IMMUNOREACTIVITY FOR GLYCOPROTEIC HORMONES AND TUMOR SIZE IN PITUITARY ADENOMAS

C. Badiu*,[1], Mona Dumbrava[1], Cristina Stancu[1], V. Ciubotaru[2], M. Coculescu[1]

[1]Department of Endocrinology, "Carol Davila" University of Medicine and Pharmacy,
[2] Bagdasar Arseni Hospital, Bucharest, Romania

At least one fifth of pituitary adenomas exhibit plurihormonality when using immunohistochemistry for anterior pituitary hormones. However, the correlation with clinical features is weak, without an agreement upon pathological predictors of tumor behavior.

The aim was to determine the immunoreactivity for anterior pituitary hormones and alpha subunit in 276 consecutive pituitary adenomas patients, aged 22-79 years (44.3 ± 8), 154 F/ 122 M: 83 acromegalics (ACM), 173 nonfunctioning adenomas (NFA) and 20 prolactinomas (PRM) submitted to surgery via transfrontal (81) or transsphenoidal (195) along 10 years (1995-2005). In addition, clinical data, hormonal secretion and tumour size were evaluated before pituitary surgery. Local ethical committee approved the study design. The immunoreactivity performed by the avidin-biotin-complex method was evaluated for beta FSH, LH, TSH, alpha subunit, PRL and GH, using a semiquantitative scale of stained cells: strong (>20%), positive (10-20%), weak (5-10%) and negative (<5%). CT or MRI tumor size (less than 1 cm, 1-2 cm, 2-4 cm and over 4 cm on maximal diameter) were considered together with the Hardy neuroradiological stage. The results showed that 16/83 ACM, 53/173 NFA and 4/20 PRM exhibited immunoreactivity for beta FSH and LH. TSH immunoreactivity was positive in 13/83 ACM, 11/173 NFA and 1/20 PRM. Tumor size in gonadotrophin - positive group (> 10% of stained cells) was between 1-2 cm in 6 ACM, 21 NFA and 2 PRM, while positive bigger tumors (2-4 cm) were in 7 ACM, 24 NFA and 2 PRM. Giant, over 4 cm tumors were positive in 3 ACM, 8 NFA and no PRM. A similar trend of the tumor size distribution was observed in the monohormonal or null cell adenomas. In conclusion, tumor size and gonadotrophin plurihormonality are independent factors in the management of pituitary adenomas.

Key words: pituitary adenomas, gonadotrophs, immunohistochemistry, neurosurgery.

INTRODUCTION

The adenomas are the most frequent pituitary lesions, in imagistic (22.5%) or autopsy (14.4%) studies with an overall prevalence of 16.7% (1). However, most of

*Correspondence to: Corin Badiu "C. I. Parhon" Institute of Endocrinology, Bd. Aviatorilor 34-36, Bucharest, Romania, fax: +4021.3198718, e-mail:badicrin@yahoo.co.uk

them are devoid of any clinical signs, since an approach for pituitary macroadenomas in a similar unselected series, showed a general population prevalence of 0.2%. Pituitary adenomas express a wide range of clinical, proliferation behavior and hormonal activities. Some are brought to the clinical attention due to autonomous oversecretion of PRL, GH, or ACTH, others are devoid of excess obvious secretory activity but lead to mass effects. Current advances in understanding of molecular biology of pituitary adenomas led to a more comprehensive classification, related to their biological behavior (2). The so-called "functionless" pituitary adenomas, classified on the basis of hormone production expressed by immunohistochemistry, are gonadotropinomas, and using the *in vitro* primary tumor cell cultures approach, the amount increased at 80% (3). Formerly known as "null cell adenomas", even the adenomas devoid of gonadotropins immunoreactivity are positive for SF-1, witnessing the relation with gonadotroph adenoma family.

Plurihormonal pituitary adenomas raised many controversies over time, beginning with their discovery, which infirmed the traditional dogma of "one cell-one hormone". With the introduction of immunohistochemical staining in the usual algorithm of diagnosis of pituitary adenomas, it became clear that the adenohypophysial cells did not have a straight specialization and the group of hormonally inactive adenomas was not entirely inactive as it was believed. After the first recognition of their existence, a more precise diagnosis was established using the monoclonal antisera (4), which obviate the false positive results of plurihormonal adenomas with gonadotroph expression related to the use of polyclonal antisera contamined with antibodies to alpha-subunit of the glycoprotein hormones.

Based on the present knowledge of the pituitary cytodifferentiation (5), the plurihormonal adenomas have been classified according to the molecular determinants of their origin: transcription factors involved (6). Although, there are yet unclassified unusual plurihormonal adenomas, which seem to arise from an early undifferentiated pituitary lineage.

Factors which might affect the clinical aggressiveness (size and invasion) of pituitary adenomas have been extensively evaluated. Proliferation markers, such as PCNA, Ki-67, anti-apoptotic proteins (Bcl-2, p53) are not related to the adenoma size. The present study aims to discern the distinctive features of plurihormonal vs monohormonal adenomas, concerning tumor size, recurrence and markers of proliferation. The relationship between the plurihormonality**,** size and invasiveness has not been evaluated in the literature yet and it represents the purpose of this study.

PATIENTS AND METHODS

The aim was to determine the immunoreactivity for anterior pituitary hormones and alpha subunit in 276 consecutive pituitary adenomas patients, aged 22-79 years (44.3 ± 8), 154 F/ 122 M: 83 acromegalics (ACM), 173 nonfunctioning adenomas (NFA) and 20 prolactinomas (PRM) along 10 years (1996-2005). In

addition, clinical data, hormonal secretion and tumor size were evaluated before pituitary surgery.

Screening investigations included general clinical and endocrine evaluation assessment of pituitary morphology by MRI or high resolution CT in coronal acquisition mode. Endocrine evaluation was performed with pituitary suppression or stimulation tests. For acromegaly, oral glucose tolerance test with GH measurement and basal IGF1 values represents the gold standard for tumor activity. Assessment for pituitary reserve was done after radical treatment, for basal gonadal hormones, TSH and free T4, as well as insulin tolerance test with 0.1 -0.15 u/kg bw (regular insulin) with cortisol measurement at 30 min after the glycemic nadir.

Consecutive cases were enrolled according to the following inclusion criteria: acceptance of the informed consent signed by the patient; age 18-80; presence of a pituitary tumor mass suggesting invasion, on MRI or coronal CT; presence of evolution signs for a pituitary mass, along previous follow-up; presence of abnormal plasma hormone levels. The main exclusion criterion was patients' decision to avoid surgery.

We aimed to determine the plurihormonality degree by a complete immunohistochemical staining and the quantification of pluri/monohormonal cells.

The patients were referred to the Department of Endocrinology at the University of Medicine and Pharmacy in Bucharest, on the basis of visual field disturbances, acromegaly, amenorrhea, pituitary failure or radiological changes on the sellar X ray. Diagnosis was established using classical clinical, biological and neuroradiological criteria.

The selected cases were submitted to neurosurgery at the "Bagdasar Arseni" Hospital in Bucharest, where adenoma removal was performed via transfrontal (81) or transsphenoidal (195) pathway. Three months after surgery, patients were sent for evaluation at the Endocrine Clinic in Bucharest Institute of Endocrinology.

The Ethical Committee of the "Carol Davila" Bucharest University of Medicine and Pharmacy approved the study protocol.

TISSUE PREPARATION

Fragments of pituitary adenomas were collected at surgery. Extreme care was taken to ensure that samples for analysis were devoid of any contaminating normal tissue. The adenoma nature as well as the lack of contamination of the samples was confirmed later by pathological analysis (Gomori stain for reticulin network) of the samples. Tissue blocks were fixed in cold 4% paraformaldehyde (PFA) in phosphate buffered saline (consisting of 130 mM NaCl, 5 mM Na_2HPO_4, 5 mM NaH_2PO_4 adjusted to pH 7.4 -PBS). After 4-6 hours, the tissue was processed for paraffin embedding. Three mm thick sections were cut, mounted on gelatine coated slides and stored until processed. Immunohistochemistry was carried out on serial sections as described below.

Antisera against human AP hormones FSH β (AFP891891), GH (AFPC11981A), LH β (AFP55951889), PRL (AFP55781789), TSH β (AFP55741789), and ACTH (AFP39032082Rb) were generous gifts from the National Hormone and Pituitary Program (Torrance, CA) and Dr. A. F. Parlow. The TSH, FSH and LH antisera are beta subunit specific, purified by affinity chromatography. A special attention was given to the association of peptide hormones GH or PRL with glycoproteic hormones: FSH, LH, TSH or alpha subunits.

Immunostaining was performed using the avidin-biotin horseradish peroxidase complex (ABC) method, according to the following protocol: the slides were washed 3× in 10 mM PBS for 10 min, incubated with 1% H_2O_2 for 10 min., then washed again with PBS. The slides were then preincubated with 5% swine serum in PBS containing 1% bovine serum albumin (BSA) and 0.3% Triton X-100 (Tx) for 1 h at room temperature (RT). Primary antibodies (polyclonal rabbit anti human), in the following dilutions: β TSH 1/2000, β FSH 1/1000, β LH 1/1000, PRL 1/3000, GH 1/2000 were added and incubated overnight at 4°C. After 3×10 min washings in PBS + 0.25% BSA + 0.1% Tx, the sections were incubated with the biotinylated swine anti-rabbit (E 353, DAKO) secondary antibody at a dilution of 1:200 for 1 hour, RT. Sections were then rinsed for 3x10 min in 10 mM PBS and incubated in avidin-biotin horseradish peroxidase complex (DAKO) 1/200 in 10 mM PBS for 1 h, RT. Finally, the slides were washed in PBS, then for 10 min with 50 mM Tris-HCl (pH 7.6), followed by incubation with 0.05% 3,3' diaminobenzidine hydrochloride (Sigma) with 0.005% H_2O_2 in Tris/HCl. Slides were air dried and coverslipped with Depex. The sections were quantified for the number of stained cells reported to the total area using image analysis software (LUCIA M version 3.0, Nikon Laboratory Imaging Ltd.) coupled with a Zeiss AXIOSKOP microscope. The results were considered as +++ when more than 20% cells were positive, ++ when 10-20% cells were stained, + when 5-10% of the cells expressed signal and 0 when <5% were stained. For the negative controls, the primary antibody was omitted and replaced with PBS. Normal pituitaries from autopsies in patients without known pituitary disease were used as a positive control.

CT evaluation was done at the Institute of Endocrinology, with a General Electric CT scanner using the coronal acquisition plane and sections at 1-2 mm every 2-3 seconds. After a first native scan (without contrast), the patients received 50 ml bolus and more 50 ml intravenous contrast fluid (sodium diatrizoate or iopromide in selected cases). Using this protocol, the scanning shows progressive pituitary contrast which ends within 1 minute delay from the first scan. Pituitary masses presented as hypodense lesions. Using this protocol we can precisely locate the tumour extension and invasion: lateral towards cavernous sinuses, upward pushing the optic chiasm or down towards the sphenoid sinus. The tumours were classified according to the maximal tumour diameter: less than 1 cm, 1-2 cm, 2-4 cm and over 4 cm. Consecutive evaluations were done in pituitary adenoma patients, before therapy and at 3 months, 6 months and then yearly. For this study, CT performed before surgery was considered in the evaluation.

RESULTS

In an attempt to characterize the immunoreactivity of pituitary adenomas, a total of 276 consecutive pituitary adenomas were evaluated by immunohistochemistry: 83 acromegalics (ACM), 173 nonfunctioning adenomas (NFA) and 20 prolactinomas (PRM) along 10 years (1995-2004). In addition, clinical data, hormonal secretion and tumor size were evaluated before pituitary surgery. The immunostain for gonadotrophins was encountered in 16/83 ACM, 53/173 NFA and 5/20 PRM (Fig. 1 a-d).

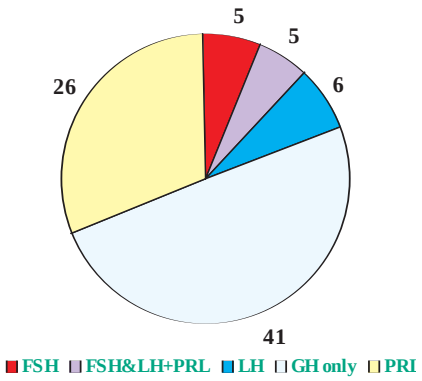

Figure 1 a. Immunostaining in 83 ACM, with co-secretion of gonadotrophins in 16/83 and PRL in 26/83.

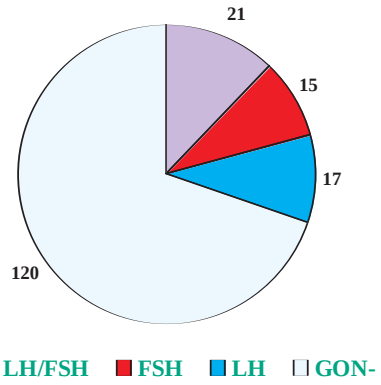

Figure 1 b. Immunostaining in 173 NFA, with co-secretion of gonadotrophins in 53/173.

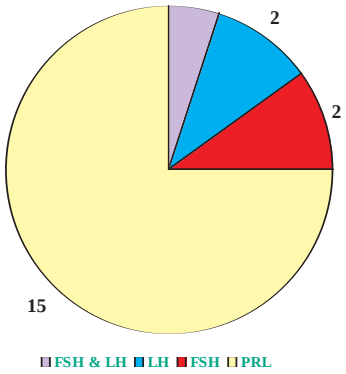

Figure 1 c. Immunostaining in 20 PRM, with co-secretion of gonadotrophins in 5/20.

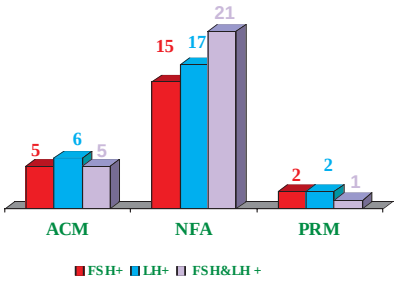

Figure 1 d. Distribution of gonadotrophin immunoreactivity among 276 consecutive cases of pituitary adenoma patients. ACM = acromegaly (n=83), NFA = non-functioning adenomas (n=173), PRM = prolactinomas (n=20). Positive staining (+) includes more than 10% of immunoreactive cells.

In addition, GH immunoreactivity was strongly positive in all but 3 cases, while PRL was positive in 26 pure GH cases and in 5 cases with FSH and LH immunoreactivity. NFA were negative for the rest 120 cases. All prolactinomas were intense positive for PRL. The immunoreactivity for TSH was positive in 13/83 ACM, 11/173 NFA and 1/20 PRM. A large nonfunctioning pituitary adenoma evaluated by MRI (Fig. 2 a, b) expresses immunoreactivity for all glycoproteic hormones by immunohistochemistry (Fig. 3 a-c).

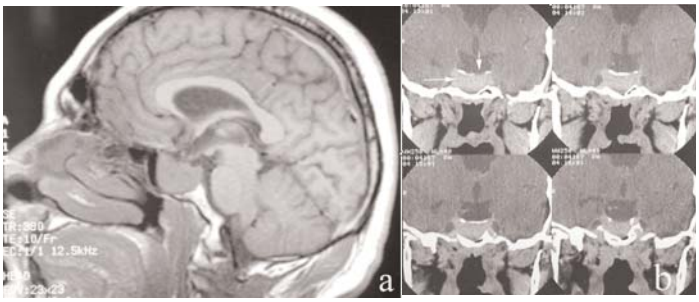

Figure 2. Large pituitary macroadenoma with optic chiasma syndrome in a 37 y aged man. Sagittal (a) and coronal (b) MRI.

Tumor size in gonadotrophin - positive group (> 10% of stained cells) was between 1-2 cm in 6 ACM, 21 NFA and 2 PRM, while positive bigger tumors (2-4 cm) were in 7 ACM, 24 NFA and 2 PRM. Giant, over 4 cm tumors were gonadotrophin positive in 3 ACM, 8 NFA and no PRM. No significant differences were observed between FSH and LH. TSH immnoreactivity was positive in 3 cases with giant NFA. In big tumors (2-4 cm), TSH was positive in 9 ACM, 5 NFA and 1 PRM, while tumors between 1-2 cm were positive for TSH in 4 ACM and 3 NFA.

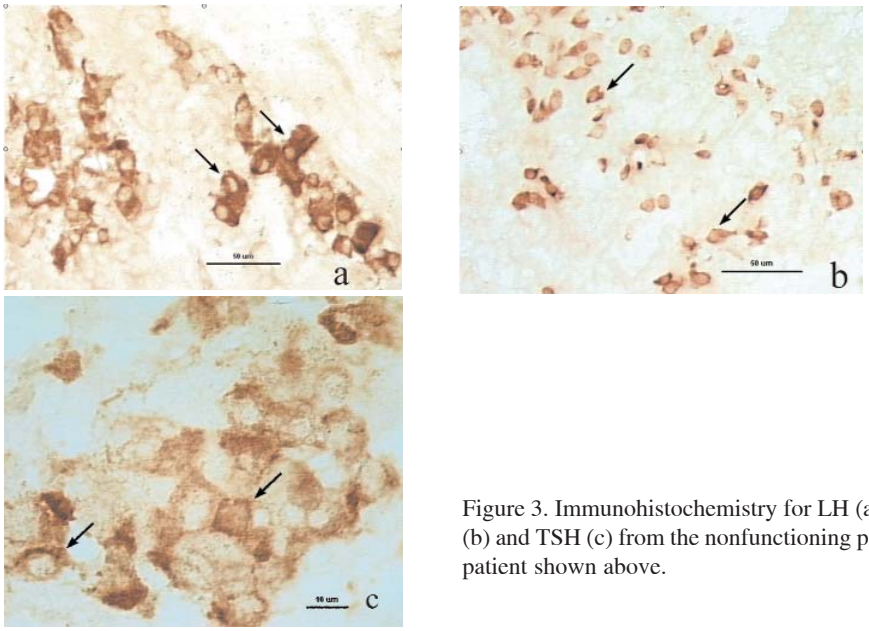

Figure 3. Immunohistochemistry for LH (a), FSH (b) and TSH (c) from the nonfunctioning pituitary patient shown above.

A similar trend of the tumor size distribution was observed in the monohormonal (ACM, PRM) or null cell adenomas.

DISCUSSION

Pituitary adenomas represent about 15% of all intracranial neoplasias, causing considerable morbidity through local invasion, hypopituitarism and tumoral secretion. The classic histopathological classification of pituitary adenomas has significantly changed since new techniques of morphological investigation were introduced. Immunohistochemistry, multiple sequential primary immunohistochemistry, immune electronmicroscopy have led to a more complex understanding of histologic types of these tumors. However, a comprehensive characterization of pituitary tumors according to their cell phenotypes is lacking.

The existence of multihormonal cells in the developing human hypophysis is now in progress, being proved in adenomas. A complete phenotypic characterization of pituitary tumor cells in respect of hormonal secretion and response to hypothalamic releasing hormones has demonstrated recently the heterogeneity of cell phenotype among different tumors and among tumors of the same type (7). The multihormonal profile was found in 20% of cells in prolactinomas, in 18% of cells in non-functioning adenomas and in 50% of cells in ACTH-secreting adenomas. Multiresponsive cells varied between 40% and 70%, an important feature in the perspective of judging their proliferative capacity. It is well known that hypothalamic releasing hormones increase also the mitotic activity of target cells. Thus, the excessive secretion of GHRH stimulates the proliferation of somatotropes, with consecutive hyperplasia or even neoplastic transformation of GH-secreting cells (8). It may presume that multihormonal/multiresponsive cells, target of multiple proliferative signals of environment, tend to proliferate more than normal adenohypophysial cells. If pituitary adenomas arose from multifunctional cells, then paracrine or hormonal signals contribute to the process of tumorigenesis and promote the expansion of tumor clone. In addition, as it was described (9), such tumors could change the phenotype along its evolution, due to the extinction of some factors and the coming up of others, which generate a different type of tumoral and hormonal response.

There are extremely few systematic studies investigating the prevalence of plurihormonal adenomas. The few available data come from studies of cases or reports of adenomas with immunohistochemical staining for a particular hormone in common, e.g. TSH, which have been found additional positive for more than one hormone. A systematic study of surgically removed pituitary adenomas over 12 years in a single department found a prevalence of 31% of plurihormonal phenotype (10). The most frequent hormonal combinations were GH + PRL (14%) and GH/PRL + one or more glycoprotein hormones (11%). Surprisingly, as we mentioned before, the combination GH+PRL+ACTH ± glycoprotein hormone,

which has been encountered in as many as 11.4% of cases, should not be found, in the perspective of the present knowledge of pituitary ontogenesis.

Concerning the cytogenesis of plurihormonal adenomas, three possible working hypotheses exist: 1. plurihormonal primordial stem cells undergo multidirectional differentiation and produce more than one hormone; 2. more differentiated monohormonal cells undergo mutation during tumor progression and begin to produce more than one hormone; 3. neoplastic transformation of two or more cell types.

Despite their benign histological appearance, pituitary adenomas are known to have a propensity to invade the surrounding structures. This capacity for aggressive local growth, defined either radiological or intraoperative evidence of gross invasion and/or histopathological evidence of dural invasion not only precludes complete surgical removal, but also imposes significant neurological and endocrinological morbidity. Hence, a clear tool to predict the tumor behavior and to dictate the therapy is mandatory.

Ki-67 is a specific antigen of the cell cycle, expressed in the G1, S, G2 and M phases, but not in the quiescent G0 phase, which is recognized by the monoclonal antibody MIB-1. The relationship between the Ki-67 expression and the tumor growth velocity has been evaluated recently in a study on non-functioning adenomas (11). This study demonstrated that the growth rate is significantly correlated with the proliferation marker Ki-67: a rapid growth (daily growth rate exceeding 0.07%, equivalent to a yearly growth rate over 25% of tumor volume) was found to have a Ki-67 index >1.5%; slowly growing adenomas (less than 0.02% daily increase) were found to have Ki-67 index lower than 1.5%. This marker is an important tool for a clinician, who can decide, after performing the surgical intervention, to add another therapeutic option, like radiotherapy, if a higher risk of rapid tumor regrowth exists. It is still controversial whether Ki-67 expression correlates with invasive behavior of pituitary adenomas (12;13).

In our study, the pituitary adenomas were evaluated for hormonal immunoreactivity versus tumor size and invasion. Tumors above 2 cm in maximal diameter were not more immunoreactive than smaller tumors, suggesting that tumor size and glycoproteic immunoreactivity are independent factors of evolution. Further studies are necessary in order to evaluate this correlation for alpha subunit immunoreactivity or Ki -67 index.

References

1. Ezzat S, Asa SL, Couldwell WT, Barr CE, Dodge WE, Vance ML, McCutcheon IE. The prevalence of pituitary adenomas: a systematic review. Cancer 2004; 101(3):613-619.

2. Al Shraim M, Asa SL. The 2004 World Health Organization classification of pituitary tumors: What is new? Acta Neuropathol (Berl) 2005.

3. Chaidarun SS, Klibanski A. Gonadotropinomas. Semin Reprod Med 2002; 20(4):339-348.

4. Labat-Moleur F, Trouillas J, Seret-Begue D, Kujas M, Delisle MB, Ronin C. Evaluation of 29 monoclonal and polyclonal antibodies used in the diagnosis of pituitary adenomas. A collaborative study from pathologists of the Club Francais de l'Hypophyse. Pathol Res Pract 1991; 187(5):534-538.
5. Asa SL, Ezzat S. Molecular basis of pituitary development and cytogenesis. Front Horm Res 2004; 32:1-19.
6. Al Shraim M, Asa SL. The 2004 World Health Organization classification of pituitary tumors: What is new? Acta Neuropathol (Berl) 2005.
7. Senovilla L, Nunez L, de Campos JM, de Luis DA, Romero E, Sanchez A, Garcia-Sancho J, Villalobos C. Multifunctional cells in human pituitary adenomas: implications for paradoxical secretion and tumorigenesis. J Clin Endocrinol Metab 2004; 89(9):4545-4552.
8. Frohman LA, Kineman RD. Growth hormone-releasing hormone and pituitary development, hyperplasia and tumorigenesis. Trends Endocrinol Metab 2002; 13(7):299-303.
9. Mazarakis N, Kontogeorgos G, Kovacs K, Horvath E, Borboli N, Piaditis G. Composite somatotroph-ACTH-immunoreactive pituitary adenoma with transformation of hyperplasia to adenoma. Pituitary 2001; 4(4):215-221.
10. Ho DM, Hsu CY, Ting LT, Chiang H. Plurihormonal pituitary adenomas: immunostaining of all pituitary hormones is mandatory for correct classification. Histopathology 2001; 39(3):310-319.
11. Honegger J, Prettin C, Feuerhake F, Petrick M, Schulte-Monting J, Reincke M. Expression of Ki-67 antigen in nonfunctioning pituitary adenomas: correlation with growth velocity and invasiveness. J Neurosurg 2003; 99(4):674-679.
12. Iuchi T, Saeki N, Osato K, Yamaura A. Proliferation, vascular endothelial growth factor expression and cavernous sinus invasion in growth hormone secreting pituitary adenomas. Acta Neurochir (Wien) 2000; 142(12):1345-1351.
13. Yokoyama S, Hirano H, Moroki K, Goto M, Imamura S, Kuratsu JI. Are nonfunctioning pituitary adenomas extending into the cavernous sinus aggressive and/or invasive? Neurosurgery 2001; 49(4):857-862.