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Original Contribution

PTTG overexpression in non-functioning pituitary adenomas: Correlation with invasiveness, female gender and younger age

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ABSTRACT

Background: Non-functioning pituitary adenomas (NFPA) are prevalent pituitary neoplasms. Because they do not present with hormonal hypersecretion, there is no marker that indicates regrowth or recurrence, as in other adenomas.
Objectives: Evaluate the immunohistochemical expression of PTTG, CD105 and Ki-67 and their relationships with age, gender, invasiveness, hormonal expression and regrowth or recurrence in the follow-up of NFPA operated and not submitted to radiotherapy.
Methods: Included 56 patients submitted to transsphenoidal surgery. Clinical data were obtained from medical records. The invasion degree was obtained by Hardy's classification.
Results: Mean age 55 ± 13.6 years, 62.5% men and 68% invasive. Lesion persistence was present in 62.2% and regrowth in 35.7%. The recurrence-free survival rate was 94.5%, 75.4% and 69.1% (1, 2 and 3 years). No patient presented recurrence. The PTTG was positive in 55.3%, with statistically significant relationship with invasiveness, age and female gender, without relation to regrowth. The microvascular density showed statistically significant relationship with male gender, negative correlation with PTTG ($r = -0.434$, $p = 0.001$), and no relation with invasiveness and regrowth. The Ki-67 showed statistically significant relationship with age, tendency towards regrowth ($p = 0.054$) and, with no relation to invasiveness.
Conclusions: It is suggested that PTTG can be used as a prognostic marker in NFPA.

1. Introduction

Pituitary adenomas are benign, slow-growing neoplasms, but a third of them exhibit invasive potential. Non-functioning adenomas (NFPA) are the most prevalent, and may lead to complications by tumor mass effects [1,38]. The treatment of choice for NFPA is surgery. Because they do not present with hormonal hypersecretion, as with the other adenomas, so far we have no markers that can predict regrowth or recurrence, despite many candidates [4,11,19,43,45,65].

PTTG is an oncogene in which overexpression leads to cellular transformation and tumor development [41,66]. Studies showed that pituitary adenomas present with increased PTTG expression compared to normal pituitary tissue [16,28,34] in association with increased invasiveness [26,62].

CD105 (cluster of differentiation number 5) is an antigen associated with endothelial cell proliferation, present in new formed vessels cells, being the most specific antigen for the evaluation of their microvascular density (MVD) [7,57]. Few studies have evaluated the MVD through CD105 in pituitary adenomas. Miao et al. (2016) described that adenomas have higher MVD than normal pituitary tissue, while Rotondo et al. (2010) found lower MVD in pituitary adenomas [33,47].

Ki-67 is a nuclear antigen expressed in proliferating cells, associated with proliferative potential and invasiveness of neoplasms [13]. According to WHO data, Ki-67 indexes equal to or > 3% are associated with aggressive behavior and potentially invasive adenomas [6]. In NFPA, some studies associated higher indexes of Ki-67 with regrowth and invasiveness [8,27,36], while others did not [4,18,51,63].

The aim of the present study was to evaluate the immunohistochemical expression of the markers PTTG, CD105 and Ki-67 and their relations with age, gender, invasiveness, hormonal

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expression, presence of regrowth or recurrence in clinical follow-up of patients with operated NFPA that were not submitted to radiotherapy.

2. Methods

2.1. Patients

In this cross-sectional study, 56 patients diagnosed with NFPA were included, submitted to transsphenoidal surgical resection by a single surgeon (N.P.F.), in Santa Casa de Misericórdia, Porto Alegre, between 2009 and 2016. Sample size was for convenience and, patients undergoing complementary radiotherapy were excluded. Surgery tissue slides were stained with hematoxylin and eosin to confirm the presence of tumor and were submitted to immunohistochemical analysis for the six adenohypophyseal hormones (GH, PRL, ACTH, FSH, LH, and TSH) and chromogranin A using commercial antibodies. The diagnosis of NFPA was made based on the absence of clinical symptoms and no biochemical evidence of hormonal excess, detected by blood tests [39].

Patients´ medical records were reviewed to obtain data on age, gender, tumor size, extension, invasion, and recurrence or regrowth. All patients signed the free and informed consent term, the study was approved by the Research Ethics Committee of Santa Casa de Misericórdia, Brazil (Report n° 1397227) and was conducted in accordance with the Helsinki Declaration.

Tumor grade and invasiveness were defined based on magnetic resonance images (MRI 1.5 T) obtained preoperatively and classified according to the criteria proposed by Hardy [17]: grade I (microadenomas, < 1 cm in diameter), grade II (≥1 cm in diameter, intrasellar or with suprasellar extension without causing bone erosion), grade III (locally invasive tumors that may be associated with diffuse sellar enlargement and bone erosion of the sella turcica), and grade IV (invasive tumors that involve extrasellar structures including bone, hypothalamus, and the cavernous sinus). Pituitary adenomas grades I and II were considered non-invasive tumors, while grades III and IV were considered invasive tumors [29].

Diagnosis of complete surgical resection was based on the absence of visible tumor in the surgical description and on the images performed 3 months after the surgery. The presence of recurrence was defined as the appearance of a new tumor after complete surgical resection and regrowth by the increase of remaining surgical residue > 2 mm in at least one diameter [2]. Patients with a medical monitoring of at least six months after surgery were included in the follow-up.

2.2. Immunohistochemistry

The streptoavidin-biotin method (LSAB + peroxidase kit; Dako®, Denmark) was used for the detection of PTTG, CD105 and Ki-67. The formalin-fixed and paraffin-embedded tumor tissues blocks were sectioned at 4 μm, deparaffinized with xylol and rehydrated with ethanol. Antigenic recovery was performed with citrate (pH = 6) for Ki-67 and CD105 and with Tris-EDTA (pH = 9) for PTTG, both at 92 °C for 40 min. Endogenous peroxidase was inactivated using H_2O_2 30 V dissolved in methanol (5%) for 10 min (three times). Non-specific proteins were blocked using BSA 1% (bovine serum albumin) for 1 h at room temperature. Primary antibodies were applied overnight at 4 °C (Table 1). Slides were washed with PBS-tx and incubated with the secondary and tertiary antibodies for 40 min each at room temperature. The reaction was revealed with 3,3-diaminobenzidine (DAB). Sections were counterstained with Harris hematoxylin and assembled with Entellan®. For the negative control, the primary antibodies were replaced with saline.

Table 1
Description of the antibodies used in the immunohistochemical analysis.

Antibdy	Manufacturer	+ control	Dilution
Anti-PTTG policlonal	Abcam, Cambridge, UK	Esophagus	1:200
Anti-CD105 Monoclonal (4G11)	NovoCastra Laboratories, Newcastle, UK	Tonsil	1:80
Anti-Ki-67 policlonal	Dako®, Carpinteria, CA, USA	Tonsil	1:100

Hot spots selection was done under a light microscope with a 100× magnification. Counts were done by two independent observers, and the final results were the average between both. Cell counts for PTTG and Ki-67 and the microvessel count for CD105 were performed in 400× and 200× magnification, respectively, with the aid of Image J® software.

PTTG expression was evaluated by cytoplasmic staining intensity (0 = absent, 1 = weak, 2 = moderate, 3 = strong) (Fig. 1) and the percentage of positive cells in 10 hot spots. The mean number of cells counted in each field was 1077.92 ± 338.9. The final score was calculated by multiplying the intensity by the percentage, being 300 the maximum value score [48].

The evaluation of MVD with the CD105 antibody was made through the Chalkley point counting method [57], with three hot spots selection on each slide, the Chalkley graticule attached to the microscope lens and a 200× magnification. Endothelial cells or stained cell groups were considered microvessels, being MVD obtained by the average of microvessels in the three hot spots.

Ki-67 index was defined by tumor cells counting with immunolabeled nuclei in relation to the total cells in at least 3 hot spots, with the result given in percentage. The mean cells number counted in each field was 1032.08 ± 292.6. The cutoff point for defining increased proliferative activity of the tumor was 3% or more Ki-67 positive cells [6].

2.3. Statistical analysis

Concordance analysis between observers were determined through the intraclass correlation coefficient, with the absolute agreement definition. Quantitative variables were described as mean and standard deviation or median and interquartile range (p25–p75), according to their distribution, according to the Kolmogorov-Smirnov normality test. Correlations between the quantitative variables were verified by the Spearman Correlation test. Chi-square or Fisher's exact test was used for qualitative variables, when necessary. Comparisons between quantitative and qualitative variables were evaluated by Student's *t*-test or Mann-Whitney *U* test, according to the distribution. The Kaplan-Meier test was used to estimate the recurrence-free survival time. The level of significance was set at $p < 0.05$. Analysis were performed by SPSS version 23.0 software (SPSS Inc., IBM Company, Chicago, IL, USA).

3. Results

Of the 56 patients with NFPA, 35 (62.5%) were men. The mean age was 55 ± 13.6 years, ranging from 23 to 80 years. In the immunohistochemical analysis, 27 (48.2%) did not show hormonal expression (hormone negative), and 29 showed hormonal expression (Table 2).

As to tumor grade, 18 (32.1%) were grade II (non-invasive), 9 (16.1%) grade III and 29 (51.8%) grade IV, totaling 38 (67.9%) invasive tumors.

Two patients died after surgery and nine patients had follow-ups in other centers, remaining 45 under follow-up. The median period of follow-ups at the time of data analysis was 29 months (4.5–42.5), ranging from 6 to 104 months. On the post-surgery, 28 (62.2%) presented with lesion persistence (18 with the stable lesion and 10 with regrowth) and 17 (37.8%) had no remaining lesion. Patients without remaining lesion did not showed recurrence. Regrowth occurred in 35.7% of the cases, with a median time of 17 months (14.2–29.2), ranging from 12 to 50 months after surgery. Of these, eight were submitted to one intervention, one had two reinterventions and one had radiotherapy alone.

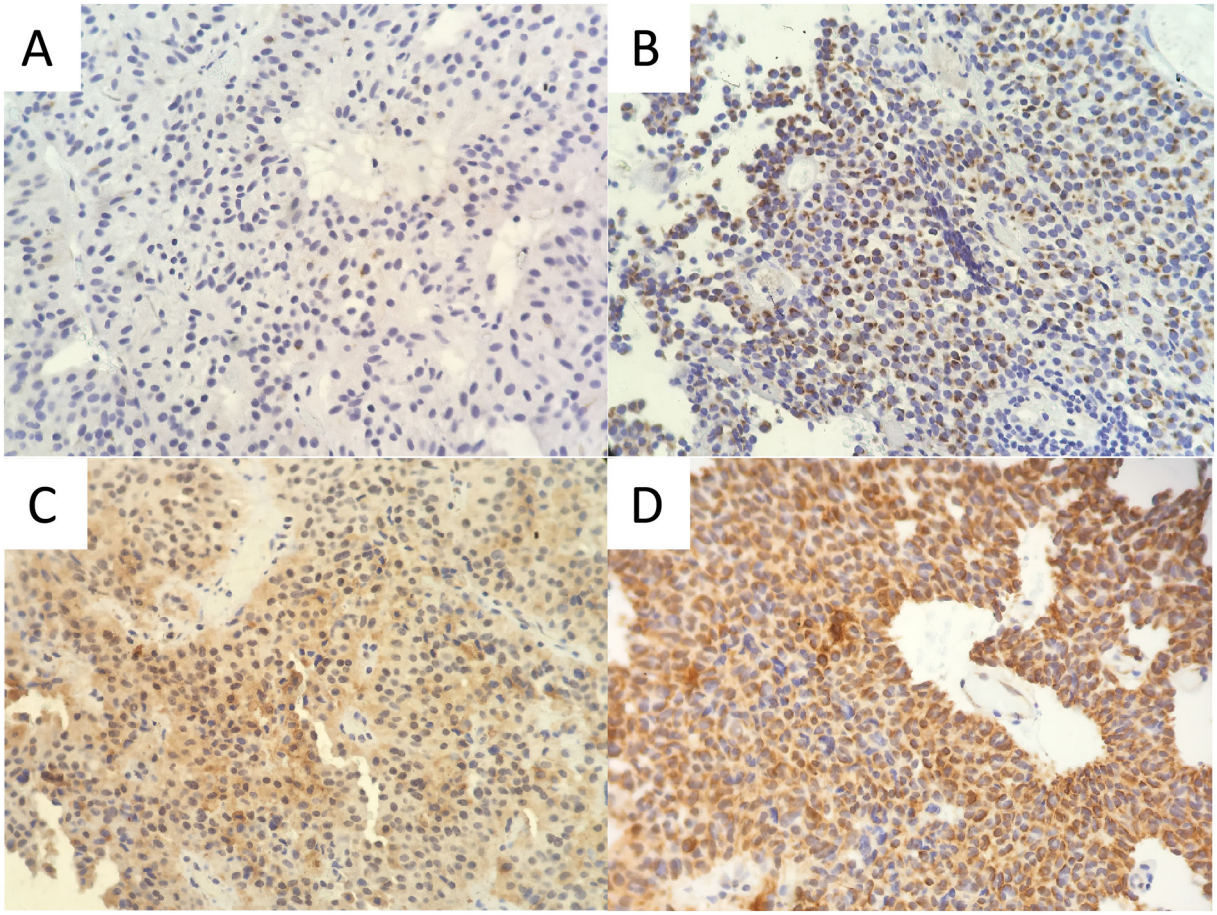

Fig. 1. Immunohistochemical expression of PTTG in nonfunctioning pituitary adenoma: cytoplasmic staining intensity. (A) absent, (B) weak, (C) moderate, (D) strong. 200×.

Table 2
Description of the hormonal expression of NFPA.

Hormonal expression	*n* (%)
Absent	27 (48,2)
Present	29 (51,8)
PRL, TSH	5 (8,9)
FSH	4 (7,1)
LH	3 (5,4)
LH, FSH	3 (5,4)
LH, FSH, TSH	3 (5,4)
GH	2 (3,6)
PRL	2 (3,6)
ACTH	2 (3,6)
GH, PRL	2 (3,6)
PRL, FSH	2 (3,6)
GH, FSH	1 (1,7)

According to the Kaplan-Meier's test, the recurrence-free survival rate was 94.5% in 1 year, 75.4% in 2 years and 69.1% in 3 years (Fig. 3).

Regarding the immunohistochemistry, there was an agreement between the two observers readings for all markers, with intraclass correlation coefficients for Ki-67 of 0.98, for PTTG of 0.99 and for CD105 of 0.94.

PTTG expresion was positive in 55.3% (31 cases), with a median of 99 (34–196), ranging from 17 to 300. Twenty cases (64.5%) showed strong intensity expression. It was found a statistically significant relationship between PTTG and invasiveness ($p = 0.022$), being higher in invasive, gender ($p = 0.002$), being higher in women, and age ($p = 0.03$) (Fig. 4b), being higher in younger patients (Fig. 4b). There was no statistically significant difference between PTTG and regrowth ($p = 0.799$) or hormonal expression ($p = 0.984$).

Regarding the MVD, 100% of the cases were positive, with a median of 27.5 (16–44.2), ranging from 1 to 93 microvessels. A statistically significant relationship was found between MVD and gender ($p = 0.01$), with MVD being higher in men. There was no statistically significant difference between MVD and invasiveness ($p = 0.61$), regrowth ($p = 0.51$), hormonal expression ($p = 0.54$) or age ($p = 0.07$).

The Ki-67 was positive in 100% of the cases, with a median of 0.7% (0.43–1.3), ranging from 0.1 to 7.6%. Six cases (10.7%) showed Ki-67 indexes equal to or > 3%, of which five patients were < 60 years old. A statistically significant relationship was found between Ki-67 and age ($p = 0.001$) (Fig. 4a), with higher rates in younger patients (Fig. 4a) and a tendency of Ki-67 relationship with regrowth ($p = 0.054$). There was no statistically significant relationship between Ki-67 and invasiveness ($p = 0.819$), hormonal expression ($p = 0.304$) or gender ($p = 0.091$).

A negative correlation was found between PTTG and MVD expression (Fig. 5). There was no correlation between Ki-67 and PTTG ($p = 0.820$), nor between Ki-67 and MVD ($p = 0.079$).

4. Discussion

The present study analyzed a representative sample of NFPA with mean age and the male gender predominance in accordance to literature [10,15,37].

The majority (67.9%) of the NFPA studied were classified as invasive, similar to other studies with surgical series that described 57% to 91.9% of invasive NFPA [25,49].

The 48.2% prevalence of adenomas without hormonal expression

(hormone-negative) was similar to other studies with NFPA, which varied from 6.8% to 78.8% [2,4,5,12,24,31,37].

Persistence of lesion after surgery was observed in 62.2% of the patients, other studies described rates from 52.3% to 79.8% [39,46,50,55]. In this study, the regrowth rate was 35.7%, also similar to other studies with patients with persistence of lesion, also not submitted to radiotherapy, in which 35% to 73% presented regrowth [39,46,50,55,61].

The regrowth median time was 17 months, ranging from 12.5 to 50 months after surgery. In the literature, tumor regrowth occurred between a period of 27 and 64 months in operated NFPA patients that were not submitted to radiotherapy [14,39,50,55,56]. The earlier detection of regrowth in our research may be due to the use of more sensitive imaging methods.

All patients that underwent total resection did not showed recurrence in the follow-up period. Other studies also found 0% of recurrence rates, with a mean follow-up of 5.6 years [39,50]. Our study had a recurrence-free survival rate of 94.5% (1 year), 75.4% (2 years) and 69.1% (3 years). Similar to our results, recurrence-free survival rates range from 92.9 to 100% (1 year), 78% (2 years), from 48.6 to 100% (5 years) and from 22 to 100% (10 years) [14,39,56].

Previous immunohistochemical studies found high PTTG indexes in most pituitary adenomas, in contrast to absent or very low PTTG indexes in normal pituitary tissue [16,28,34].

In the present study, the PTTG expression was detected in tumor cells cytoplasm, with a paranuclear staining pattern predominance (Fig. 2), like other authors [40,48,53]. The PTTG median expression was 99, ranging from 17 to 300. Studies of Salehi et al. (2010) and Ozkaya et al. (2016) used the same counting method used in the present study and found medians of 110 and 225, ranging from 34 to 266, being the first study with functioning and non-functioning adenomas and the second with GH secreting adenomas [40,48].

Regarding the positive PTTG expression cases, 64.5% of them showed strong intensity. Tena-Suck et al. (2008), when evaluating the cytoplasmic expression of NFPA, found a strong intensity in only 5% of the cases [53]. The group of Wierinckx (2007) observed that 20% of the adenomas studied showed aggressive characteristics and also, expressed higher PTTG indexes in the nucleus and the cytoplasm, while those without these characteristics showed only lowers indexes of cytoplasmic expression suggesting that the PTTG overexpression and its translocation from the cytoplasm to the nucleus may be related to aggressiveness [59].

To date, only studies by Noh et al. (2009) and Ramirez et al. (2012) analyzed the PTTG expression in exclusive series of NFPA. The study by Noh et al. (2009) showed PTTG expression in 100% of the cases and a

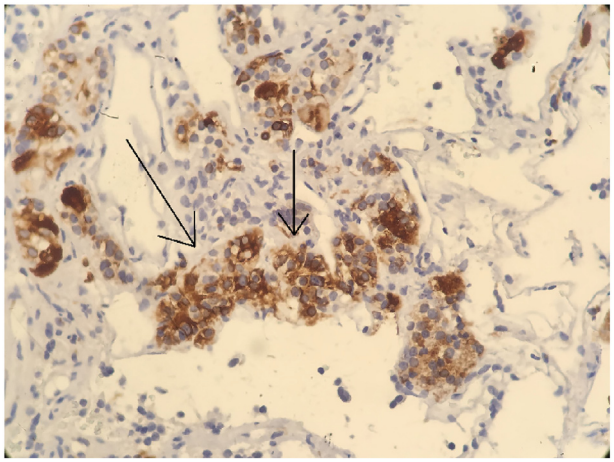

Fig. 2. Immunohistochemical expression of PTTG in nonfunctioning pituitary adenoma with predominance of paranuclear staining pattern (arrows). 400×.

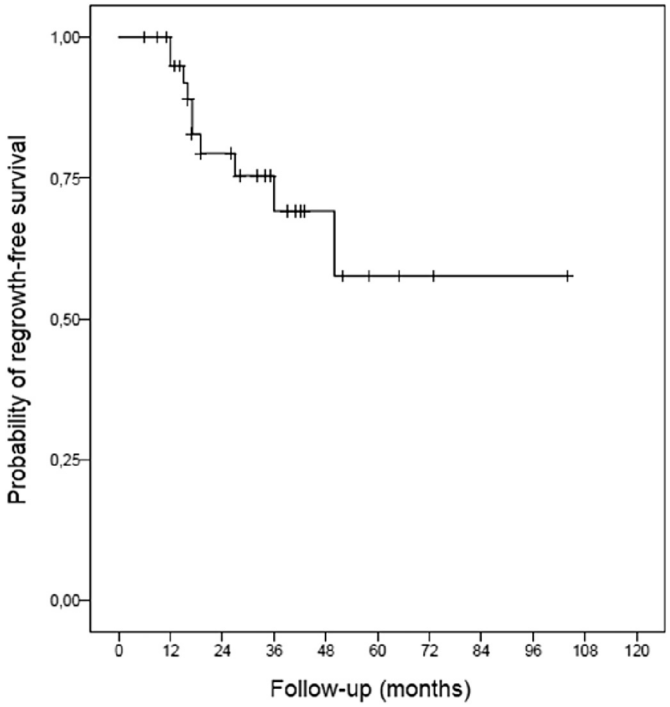

Fig. 3. Kaplan-Meyer curve showing the recurrence-free survival rate in NFPA.

statistically significant relationship with tumor regrowth and with a higher expression in the regrowth adenomas [36]. Ramirez et al. (2012) found positivity in 99% of the samples, but no relation with invasiveness or hormonal expression [44]. In our study, we observed positivity in > 50% of sample.

In relation to invasiveness, Jia et al. (2013), studied functioning and non-functioning pituitary adenomas and found significantly higher PTTG indexes in invasive adenomas [21]. Meta-analysis published by Xiao et al. (2014) and Li et al. (2014), showed that patients with invasive adenomas have higher PTTG indexes compared to non-invasive ones, this data is similar to our findings [26,62].

The only studies that describe gender-specific analysis include functioning and non-functioning adenomas, and, unlike our findings, found no PTTG and gender relationship [11,28,40]. Analyzing the influence of age, studies involving functioning and non-functioning adenomas found higher PTTG indexes in younger patients [16,60], as observed here.

Angiogenesis is important for tumor growth and development, correlated with metastasis, progression, and survival. Benign neoplasms are usually poorly vascularized, but have abnormal and fragile vessels [9]. The role of angiogenesis in pituitary adenomas remains controversial.

Studies fail to show uniformity in microvessel assessment methods. Some use manual microvessel counting in hot spots, considering the highest value or average of values found, and there are variations in microscopic magnifications. The Chalkley method, in which a grid is attached to the lens in the magnification of 200×, is considered the gold standard for microvessel counting [57]. Few studies have evaluated the CD105 expression through this method in pituitary adenomas. Lee et al. (2011) and Miao et al. (2016), studied samples of functioning and non-functioning adenomas and found a mean of 53.4 ± 17.2 and 48.2 ± 24.4 new vessels per field, respectively [23,33]. In our study with NFPA, the median was 27.5 (16–44.2) new vessels per field. We found a statistically significant relationship between MVD and gender, with higher MVD in men. The study by Pizarro et al. (2008) found a similar result, but with functioning and non-functioning adenomas samples [43].

Some studies reports that pituitary adenomas are less vascularized

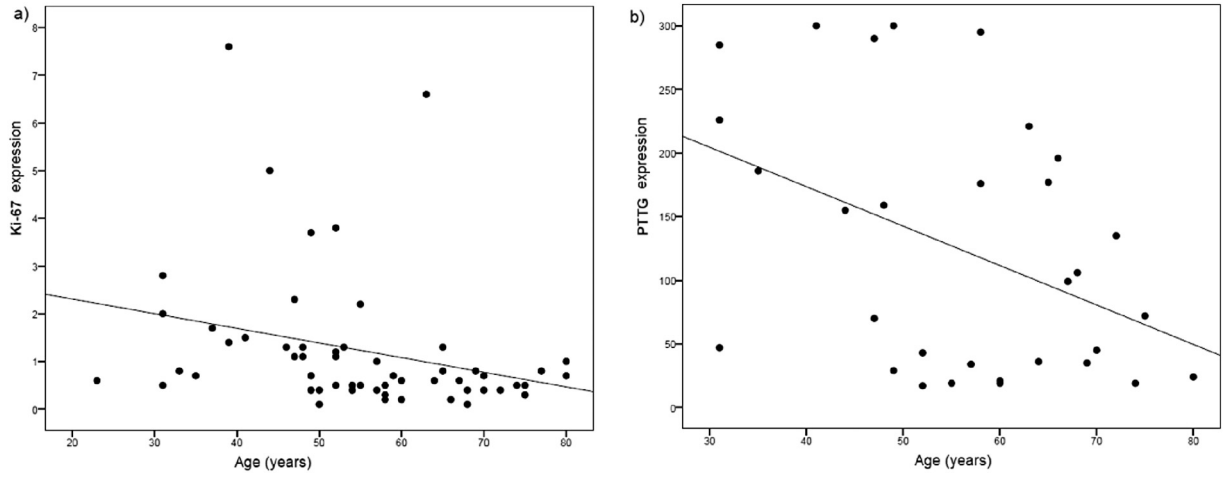

Fig. 4. a) Correlation between a) Ki-67 and age ($r = -0.440$, $p = 0.001$). b) PTTG and age ($r = -0.376$, $p = 0.037$).

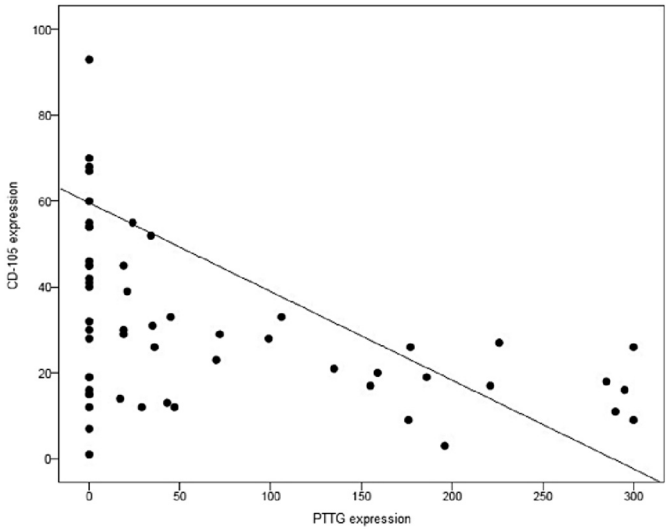

Fig. 5. Correlation between PTTG and CD105 ($r = -0.434$, $p = 0.001$).

than the normal pituitary tissue and this led to the hypothesis that the lack of angiogenesis could be a factor in the infrequency of metastases and slow growth of these tumors [7]. Evaluating MVD through CD105, Miao et al. (2016) found a higher mean of new vessels in the normal pituitary compared to tumor tissue, unlike Rotondo et al. (2010) and Pizzaro et al. (2008) [33,43,47]. Our findings, with an exclusive NFPA sample, showed negative correlation between PTTG and MVD, being at our knowledge the first study to relate these markers with MVD evaluated through CD105, a specific marker of neoangiogenesis. Other researches have studied factors related to angiogenesis, such as CD34, FGF (basic fibroblast growth factor) and VEGF (vascular endothelial growth factor) [3,21,32,34], being the MVD higher since they are not exclusive new vassels markers.

In the current study, Ki-67 was positive in 100% of cases. In the literature, the positivity of NFPA samples ranges from 95 to 100% [4,22,58]. The median was 0.7%, ranging from 0.1 to 7.6%. Previous studies with NFPA showed similar results, with medians of 0.88; 1.49 and 2.15% [4,44,58].

According to WHO data, Ki-67 indexes equal to or > 3% are associated with adenomas with aggressive behavior and invasive potential [6]. In our study, 10.7% of the cases presented Ki-67 equal to or > 3%. Studies with NFPA describe from 9.75 to 14.28% of samples with Ki-67 equal to or > 3% [4,22]. We found statistically significant relationship between Ki-67 and age, with a higher proliferation index in younger patients. When assessing the influence of age on the Ki-67 index in NFPA, other studies also demonstrated inverse relationship between Ki-67 and age [20,30,52,64].

We observed no statistically significant relationship between Ki-67 expression and invasiveness, as well as most of the NFPA studies [4,18,42,51,52]. Liu et al. (2016) found higher Ki-67 in invasive NFPA [27].

Regarding the regrowth of the remaining lesion, no statistically significant relationship was observed with the Ki-67 index, data also obtained by Yao et al. (2017) [63]. In other NFPA studies, regrowth was related to higher Ki-67 indexes [8,36].

We found no statistically significant relationship between Ki-67 and hormonal expression, as well as the Ramirez group (2012) found no statistically significant relationship between Ki-67 and hormone negative and gonadotropic adenomas [44]. Nishioka et al. (2012) observed a statistically significant relationship between Ki-67 and hormone negative and silent corticotropic adenomas, with Ki-67 indexes lower than the other NFPA [35].

The present study demonstrated no statistically significant relationship between Ki-67 and gender. Studies with NFPA patients showed no influence of gender on the proliferation rates of Ki-67 [30,35].

We found no correlation between PTTG and Ki-67 expressions in NFPA, similarly to Ramirez et al. (2012) [44]. Minematsu et al. (2006) and Ozkaya et al. (2016), studied functioning and non-functioning adenomas, also found no correlation between PTTG and Ki-67 expressions [34,40].

No correlation was found between Ki-67 and CD105 expressions in NFPA. Pizarro et al. (2008) studied the relationship between Ki-67 and CD105 in functioning and non-functioning pituitary adenomas and fail to find a, correlation between these markers [43], as well as our results. These findings suggest the hypothesis that microvascular density is not necessarily a pituitary cell proliferation modulator, although it may be indicative of antiapoptotic activity [54].

Our study had some limitations. Firstly, the retrospective nature of data collection may have limited the study strength. The follow up was 29 months, without recurrence and with a regrowth of 35.7%. This follow-up time is in agreement with other studies, as well as rates of recurrence and regrowth. We did not assess PTTG gene expressions, but immunohistochemical PTTG protein expression was evaluated by cytoplasmic staining intensity and the percentage of positive cells in 10 hot spots. The final score was calculated by multiplying the intensity by the percentage.

5. Conclusions

In an exclusive NFPA sample, we found PTTG expression in > 50% of the cases, with higher indexes in invasive adenomas and in younger women, with no relation to regrowth. MVD was not related to invasiveness or tumor regrowth. Ki-67 was found to be higher in younger patients, with a trend towards tumor regrowth, with no relation to invasiveness. The PTTG expression showed a negative correlation with MVD and no correlation with Ki-67. PTTG was related to invasiveness, possibly due to its role as cell cycle regulator and transcriptional activator, suggesting its use as a prognostic marker in NFPA patients.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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