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Over-expression of vascular endothelial growth factor in pituitary adenomas is associated with extrasellar growth and recurrence

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Abstract Some pituitary adenomas (PA) demonstrate aggressive behavior with local invasion and recurrences. Angiogenesis is regarded as an essential step in the formation of solid tumors. The aim of this study is to find out whether angiogenic factors may have information about the aggressiveness of PA that could be useful in determining the frequency of follow-up and whether adjuvant therapy is necessary. In this retrospective descriptive study, we evaluated vascular endothelial growth factors (VEGF) and VEGF receptor (KDR) mRNA expression by RT-PCR analysis on 46 human PA samples. Clinical data, histological subtype and radiologic characteristics were studied to determine the associations between the variables and the pre-operative behavior of the tumor. In addition, we monitored 12 patients without adjuvant post-operative therapies over 46 months after surgery, determining progression of tumor remnants and its association with these markers. VEGF expression correlates with KDR expression ($r = 0.40$, $p = 0.006$). VEGF demonstrates different expression between histological subtypes ($p = 0.036$). The extension at magnetic resonance imaging showed that VEGF expression was related to suprasellar extension ($p = 0.007$), being expressed more on tumors with extrasellar growth than intrasellar ones ($p = 0.008$). Our results demonstrate a 27.5 times increased risk of extrasellar growth when VEGF expression exceeds 0.222 normalized copy number (NCN) ($p = 0.002$). Likewise, tumors with KDR greater than 0.750 NCN had less recurrence-free survival time ($p = 0.032$). Our results suggest that the expression of VEGF and its receptor could be a marker for poor outcome after partial tumor resection. These data should be considered in future studies evaluating angiogenic factors as therapeutic targets in patients with PA.

Keywords Pituitary adenoma · VEGF · KDR Tyrosine kinase · Recurrence

Introduction

Angiogenesis is an essential process of solid tumors allowing their growth and determining tumor behavior. Increased angiogenesis has been shown to be associated with the development of metastases, poor prognosis, and reduced survival in several human tumors [1]. However, the role of angiogenesis in the pituitary gland is not clear, because a few studies have shown lower vascularization in pituitary adenomas (PA) compared with the normal gland; however, there are some discordant results [2–5].

The process of angiogenesis involves the balance between stimulating and inhibiting factors. Cytokines and growth factors are important modulators of angiogenesis. Vascular endothelial growth factors (VEGF) are a family of angiogenic and lymphangiogenic growth factors that

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comprise 6 glycoproteins [6, 7]. Enhanced VEGF expression has been associated with vascular tumors [8]. VEGF-A, commonly referred to as VEGF, is a 45-kDa homodimeric polypeptide that is present specifically in vascular endothelial cells, playing a pivotal role as an angiogenic promoter by stimulating cell proliferation, migration, and enhancing vascular permeability [9]. It has been demonstrated that VEGF mRNA is up-regulated in several human tumors [1]. Furthermore, overexpression of VEGF has been correlated with poor outcome in tumors such as breast, lung or gastrointestinal cancer [4]. The biological activities of VEGF are mediated mainly by 2 tyrosine-kinase receptors: VEGFR2 or Flk-1/KDR (fetal liver kinase 1/kinase insert domain receptor) and VEGFR1 or Flt-1 (fms-like tyrosine kinase) [10]. VEGFR-2 is responsible for signaling that promotes proliferation, migration, survival and angiogenesis; whereas VEGFR-1 may play a regulatory role [10].

Vascular endothelial growth factors (VEGF) was first discovered in pituitary gland extracts [11] and later was described in PA [12]. Some studies have shown higher VEGF expression in PA than in normal pituitary tissue [3], although there are some discordant results [2, 5]. The association of VEGF expression and tumor subtype or extension has been studied with discordant results, some authors found higher VEGF and KDR expression in non-functioning adenomas [3, 4] although others did not find an association [13–15]. Some of these discrepancies could be attributable to different methodology employed. When immunohistochemistry (IHC) was used, the expression of VEGF was found unaltered or decreased and when western-blot or other quantification were used, the expression was increased [2–5].

The aim of the present study is to evaluate the relationship between mRNA VEGF and KDR expression and the clinical parameters of tumor behavior, such as tumor size, extrasellar growth and recurrence, in order to find out whether they could be used as prognostic markers.

Materials and methods

Subjects

We studied 46 PA samples randomly selected from 133 patients who were surgically treated between 1995 and 2008 at Hospital General Universitario de Alicante (HGUA). The inclusion criteria were the availability of enough tumor tissue for carrying out molecular analysis and complete presurgical evaluation (minimal data required were age, sex, presurgical magnetic resonance imaging (MRI) test and hormonal status). Tumors were diagnosed with immunohistochemical assays according to the WHO 2004 classification. Tumor tissue samples were previously formalin-fixed and paraffin-embedded (FFPE) for pathology diagnostic and the remaining fragments were used for our study. Clinical data were retrieved from medical files. Table 1 summarizes the baseline characteristics. This study was approved by the Ethics Committee of the HGUA.

We considered extrasellar growth as the affection of the surrounding structures, such as the cavernous sinus, sphenoidal sinus or optic chiasm in the extension at presurgical MRI. Functioning pituitary tumors (FPA) were considered according to hormonal hyper-secretion associated with a specific syndrome. The lack of a hormone-related syndrome, whether associated or not with a biologically inactive hyper-secretion, was defined as a non-functioning pituitary adenoma (NFPA). We also considered immunohistochemical classification of the tumors: gonadotrophic adenomas (GT), somatotrophic (ST), corticotrophic (CT), lactotrophic (LT), thyrotrophic (TT) and null-cell adenoma (NC). We assessed the recurrence of PA after surgery in GT tumors who did not receive pre- or post-surgical treatment. We considered the follow-up of patients as the

Table 1 Baseline characteristics

Age (years)	53 ± 15
Men	25/46
Largest diameter (mm)	30 ± 16
Invasiveness of surrounding structures	86.7 %
Cavernous sinus	55.6 %
Sphenoidal sinus	22.2 %
Chiasmal compression	75.6 %
Histological classification [N (%)]	
GT	27 (58.7)
ST	10 (21.7)
CT	4 (8.7)
LT	3 (6.5)
TT	1 (2.2)
NC	1 (2.2)
Functioning status [N (%)]	
FPA	13 (28.3)
NFPA	33 (71.7)
Pre-surgically therapies	
Dopamine agonist (DA)	3
Somatostatin analogs (SSa)	5
Post-surgically therapies	
DA	1
SSa	9
Ketoconazole	2
Radiotherapy	13
Second surgery	12

GT gonadotrophic adenomas, *ST* somatotrophic, *CT* corticotrophic, *LT* lactotrophic, *TT* thyrotrophic, *NC* null-cell adenoma, *FPA* functioning pituitary tumor, *NFPA* non-functioning pituitary adenoma

time between surgery and last pituitary scan. Poor prognosis was defined as the growth of tumor remnants. All the MRI scans performed during the follow-up period were evaluated in order to establish the time of the recurrence (increase of over 20 % in any of the tumor diameters). Twelve patients were included in this analysis with a 46 ± 36 month follow-up.

RNA source, total RNA extraction and retrotranscription

From 2 FFPE cylindrical cores (0.6 mm-thick) from preselected tumor areas, we extracted and isolated total RNA using an RNeasy FFPE Kit (QIAgen) according to the protocol and working under the conditions recommended by the manufacturer. We assessed the quality and quantity of RNA extracted from them using the NanoDrop. Retrotranscription of 2 μg of RNA was performed in 20 μl reaction volumes with random hexamer primers, using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and stored at −20 °C until used.

Real-time quantitative PCR (qPCR)

To select the endogenous genes, we analyzed the expression of 32 housekeeping genes in three pools consisting of 10 samples, mixing each one on a commercial endogenous plate based on TaqMan assay technologies (Applied Biosystems®). The results analyzed using GeNorm found that the most stable genes were GAPDH and YWHAZ.

To analyze the gene expression of VEGF and KDR genes, we chose the TaqMan assays for each gene under the conditions recommended by the manufacturer (YWHAZ: Hs00237047_m1, GAPDH: Hs99999905_m1, PTTG1: Hs00851754_u1, VEGFA: Hs00173626_m1 and KDR: Hs00911700_m1). We constructed standard curves that were used to check that the amplification efficiency of the endogenous genes selected and the target genes were similar (with a difference of less than 10 %) and to estimate the number of copies of mRNA. Specific primers were designed to amplify cDNA fragments detected by each TaqMan assay using Primer-BLAST. The chosen primers were subject to the Basic Local Alignment Search Tool (BLAST®) database, which searches to find any sequence similarity. We used commercial total RNA from the brain (Ambion®), retrotranscribed together with adenoma samples as a template. PCR amplification was performed in a 25-μl reaction volume containing 2.5 mM $MgCl_2$, 0.5 μM of each primer, 1 unit of KAPA2G Robust HotStar Taq polymerase (KAPABiosystems®) and 25 ng of cDNA. PCR reactions were amplified for 35 cycles with an annealing temperature of 55 °C. The amplified fragments were purified using the Qiaquick Purification Kit (Qiagen®). We confirmed the specificity of the primers and obtained the concentration of the amplified fragments using the 2100 Bioanalyzer (Agilent®). Dilutions were made for standards such as 10^9, 10^{10}, 10^{11}, 10^{12}, 10^{13} and 10^{14} copies of mRNA calculated from total ng of PCR product (according to the formula MW = [Number of nucleotides × 607.4] + 157.9). qPCR of serial dilutions in triplicate were performed in 12.5-μl reaction volumes containing 6.25 μl of TaqMan Gene Expression Master Mix (Applied Biosystems®), 0.625 μl of TaqMan Gene Expression Assay, and 2.5 μl of template in the 7500 Real Time-PCR System (Applied Biosystems®) and were analyzed using SDS software (Applied Biosystems®).

The R2 values for all standard curves generated were higher than 0.99 and all the efficiencies of TaqMan assays were higher than 90 %. We conducted an RT-minus control using Total Human DNA (Roche®) to ensure that the TaqMan assays did not detect amplification of genomic DNA.

The analysis of the samples was carried out following the same protocol. We included two dilutions of standard curves and no template control (NTC) on all qPCR plates to ensure the maintenance of efficiencies and that these were contamination-free. All analyses were performed in duplicate. Only when the NTC was undetermined and the dCt maintained a lower standard deviation of 0.33 between duplicates and between both dilutions as compared to the initial results did we validate the results of each plate.

Since both endogenous genes were highly correlated (Spearman rank correlation coefficient: $r = 0.82$; $p < 0.000$), all the results were normalized to YWHAZ. We used standard curves to estimate the normalized copy number (NCN) of VEGF and KDR.

Statistical analyses

Data were tested for statistical significance using SPSS 11.0 software (SAS Institute®). Molecular variables showed a non-normal distribution (Kolmogorov–Smirnov test). Associations between the molecular assays and the clinic-pathological features were calculated. Mann–Whitney U and Kruskal–Wallis tests were used for comparison between two or more groups. The Chi-square test, followed by Fisher's exact test, where appropriate, was used to identify the correlations between categorical parameters. Kapplan–Meier survival analysis and the log-rank test were used to compare recurrence-free interval curves. A non-parametric Spearman's correlation test was used to test the association between molecular variables and age or tumor size. The effect of a variable was estimated using the model's odds ratio (OR), and a 95 % confidence interval (95 % CI) was calculated for each OR. Data shown as median (quartiles). p Values <0.05 were considered statistically significant.

Results

Histological subtypes

Vascular endothelial growth factors (VEGF) demonstrated different expression between histological subtypes ($p = 0.036$) (Fig. 1). ST had the lowest VEGF expression (0.271 (0.145–0.511) vs. 0.531 (0.256–0.747) NCN, $p = 0.05$). GT had statistically more VEGF expression than other PAs on the whole (0.629 (0.333–0.747) vs. 0.236 (0.175–0.610) NCN, $p = 0.03$) (Fig. 2). There was a trend toward lower VEGF expression in FPA compared with NFPA (0.337 (0.176–0.610) vs. 0.566 (0.270–0.758) NCN, $p = 0.079$). KDR did not differ between subtypes or functional status. Other parameters such as age, sex or years of progression were not associated with PA subtypes.

VEGF and KDR were correlated (r = 0.40, $p = 0.006$). The molecular variables studied were not related to age or sex.

Extrasellar growth

Thirty-nine out of the forty-six tumors had any of the surrounding structures affected, so they were classified as tumors with extrasellar growth. Age, sex or KDR were not able to determine extrasellar growth; however, tumors with extrasellar growth showed higher expression of VEGF (0.566 (0.291–0.733) vs. 0.200 (0.128–0.207) NCN, $p = 0.008$) (Fig. 3) and a cut-off of 0.222 NCN entailed 27.5 more risk (CI 95 % 2.7–278.9) ($p = 0.002$) for extrasellar growth. This was related to suprasellar extension (0.645 (0.323–0.758) vs. 0.221 (0.153–0.456) NCN, $p = 0.007$). None of the variables were associated with the largest tumor diameter .

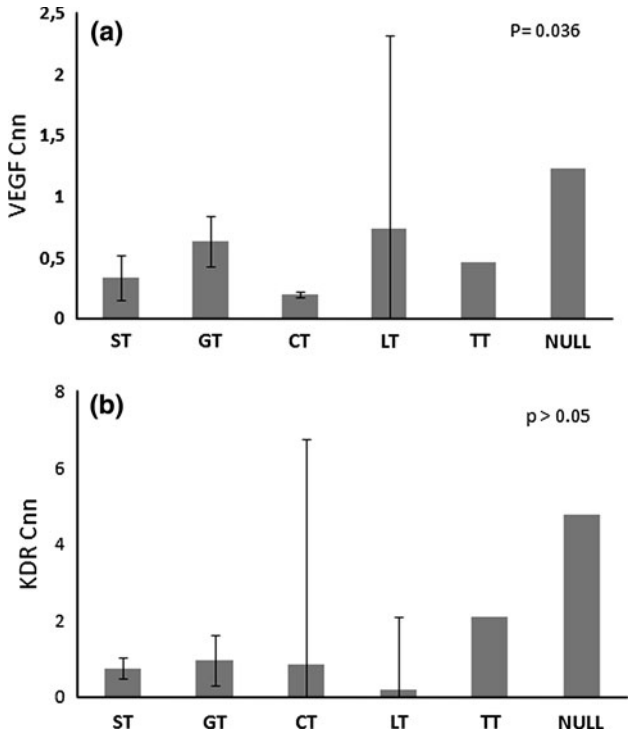

Fig. 1 VEGF (**a**) and KDR (**b**) NCN expression between histological subtypes of PA. Data shown as median and quartiles. Kruskal–Wallis test used and p values <0.05 were considered to be statistically significant. The distribution of LT in **a** and CT in **b** is due to the low number of cases included

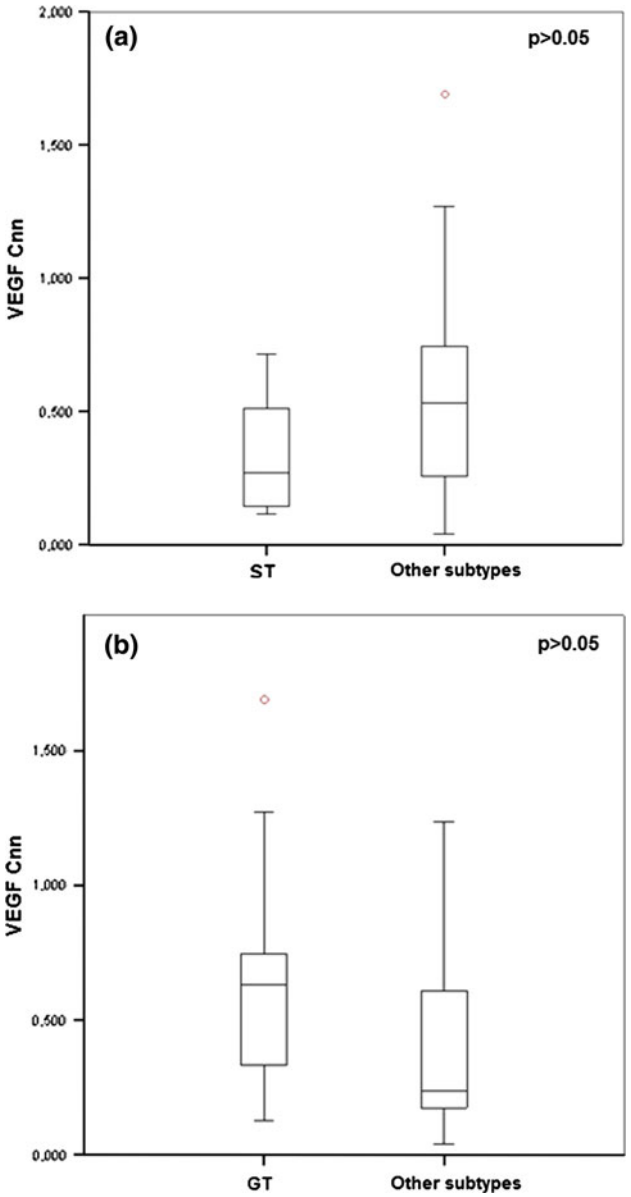

Fig. 2 Differences between VEGF NCN expression on somatotropic (ST) (**a**) or gonadotropic adenomas (GT) (**b**) and other histological types. Data shown as median and quartiles. Mann–Whitney U test used and p values <0.05 were considered to be statistically significant

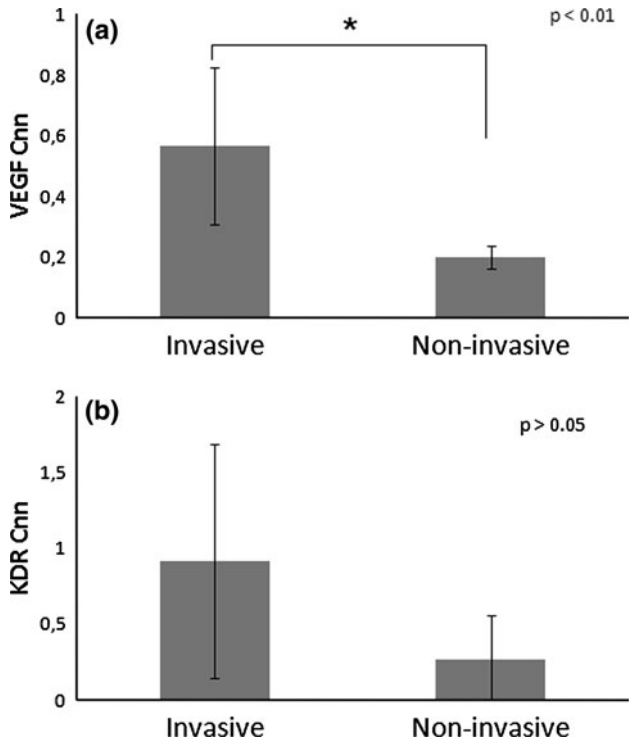

Fig. 3 VEGF and KDR expressions related to extrasellar growth, considered as the affection of the surrounding structures, such as the cavernous sinus, sphenoidal sinus or optic chiasm in the entension at MRI. Data shown as median and quartiles. Mann–Whitney U test used and p values <0.05 were considered to be statistically significant

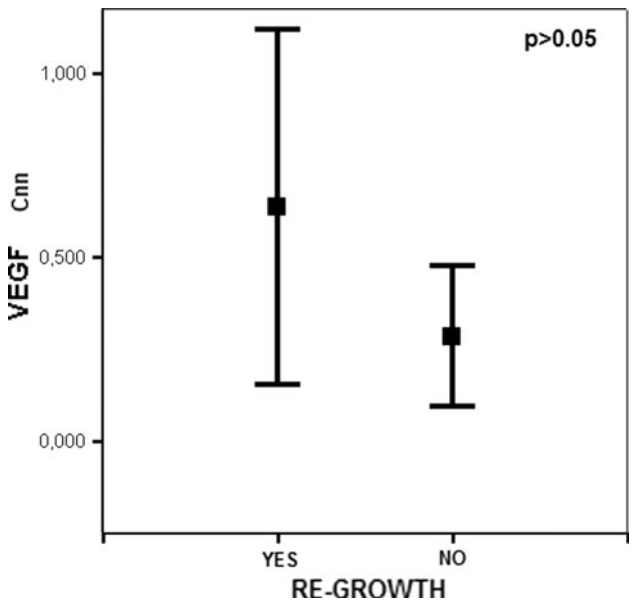

Fig. 4 Effect of pre-surgical treatment with somatostatin analogs (Ssa) on VEGF NCN expression in 10 acromegalic patients. Data shown as median and quartiles. Mann–Whitney U test used and p values <0.05 were considered to be statistically significant

Medical therapies

Five out of ten ST were previously treated with somastostatin analogs (Ssa) and this group had less VEGF expression than non-treated ST, although this was not statistically significant (0.157 (0.114–0.200) vs. 0.338 (0.271–0.610) NCN, $p = 0.079$) (Fig. 4). We did not find any difference in KRD expression between these two groups of ST. There were no differences between molecular variables in pre-surgically treated LT (1/3) or with dopamine agonist (DA) (2/3).

Regrowth

Five out of twelve patients with tumoral remnants who did not receive post-surgical therapies had growth of tumor remnants. All were GT, and progression was diagnosed between 11 and 53 months after surgery (Table 2). Tumors that grew were smaller at diagnosis than those without growth of remnants (27 ± 11 vs. 42 ± 8 mm, $p = 0.019$), although there were no differences in the size of the remnants. There were no statistical differences in terms of VEGF or KDR expression. However, tumors with more than 0.460 VEGF NCN had 2.667 more risk (CI 95 % 1.09–6.524) of growth during follow-up and had less recurrence-free time: 20 ± 6 versus 38 ± 9 months ($p > 0.05$) (Fig. 5). Tumors with more than 0.750 KDR NCN had less recurrence-free time: 16 ± 3 versus 42 ± 5 months ($p = 0.032$).

Discussion

Solid tumors are dependent on the process of angiogenesis for growth and development. However, this process does not appear to be a significant step in PA tumorigenesis, due to lower vascular densities found in this kind of tumor. This could be explained, at least in part, by the lower metabolic demands of slow-growing adenomas [1]. Likewise, PA demonstrated less VEGF expression, the most pro-angiogenic factor studied, than normal pituitary tissue in several studies [2, 5, 16], although there are some discordant results [3, 15]. These inconsistencies may be attributed to the use of different VEGF expression measurement techniques and the different criteria for selecting PA samples, which implies a change in the percentage of histological subtypes. Although there are three studies that found no differences in VEGF expression between PA subtypes [13–15], Niveiro [4] and McCabe [3] found higher expression in NFPA. The small sample size and its heterogeneity could compromise the extrapolation of our results, but these are consistent with the findings of McCabe [3]. We also found a trend toward higher VEGF expression in NFPA and GT than in other PAs.

Some authors attempt to assess the association of this angiogenic factor with PA outcome as high VEGF

expression correlating to poor prognosis in several tumors, such as breast cancer and non-small cell lung cancer [17, 18]. None have been able to correlate VEGF expression to tumor diameter [2, 4, 13–15, 19] or recurrence [13, 15, 20]. Our results also corroborate the independence of VEGF expression and tumor diameter. However, when we follow-up patients without post-surgical therapies, expression above the median of VEGF NCN of this sample (corresponding to 0.460 VEGF NCN) had higher risk (RR = 2.667; 95 % CI 1.09–6.524) for the growth of tumor remnants during follow-up. Nevertheless, our results are not comparable to those of other authors, probably because there are methodological differences, as we quantified VEGF mRNA expression by qPCR, the current gold standard; but Viacava et al. [15] and Fukui et al. [13] used the IHC technique and Cristina et al. [20] used Western Blot. KDR expression was correlated with VEGF in our series and this may also be related to poor prognosis, thus tumors with more than 0.750 KDR NCN had less recurrence-free time. Another factor related to poor prognosis of a PA is the invasion of surrounding structures, and although some authors found no differences in VEGF expression [2, 4, 19, 21, 22], Pan et al. [23] and Yarman et al. [24] described higher expression of VEGF in invasive PA. At this point, there are also methodological differences: some authors assessed cavernous sinus invasion using intraoperative findings [21], others employed Hardy's [19, 22] or Knosp [23] classifications on pre-operative MRI. Considering extrasellar growth as the affection of the surrounding structures, such as the cavernous sinus, sphenoidal sinus or optic chiasm in the extension at presurgical MRI, we found the expression of VEGF was more than twice higher in tumors with extrasellar growth versus intrasellar tumors. This finding may have an impact on regrowth analysis, as tumors with extrasellar growth are less likely to be cured by surgery due to the presence of tumor remnants. However, all tumors included in regrowth study had extrasellar growth.

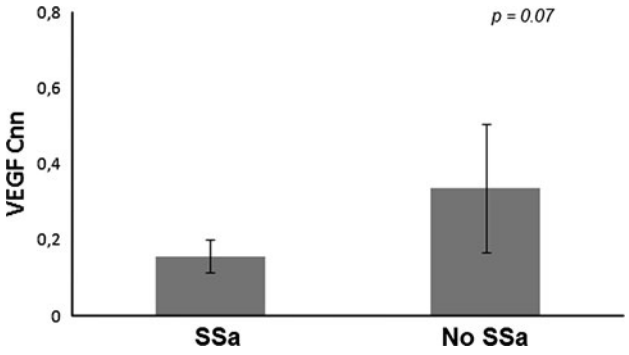

Fig. 5 VEGF NCN expression related to the growth of tumor remnants more than 20 % on fourteen patients without post-operative therapies. Data shown as *mean* and *error bars* (95 % CI)

Should it be definitively demonstrated that VEGF and KDR play a role in PA aggressiveness, drugs that decrease VEGF or block its action could be useful in PA treatment. In fact, there are six studies that determine the effect of Ssa on VEGF expression [2, 15, 16, 24–26]. All authors, except for Viacava et al. [15], found that VEGF decreases its expression after Ssa treatment and this was related to the expression of somatostatin receptor subtypes sst1 and sst2 [25, 26]. These results are consistent with our findings, as 5/10 ST pre-surgically treated with Ssa had lower VEGF expression than those not treated (0.157 (0.114–0.200) vs. 0.338 (0.271–0.610) NCN, $p = 0.079$). To date, there is no published study which analyzes the effect of anti-VEGF drugs on PA, although there are a lot of in vitro and animal studies showing promising preliminary results on the

Table 2 Clinical characteristics of patients without adjuvant therapies

Patient	Age (years)/sex	Histological subtype	Tumor size (mm)	Tumoral rest	Follow up (months)	Progression free time post surgery (months)	VEGF (NCN)	KDR (NCN)
1	34/f	GT	15	Yes	60	11	0.758	1.216
2	70/f	GT	22	Yes	30	12	0.660	1.794
3	71/m	GT	20	Yes	36	21	0.686	0.914
4	67/f	GT	20	Yes	108	23	0.311	2.805
5	64/m	GT	32	Yes	48	37	1.689	0.348
6	67/m	GT	30	Yes	52	37	0.241	0.387
7	70/m	GT	50	Yes	88	53	0.135	0.239
8	37/m	GT	43	Yes	114	–	1.270	1.726
9	72/m	GT	28	Yes	3	–	0.270	1.534
10	72/f	GT	15	Yes	20	–	0.343	0.457
11	67/f	GT	50	Yes	39	–	0.671	0.529
12	63/f	GT	46	Yes	6	–	0.450	0.629

f female, *m* male, *GT* gonadotrophic adenomas, *ST* somatotrophic, *LT* lactotrophic

potential effects of antiangiogenic drugs. Theoretically at least, treatments such as sorafenib or sunitinib, which inhibit cellular signaling by targeting the receptor tyrosine kinases of VEGF, may have a role in the treatment of aggressive PA.

Some limitations of this study can be noted, the sample size is too small and it is a retrospective evaluation which makes difficult to draw firm conclusions. Also, the study of mRNA expression from FFPE cylindrical cores can lead to inaccuracies but our protocol was designed to ensure the maintenance of efficiency. On the other hand, it has not been possible to use the relative quantification by qPCR using ddCT method because it was not possible to obtain normal tissue and compare the levels of expression with the tumors. Nevertheless, we have adapted our protocol to estimate the number of copies of the different gene' transcripts and we have only compared the behavior of these genes among different subtypes of PA or among the different variables studied. This quantification method has been used before by other researchers as Taboada et al. [27].

In conclusion, VEGF and its receptor may not only have prognostic implications, but could also be the target of medical therapies. In order to verify these findings, prospective studies must be conducted on a large number of patients with validated criteria for poor prognosis.

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Conflict of interest There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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