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Localization of Inhibin/Activin Subunits in Normal Pituitary and in Pituitary Adenomas

Silvia Uccella[1], **Stefano La Rosa*[2], *Anna Genasetti*[1], *and Carlo Capella*

[1]*Department of Clinical and Biological Sciences, University of Insubria, Varese, Italy;* [2]*Department of Pathology, Ospedale di Circolo, Varese, Italy*

Abstract. **The localization of inhibin/activin (I/A) subunits was investigated in human normal adenohypophysial cells and in 87 pituitary adenomas of different types, using immunohistochemistry. Monoclonal antibodies directed against α, βA and βB subunits of I/A were employed. In normal pituitary, α subunit of inhibin was detected only in FSH-positive gonadotrophs, while βA subunit of I/A was expressed in FSH-positive gonadotrophs, GH-cells and in a few PRL-cells. βB subunit was found in FSH-positive gonadotrophs, TSH-cells and a few LH-positive gonadotrophs. The three subunits of I/A were detected in the majority of nonfunctioning tumors, while functioning adenomas showed a significantly lower expression. This study shows that α, βA and βB subunits of I/A are expressed by specific adenohypophysial cell types and that they are characteristically present in nonfunctioning adenomas. These results suggest that inhibins and activins may play a role in the local regulation of pituitary hormonal secretion both in normal adenohypophysial cells and in pituitary adenomas.**

Keywords. **inhibin, activin, pituitary, immunohistochemistry**

Introduction

Inhibins and activins are regulatory factors initially isolated from gonadal fluids on the basis of their ability to inhibit and stimulate FSH synthesis and secretion, acting with a long loop feedback on pituitary gonadotrophs [1]. Both these factors are disulphide-linked dimeric proteins belonging to the transforming growth factor β (TGFβ superfamily, which consists of 5 families of functionally different, but structurally related, regulatory molecules: TGFβ and its isoforms, Müllerian inhibiting substance, decapentaplegic gene complex polypeptide, cartilage-inducing factos and inhibins/activins [2]. Inhibins are heterodimers composed of an α subunit and one of two β subunits, βA or βB; up to now, two forms of inhibins have been characterized: inhibin A (α**A**) and inhibin B (αβB) [1,3]. Activins are homo- or heterodimers of inhibin β subunits: activin A is a βAβA homodimer, activin B is a βBβB homodimer and activin AB is a βAβB heterodimer [1,4,5]. Although the first isolation of activins and inhibins dates back to 1932 [6], molecular and functional characterization of these proteins has been accomplished only in the last fifteen years [1,7–9]. Recent investigations have shown that inhibin/activin subunits and their mRNAs are not only restricted to the gonads, but they are also present in a wide variety of reproductive and non-reproductive organs and tissues, including the placenta, the endometrium, the adrenal glands, the bone marrow, the kidney, the spinal cord, the brain, and specific types of endocrine cells of the gut and pancreas [10–14]. In these sites, activins and inhibins can locally regulate a number of cellular functions, exerting their activities through autocrine/paracrine mechanisms, in addition to the well known endocrine ones [9,15].

Inhibins and activins have also been localized in the anterior pituitary gland, where their best known function is the local modulation of gonadotroph function. In particular, inhibin inhibits both basal and GnRH-mediated FSH synthesis and release, while activin acts in an opposite way [1,6]. Recent studies, have provided further details about this FSH-secretagogue activity, demonstrating that inhibins are able to reduce transcription of FSH gene and to down-regulate GnRH receptors [16]. Further investigations, however, have pointed out that these proteins, and particularly activin, play also a role in the regulation of synthesis and release of growth hormone (GH) [17] and prolactin [18]; in addition, they are able to modulate the proliferation of GH- and PRL-cells [18,19]. It has also been suggested that activin may influence the phenotype of pituitary cells, intervening in the temporary conversion from somatotrophs to gonadosomatotrophs during proestrus and diestrous of female rats [20]. However, most of the studies, which have investigated the function and localization of inhibins and activins in specific pituitary cell types, were performed on animal tissues and their conclusions cannot be easily applied to humans. Moreover, the results concerning the cellular localization of activins and inhibins are often conflicting and sometimes incomplete, with special reference to βB subunit expression, which has been investigated only in one study [21].

Address for correspondence: Prof. Carlo Capella, Servizio di Anatomia Patologica, Ospedale di Circolo, v.1e Borri, 57, I-21100 Varese, Italy. Tel.: (+39) 0332 278231; Fax: (+39) 0332 278599; E-mail: Carlo.Capella@ospedale.varese.it

The expression of inhibins and activins and their subunits has also been investigated in pituitary adenomas [22–24]. However, the localization of inhibin and activin subunits in the different functional types of pituitary adenomas has not been fully clarified.

The aim of our study was to clarify, using immunohistochemical methods, whether inhibins/activin subunits α, βA, and βB are differently expressed in the various types of adenohypophysial cells and related tumors.

Materials and Methods

Four normal pituitary glands were obtained at autopsy from subjects without endocrinological abnormalities (age range: 54–84 years), while samples of 87 pituitary adenomas were collected at transsphenoidal surgery. Pituitary adenomas were subdivided into 47 functioning adenomas and 40 nonfunctioning adenomas, on the basis of clinical symptoms and hormonal measurements, and classified according to the prevalent hormonal product detected immunohistochemically.

All tissue specimens were fixed in buffered formalin (formaldehyde 4% w/v and acetate buffer 0.05 M) and embedded in paraffin. Routinary histopathologic evaluation was performed on 4μm-thick sections stained with hematoxylin-eosin, Grimelius' silver stain and Alcian blue/periodic acid-Schiff (AB/PAS). For imunohistochemical study, 3μm-thick sections mounted on poly-L-lysine slides were employed. After deparaffinization and rehydratation through graded alcohols, endogenous peroxidase activity was inhibited by treatment with 3% hydrogen peroxide for 10 minutes. Incubation with primary antibodies and antisera directed against inhibin/activin α, βA and βB subunits and against pituitary hormones (Table 1) was done at 4°C for 18–20 hours. Reactions were amplified employing the avidin-biotin-peroxidase technique and developed using 0.03% 3,3′ diamino-benzidine. Colocalization studies were performed with double-label immunostaining according to Mason and Sammons [25] and to Lan et al. [26]. The localization of various subunits of inhibin/activin was obtained using specific monoclonal antibodies, as reported in Table 1. In detail, the anti-α-subunit antibody was a mouse monoclonal antibody directed against a synthetic protein corresponding to the 1-32 peptide sequence of the α-subunit of 32kd human inhibin. The mouse monoclonal antibody directed against the βA-subunit recognized a synthetic peptide corresponding to the 82-114 protein sequence of the βA-subunit of 32 kd human inhibin A and activin A. The anti-βB-subunit mouse monoclonal antibody was directed against a synthetic peptide corresponding to residues 82-114 of the inhibin βB-subunit. Sections stained with antibodies directed against inhibin/activin α and βA subunits and against FSH were pretreated with 0.01M citrate buffer pH 6 for 10 minutes in a microwave ovem at 750W; sections stained with anti-βB antibody were pretreated with etylendimitrilo tetracetic acid (EDTA) buffer pH 8 for 10 minutes in a microwave oven at 750W; sections stained with anti-ACTH antiserum were pretreated for 10 minutes with 0.003% subtilisin (protease type XXVII or Nagarse preotease; P4789, Sigma, St. Louis, MO, USA).

Table 1. *Antibodies and antisera employed*

Antibodies/ antisera*	P/M	Dilution	Source
FSH	M	1/20	Dako, Copenhagen, DK
LH	M	1/100	BioGenex Laboratories, San Ramon, CA, USA
GH	M	1/200	BioGenex
PRL	P	undiluted	Biomeda, Foster City, CA, USA
ACTH	P	1/250	Dako
αhCG	M	1/5000	Dr. Ghielmi, University of Brescia, Italy
TSH	M	1/2	Biomeda
α subunit of inhibin	M	1/100	Serotec, Oxford, UK
βA subunit of I/A	M	1/100	Serotec
βB subunit of I/A	M	1/100	Serotec

M/P = monoclonal/polyclonal; I/A = inhibin/activin; * = all antibodies and antisera used were directed against human peptides.

Specificity controls consisted of absorption of antibodies and antisera with 10–20 nMol of the related antigens, substitution of the primary antibodies with non immune serum of the same species at the same dilution, and use of control tissues with or without the pertinent antigen. These tests confirmed that the antibodies and antisera employed were specific.

To correlate the expression of α, βA and βB subunits of inhibin/activin in pituitary adenomas with their clinico-pathologic features, statistical analysis was performed using the Fisher's exact test.

Results

In normal anterior pituitary we observed an intense positivity for all inhibin/activin (I/A) subunits, α, βA and βB, with a fraction of immunoreactive (IR) cells variable according to the type of subunit. The positivity was granular and was located in the cytoplasm, with a membrane reinforcement in some cells. The posterior pituitary lobe did not show any positive reaction. α subunit of inhibin was detected in about 10–20% of adenohypophysial cells and colocalization studies showed that α subunit of inhibin-IR cells corresponded almost exclusively to FSH-positive gonadotrophs (Figure 1). βA subunit of I/A was more widely expressed, being present in about 40–50% of anterior pituitary cells. βA subunit-positive cells included about 20% of FSH-producing gonadotrophs, very few (less than 5%) PRL-cells and the vast majority (>90%) of GH-cells (Figure 2). βB subunit of I/A was found in about 40% of adenohypophysial cells and double label immunohis-

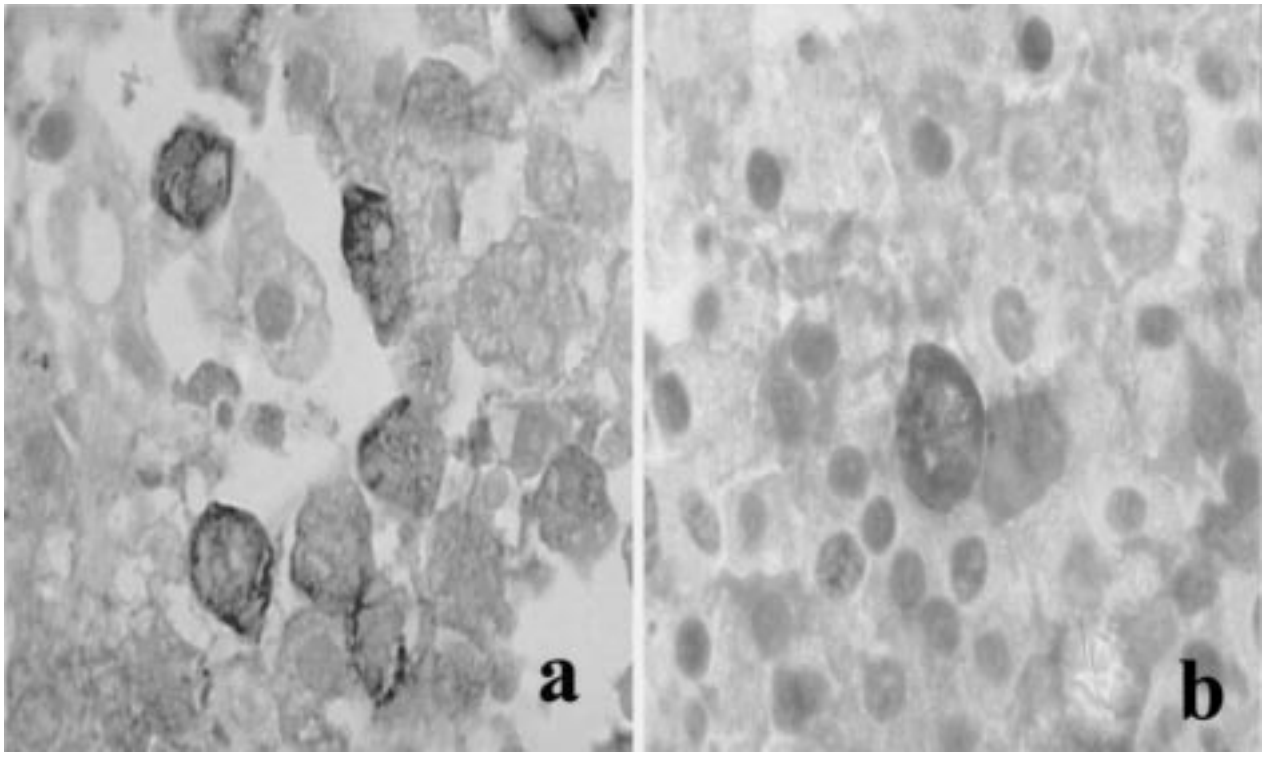

Fig. 1. *Normal autoptic pituitary gland. Double label immunostainings showing that α-subunit of inhibin (brown) is colocalized with FSH (a), but not with GH (b). Hormones are stained in red. (Avidin-biotin-peroxidase method for brown staining and alkaline phosphatase method for red staining, original magnification × 1000).*

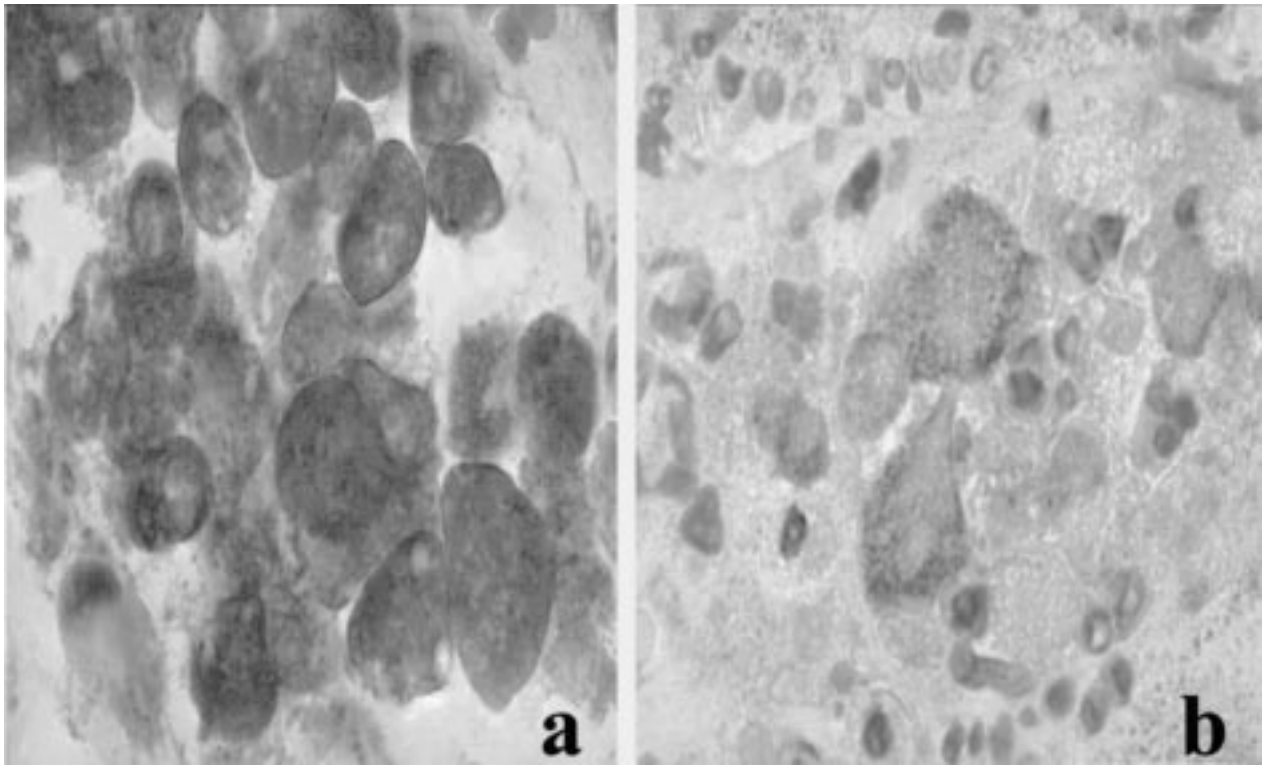

Fig. 2. *Normal autoptic pituitary gland. Double label immunostainings showing that βA-subunit of inhibin/activin (brown) is colocalized with GH (a), but not with ACTH (b). Hormones are stained in red. (Avidin-biotin-peroxidase method for brown staining and alkaline phosphatase method for red staining, original magnification × 1000).*

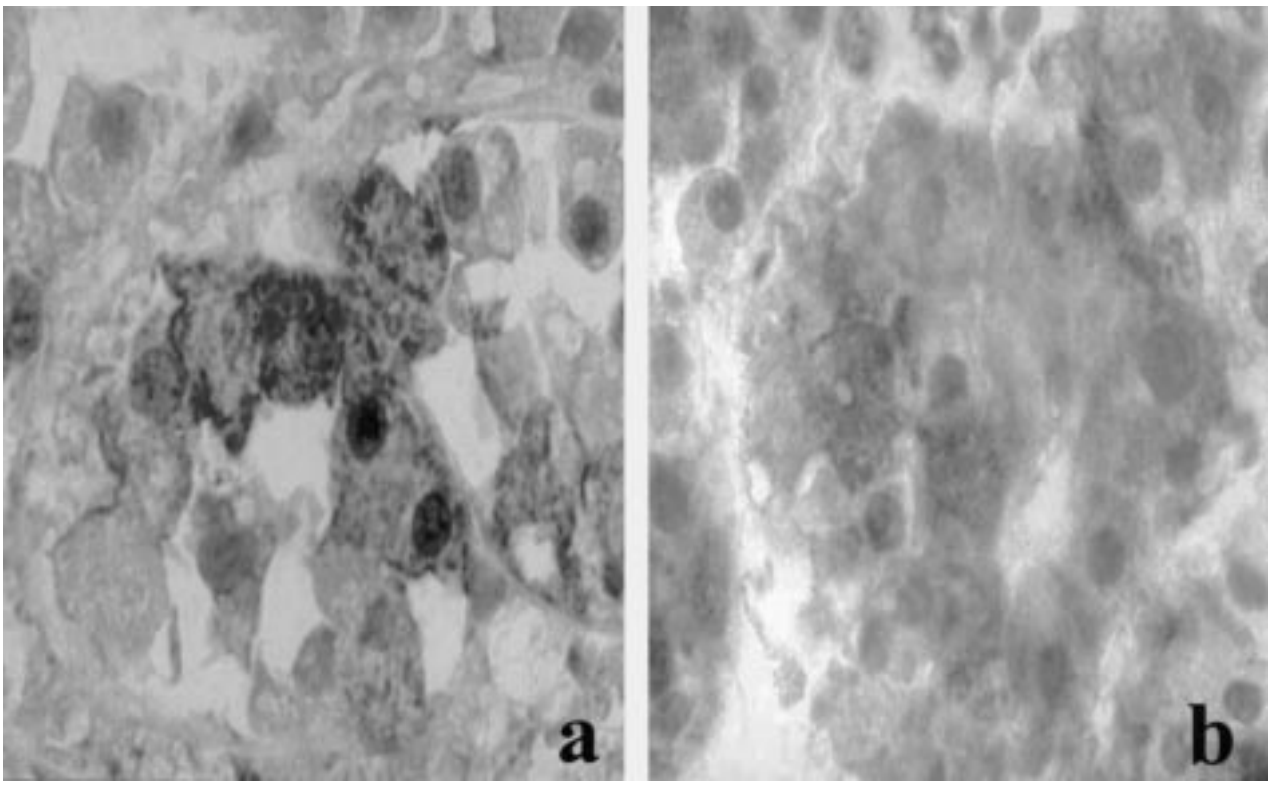

Fig. 3. *Normal autoptic pituitary gland. Double label immunostainings showing that βB-subunit of inhibin/activin (brown) is colocalized with TSH (a), but not with ACTH (b). Hormones are stained in red. (Avidin-biotin-peroxidase method for brown staining and alkaline phosphatase method for red staining, original magnification × 1000).*

tochemical stains revealed that all FSH-producing gonadotrophs, a few LH-producing gonadotrophs, and nearly all TSH-cells were βB-IR (Figure 3). ACTH-cells were not immunoreactive for any I/A subunits. Table 2 summarizes the immunohistochemical results obtained in the normal pituitary gland.

The 87 pituitary adenomas were subdivided in two groups on the basis of their functional activity: forty-seven tumors were associated with clinical symptoms caused by hormonal hypersecretion and were classified as functioning, while the remaining 40, not associated with typical endocrine syndromes, were classified as nonfunctioning. The mean age of the patients was 46.5 years (age range: 12–71); in the group of functioning adenomas, the mean age was 41 years, while, in the nonfunctioning group it was 53 years. Thirty-seven patients were males and 46 females, for 4 patients data about sex were not available. On the basis of immunohistochemical results, the 47 functioning adenomas were further classified as 19 GH-cells, 9 mixed GH/PRL-cells, 12 PRL-cells and 7 ACTH-cells, while the 40 nonfunctioning adenomas were classified into 19 gonadotroph, 9 α subunit of gonadotropin (α SU-GO)-producing-cells, 5 null cell, 3 ACTH-cell and 4 TSH-cell adenomas.

Table 2. *Expression of α, βA and βB subunits of inhibin/activin in normal adenohypophysial cell types*

Cell type	α	βA	βB
FSH	+	+	+
LH	–	–	+
GH	–	+	–
PRL	–	+	–
ACTH	–	–	–
TSH	–	–	+

Tables 3 and 4 show immunohistochemical results of pituitary adenomas, in relation to functional activity and immunophenotype. Positivity for α subunit of inhibin was observed in 23/87 (26%) adenomas, most of which were represented by nonfunctioning tumors (22/40, 55%). In particular, 15/19 (79%) gonadotroph (Figure 4), 4/9 (44%) α SU-GO-cell, 2/5 (40%) null-cell and 1/4 (25%) TSH-cell adenomas were positive for the α subunit of inhibin. The mean percentage of positive cells in nonfunctioning adenomas ranged from 15% to 35%. On the contrary, among 47 functioning tumors, only one mixed GH/PRL-producing adenoma (2%) contained rare cells (about 5%) immunoreactive for α subunit of inhibin. The difference of the expression of α subunit of inhibin between functioning and

Table 3. *Expression of inhibin/activin subunits in nonfunctioning and functioning pituitary adenomas*

	α		βA		βB	
	n.(%)	m.p.	n.(%)	m.p.	n.(%)	m.p.
Nonfunctioning adenomas	22/40 (55%)	23.7	22/39 (56%)	65	20/35 (57%)	17.4
Functioning adenomas	1/47 (2%)	5	11/47 (23%)	54	9/36 (25%)	52

m.p. = mean percentage of immunoreactive cells in positive tumors

nonfunctioning adenomas was highly significant ($p < 0.00001$).

βA subunit of I/A was expressed by 33/86 (38%) adenomas, of which 22 (66%) were nonfunctioning and 11 (33%) were functioning. In the nonfunctioning group, 12/18 (67%) gonadotroph cell, 4/9 (44%) αSU-GO-cell (Figure 5), 2/5 (40%) null cell and 4/4 (100%) TSH-cell adenomas were βA subunit of I/A-IR. In these tumors, the mean percentage of βA subunit-positive cells ranged from 45% to 77%. Among functioning tumors, 3/12 (25%) PRL-cell, 4/19 (21%) GH-cell and 4/9 (54%) mixed GH/PRL-cell adenomas expressed βA subunit, with a mean percentage of immunoreactive cells ranging from 51% to 59%. The different expression of βA subunit of I/A between functioning and nonfunctioning adenomas was statistically significant ($p < 0.00002$).

The βB subunit was expressed in 29/71 (41%) adenomas. Twenty of 35 (57%) nonfunctioning adenomas were βB-immunoreactive, including 7/15 (47%) gonadotroph (Figure 6), 8/8 (100%) αSU-GO cell, 1/5 (20%) null cell, 2/3 (67%) ACTH-cell, and 2/4 (50%) TSH-cell adenomas. The mean percentage of βB-immunoreactive cells in nonfunctioning adenomas ranged from 10% to 37%. Nine of 36 (25%) functioning adenomas were positive for βB subunit of I/A, including 4/15 (27%) GH-cell, 3/8 (37%) PRL-cell and 2/6 (33%) mixed GH/PRL cell adenomas. Also for βB-immunoreactivity, Fisher exact test revealed a statistically significant difference between functioning and nonfunctioning adenomas ($p < 0.003$).

At statistical analysis, α, βA and βB subunit immunoreactivities were not significantly related to the sex or age of the patients.

Discussion

We have investigated the immunohistochemical expression of α, βA and βB subunits of inhibin/activin (I/A) in normal human anterior pituitary gland and in a series of 87 pituitary adenomas. The comprehensive study of all three subunits composing inhibins and activins molecules allowed us to identify the profile of expression of the various forms of these proteins both in normal and neoplastic pituitary cells. In fact, the coexpression of α subunit with βA subunit of I/A was indicative of the presence inhibin A (αβA) and the coexpression of α subunit with βB subunit was indicative of the presence of inhibin B (αβB). On the other hand, the sole immunoreactivity for βA or βB chains was consistent with the presence of activin A (βAβA) or activin B (βBβB), respectively. Finally, when βA and βB subunits were co-expressed, the presence of activin AB (βAβB) was suggested. These data, combined with the immunohistochemical identification of normal and neoplastic pituitary cell types, provided a survey of the differential distribution of activins and inhibins in dif-

Table 4. *Expression of inhibin/activin subunits in different types of pituitary adenomas*

	α		βA		βB	
	n.(%)	m.p.	n.(%)	m.p.	n.(%)	m.p.
Nonfunctioning adenomas						
Gonadotroph	15/19 (79%)	24	12/18 (67%)	71	7/15 (47%)	12.5
αSU-GO cell	4/9 (44%)	21	4/9 (44%)	67.5	8/8 (100%)	37
null Cell	2/5 (40%)	35	2/5 (40%)	45	1/5 (20%)	10
ACTH cell	0/3	0	0/3	0	2/3 (67%)	15
TSH cell	1/4 (25%)	15	4/4 (100%)	77	2/4 (50%)	12.5
Functioning adenomas						
GH cell	0/19	0	4/19 (21%)	51	4/15 (27%)	50
PRL cell	0/12	0	3/12 (25%)	59	3/8 (37%)	47
GH-PRL cell	1/9 (11%)	5	4/9 (44%)	52	2/6 (33%)	60
ACTH cell	0/7	0	0/7	0	0/7	0

m.p. = mean percentage of immunoreactive cells in positive tumors

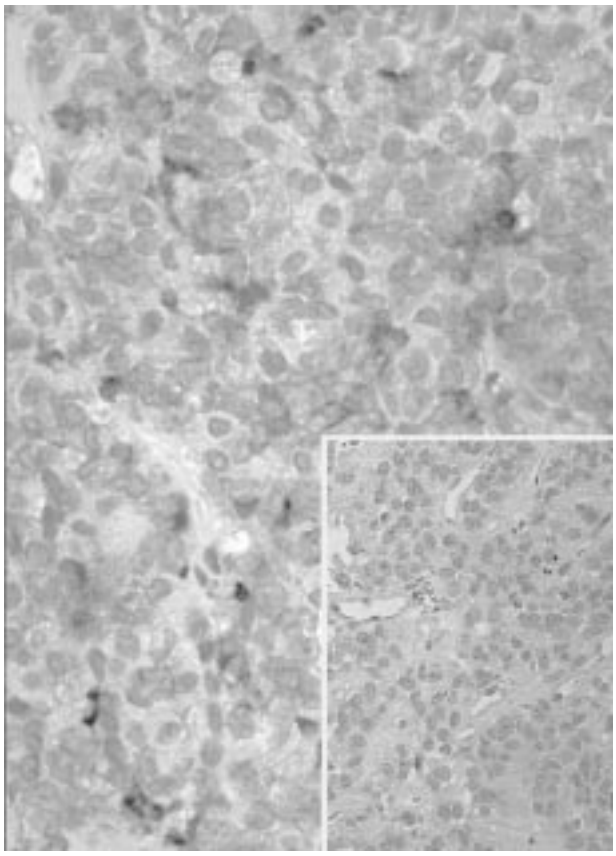

Fig. 4. *Immunoreactivity for α-subunit of inhibin in a gonadotroph adenoma. (Avidin-biotin-peroxidase method, original magnification × 400). The inset shows that after absorption with the related antigen the immunoreactivity is lacking.*

ferent adenohypophysial cell types and in various pituitary adenomas.

Our results show that, among adenohypophysial cells, FSH-producing gonadotrophs represent the main site of production of inhibins and activins, since they are positive for all three subunits and therefore express all forms of inhibins and activins. In the other adenohypophysial cell types, I/A subunits were less widely expressed. Nearly all GH-cells and few PRL-cells were strongly immunoreactive only for βA subunit, which indicates the presence of activin A. In TSH-cells and in a fraction of LH-producing gonadotrophs an exclusive immunostain for βB subunit was found, suggesting the presence of activin B. It is worth noting that α subunit was completely lacking in cells other than FSH-producing gonadotrophs, indicating that gonadotrophs are the only pituitary cell type in which inhibin is present.

The expression of inhibins, activins and their subunits in pituitary gland has been previously investigated employing different experimental techniques, including immunohistochemistry [12,21,24,27] and mRNA in situ hybridization [24]. In a recent work, Sugiyama et al. [24], investigated α and βA subunit expression in human pituitary, using both immunohistochemistry and mRNA in situ hybridization. In this study, the α subunit of inhibin was reported to be present in all adenohypophysial cell types, while βA subunit was only found in FSH/LH-producing gonadotrophs and in PRL-cells. The difference between our results and those obtained by the authors reported above, concerning the localization of the I/A subunits in normal pituitary gland, may be due to the different antibodies employed or the different species examined [12,21,27].

The presence of I/A subunits in anterior pituitary gland suggests that these proteins may play a role in local regulation of pituitary function via paracrine and/or autocrine mechanisms, in addition to their well-known endocrine function. In particular, the expression of inhibins and activins in FSH-producing gonadotrophs supports the hypothesis that they may locally modulate FSH synthesis and secretion [15], as it has been shown by investigations on rat adenohypophysial cell lines [28]. A noteworthy result of our investigation is that α subunit of inhibin is localized only in FSH-producing gonadotrophs, but not in other pituitary cell types, suggesting that the expression of inhibins is a peculiarity of this cell type. In the pituitary gland, inhibin activity seems to be specifically directed to regulate gonadotroph functions, and no other functional role has been identified for this regulatory factor to date [15,17,19]; thus, the exclusive localization of inhibins in FSH-producing cells is not surprising. On the contrary, activins have been reported to modulate GH and PRL synthesis and secretion [17,18,29], and to influence the proliferation rate of GH- cells and PRL-cells [18,19] and these data are in agreement with our results showing that most GH-cells and a few PRL-cells express βA subunit which is indicative of the presence of activin A in these cell-types.

The results of our study on pituitary adenomas show that the subunits of I/A are more frequently expressed in nonfunctioning tumors, which are mainly represented by gonadotroph and null cell adenomas. In particular, α subunit of inhibin was exclusively expressed by functionally inactive adenomas, while βA and βB chains were also detected in functioning tumors, although in a significantly lower number of cases, in comparison to functioning adenomas. Sugiyama et al. [24] analyzing a series of 79 pituitary adenomas, with immunohistochemical (using polyclonal antibodies directed against porcine subunits of I/A) and in situ hybridization techniques, reported that α subunit was found in all types of adenomas, without any cell type specificity and any differential expression in functioning and nonfunctioning tumors. On the contrary, βA subunit was found to be preferentially expressed in nonfunctioning gonadotroph adenomas and in functioning TSH- and ACTH-cell tumors [24]. Previous investigations on the detection of activin/inhibin

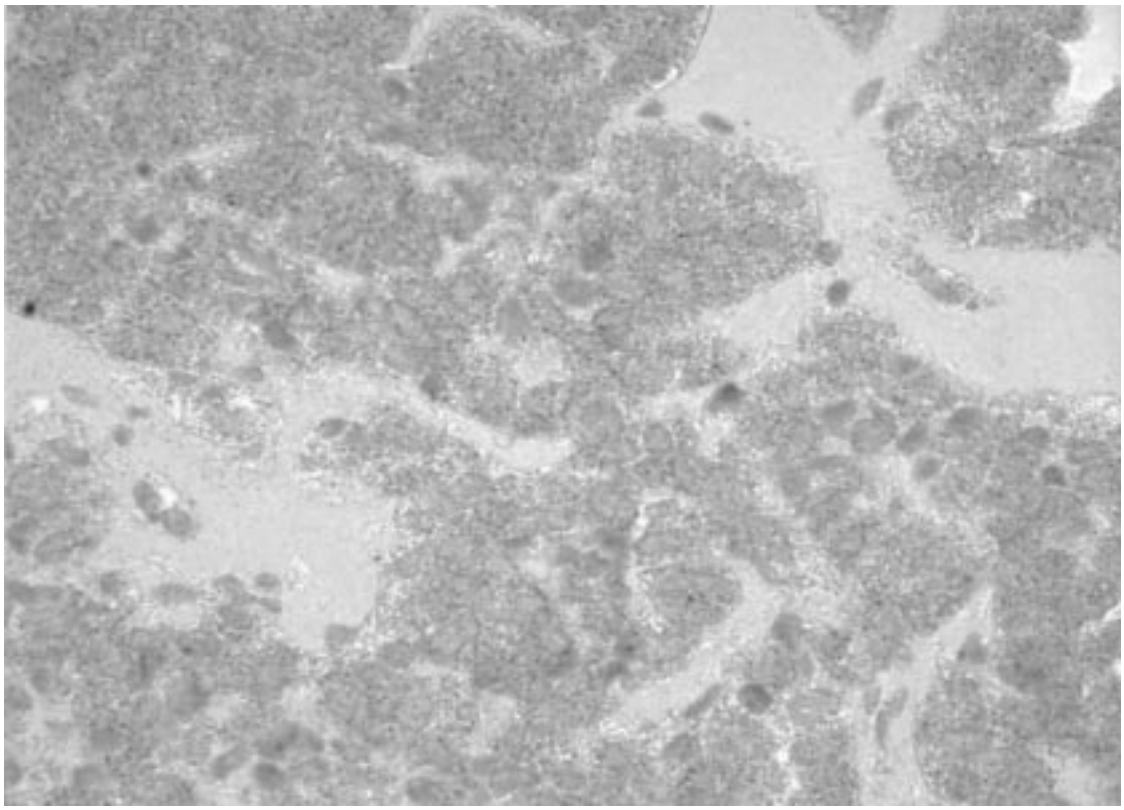

Fig. 5. *Immunoreactivity for βA-subunit of inhibin/activin in a α-SU-GO cell adenoma. (Avidin-biotin-peroxidase method, original magnification × 400).*

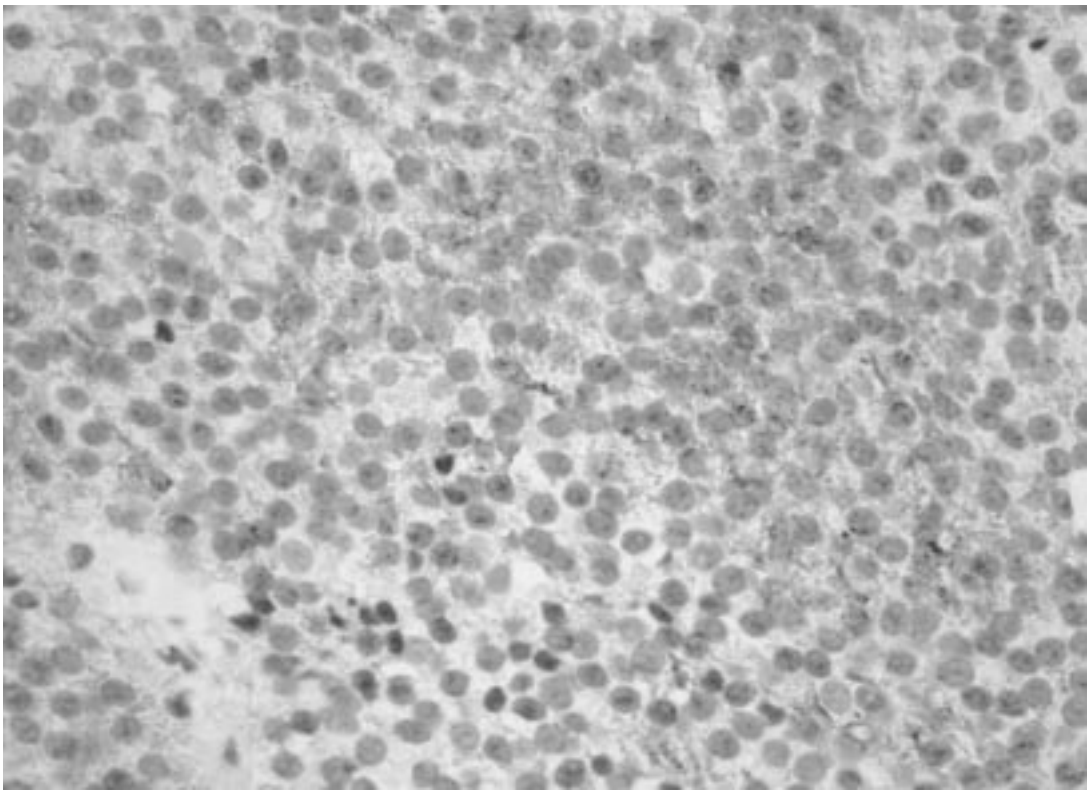

Fig. 6. *Immunoreactivity for βB-subunit of inhibin/activin in a gonadotroph adenoma. (Avidin-biotin-peroxidase method, original magnification × 400).*

in pituitary adenomas were performed employing molecular biology techniques, such as polymerase chain reaction (PCR) or reverse transcriptase PCR (RT-PCR), but the results reported were rather conflicting [22,23,30,31]. The disagreement between our results and those previously reported may be due to the different techniques employed. Molecular biology methods have a higher sensitivity than immunohistochemistry, but, on the other hand, they refer only to the presence of the mRNA and do not provide information about the actual synthesis and expression of the protein. In addition, the differences in immunohistochemical results may depend on the different antibodies employed in the various studies.

Our study shows that the subunits of I/A are preferentially expressed in gonadotroph adenomas. This finding, however, seems to be *per se* unrelated to the pathogenesis of neoplastic proliferation, since in normal gonadotrophs we observed the same pattern of immunoreactivity as in adenomas. However, it has been recently demonstrated that activin has an antiproliferative effect only in a subset of nonfunctioning pituitary adenomas, while the majority seems to be insensitive to this antimitogenic action [32]. D'Abronzo et al. [33] have hypothesized that the lack of responsivity to exogenous activin of a subset of human gonadotroph adenomas could be due to alterations in the expression of cell surface activin receptors.

In the present study, we found that GH-cell adenomas, unlike normal GH-cells, were mostly negative for βA chain of I/A and that, in the few positive cases, the number of immunoreactive cells was low. These findings might indicate a role of loss of activin A in the tumorigenesis of GH-cell adenomas or in uncontrolled cellular proliferation and hormonal secretion of these adenomas.

In conclusion, our study has shown that α, βA and βB subunits of I/A are expressed by specific endocrine cell type in normal human pituitary. Furthermore, we have demonstrated that gonadotroph adenomas, as their normal counterpart, retain the expression of subunits of I/A, while GH-cells adenomas, unlike normal somatotroph cells, lack βA subunit expression. Finally, we reported that nonfunctioning pituitary adenomas characteristically express both inhibins and activins.

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