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EXPRESSION OF P53, KI-67 AND C-ERB B2 IN GROWTH HORMONE- AND/OR PROLACTIN-SECRETING PITUITARY ADENOMAS

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ABSTRACT - The subcellular events implicated on the formation and behavior of pituitary adenomas are not fully understood. In this study we investigated the presence of p53, Ki-67 and c-erb B2 in 38 pituitary adenomas with immunohistochemical positivity for GH and prolactin (n=26; 68.4%), for prolactin (n=9; 23.7%) and for GH (n=3. 7.8%). The analyses revealed the following results: 24 (63.2%) tumors expressed variable positivity for c-erb B2, 11 (28.9%) expressed p53 positivity and 11 (28.9%) tumors were variably positive for Ki-67. Our results demonstrated a high percentage of GH/prolactin-, prolactin- and GH-secreting tumors with immunohistochemical positivity for c-erb B2. Once this membrane receptor is related to growth factors EGF and TGFα and both have a definite effect on tumor growth, our data suggest a possible role for c-erb B2 on the evolution of these tumors.

KEY WORDS: pituitary adenomas, growth hormone, prolactin, p53, Ki-67, c-erb B2, immunohistochemistry.

Expressão de p53, Ki-67 e c-erb B2 em adenomas hipofisários secretores de prolactina e/ou hormônio de crescimento

RESUMO - Os eventos subcelulares implicados na formação e comportamento dos adenomas hipofisários não são completamente compreendidos. Neste estudo nós investigamos a presença de p53, Ki-67 e c-erb B2 em 38 adenomas hipofisários com positividade imuno-histoquímica para GH e prolactina (n=26, 68,4%), para prolactina (n=9, 23,7%) e para GH (n=3, 7,8%). A análise revelou os seguintes resultados: 24 tumores (63,2%) expressaram positividade variável para c-erb B2, 11 (28,9%) expressaram positividade para p53 e 11 tumores (28,9%) foram variavelmente positivos para Ki-67. Nossos resultados demonstraram elevada percentagem de tumores secretores de GH/prolactina, prolactina e GH com positividade imuno-histoquímica para c-erb B2. Desde que este receptor de membrana está relacionado aos fatores de crescimento EGF e TGFα e ambos têm efeito definido no crescimento tumoral, nossos dados sugerem possível função para o c-erb B2 na evolução destes tumores.

PALAVRAS-CHAVE: adenomas hipofisários, hormônio de crescimento, prolactina, p53, Ki-67, c-erb B2, imuno-histoquímica.

Human pituitary originates from Rathke's pouch during the initial stage of embryonal development. Its ventral epithelium differentiates in the direction of anterior pituitary, while dorsal epithelium originates the intermediate lobe. A process of cellular differentiation gives rise to different cells: acidophil cells, that are the progenitor cells for GH- and prolactin-secreting cells, and basophilic cells, originating ACTH-, TSH-, LH- and FSH-secreting cells[1]. There are experimental evidences that most of the lactotrophic cells come from somatotrophic cells. Destruction of somatotrophic cells results on the elimination of most lactotrophs, despite persistence of a small percentage of these cells[1]. Besides that, most prolactin is secreted by lactotrophs in normal subjects, but there is a small fraction that is secreted by mamosomatotrophic cells, also capable of GH secretion[2]. Among human foetal cells, between 18 and 22 weeks, 70% secrete GH only, 9% prolactin only and 21% both hormones[3]. Other connection between

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GH- and prolactin-secreting is related to Pit 1, a transcription factor expressed in lactotrophs and somatotrophs (and also thyrotrophs) that is a critical element for activating the expression of prolactin, GH and TSH genes[4]. Reinforcing this idea, acromegalic patients disclose adenomas that secrete GH or mixed adenomas, composed of two different types of cells, GH- and prolactin-secreting cells, or adenomas secreting both, GH and prolactin, in the same cell[5]. Pituitary adenomas are monoclonal. An intrinsic genetic alteration gives rise to a clonal expansion of only one cell, resulting in adenoma formation[6]. Point mutations on the α chain of stimulating G protein gene, the Gsp oncogene, are present in 40% of the GH-secreting adenomas[7]. Also associated with this group of tumors are the phosphorilation of the c-AMP binding protein (with subsequent increased secretion of GH and increased somatotrophic proliferation) and the increased expression of pituitary tumor transforming gene (PTTG), with its cell transforming effect. Fibroblast growth factor 2 (FGF-2) is abundantly found in normal pituitary, stimulating prolactin secretion by normal and adenomatous cells[8].

The sub-cellular events involved in the formation and evolution of pituitary adenomas are not fully understood. Proto-oncogene c-erb B2 codifies a transmembrane protein and is a receptor for the epidermal growth factor (EGF). This growth factor has an important role on the evolution of pituitary tumors[8]. Despite this fact, we did not find references to possible associations between c-erb B2 and pituitary tumors. Tumor suppressor gene p53 controls entry of the cells on the cell cycle, on phase G1, and mutations on this gene permit progression of tumor cells in the same cell cycle. The presence of p53 mutations in pituitary tumors is rarely described[9,10], but some authors found a relationship with proliferative states[11,12] or mediating bromocriptine action in prolactinomas[13]. The nuclear antigen Ki-67 has not been demonstrated to be associated with GH- and/or prolactin-secreting tumors[14], but may have some relationship with invasiveness of pituitary tumors[15] or their proliferative potential[16], observations contested by Mastronardi et al.[17]. Pituitary adenomas are almost always benign. They grow slowly, may be confined to the sellar compartment or extend superiorly through sellar diaphragm, compressing optic quiasm and invading cavernous as well as sphenoidal sinuses. In up to 30% of cases they infiltrate adjacent bone and, more rarely, the brain, characterizing an invasive adenoma[18]. Due to this particular behavior, the search for cell proliferation markers could be useful for understanding their clinical evolution. Up to this time, no parameters are available as dependable tools for identifying aggressive tumors on risk of recurrence[19].

Due to the common origin of GH- and prolactin-secreting cells, the presence of lesions secreting both hormones and the need for understanding the intrinsic cell factors that may be involved in cell proliferation of these tumors, we studied the presence of tumor markers (p53, Ki-67 and c-erb B2) in a series of 38 GH- and prolactin-secreting tumors.

METHOD

Thirty-eight patients presenting with GH- and prolactin-secreting pituitary adenomas (13 male - 34.2% - and 25 female - 65.8%) were selected for this study based on the availability of clinical information and of histological samples. In the male population the mean age was 37 years, varying from 18 to 60 years (median: 39 years); in the female population the mean age was 40 years, varying from 18 to 62 years (median: 41 years). All patients were submitted to adenomectomy at the Hospital Universitário de Brasília.

Nine patients were re-operated, for periods that lasted from 1 to 95 months after the first operation. Four male patients (44.5%) presented with tumors from 1 to 95 months after the first surgery (mean: 30 months; median: 12 months). One of the patients (case 27) was re-operated twice (7 and 20 months, respectively, after the initial procedure). He was recently seen and considered inoperable, waiting for complementary radiotherapy. Five female patients (55.5%) with tumors detected from 2 to 96 months, respectively, from the first intervention (mean: 28 months; median: 14 months).

In this study we used paraffin block-embedded material stored at the archives of the Department of Pathology, Universidade de Brasília. The specimens were obtained during operative procedures intended to cure or limit the extension of the disorders, for which informed consent was

Table 1. Used antibodies, clones and dilutions.

Antibodies (all Dako Corporation products)	Clones	Dilution
1. Anti-PRL	Polyclonal	1:2000
2. Anti-GH	Polyclonal	1:2000
3. Anti-FSH	C10	1:50
4. Anti-LH	C93	1: 50
5. Anti-ACTH	O2A3	1:100
6. Anti-TSH	42	1: 50
7. p53	DO7	1:100
8. Ki-67	Mib 1	1:100
9. c-erb B2	Oncoprotein C	1:400

Table 2. Immunohistochemistry. Data analyses.

A. GH, PRL, FSH, LH, TSH and ACTH antibodies (positive: cytoplasmic staining):

1 less than 10% of cells with positive staining
2 between 10 and 20% of cells with positive reaction
3 between 21 and 50% of cells with positive reaction
4 more than 51% of cells with positive reaction

Note: We considered, as secreting, tumors with any staining intensity.

B. c-erb B2 (positive:membrane staining):

0 lack of positivity in more than 90% of tumor cells
1 discrete immunopositivity in more than 10% of tumor cells
2 discrete to moderate positivity in more than 10% of tumor cells
3 intense and complete immunoreactivity in more than 10% of tumor cells

C. Ki-67 and p53 (positive:nuclear staining):

0 complete lack of positivity in tumor cell
1 immunoexpression in less than 10% of tumor cells
2 immunoexpression between 10 and 25% of tumor cells
3 immunoexpression between 25 and 50% of tumor cells
4 immunoexpression in more than 50% of tumor cells

Note: Lesions with less than 10% of nuclear staining for Ki-67 were considered of low proliferative potential; lesions with more than 25% of nuclear staining as highly proliferative, and intermediate values as moderate or intermediate proliferative potential.

obtained from all patients. Approval by the Committee on Ethics in Research of the Universidade de Brasília was an obligatory requirement for this study, being obtained after fulfilling extensive prerequisites. Techniques were performed both at the Department of Pathology, Universidade de Brasília, Brazil, and IPATIMUP, O Porto, Portugal.

Specimens were fixed in 10% formalin and submitted to embedding in paraffin according to standard histological procedures. Hematoxylin and eosin staining was performed in all sections. Immunohistochemical evaluation (using Streptavidin-Biotin systems) included hormonal as well as "prognostic" profiles. The used antibodies, clones and respective dilutions are depicted in Table 1. Reactions were developed with diaminobenzidine (DAB) and counterstained with hematoxylin[20].

Immunohistochemistry results were interpreted according to Table 2.

Immunohistochemical evaluation was done according to standard techniques and by "tissue microarray". In this last modality, only the most representative areas of the lesions were selected and extracted from the original paraffin block using specially designed syringes. Samples from different cases were then joined in new paraffin blocks, permitting simultaneous analysis of many surgical specimens (Figs 1 and 2).

RESULTS

Data from immunohistochemical evaluation con-

Table 3. Immunohistochemical semi-quantitative analysis of pituitary hormones PRL and GH and associated Ki-67, p53 and c-erb B2 expression.

Patient	PRL	GH	P53	Ki 67	C-erb B2
1	2	2	0	0	0
2	3	4	0	0	0
Reoperation	0	4	0	0	0
3	3	4	0	0	3
4	4	0	0	0	0
5	2	4	0	0	0
Reoperation	0	2	0	0	0
6	1	3	0	0	0
7	2	3	0	0	0
Reoperation	2	3	0	0	1
8	4	4	0	0	0
Reoperation	4	4	0	0	2
9	4	0	3	0	2
10	3	0	3	0	1
11	4	4	2	1	3
12	2	3	0	0	0
13	4	0	0	0	0
14	4	2	0	0	2
15	0	4	1	0	1
16	4	4	0	0	0
17	3	4	1	0	2
18	2	3	0	1	0
19	4	4	3	2	3
20	4	4	3	3	3
Reoperation	2	4	0	1	0
21	2	0	0	4	3
22	2	0	0	1	3
23	4	0	1	0	3
24	2	4	1	0	0
25	4	2	0	0	0
26	2	3	0	1	3
27	0	2	0	0	3
Reoperation	-	-	-	-	-
28	4	4	4	1	3
Reoperation	4	4	0	1	0
29	4	0	0	1	0
30	3	3	0	0	3
31	2	3	0	0	2
Reoperation	0	4	0	0	2
32	2	3	0	0	3
33	0	4	0	0	3
34	3	2	0	0	0
Reoperation	-	-	-	-	-
35	3	3	0	3	0
36	3	2	2	0	3
37	2	3	1	1	3
38	1	0	0	0	0

cerning hormonal as well as prognostic profiles are presented in Table 3.

Immunohistochemical analysis of the lesions obtained from the first operation revealed that 26 (68.4%) were positive for GH and prolactin, nine (23.7%) were positive for prolactin and three (7.8%) for GH. Immunohistochemical profiles of the tumors operated for the second time disclosed concordant results with first operation in four cases; in two cases analysis was not done (one of them because of lack of tumoral tissue at reoperation - case 34), and in three cases results were discordant (originally GH- and PRL-positive, and only GH positivity in the second evaluation). Two of the GH- and prolactin-positive cases showed also LH- and TSH-positivity; other case disclosed simultaneous positivity for ACTH. A PRL-positive case and a GH-positive case disclosed also positivity for LH.

Among the cases operated on for the first time, 20 (52.6%) showed c-erb B2 positivity from 2 to 3, and two were 1 (Fig 3). Considering the hormonal expression, the distribution was 14/26 (53.8%) GH/PRL-positive, 6/9 (66.6%) PRL-positive (Fig 4) and 1/3 (33.3%) GH-positive (Fig 5). These differences were not statistically significant (Fischer test p=0.394 GH/PRL and prolactin; p=0.363 PRL and GH). From seven tumors analysed for c-erb B2 after the second operation, three were concordant with the first profile, two were not analyzed and four were discordant; the discordant ones were two negatives on first and 1 or 2 on the second intervention, and two 3 on the first and negative on the second.

Concerning p53, 11 (28.9%) tumors showed expression, but only six (15.9%) from 2 to 3 and five 1 (Fig 6). The distribution, according to the histochemical type of tumor, was similar: 8/26 (26.9%) GH-/PRL-secreting, 3/9 (33.3%) PRL-secreting and 1/3 (33.3%) GH-secreting. For Ki-67 we observed 11 positive cases (28.4%), from which four (10.5%) from 2 to 4, and seven 1 (Fig 7). The distribution according to tumor type was 8/26 of GH-/PRL-secreting (30.7%) and 3/9 (33.3%) of PRL-secreting type. None of the isolated GH-secreting tumors was positive for Ki-67.

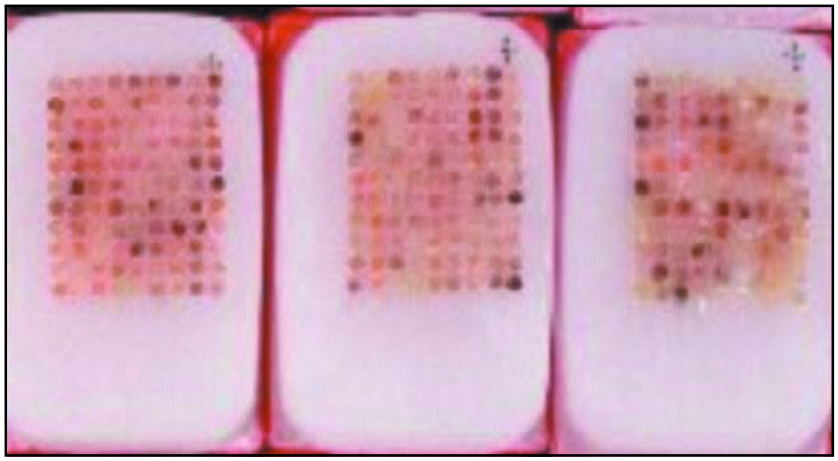

Fig 1. Microarray prepared paraffin blocks.

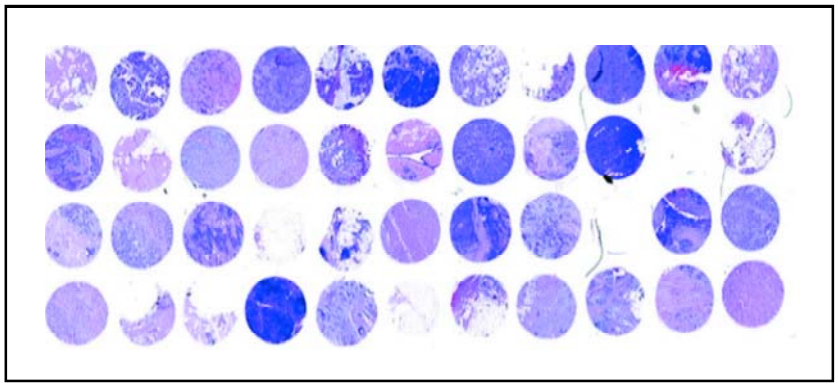

Fig 2. Tissue microarray slide sections. Up to 600 different specimens may be displayed in the same slide using modern technology.

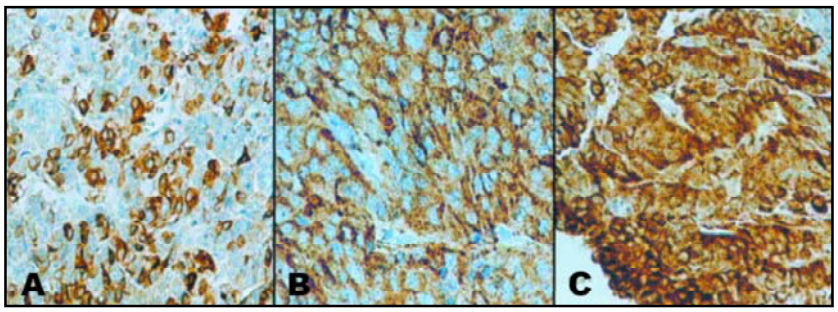

Fig 3. Grade 3 intensity of membrane staining for c-erb B2. A:200X; B:400X; C:200X.

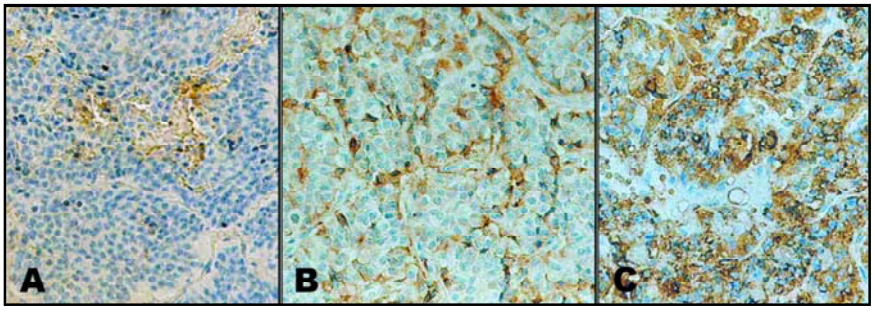

Fig 4. Intensity of cytoplasmic staining for prolactin: A:(0), 100X; B:(2), 200X; C:(4), 200X.

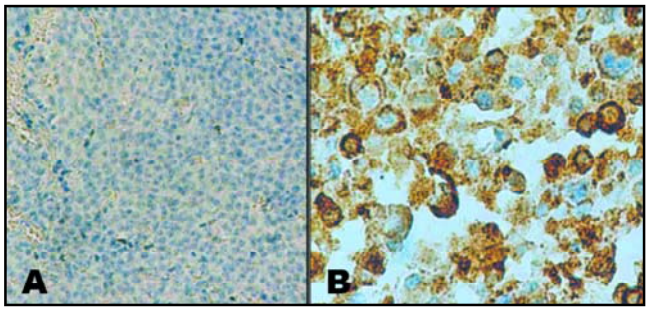

Fig 5. Intensity of cytoplasmic staining for GH. A: negative (0), 200X; B: positive (4), 400X.

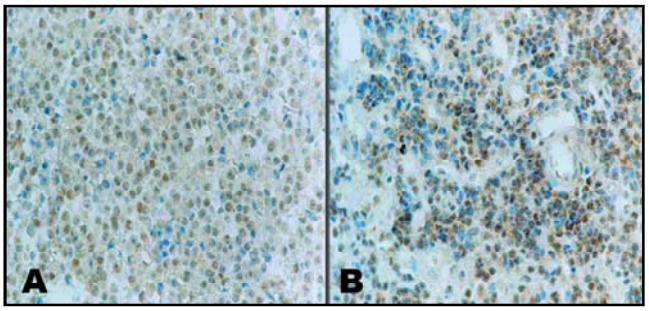

Fig 6. Intensity of nuclear staining for p53 (200X). A:(1); B:(2).

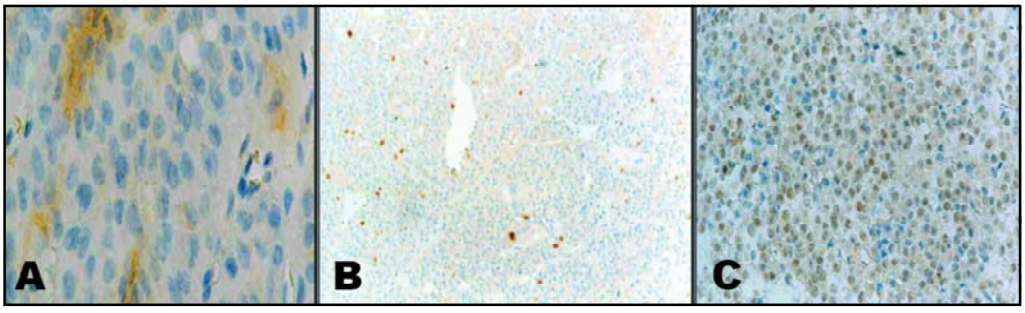

Fig 7. Intensity of nuclear staining for Ki-67. A: negative (0), 400X; B:(1), 100X; C:(1), 200X.

DISCUSSION

Acromegaly is due, in more than 95% of cases, to GH-secreting pituitary adenomas, that may be of different cell types. Exclusively GH-secreting adenomas are identified in 60% of acromegalic patients[5]. In our study, however, these adenomas represented only 7.8% of cases. Mixed adenomas (composed of two different populations of GH- and PRL-secreting cells) cause acromegaly with equally high prolactin levels. These are fast-growing, aggressive lesions. These

were the most common adenomas among our cases (68.4%), a finding not confirmed by other authors[5]. Adenomas expressing both, GH and PRL, in the same cell, present with, generally, normal or lowly increased prolactin levels[5].

A mutation in the somatotrophic cell is a prerequisite for the development of a GH-secreting tumor. All adenomas composed exclusively of GH-secreting cells are of monoclonal origin[6]. Many alterations were described in the GH-secreting adenomas, such as irregularities of the Gs α protein (gsp) that simulates the function of GHRH. Patients carrying these adenomas have smaller tumors and keep GH suppressibility to glucose overcharge[21]. Despite the evidences that a somatotrophic cell may originate GH-secreting adenomas, the events leading to this tumoral expansion are not clear. The presence of an activating oncogene may be necessary for initiating these adenomas.

The pathogenesis of prolactinomas is not equally understood. Due to the inhibitory influence of hypothalamus (through dopamine) on PRL secretion, it has been considered that the decreased dopaminergic tonus should participate in this process. It has been described that prolactinomas are monoclonal, but that hypothalamus would exert a facilitating action on the clonal expansion of a genomically altered cell[6]. It was recently described that tuberoinfundibular dopaminergic (TIDA) neuronal lesion by estrogen taken as a contraceptive method was not associated with a pituitary tumor, despite associated high PRL levels, probably for not being carrier of a mutant lactotrophic cell[22].

Proto-oncogene c-erb B2 is localized in chromosome 17, which encodes a transmembrane receptor protein with tyrosine kinase activity belonging to the epidermal growth factor receptor (EGFR) family. A positive membrane staining for the c-erb B2 product is in most cases associated with real gene amplification, such as demonstrated by molecular biology techniques[20]. The proportion of positivity for c-erb B2 was significative in the GH/PRL-, PRL- and GH-secreting tumors included in our study. This finding was not paralleled in the consulted literature. We did not find papers that described the presence of c-erb B2 in pituitary tumors. However, it is possible that the detection of c-erb B2 in the selected cases might have some influence on their evolution, considering the intimate relationship between c-erb B2 and epidermal growth factor (EGF), as well as the fact that most pituitary adenomas disclose EGF receptors (the expression being higher in non-functioning tumors as compared to functioning ones). High levels of EGF receptor in pituitary tumors suggest that they are involved in their progression[23]. Besides that, serum levels of EGF receptor were higher in patients presenting with macroadenomas and giant adenomas, as compared to pituitary hyperplasia, Rathke's cyst and normal controls. There was a positive correlation between levels of EGF receptors and tumor size[24].

Other growth factor related to EGF, the transforming growth factor alfa (TGF α), that exerts biological effects on EGF receptor, has also been localized in prolactin-secreting cells. Both functioning and non-functioning adenomas, as well as normal pituitary, express this growh factor, suggesting that it might have some role in the pathogenesis of these tumors[8].

In our cases, we identified p53 positivity in only 28.9% of tumors, with similar proportions among the three types of lesions (GH/PRL, PRL and GH). Besides that, only 6 (15.7%) expressed positivity higher than 2. This suggests that p53 would not have an important role in our patients' tumor evolution.

This finding was confirmed by other authors. Levy et al.[9] did not find p53 mutations on secreting or non-secreting adenomas, suggesting that this gene would not have a role in the development of pituitary tumors. The high p53 protein levels identified through immunohistochemistry in pituitary tumors could be a consequence of binding to other cell proteins in these tumors[9]. Oliveira et al.[10] also observed low positivity frequency (1.3%) for p53 among their cases. The tumors studied by these authors were highly invasive, implying that p53 alterations would not be implicated in pituitary tumors aggressiveness. However, other authors identified p53 expression in 61% of pituitary adenomas, associating these finding with the proliferative status of these tumors, but not with their invasiveness or volume[11]. Alterations on p53 were identified in higher proportions of aggressive[12] or invasive[25] neoplasms.

Ki-67 is a nuclear antigen expressed in phases G1, S, G2 and M of the cell cycle and recognized by the commercially available antibody MIB-1. It has been referred that MIB 1 values are not related neither to serum levels of prolactin and GH in patients with prolactinoma and acromegaly, respectively, nor to invasiveness of GH-secreting tumors. However, there is an association with positivity in staining for prolactin and growth hormone[14].

Other studies showed greater MIB-1 positivity in tumors with dural invasion, compared to those in which no invasion was verified[15]. In non-functioning adenomas and oncocytomas (specially the invasive

ones) high levels of Ki-67 expression were detected, as well as a positive relationship between p53 and Ki-67 with invasiveness and tumor recurrence[25]. It was also suggested that invasive adenomas would have a higher proliferation rate, compared to non-invasive, and would express greater amounts of Ki-67[26]. *In vitro* Ki-67 positivity was detected in ten out of twelve pituitary adenomas and, in primary cultures of these tumors, in eleven out of twelve adenomas[16]. The expression of cyclin A and the presence of Ki-67 were significant survival and progression factors in pituitary adenomas[27].

The low expression frequency observed in our cases suggest that Ki-67 would not be involved in the development of GH/PRL, GH and PRL adenomas. Other authors did not found a significant relationship between the presence of Ki-67 and pituitary tumors[28]. These authors were not able to find a positive relationship between Ki-67 and tumor size and its invasiveness potential in neighboring structures, but verified that tumors of older patients had greater Ki-67 positivity than tumors of younger ones[17].

In conclusion, we found a high percentage of GH/PRL-, GH- and PRL-secreting adenomas with immunohistochemical expression for c-erb B2 not previously identified in the literature. Once this membrane receptor is associated to growth factors EGF and TGF α, that have a well-known effect on tumor growth, we suggest that c-erb B2 might have a role in the evolution of these lesions. Complementary studies are needed to confirm this hypothesis.

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