

## 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:** Adrenocorticotrophic 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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### 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

■ **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, over-staining 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 over-staining 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.

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 trilocula. 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 adeno-hypophysial 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 adeno-hypophysial 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 medial 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 adeno-hypophysial 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- $\mu$ m 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

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