with suprasellar

Table 8. Multivariate Analysis of Predisposing Factors for Recurrence in Functioning Pituitary Adenoma by Use of Cox Proportional Hazard Regression Analysis

Variables	Hazard Ratio	95% CI	*P*-Value
Knosp classification (III-IV vs. 0-II)	4.02	2.69–5.35	< 0.001*
Size of adenoma (≥25 mm vs. <25 mm)	1.52	0.94–2.10	0.072
IHC staining for p16 (<5% vs. ≥5%)	3.16	1.70–4.62	< 0.001*
IHC staining for p15 (<8% vs. ≥8%)	1.54	0.96–2.12	0.067
IHC staining for p21 (<10% vs. ≥10%)	1.29	0.61–1.97	0.526
IHC staining for pRB protein (≥20% vs. <20%)	2.45	1.75–3.15	0.008*
IHC staining for cyclin D1 (≥5% vs. <5%)	2.13	1.33–2.93	0.029*
MIB-1 (≥2% vs. <2%)	2.74	1.80–3.68	0.002*
Mitotic index (≥1.0 vs. <1.0)	1.59	0.98–2.20	0.052
IHC staining for p53 (≥3% vs. <3%)	2.21	1.48–2.94	0.022*
ACTH-secreting adenoma vs. others	1.65	1.02–2.28	0.047*
PRL-secreting adenoma vs. others	1.26	0.63–1.89	0.581
GH-secreting adenoma vs. others	1.34	0.88–1.80	0.272
Mixed PRL- and GH-secreting adenoma vs. others	2.08	1.35–2.81	0.032*

ACTH, aderenocorticotropic hormone; CI, confidence interval; GH, growth hormone; IHC, immunohistochemical; pRB, phosphorylated retinoblastoma; PRL, prolactin; TSH, thyroid-stimulating hormone.
*Statistically significant.

extension (41), but its influence on the risk of recurrence is uncertain (32).

Although this study included surgically obtained sample of FPA, in terms of surgical indication for FPA, there has been no standard guideline, especially for the prolactinomas. Transsphenoidal microsurgery has been the mainstream treatment prolactinomas since its introduction in the 1960s, but fell out of favor as the result of a considerable rate of disease recurrence, ranging from 26 to 50% (42). Although more recent reports with rigorous long-term follow-up review contained a reduced postoperative recurrence rate (12–15%) (33, 51), an alternative treatment of prolactinomas with a dopamine-agonist agent is now the treatment of choice for most patients with hyperprolactinemia.

However, Tamasauskas et al. (47) suggested that preoperative dopamine-agonist treatment should be even the main factor associated with worse outcomes after prolactinomas surgery in women. Therefore, our indication for surgery in the population with prolactinoma is primarily dependent on the patient's preference after receiving sufficient information on each therapeutic modality. However, we strongly recommended surgical resection in the following cases: sudden deterioration of clinical condition suggesting pituitary apoplexy, worsening neurologic and ophthalmologic symptoms of mass effect, large size (≥25 mm) with <200 mL of serum PRL level. We also included only patients who underwent surgical resection for prolactinomas without any history of medical treatment for prolactinoma.

Despite the interesting results of this study, it has its limitations. The first concern is the pathological classification of APAs. Although there is a World Health Organization Classification of pituitary adenoma (36), the classifications of pituitary adenoma in terms of their clinical, hormonal, and histological characteristics are not always unequivocal. Importantly, modern histopathologic classifications are determined by sophisticated morphologic techniques, such as immunohistochemistry, transmission electron microscopy, immunoelectron microscopy, morphometric analysis, and molecular methods such as in situ hybridization.

Nosè et al. (35) proposed a protocol for the examination of specimens from pituitary adenoma that evolved to include clinical, radiographic, morphologic, immunohistochemical and molecular results in an effort to guide clinical management. According to the derived protocol, immunohistochemical study included Pit1, Tpit, SF1, Ki-67 labeling index, and p53. However, more recent studies have sought a correlation between mitotic activity, MIB-1, proliferating cell nuclear antigen, p53, and $p27^{Kip1}$ labeling indices on the one hand and tumor invasion, recurrence, and metastatic potential on the other (20, 49, 50, 55, 57).

Therefore, our classification of pituitary adenoma, on the basis of clinical manifestations, biochemical study of serum adenohypophysial hormone, and immunohistochemical staining, limits interpretations of the results obtained. In fact, this study showed that mixed PRL- and GH-secreting adenomas have a significant association with the recurrence of FPA. However, we could not subdivide them into acidophilic stem cell adenoma, mixed somatotroph and lactotroph adenoma, mammosomatotroph adenoma, and plurihormonal tumor due to omitting electron microscopic analysis. Although they can secrete both PRL and GH simultaneously, they have quite different clinical and histopathological characteristics, especially the tendency to invade into adjacent structures around pituitary gland. Clinically, acidophilic stem cell adenoma is more aggressive than other adenoma and always is invasive and mostly resistant to drug therapy (39). Because the immunohistochemistry in acidophilic stem cell adenoma may be similar to other mixed PRL- and GH-secreting adenomas, the detection of giant mitochondria with the use of electron microscopy is essential to confirm the diagnosis of acidophilic stem cell adenoma (1, 18). The mixed PRL- and GH-secreting adenoma in this study had a significantly greater rate of invasion into cavernous sinus than other FPAs. Although they were not categorized into subtype, we could imagine that much portion of acidophilic stem cell adenoma should be included in total samples of mixed PRL- and GH-secreting adenomas.

The second limitation, we thought, is that we did not examine all cell-cycle regulatory proteins. The cell-cycle control system is a cyclical biochemical device constructed from a set of interacting proteins that induce and coordinate proper progression through the cycle and includes cyclins, CDKs, and their CDK inhibitors (CDKIs). There are two main families of

CDKIs, the INK family (INK4a/p16; INK4b/p15; INK4c/p18 and INK4d/p19) and the WAF/KIP family (WAF1/p21; KIP1/p27; KIP2/p57). Progression through the cell cycle is mainly dependent on fluctuations in the concentration of cyclins, and CDKIs achieved via the programmed degradation of these proteins by proteolysis within the ubiquitin-proteasome system. However, we studied a part of the cell-cycle control system, especially the transmission from G1 to the S phase, which may have biased our interpretations of results, because, for example, some interactions between regulatory proteins were not considered.

The third limitation is that this study does not reflect modern molecular and biological interpretations of APAs. Recently, numerous studies have sought to identify prognostic parameters related to adenoma subtype, invasiveness, and metastasis. For example, differences in invasiveness have been documented among major adenoma categories (40). The significance of flow cytometric data relative to recurrence has also been examined (4, 10, 17). Collectively, these modern and high-technologic studies have increased our understanding of the pathobiology of this tumor spectrum.

CONCLUSIONS

In this study, we investigated the prognostic value of several cell-cycle regulatory proteins for predicting the recurrence of FPA after surgical resection. We found that p16, RB protein, and cyclin D1 are associated with recurrence of FPAs. Also, we confirmed that proliferative markers such as MIB-1 labeling index and p53 have a significant association with recurrence FPAs. Radiologically, adenomas with cavernous sinus invasion were found to be associated with higher recurrence rate. In terms of FPA classification, ACTH-secreting adenoma and mixed PRL- and GH-secreting adenoma were found to have a high rate of recurrence. Nonetheless, despite our results, further studies using sophisticated and systemically developed molecular biology techniques are required to scrutinize these adenomas further and to characterize the pathobiological process of FPA recurrence.

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