Expression of Ki-67, p53 and vascular endothelial growth factor (VEGF) concomitantly in growth hormone-secreting pituitary adenomas; which one has a role in tumor behavior?

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Abstract

OBJECTIVE: In many pituitary tumor, immunohistochemical studies have been shown to be correlated with different aspects of tumor behavior. There is no study up to date in which markers of Ki-67, p53, VEGF were evaluated concomitantly in GH-secreting adenomas. This study aims to determine which marker has a major role in tumor behavior and whether these markers have a cut-off value to distinguish invasive adenoma from non-invasive pituitary adenoma.

METHODS: Fourty-seven acromegalic patients operated by the same neurosurgeon were included in this study. Twenty-one patients (5 micro/16 macro) had non-invasive adenomas and 26 had invasive macroadenomas. Eight patients (6 invasive macroadenomas, 2 microadenomas) were treated with OCT-LAR until one month prior to surgery with treatment duration range of 3–11 months. These patients were excluded from the study group as the noninvasive and invasive adenomas were compared. A separate analysis was performed in invasive adenomas to compare OCT(+) and OCT(–) patients.

RESULTS: Both Ki-67 and p53 expressions showed no correlation with the invasive character of adenomas, but VEGF expression in invasive adenomas was significantly higher with respect to noninvasive group. Our study has taken intermediate staining (>25%) for VEGF as a cut off value for invasive adenomas. It was also observed that the decrease in VEGF staining in OCT pretreated invasive adenomas was significantly more than those not treated with OCT.

CONCLUSION: VEGF becomes an independent stimulator of angiogenic growth and progression for GH-secreting adenomas with >25% cytoplasmic immunoreactivity. This cut-off value may be useful in determination of prognosis and appropriate treatment strategy. A short term preoperative OCT treatment may be useful as adjunctive therapy especially for locally invasive GH-secreting adenomas.

ORIGINAL ARTICLE

INTRODUCTION

Most pituitary adenomas grow slowly, but despite their mostly benign histopathological features, underlying mechanism(s) of aggresive biological behavior in some of them are poorly understood. Although studies examining the markers of Ki-67 and P53 in different pituitary tumors have yielded contradictory results; angiogenesis has been described as an important mechanism of growth in human pituitary adenomas and carcinomas (Abe & Lüdecke 2001; Lloyd *et al.* 1999; Pan *et al.* 2005; Thapar *et al.* 1996; Turner *et al.* 2000). Angiogenesis is thought to be regulated by a number of growth factors; vascular endothelial growth factor (VEGF) is one of these angiogenic factors, and it's expression in pituitary adenoma immunohistochemically, is known (Fukui *et al.* 2003; Niveiro *et al.* 2005; Takata *et al.* 2004). Recently, VEGF was demonstrated in a diffuse cytoplasmic pattern in GH-secreting adenoma (Kurosaki *et al.* 2008). However, there is no study up to date in which markers of Ki-67 and p53, and angiogenesis (VEGF) were evaluated concomitantly in GH-secreting adenomas. This study aims to determine the influence of age, sex, hormonal status on cell proliferation and angiogenesis, and the influence of cell proliferation and angiogenesis on tumor invasiveness and clinical course, and also to investigate whether these markers have a cut-off value to distinguish invasive adenoma from non-invasive pituitary adenoma.

MATERIAL AND METHODS

Subjects

Fourty-seven patients with GH-secreting adenomas were included in our study. Twenty-six were female and 21 were male, with mean age of 43.6 years (range 19–67 years). The mean size of adenomas was 1.79 ± 1.14 cm (range 0.8–6.0 cm). All of the patients were operated by the same neurosurgeon with transsphenoidal approach (Hardy 1973). Status of invasiveness was determined by intraoperative and radiological findings. On the basis of MRI scans, tumor size and extension were cassified according to Lüdecke's classification (Ludecke 1945) (the maximum diameter of tumor was used as a basis). The tumors were divided into two groups: invasive and non-invasive. The non-invasive group consisted of 21 patients (5 micro and 16 macroadenomas), and the invasive group consisted of 26 patients with macroadenomas. Eight patients (2 non-invasive microadenomas and 6 invasive macroadenomas) were treated with OCT-LAR until one month prior to surgery with treatment duration range of 3–11 months. These OCT(+) patients were excluded from the study group. Nineteen non-invasive and 20 invasive adenomas were compared. A separate analysis was performed in 26 invasive adenomas to compare six OCT(+) and twenty OCT(–) patients. Postoperatively, biochemical controlled disease was confirmed by GH concentrations less than 1 μg/L on OGTT, and normalisation of IGF-1 levels to the age-and sex matched controls. All the tumor specimens obtained from surgery were histologically diagnosed as pituitary adenoma, and were confirmed with immunohistological staining for only growth hormone. In five cases, non-tumoral adenohypophyseal tissue adjacent to adenomas was obtained. One pituitary obtained during autopsy was also investigated. The mean postoperative follow-up period was 41.97 ± 37.22 months (range 5–168 months).

Immunohistochemical analysis

For immunohistochemical studies, formaline fixed paraffin embedded material from the archives of the Department of Neuropathology were used. Serial sections were stained with Hematoxyline-eosin, PAS, Masson trichrome and Gomori reticulin. Immunohistochemical procedure was performed using the Streptavidin-Biotin method. Following antibodies were applied; Ki-67 (monoclonal, dilution 1:200, incubation time: 1hour; Immunovision USA); p53 (monoclonal, ready to use, incubation time: 1 hour; Scytech, UTAH USA); VEGF (polyclonal, dilution 1:100, incubation time: 1 hour; Santa Cruz Biothechnology, CA USA. Hormonal staining were performed; ACTH (dilution: 1:75, monoclonal, DAKO, antigen retrieval with pressure cooker, incubation time: 1 hour), FSH (dilution: 1:50, monoclonal, DAKO, antigen retrieval with pressure cooker, incubation time: 1 hour), LH (dilution: 1:50, monoclonal, DAKO, antigen retrieval with pressure cooker, incubation time: 1), TSH (dilution: 1:100, monoclonal, BIOGENEX, antigen retrieval with pressure cooker, incubation time:1 hour), Prolactin (dilution: 1:60, monoclonal, BIOGENEX, antigen retrieval with pressure cooker, incubation time: 1 hour), GH (dilution: 1:100, monoclonal, BIOGENEX, antigen retrival with pressure cooker, incubation time:1 hour). Histopathologocical scoring was performed by two different pathologists several times. Intra- and interobserver reliability were high. The staining patterns of each specimen were examined under light microscopy and graded as previously described. Quantification of Ki-67–labelled cells was determined by counting 500 nuclei in three or more randomly selected high power fields (x400); positive nuclei were reported as the percentage of immunopositive cells (Wakimoto *et al.* 1996). The staining pattern for p53 was reported either positive or negative (without giving any percentage). Vascular endothelial growth factor (VEGF) was evaluated by a score corresponding to the sum of both (a) staining intensity (0=negative; 1=weak; 2=intermediate; 3=strong) and (b) percentage of positive cells (0=0% positive cells; 1<25% positive cells; 2=26–50% positive cells; 3=>50% positive cells). The sum of (a) + (b) reached a maximum score of 6. A score greater than 2 was considered as immunohistochemical positive. In each case, scoring was performed from 10 randomly

selected areas (×400, ensuring that the whole section was scanned (Pan *et al.* 2005).

Statistical analysis

Statistical analysis was performed using SPSS 12.0 software with appropriate tests (Pearson chi-square, Fisher's exact test) and the results are expressed as mean ± S. E. M. differences between groups were considered as significant if $p<0.05$.

RESULTS

In this study, age, sex, hormonal status and clinical course (remission/activity) did not influence Ki-67, p53 and VEGF. Although Ki-67 and p53 expression have no association with invasiveness of adenomas ($p=0.294$, and $p=0.511$, respectively; Tables 1 and 2), there was an association between the invasion of adenomas and the expression of VEGF ($p=0.016$; Table 3).

Our result showed that there is no cut-off value for Ki-67% indicating tumor invasiveness. The intensities and numbers of immunoreactive cells for VEGF staining are summarized in Table 3.

Strong positive staining with VEGF is shown in Figure1. In contrast, negative staining was observed in both non-tumoral adenohypophyseal pituitary tissues adjacent to adenoma and normal pituitary (Figure 2A, and 2B). They are also negative stainings with both Ki-67 and P53.

If we accept more than 25% staining of VEGF, as a cut- off level, the expression of VEGF in invasive adenomas was significantly more than non-invasive adenomas ($p=0.002$) (Table 4).

Furthermore, it was observed that the decrease in VEGF staining in OCT pretreated invasive adenomas was significantly more than those not treated with OCT ($p=0.029$) (Table 5).

There was no statistical difference for Ki-67 index between OCT pretreated and not-treated adenomas.

Tab. 1. The expression of Ki-67 in non-invasive and invasive adenomas was not statistically different ($p=0.294$).

Ki-67 Staining count (%)	Non-invasive n (%)	Invasive n (%)
0 (-)	0 (0.0)	1 (5.0)
<1%	3 (15.8)	6 (30.0)
1-3%	9 (47.4)	5 (25.0)
>3%	7 (36.8)	8 (40.0)
Total	**19 (100.0)**	**20 (100.0)**

Tab. 2. The expression of p53 in these two groups was similar ($p=0.511$).

p 53	Non-invasive (n=19)	Invasive (n=20)
Staining	% within group	% within group
Negative	64.3%	68.4%
Positive	35.7%	31.6%

Tab. 3. The expression of VEGF in invasive as compared with non-invasive adenomas was statistically different ($p=0.016$)

VEGF Staining	Count %	Non-invasive n (%)	Invasive n (%)
Negative	0	2 (10.5)	0 (0.0)
Weak	<25%	9 (47.4)	2 (10.0)
Intermediate	26-50%	1 (5.3)	2 (10.0)
Strong	>50%	7 (36.8)	16 (80.0)
Total		19 (100.0)	20 (100.0)

Tab. 4. VEGF in invasive and non-invasive adenomas as a cut-off level 25%.

VEGF Staining count (%)	Non-invasive n (%)	Invasive n (%)
<25%	11 (57.9)	2 (10.0)
≥25%	8 (42.1)	18 (90.0)
Total	19 (100.0)	20 (100.0)

Tab. 5. VEGF in OCT pretreated and not treated invasive adenomas.

VEGF Staining count (%)	OCT(–) invasive n (%)	OCT(+) invasive n (%)
<25%	2 (10.0)	3 (50.0)
≥25%	18 (90.0)	3 (50.0)
Total	20 (100.0)	6 (100.0)

DISCUSSION

Pituitary tumorigenesis is a poorly understood process involving dysregulation of the cell cycle, proliferation, and angiogenesis, and more detailed analyses are needed to elucidate the molecular mechanisms. Although studies examining the marker of Ki-67 and P53 in different pituitary tumors have yielded contradictory results, angiogenesis has been described as an important mechanism of growth in human pituitary adenomas and carcinomas (Kawamato *et al.* 1995; Landolt *et al.* 1987; Schreiber *et al.* 1999; Turner *et al.* 2000). Up to this time, there is no study in which markers of cell proliferation (Ki-67, p53) and angiogenesis (VEGF) were evaluated concomitantly in GH-secreting adenomas. In this study, the presence of these markers in a series of 47 GH-secreting tumors were evaluated. Using the MIB-1 antibody in paraffin-embedded sections, values of 3.6 through 16.4% have been reported (Gandour-Edwards *et al.* 1995; Krämer *et al.* 1994; Lübke *et al.* 1995). In the studies by Knosp *et al.* (1989), Pizarro *et al.* (2004), Zhao *et al.* (1999), and Mastronardi *et al.* (1999), the mean positive reaction to Ki-67 was 1.1, 1.22, 1.4, and 2.64%, respectively. In the study of Pizarro *et al.* (2004), the Ki-67 index was above 3% in 15 cases, and in two of them it was above 10% i. e.,

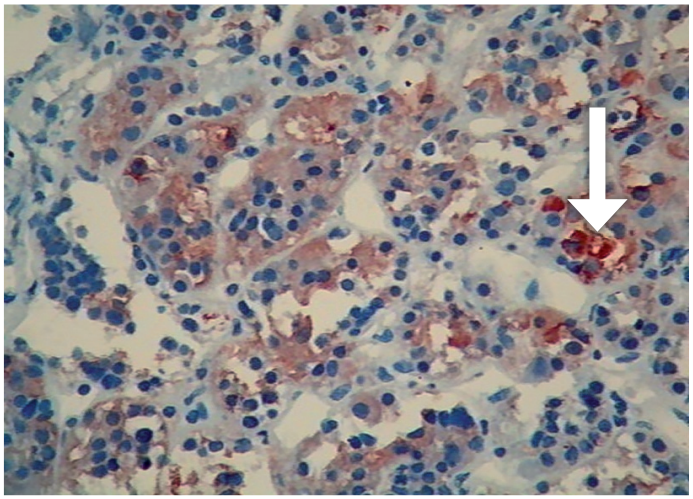

Fig. 1. Strongly positive staining (arrow) with VEGF appeared in the adenomatous cells in a case. Counterstained with hematoxylin (×400).

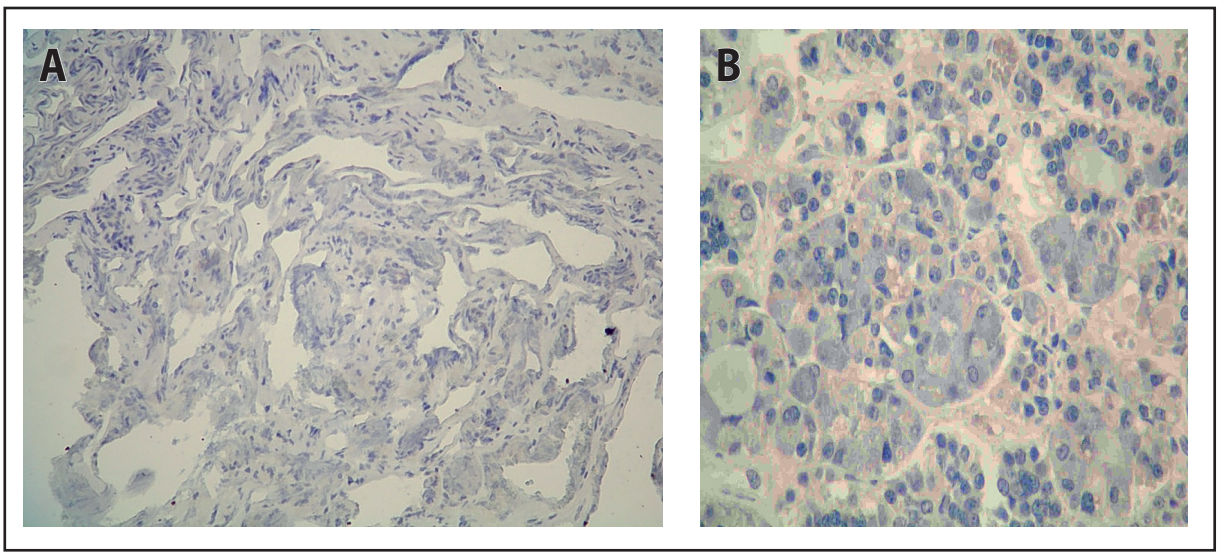

Fig. 2. As a negative control, (**A**, ×200) nontumoral adenohypophysis tissue adjacent to adenoma, and (**B**, ×200) a normal pituitary tissue were selected for VEGF immunoreactivity. Counterstained with hematoxylin.

12.71 and 15.48%. Other investigators have reported values of 16.45% (Thapar *et al.* 1996) and even 23.37% (Kawamato *et al.* 1995) in their series, values which have been usually found in pituitary carcinomas. In the present study, the Ki-67 index was above 3% in 7 non-invasive and 8 invasive adenomas. On the other hand, a significantly higher Ki 67 index in patients younger than 30 than in older patients had been reported (Jaffrain-Rea *et al.* 1998; Tsanaclis *et al.* 1991; Yonezawa *et al.* 1997). But other authors compared different age groups, and found no significant differences between Ki-67 index and different age groups analyzed (Mastronardi *et al.* 1999; Pizarro *et al.* 2004). In the present study, we did not find any significant differences in Ki-67 index regarding patient age. On the bases of literature, there is no agreement about Ki-67 index among the different age groups. In other studies, an overlap of Ki-67 index values was found in non-invasive adenomas, invasive adenomas, and pituitary carcinomas (Kawamato *et al.* 1995; Mastronardi *et al.* 1999; Pizarro *et al.* 2004; Thapar *et al.* 1996; Uozumi *et al.* 1995). Our results showed that the expression of Ki-67 in non-invasive and invasive adenomas were not different, moreover, one invasive adenoma has a negative Ki-67 index. Therefore, neither negative staining nor low values do not necessarily indicate the non-aggressive evolution of the adenoma. Landolt *et al.* (1987) demonstrated a significant higher Ki-67 index in invasive pituitary adenomas than in non-invasive adenomas. An effort has been made to define an index value that could serve as a cut-off to distinguish between invasive and non-invasive behavior of adenoma. Thapar et al (1996) reported that Ki-67 index of 3% was successful in distinguishing between invasive and non-invasive adenomas with a specificity of 97% and a sensitivity of 73%. But, we couldn't show a Ki-67 index of 3% as a cut-off level for invasiveness of tumor behavior. In the literature, however, there is no consensus about cut-off level concerning tumor inva-

siveness (Mastronardi *et al.* 1999; Thapar *et al.* 1996). The establishment of cut-off level associated with aggressive tumor behavior could be used in the future as an aid for therapeutic decision making. Losa *et al.* (2001) demonstrated that acromegalic patients receiving chronic octreotide treatment had a lower value of the proliferation marker Ki-67 index compared with matched untreated patients. In this study, we could not find any differences between groups. Alterations on p53 were identified in higher proportions of aggressive or invasive pituitary tumors (Mastronardi *et al.* 1999, Thapar *et al.* 1996). Schreiber *et al.* (1999) showed that p53 was found only 20% of all invasive adenomas, but they concluded negative results did not exclude clinically relevant invasive behavior. In another study, GH-secreting adenomas showed p53 positivity in only 28.9% (Pizarro *et al.* 2004). In this study, the expression of p53 was identified as 35.7% for non-invasive, and 31.6% for invasive adenomas. In our study, p53 expression was not related neither to clinical parameters nor to invasiveness of GH-secreting adenomas. It was suggested that p53 would not have an important role in invasiveness of tumor. Sautner & Saeger (1991) identified the high p53 levels through immunohistochemistry in pituitary tumors, and they suggested that this result could be a consequence of binding to other cell proteins in the tumors. Angiogenesis is thought to be regulated by VEGF, and it is known to be present immunocytochemically in pituitary adenomas (Fukui *et al.* 2003; Kurosaki *et al.* 2008, Lloyd *et al.* 1999; Niveiro *et al.* 2005; Takata *et al.* 2004; Viacava *et al.* 2003). LIoyd *et al.* (1999) found that GH-secreting adenomas has the strongest immunoreactivity with anti-VEGF. Morever, Kurosaki *et al.* (2008) reported that VEGF was demonstrated in a diffuse cytoplasmic pattern in GH-secreting adenoma; but age, gender, tumor size and invasiveness did not influence VEGF expression. In the present study, we observed the significant association between tumor invasiveness and expression of VEGF, but not with age, gender, and hormonal status. On the otherhand, our results showed that difference in pattern of staining in noninvasive and invasive adenomas is significantly different, a finding not reported previously. Viacava *et al.* (2003), and Kurosaki *et al.* (2008) reported that VEGF strong staining was observed in the cytoplasm in 3 and 19 specimens of normal anterior pituitary gland, retrospectivelly. But they did not observed cytoplasmic staining in a normal pituitary and nontumoral adenohypophysis tissues adjacent to the adenoma in this study. LIoyd *et al.* (1999) reported GH adenomas treated with octreotide stained less intensely than did untreated adenomas. Kurosaki *et al.* (2008) reported that moderately positive staining with VEGF was seen in 18% of the octreotide group, and in 67% of the control group. In contrast, weakly positive staining was observed in 76% of the octreotide group, and in 25% of the control group. This staining pattern differed statistically between the octreotide and control group, and they concluded that octreotide may inhibit the angiogenesis through down-regulation of VEGF. GH adenomas treated with OCT had weaker staining than untreated adenomas in our cases. In contrast to our study, Iuchi *et al.* (2000) reported VEGF expression showed no significant correlation with MR grades. We could not find any significant association between VEGF and the markers of cell proliferation. On the other hand, with a similar approach to Thapar *et al.* (1996), VEGF staining of > 25% could be used as a cut-off for distinguishing invasive from non-invasive GH-releasing adenomas, which we accepted and utilized in this study. On the basis of this cut-off level, GH adenomas treated with octreotide stained significantly less intensely than did untreated adenomas for VEGF. Particularly in locally invasive GH-secreting adenomas, preoperative medical debulking was improving of the surgical outcome confirmed by some studies (Iuchi *et al.* 2000; Oshino *et al.* 2006; Stevenaert & Beckers 1996; Thapar *et al.* 1996). This observation could be related to OCT effect, such as inhibition of angiogenesis via down-regulation of VEGF expression which was reported by Kurosaki *et al.* (2008). Therefore, a short term (such as 3 to 6 months) preoperative OCT treatment may be useful as adjunctive therapy especially for invasive GH-secreting adenomas. So, this cut off value may be useful in determination of prognosis and appropriate treatment strategies for disease follow-up and postoperative treatment. Further complementary studies are needed to confirm this observation.

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