ORIGINAL PAPER

RJME
Romanian Journal of Morphology & Embryology
http://www.rjme.ro/

Molecular markers in the diagnosis of invasive pituitary adenomas – an immunohistochemistry study

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Abstract

Pituitary adenomas are benign tumors of the brain, with a relatively high prevalence in the general population, being responsible for 14.4–16.7% from all brain tumors. These tumors, although benign, have a local invasive behavior in approximately 35% of the cases. The aim of this study was to identify the differences in expression of molecular markers between primary and relapsed pituitary adenomas (as an aggressiveness indicator), as well between secreting and non-secreting pituitary adenomas. Tumor fragments were collected from 51 patients with invasive pituitary adenomas. Of these, 10 cases were operated a second time due to tumor recurrence. The tumor fragments were retrieved from the archives of the Department of Pathology, Emergency County Hospital, Cluj-Napoca, Romania. Immunohistochemical staining was performed for nine markers on 51 invasive pituitary adenomas: Ki-67, β-catenin, E-cadherin, Bcl-2, galectin-3, p53, p27, CD117, and CD44. We compared the expression differences between two groups: the first one including primary and relapsed invasive pituitary adenomas, and another one including prolactin (PRL)-secreting and non-secreting invasive pituitary adenomas. Ki-67, p53 and Bcl-2 expressions were found significant in the PRL-secreting group. CD44 immunostaining was significant only in relapsed invasive pituitary adenomas. For the β-catenin, E-cadherin, galectin-3, p27 and CD117 expression levels were not registered statistically significant differences between our groups. Our study is the first one to report a statistically significant difference between the expression of CD44 in primary and relapsed invasive pituitary adenomas and it could be used as a negative impact prognostic marker.

Keywords: pituitary adenomas, invasive, prolactin, CD44, Ki-67, Bcl-2.

Introduction

Pituitary adenomas are usually slowly progressing benign endocrine tumors of the brain, ranking third in the order of occurrence, after gliomas and meningiomas [1, 2]. The incidence of pituitary adenomas is found to be between 14.4–16.7% from all brain tumors [3]. Although these tumors are non-metastasizing tumors, invasive local growth occurs in 35% of the cases [4]. Consequently, most neurosurgeons agree that a key feature in defining an "aggressive" pituitary tumor is the rapidity of growth or their transformation into carcinoma. These pituitary tumors may often cause an early recurrence and resistance to multimodal therapy, *e.g.*, surgery and radiotherapy. It is recognized that there is no clear distinction between adenoma and carcinoma based on distinct standard histological criteria or electronic microscopic features [5–7]. Such a distinction would permit a reliable early prediction of a future invasive, even aggressive, behavior of a pituitary adenoma. It may allow a therapeutic approach that might prevent clinical recurrence or metastasis.

The 2004 *World Health Organization* (WHO) Classification categorized pituitary tumors as typical adenoma, atypical adenoma (tumors with uncertain behavior) and carcinoma [8]. Atypical adenoma has an aggressive behavior, such as invasive growth, an elevated mitotic index, a Ki-67 labeling index greater than 3%, and an extensive nuclear staining for p53 immunoreactivity [9]. When discussing clinically aggressive adenomas, the most recent WHO Classification (August 2017) takes into account tumor size, anatomical areas of tumor invasion such as cavernous and sphenoid sinuses [evaluated by magnetic resonance imaging (MRI), intraoperative impression or histology], immunocytochemical type, and tumor cell proliferation markers (increased mitotic activity and higher index of Ki-67) [10, 11]. According to this classification, the term of atypical adenoma has been eliminated [11].

More markers are needed to refine and improve the present classification of pituitary tumors to have an early diagnosis and management strategies for patients with invasive pituitary tumors.

Thus, in the present study, we tried to identify the differences in expression of molecular markers between primary and relapsed pituitary adenomas (as an aggressiveness indicator), as well as between secreting and non-secreting pituitary adenomas.

Patients, Materials and Methods

Patients

Tumor fragments from 51 patients with invasive pituitary adenoma (tumors' size larger than 10 mm) were retrieved from the archives of the Department of Pathology, Emergency County Hospital, Cluj-Napoca, Romania. Ten of these cases were operated a second time due to tumor recurrence. These adenomas were operated in the Department of Neurosurgery, by the same surgeon. The cases stained with Hematoxylin–Eosin (HE) were independently reviewed by two pathologists called upon to confirm the diagnosis.

None of the patients had received pre-operative chemotherapy. We examined the medical records of each patient, in order to determine gender, age at surgery, tumor type according to size (macroadenomas extended preferentially suprasellar, infrasellar, laterosellar, respectively in all directions), and secretion [functioning growth hormone (GH), prolactin (PRL) hormone, adrenocorticotropic hormone (ACTH), follicle-stimulating hormone/luteinizing hormone (FSH/LH), thyroid-stimulating hormone (TSH) by immunocytochemistry, and non-functioning tumors]. Tumor invasion was evaluated with the pre-operative MRI for all patients. Laterosellar invasion, in the cavernous sinus was considered when the percentage of encasement of the internal carotid artery by the tumor was of 67% or greater for grades 3 or 4 of Knosp's classification [12–14]. Patients included in the study underwent MRI immediately postoperative, three months later, and on a yearly basis after surgery. Postoperative results, progression and recurrence were also evaluated with MRI.

Construction of tissue array blocks

The immunohistochemical (IHC) analysis of the samples was performed on tissue microarray (TMA) blocks. This was done with the help of an automatic tissue microarrayer Alphelys MTA Booster 0.1. Four cores (0.6 mm in diameter, 4 mm in length) were extracted for each patient's formalin-fixed paraffin-embedded block. The cores featured the optimum number of tumor cells (minimum 60%). The areas rich in stroma or with necrosis were avoided. Several cores coming from a positive control were inserted into the tissue blocks in order to validate the staining pattern.

Immunohistochemistry

The IHC staining was performed automatically with DAKO Omnis®, using the ethylenediaminetetraacetic acid (EDTA), pH 9, for antigen retrieval. Mouse anti-human monoclonal antibodies were used at the following dilutions: 1:200 for β-catenin (Dako, clone β-catenin-1), 1:100 for galectin-3 (Leica, clone GAL3), 1:50 for E-cadherin (Invitrogen, clone 4A2C7), 1:200 for Bcl-2 (Dako, clone 124), 1:200 for CD44 (Abcam, clone 51037), 1:250 for Ki-67 (Dako, clone MIB-1), 1:200 for p53 (Dako, clone DO-7), 1:40 for p27 (Leica, clone 1B4). For CD117, rabbit anti-human monoclonal antibody (Dako, clone 104D2) at 1:400 dilution was used.

We assessed the percentage of tumor cells (0–100%). The immunostaining was classified according to location (membrane, nucleus, or cytoplasm) and intensity (evaluated qualitatively as absent, poor, moderate or intense). For Ki-67, a 3% positivity of tumors cells was used as a cutoff. For each marker, if all the cores per subject were negative, we considered a negative expression, respectively a positive expression, otherwise. For immunohistochemistry, the *H*-score was calculated for all subjects. A score was computed for each core by multiplying the percentage of cells of the same intensity with their intensity (absent: 0, poor: 1, moderate: 2, intense: 3). Finally, for each subject, the mean of the scores of each of their cores was computed. The slides were independently read by two pathologists. In the case of divergent results, the slides were reviewed by both pathologists working together, and consensus was reached.

Statistical analysis

Categorical data were presented as absolute and relative frequencies. Normally distributed continuous data were presented as mean ± standard deviation (SD); skewed continuous data were presented as median and quartiles. Comparisons between groups for categorical data were performed with the Fisher's exact test (*e.g.*, we compared the PRL-secreting adenomas with those non-secreting, respectively the primary with the relapsed invasive pituitary adenomas, regarding the presence or absence of positive or negative immunohistochemistry of different markers), while for skewed continuous data the Wilcoxon rank-sum test was used. For all statistical tests, we used the two-tailed *p*-value with a 0.05 level of significance. All analyses were performed in R environment for statistical computing and graphics, version 3.2.3.

Results

The study group included 51 patients: 29 (56.86%) women and 22 (43.14%) men (with a mean age of 63± 11.71 years). Ten (19.61%) patients had relapses for which they underwent surgery. Out of 51 cases, 31 (60.78%) were non-secreting adenomas and 20 were identified as functional adenomas: 12 (23.53%) PRL-secreting adenomas, five (9.8%) TSH-secreting adenomas, three (5.88%) ACTH-secreting adenomas and one (1.96%) FSH/LH-secreting adenoma (Figures 1 and 2).

All tumors from our group are macroadenomas (size larger than 10 mm) with the following characteristics of invasion: 17 (33.33%) adenomas with unilateral extension (one laterosellar and 16 suprasellar), 18 (35.29%) with bilateral extension (five supra-/infrasellar and 13 supra-/laterosellar) and 16 (31.37%) extended in all directions (Figure 3, a and b).

Within our immunohistochemistry study, we have evaluated a set of nine markers (Ki-67, β-catenin, E-cadherin, Bcl-2, galectin-3, p27, p53, CD117, and CD44) in a group of 51 invasive pituitary adenomas. We compared the expression levels between primary and relapsed tumors and between PRL-secreting and non-secreting invasive pituitary adenomas.

Ki-67 immunoexpression was present in 26 (63.41%) of the primary tumors (Table 1) and in eight (66.67%) of the PRL-secreting tumors. We found a higher percentage of Ki-67 ≥3 (66.67%, in eight cases) of PRL-secreting adenomas compared to the other adenomas (23.08%, in nine cases), the result being statistically significant (p=0.012). Also, the Ki-67 *H*-scores were higher in the PRL-secreting adenomas compared to the other adenomas (p=0.036) (Table 2). Ki-67 expression was localized in the cell nucleus (Figure 4). Ki-67 index over 3% was present in 17 (33.33%) of our cases, eight

functional and nine non-secreting adenomas. *β*-catenin and E-cadherin immunopositivity were detected in both subgroups of invasive adenomas and the results were not statistically significant. Immunoreaction was present at membrane level for both *β*-catenin and E-cadherin (Figure 5, a and b).

Bcl-2 immunostaining was positive in six (15%) of the primary tumors and in five (41.67%) of the PRL-secreting tumors, immunostaining being found at the level of the cytoplasm (Figure 6). We found a higher percentage of Bcl-2-positive immunostaining (41.67%, in five cases) in PRL-secreting adenomas compared to other adenomas (5.26%, in two cases), the result being statistically significant (p=0.006). Also, for Bcl-2, the *H*-scores were higher in the PRL-secreting adenomas compared to the other adenomas (p=0.036) (Table 2).

p53 was positive more often (11 cases – 91.67%) in PRL-secreting adenomas compared to other adenomas (25 cases – 64.1%) but did not reach statistical significance (p=0.083), still the p53 *H*-score was statistically significant higher in PRL-secreting adenomas compared to other adenomas (p=0.005) (Table 2). p53 expression was localized in the cell nucleus (Figure 7).

For galectin-3, p27 and CD117 markers we did not observe a difference in expression of IHC characteristics of invasive pituitary adenomas. Immunostaining was positive at the level of the cytoplasm for galectin-3 (Figure 8). p27 expression was positive at nuclear level (Figure 9). CD117 immunopositivity was detected at cytoplasm and membrane levels (Figure 10).

In the *de novo* group of adenomas, CD117 was more frequently met than in the recurrence group (no subject in the recurrence group had the positive marker), being statistically insignificant. In our study, the immunoreactivity of CD117, marker of hematopoietic stem cells, was identified in two (4.88%) cases. One case was non-secretory and the other (with CD117 immunoexpression positive) was a prolactinoma.

CD44 expression was detected more frequently in relapsed invasive pituitary adenomas (three cases, 30%), compared to primary tumors where it was absent in (p=0.006). Concordant with this finding, the CD44 *H*-scores were higher in the relapsed invasive pituitary adenomas, compared to the primary tumors (p<0.001). We did not observe statistically significant differences between secreting and non-secreting prolactin adenomas. Immunostaining, when present, was highly positive at the level of the cellular membrane of tumor cells (Figure 11).

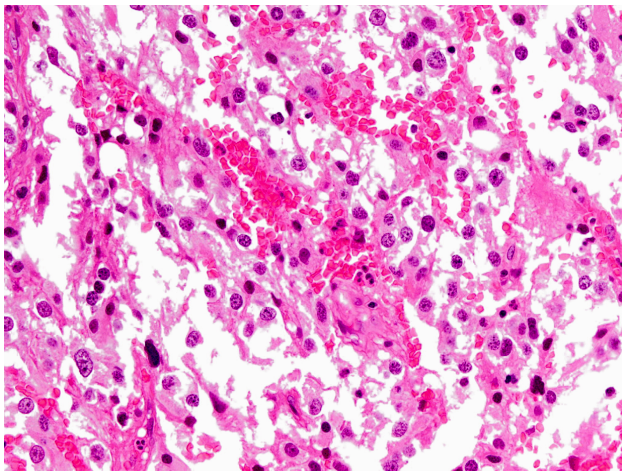

Figure 1 – ***Atypical pituitary adenoma (prolactinoma) with elevated mitotic activity and invasiveness. HE staining, ×40.***

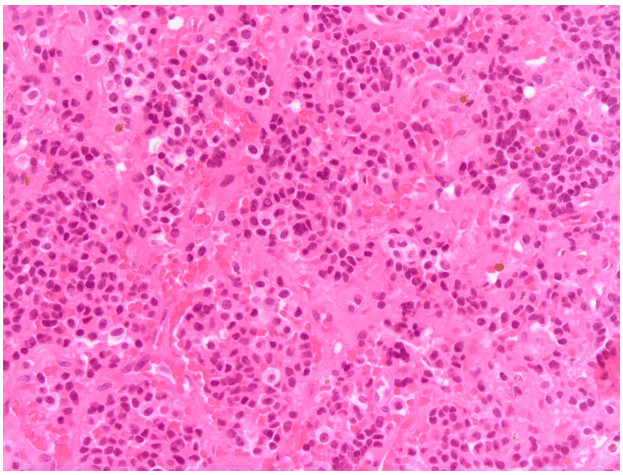

Figure 2 – ***Pituitary adenoma (non-secretory). The tumor growth pattern is diffuse, adenoma cells are small to medium sized with moderate nuclear atypia. HE staining, ×40.***

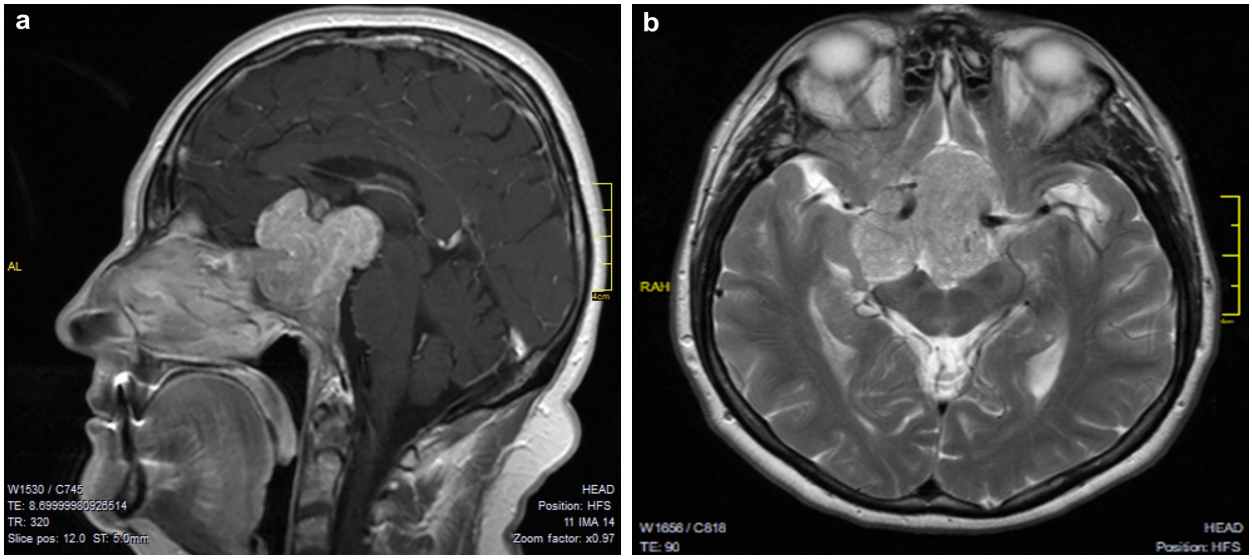

Figure 3 – ***MRI imaging: (a) Sagittal section, invasive pituitary adenomas with infra- and suprasellar invasion; (b) The same case, axial section, demonstrating the laterosellar invasion, too.***

Table 1 – ***Immunohistochemistry positivity in primary and relapsed invasive pituitary adenomas***

Group	Primary (*n*=41)	Relapse (*n*=10)	*p*-value
Ki-67 positive, *n* (%)	26 (63.41)	8 (80)	0.463
Ki-67 (*H*-score), median (IQR)	1 (0–4.25)	1.75 (0.44–5.25)	0.699
β-catenin positive, *n* (%)	34 (85)	9 (90)	1
β-catenin (*H*-score), median (IQR)	154.58 (48.75–300)	173.33 (115.21–245)	0.932
E-cadherin positive, *n* (%)	10 (24.39)	1 (10)	0.428
E-cadherin (*H*-score), median (IQR)	0 (0–0)	0 (0–0)	0.4
Bcl-2 positive, *n* (%)	6 (15)	1 (10)	1
Bcl-2 (*H*-score), median (IQR)	0 (0–0)	0 (0–0)	0.688
Galectin-3 positive, *n* (%)	24 (61.54)	9 (90)	0.135
Galectin-3 (*H*-score), median (IQR)	1.5 (0–19.38)	4.75 (1.62–62.25)	0.266
p53 positive, *n* (%)	29 (70.73)	7 (70)	1
p53 (*H*-score), median (IQR)	1.25 (0–6.67)	2.25 (0.31–4.29)	0.755
p27 positive, *n* (%)	27 (67.5)	8 (80)	0.702
p27 (*H*-score), median (IQR)	6.25 (0–48.12)	31.25 (5.5–78.33)	0.228
CD117 positive, *n* (%)	2 (4.88)	0 (0)	1
CD117 (*H*-score), median (IQR)	0 (0–0)	0 (0–0)	0.503
CD44 positive, *n* (%)	0 (0)	3 (30)	0.006
CD44 (*H*-score), median (IQR)	0 (0–0)	0 (0–60)	<0.001

IQR: Interquartile range.

Table 2 – ***Immunohistochemistry positivity of molecular markers in prolactin-secreting invasive pituitary adenomas***

Prolactin-secreting adenomas	Yes (*n*=12)	No (*n*=39)	*p*-value
Ki-67 ≥3, *n* (%)	8 (66.67)	9 (23.08)	0.012
Ki-67 positive, *n* (%)	9 (75)	25 (64.1)	0.728
Ki-67 (*H*-score) median (IQR)	6.5 (0.25–8.9)	1 (0–3)	0.036
β-catenin positive, *n* (%)	11 (91.67)	32 (84.21)	1
β-catenin (*H*-score), median (IQR)	112.5 (74.58–300)	173.33 (63.33–291.88)	1
E-cadherin positive, *n* (%)	5 (41.67)	6 (15.38)	0.102
E-cadherin (*H*-score), median (IQR)	0 (0–20)	0 (0–0)	0.052
Bcl-2 positive, *n* (%)	5 (41.67)	2 (5.26)	0.006
Bcl-2 (*H*-score), median (IQR)	0 (0–3.75)	0 (0–0)	0.002
Galectin-3 positive, *n* (%)	7 (58.33)	26 (70.27)	0.492
Galectin-3 (*H*-score), median (IQR)	1.75 (0–143.33)	3 (0–18.75)	0.925
p53 positive, *n* (%)	11 (91.67)	25 (64.1)	0.083
p53 (*H*-score), median (IQR)	10.83 (2.5–30.19)	1 (0–4.08)	0.005
p27 positive, *n* (%)	9 (75)	26 (68.42)	1
p27 (*H*-score), median (IQR)	6.33 (1.88–22.81)	8.75 (0–62.5)	0.854
CD117 positive, *n* (%)	1 (8.33)	1 (2.56)	0.419
CD117 (*H*-score), median (IQR)	0 (0–0)	0 (0–0)	0.409
CD44 positive, *n* (%)	1 (8.33)	2 (5.26)	1
CD44 (*H*-score), median (IQR)	0 (0–0)	0 (0–0)	0.679

IQR: Interquartile range.

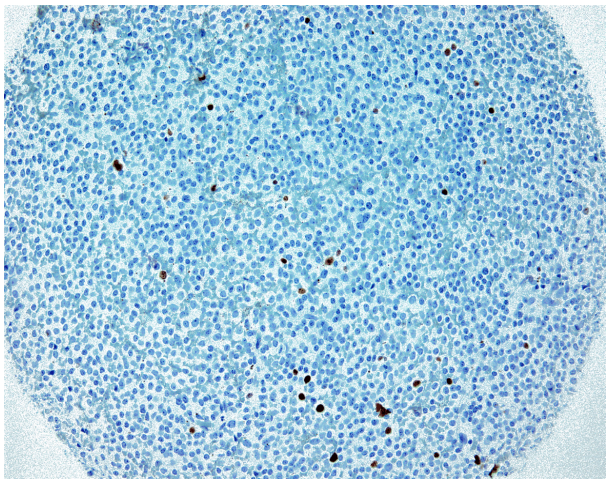

Figure 4 – ***Tumor cells of an atypical pituitary adenoma with strong nuclear expression of Ki-67. IHC staining, Ki-67, ×40.***

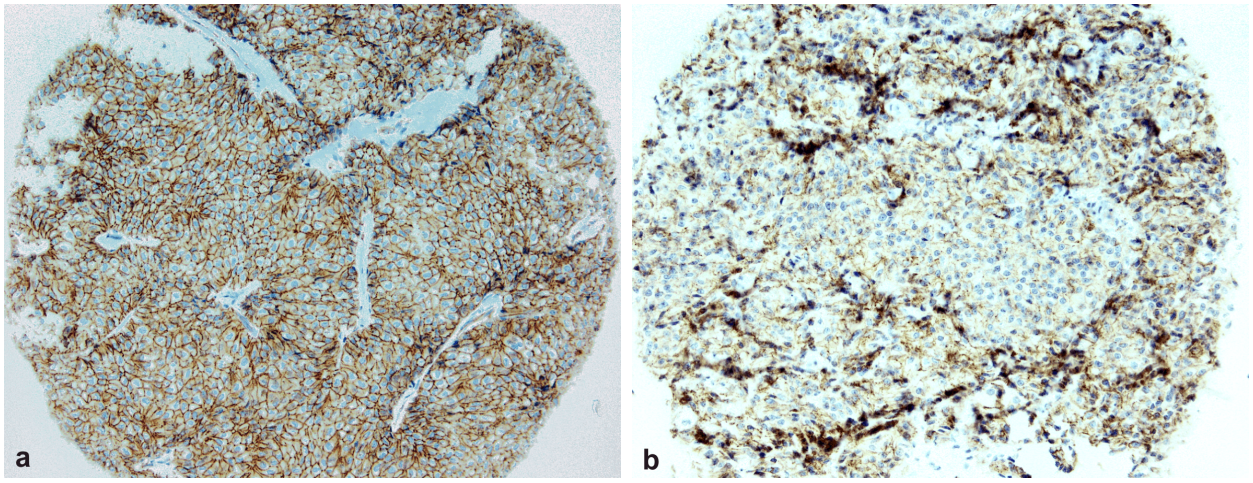

Figure 5 – ***(a) Tumor cells with moderate membrane staining for: (a) β-catenin (IHC staining, β-catenin, ×40); (b) E-cadherin (IHC staining, E-cadherin, ×40).***

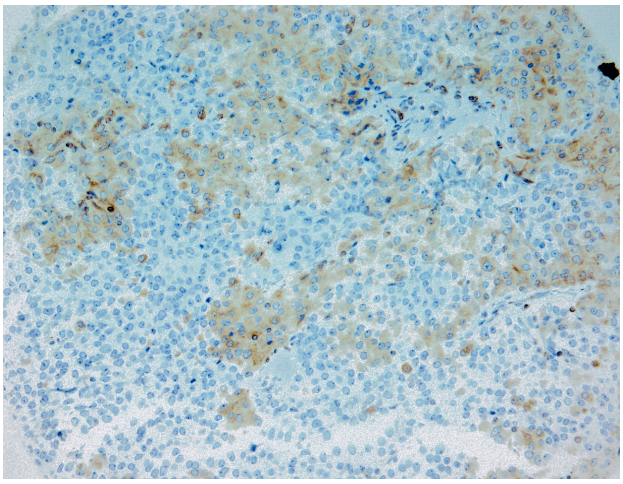

Figure 6 – ***Tumor pituitary cells with weak cytoplasmic staining of Bcl-2. IHC staining, Bcl-2, ×40.***

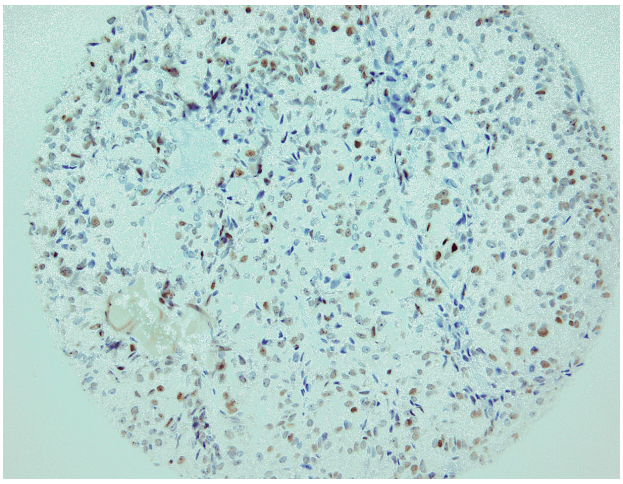

Figure 7 – ***p53 immunopositivity detected with a strong nuclear staining. IHC staining, p53, ×40.***

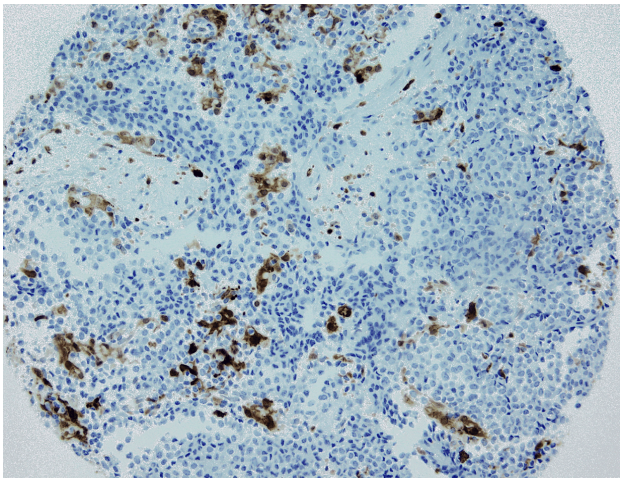

Figure 8 – ***Galectin-3 immunopositivity detected with a strong cytoplasmic staining. IHC staining, galectin-3, ×40.***

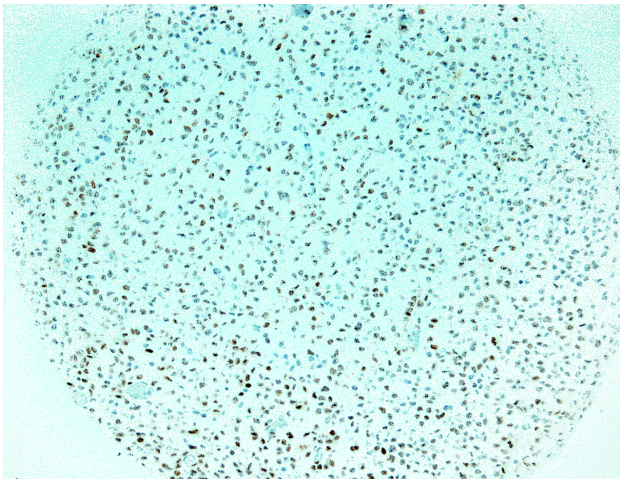

Figure 9 – ***Tumor pituitary cells with weak and moderate p27 staining at nuclear level. IHC staining, p27, ×40.***

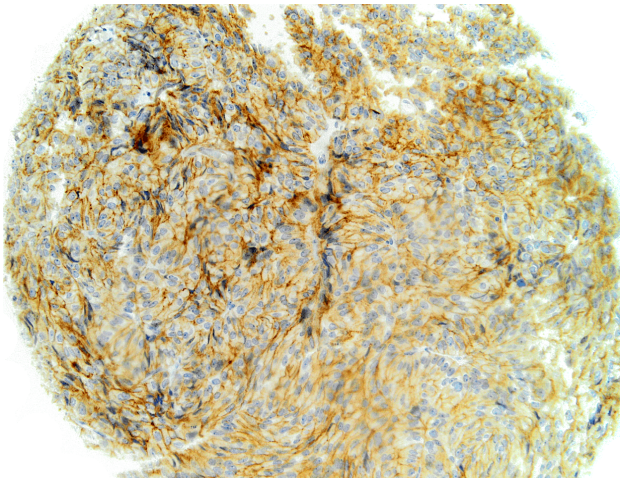

Figure 10 – ***Tumor pituitary cells with CD117 staining at cytoplasm and membrane levels. IHC staining, CD117, ×20.***

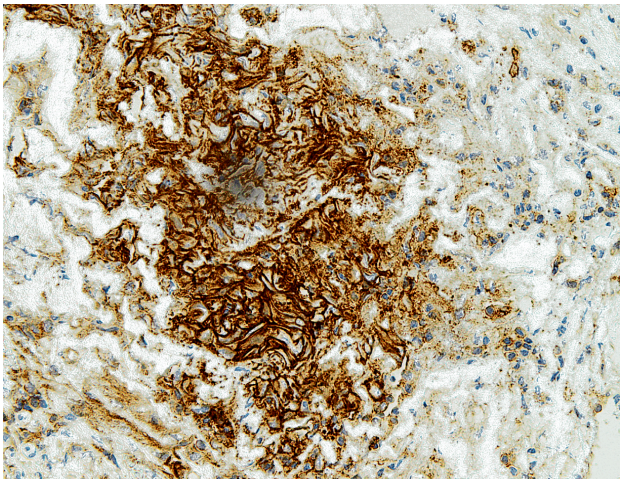

Figure 11 – ***Tumor cells of a pituitary adenoma with moderate CD44 expression at the membrane level. IHC staining, CD44, ×40.***

Discussion

Recently, the number of studies, which evaluate the role of molecular markers in brain tumors, has seen a huge increase. This is the result of the development of genetic and molecular techniques and also of the increased access to such techniques in pathology laboratories around the globe [15]. Despite finding important prognostic markers for gliomas [isocitrate dehydrogenase (IDH)-1, α-thalassemia/mental retardation syndrome X-linked (ATRX), co-del 1p/19q] [16, 17], no marker was found to predict the tumor's aggressiveness behavior in pituitary neoplasms [18, 19].

The 2017 WHO Classification relies on the pituitary cell lineage (using the pituitary transcription factors) rather than on the hormone-producing pituitary adenoma [11]. Thus, the pituitary-specific POU-class homeodomain transcription factor (PIT-1) is expressed in the cells of GH, PRL and TSH adenomas. Further, the steroidogenic factor 1 (SF-1) is specific for gonadotroph cell differentiation, while the T-box family member TBX19 (T-PIT) transcription factor generating the pro-opiomelanocortin lineage with differentiation of corticotroph cells [11, 20]. Based on many clinical studies, certain subtypes of "high-risk" pituitary adenomas, with aggressive behavior, are introduced by the new classification (the sparsely granulated somatotroph adenoma; the lactotroph adenoma occurring in the case of men; the Crooke's cell adenoma; the silent corticotroph adenoma; and the plurihormonal Pit-1-positive adenoma, formerly known as silent subtype III pituitary adenoma) [20].

In the present study, we tried to assess the differences in expression of molecular markers in invasive pituitary adenoma in order to identify markers with a prognostic role. We obtained for the first time a statistically significant result between primary and relapsed pituitary adenomas for the CD44 marker. CD44 is a transmembrane glycoprotein involved essentially in the processes of the cancer stem cell, such as cellular adhesion, migration, proliferation and maintenance of stem cell characteristics. Its expression was found in adamantinomatous craniopharyngioma and breast cancer [21, 22]. CD44 positivity was correlated with a high tumorigenic status and with a negative impact prognostic in this type of tumors [23–25]. Also, CD44 is associated with an early development of metastases, facilitating the adhesion of tumor cells to the endothelium [26]. These findings are coherent with the clinical observations of our patients. Prior studies evaluated the CD44 expression only among invasive and non-invasive pituitary adenomas [27–29] without comparing the difference between primary and relapse invasive pituitary adenomas. Xing *et al.* [27] and Chang *et al.* [28] have not found a high expression of CD44 in invasive and non-invasive pituitary adenomas, while Duan Bo & Xinjian [29] reported significant results for CD44 expression between these two groups. This difference might be associated with the various classification methods for invasive adenomas or because of the sample sizes. The high expression level of the CD44 marker found in our series can be explained by the separation of pituitary adenomas from our series in two groups, primary and relapsed. Our findings suggest that CD44 can be used in invasive pituitary adenomas as a marker of tumor aggressiveness associated with the risk of relapse.

As opposed to the data found in the literature that indicate a decrease in the expression of Bcl-2 in carcinomas and pituitary adenomas, our study found an increase in Bcl-2 expression correlated with the aggressiveness of invasive adenomas [30]. We found Bcl-2 overexpressed in PRL-secreting invasive pituitary adenomas. The increase in the expression of the anti-apoptotic factor Bcl-2 stops apoptosis (which increases stability of the mitochondrial membrane) and favors tumor progression by increasing the mitotic activity of the neoplastic cells [31]. In our study, the expression of Bcl-2 is coherent with the results obtained for Ki-67, also associated with tumor proliferation. Also, the results were comparable with those of other authors who proved the higher invasiveness, recurrence and risk of malignant transformation of prolactinomas [32].

A threshold of 3% Ki-67 index can distinguish between invasive and non-invasive adenomas, with 97% specificity and 73% sensitivity, having indeed an important a prognostic value [33]. The mean values of Ki-67, reported by Thapar *et al.* (1996) were 1.37% in non-invasive pituitary adenomas, 4.66% in invasive adenomas and 11.91% in carcinomas [33]. Also, there are authors who found no correlation between Ki-67 index and pituitary tumor invasiveness [34]. In clinical practice, Ki-67 index is used to select the patients who need a strict follow-up, because a very high proliferation index suggests the presence of a carcinoma *in situ* or of a premetastatic carcinoma, with a rapidly progressive potential and a negative prognostic.

For p53, a well-known tumor suppression gene, statistically significant differences in expression were found between PRL-secreting and other adenomas.

In our study, for the galectin-3 expression, no statistically significant differences were registered between the two types of pituitary adenomas analyzed. Galectin-3 is an IHC marker characteristic of the malignant papillary tumors of the thyroid gland [35]. Galectin-3 is a protein encoded by the LGALS3 gene, expressed in the pituitary gland by folliculostellate cells, normal PRL, and ACTH-producing cells but not in most of the other cell types. LGALS3 represents a reliable predictive marker for the aggressive tumor behavior (assessing a high risk of progression or recurrence) [5]. In both PRL- and ACTH-functioning pituitary tumors (adenomas and carcinomas), it was shown that LGALS3 has a higher expression, with the highest level in ACTH carcinomas [36]. PRL- and ACTH-functioning pituitary tumors (adenomas and carcinomas) are aggressive subtypes of tumors in which there is an invasive growth with supra-/parasellar extension, a high Ki-67 index, and LGALS3 expression levels. These are the most important pathologic features; therefore, a target therapy against galectin-3 protein may be useful.

In our study, a positive expression of CD117 was observed for one prolactinoma, result that was not statistically significant; still, another study, among the few that describe this marker in pituitary adenoma, does not identify the CD117-positive expression in the case

of prolactinomas [37]. The fact that the CD117-positive expression was registered in only two cases, can suggest that this mutation can be sporadic and not directly involved in the development of pituitary adenoma.

We analyzed the expression levels of the adhesion molecules E-cadherin and β-catenin to identify a possible correlation with the differentiation status, epithelial–mesenchymal transition and resistance to treatment. In their study, van Roy & Berx observed that in pituitary aggressive/invasive adenoma cells, E-cadherin and β-catenin expression was down regulated, furthermore, a decreased E-cadherin expression can lead to the development of metastases [38]. In our study, in the group of recurring adenomas, E-cadherin expression was most frequently negative; even though it did not reach a significant statistical level, E-cadherin can be associated with the progression of the tumor. There are studies that have demonstrated statistically significant decreases of E-cadherin in invasive prolactinomas and define this marker as an aggressiveness marker [39]. These molecules were selected because they were found to have a significantly lower expression in invasive prolactinomas and could be used as aggressiveness markers [39].

Conclusions

Our study is the first one to report a statistically significant difference between the expression of CD44 in primary and relapsed invasive pituitary adenomas. CD44 could be used as a negative impact prognostic marker. Also, the molecular profile obtained in this study displays a high proliferative potential (increased Ki-67) and anti-apoptotic potential (increased Bcl-2) for PRL-secreting invasive pituitary adenomas. The invasiveness of these types of tumors is at least partially explained by the low expression of the adhesion molecules (E-cadherin and β-catenin), all tumors being invasive. We also found an increased expression of p53 in PRL-secreting invasive pituitary adenomas. Because the low number of cases included in the study is a limiting factor, a study on a larger group is needed to confirm these results and to prove the practical utility of our findings.

Conflict of interests

The authors declare that they have no conflict of interests.

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Received: September 28, 2017

Accepted: January 19, 2018