Expression of inhibin α in adrenocortical tumours reflects the hormonal status of the neoplasm

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Abstract

Inhibins are gonadal glycoprotein hormones whose main endocrine function is to inhibit pituitary FSH secretion. In addition to testes and ovaries, other steroid-producing organs are sites of inhibin α subunit expression. To study the role of inhibins in human adrenal gland, we screened a panel of 150 adrenals (10 normal adrenals, 25 adrenocortical hyperplasias, 65 adrenocortical adenomas, 30 adrenocortical carcinomas and 20 phaeochromocytomas) for inhibin α expression. mRNA levels of inhibin α subunit were studied in 57 samples and all tissues were stained immunohistochemically with an inhibin α subunit-specific antibody. Inhibin α mRNA was detected in all adrenocortical tissues. Virilizing adenomas possessed a 10-fold higher median inhibin α mRNA expression than did normal adrenals. Bilaterally and nodularly hyperplastic adrenals and other than virilizing adrenocortical tumours had their median inhibin α mRNA levels close to those of normal adrenals. Immunohistochemically, inhibin α subunit was detectable in all normal and hyperplastic adrenals, as well as in 73% of the adrenocortical tumours. However, the percentage of inhibin α-positive cells varied greatly in different tumour types. The median percentage of positive cells was 10 in non-functional and Conn's adenomas, 30 in Cushing's adenomas and 75 in virilizing adenomas. In malignant adrenocortical tumours the median percentage of inhibin α-immunopositive cells was 20 in non-functional carcinomas, 30 in Conn's carcinomas, 65 in Cushing's carcinomas and 75 in virilizing carcinomas. All phaeochromocytomas were negative for inhibin α subunit both at the mRNA level and immunohistochemically.

Our data show that inhibin α subunit is highly expressed in both normal and neoplastic androgen-producing adrenocortical cells, with less expression in cortisol-producing and hardly any in aldosterone-producing cells. This suggests a specific role for inhibins in the regulation of adrenal androgen production. We did not find any significant difference in inhibin α expression between benign and malignant adrenocortical tumours. Thus inhibin α gene does not seem to have a tumour suppressor role in human adrenal cortex.

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Introduction

Inhibins are heterodimeric glycoproteins whose main endocrine function is supposed to be the regulation of pituitary follicle-stimulating hormone secretion. They consist of an α subunit linked to either a βA subunit (inhibin A) or a βB subunit (inhibin B) (Ying 1988). The inhibin α gene is located on human chromosome 2q (Barton *et al.* 1989). Steroid-producing organs, pituitary gland, placenta and the central nervous system are the main sites of inhibin α subunit gene expression (Meunier *et al.* 1988, Voutilainen 1995). In humans, inhibin α subunit gene is expressed in both foetal and adult adrenal cortex (Voutilainen *et al.* 1991, Spencer *et al.* 1992), and the inner zones, particularly the zona reticularis, have the strongest immunoreactivity with anti-inhibin α antibody (Chivite *et al.* 1998, McCluggage *et al.* 1998). The role of adrenal inhibins is not fully understood. Adrenocorticotrophic hormone (ACTH) has been shown to upregulate the expression of adrenal inhibins *in vitro* (Crawford *et al.* 1987, Voutilainen *et al.* 1991). A negative autoregulation of inhibin α subunit expression was suggested in a transgenic mouse study, where gonadal inhibins were shown to downregulate the expression of the inhibin α subunit gene in the adrenal gland (Kananen *et al.* 1996). Although gonads are the main source of circulating inhibins, adrenal venous blood has a higher concentration of inhibins than peripheral blood suggesting some contribution of adrenals as well (Nishi *et al.* 1995).

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Assessment of the growth potential of adrenocortical tumours can be complicated. Microscopic criteria of malignancy consider nuclear grade, mitotic rate, existence of atypical mitoses, diffuse architecture, necrosis and capsular or vascular invasion and absence of clear cells (Weiss 1984, Weiss *et al.* 1989). Flow cytometry has not offered diagnostic help in the evaluation of the malignant nature of the tumour (Cibas *et al.* 1990, Padberg *et al.* 1991, Medeiros & Weiss 1992). Proliferation marker Ki-67 and tumour suppressor gene p53 have been found helpful in distinguishing between adrenocortical adenomas and carcinomas (McNicol *et al.* 1997, Nakazumi *et al.* 1998). Abrogation of the MHC class II expression from adrenocortical tumours has been suggested to be a sign of the malignant nature of the tumour (Marx *et al.* 1996). Very frequent deletions in 11q13 and in 2p16 were revealed in carcinomas, when genotyping a number of adrenocortical tumours (Kjellman *et al.* 1999). Inhibins have been suggested to have a tumour suppressor role for the adrenal gland, since gonadectomized inhibin-deficient mice develop adrenocortical tumours (Matzuk *et al.* 1992). Recently two papers reported immunoreactivity against inhibin α in adrenocortical tumours (Chivite *et al.* 1998, McCluggage *et al.* 1998). Further studies discovered a considerable number of adrenocortical tumours exhibiting no immunostaining for inhibin α (Pelkey *et al.* 1998, Renshaw & Granter 1998).

To shed more light on the role of inhibins in human adrenal pathophysiology we studied the expression in adrenal tumours of inhibin α subunit gene by Northern blots, and of peptide by immunohistochemistry. The analysis of inhibin α expression in a large series of hormonally active and inactive adrenocortical neoplasms allows us to estimate the correlation of inhibin expression with adrenal steroidogenesis. The comparison of inhibin α expression in benign and malignant adrenocortical samples should reveal if inhibin α could have a tumour suppressor role in human adrenals.

Materials and Methods

Tissues

Tissue materials were obtained during operations performed at the Department of Surgery, Helsinki University Central Hospital. The tissue specimens were dissected and visible medullar parts were removed from normal and hyperplastic adrenals within 0·5 h, if used for RNA analysis. Normal adrenal glands were obtained from ten patients who underwent nephrectomy for kidney tumours. Pathological adrenal tissues included 10 diffuse and 15 nodular adrenocortical hyperplasias, 15 non-functional adrenocortical adenomas, 23 Conn's adenomas, 21 Cushing's adenomas, 6 virilizing adenomas, 12 non-functional adrenocortical carcinomas, 4 Conn's carcinomas, 10 Cushing's carcinomas, 4 virilizing carcinomas and 20 phaeochromocytomas. All cases were re-reviewed histologically. Malignancy of the adrenocortical tumours was assessed according to the criteria of Weiss (1984).

Immunohistochemistry

Sections were cut from formalin-fixed paraffin-embedded blocks. They were deparaffinized in xylene and rehydrated in a series of graded alcohols. The sections were pre-treated in a microwave oven in 10 mmol/l citrate buffer, pH 6·0, at 600 W for 20 min. Endogenous peroxidase activity was blocked in 0·5% H_2O_2 for 30 min. Sections were incubated overnight with the primary antibody for inhibin α (MCA9515; Serotec Ltd, Oxford, UK) at 1:50 dilution. The detection was performed using a Vecastain ABC kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions. The sections were lightly counterstained with haematoxylin. To exclude the effect of possible endogenous biotin on immunohistochemical staining, biotin blocking (Avidin-Biotin Blocking Kit; Vector Laboratories) was performed in at least one sample of each diagnostic group prior to the addition of the primary antibody. Immunoreactivity of inhibin α was assessed separately by two trained pathologists (J A and K S). Twenty representative high-power fields were chosen from one slide per tumour, with a minimum of 1000 cells to be counted per tumour. The percentage of positively staining tumour cells was rounded up to the nearest 10%.

RNA analysis

Total RNA was isolated from the frozen tissues by ultracentrifugation through a caesium chloride cushion (Chirgwin *et al.* 1979). Northern blotting and hybridizations were performed as previously described (Liu *et al.* 1995). The relative intensities of autoradiographic signals were quantified by densitometric scanning. All data shown are normalized to the respective 28S RNA values.

Probes

The probes for human inhibin α subunit mRNA were two synthetic antisense 27-mer oligonucleotides. The sequence of the first oligonucleotide was 5′-CTC CGG AGG CCT CTG CAG CAG GCG CAG-3′ corresponding to the nucleotides 693–719, and that of the second was 5′-CCA GCC CAG CTC CTG GAA GGA GAT GTT-3′, corresponding to the nucleotides 759–785 of the human inhibin α mRNA (Mason *et al.* 1986). Two separate ^{32}P-labelled oligonucleotides were used simultaneously to increase the sensitivity in the detection of inhibin α mRNA expression. Mouse ribosomal 28S RNA cDNA probe (Arnheim 1979) was used as an RNA loading control.

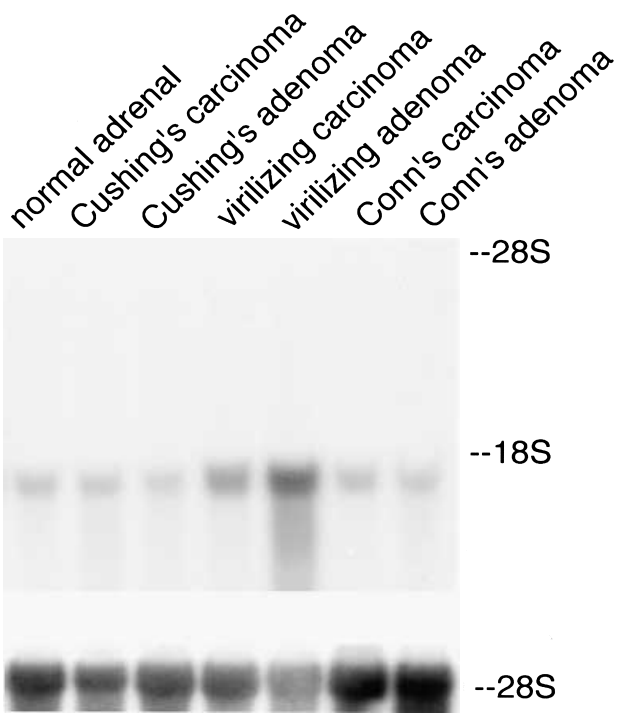

Figure 1 Expression of inhibin α subunit mRNA in normal adrenal gland and adrenal tumours *in vivo*. Total RNA was extracted from the frozen tissues indicated in the figure. The Northern blot was prepared with 20 μg RNA for each lane, and the RNA was transferred onto a nylon membrane. The filter was then sequentially hybridized with the inhibin α subunit (upper panel) and 28S ribosomal RNA (lower panel) probes. The migration of 28S and 18S ribosomal RNAs is indicated.

Statistics

Differences in the mRNA levels and in the numbers of the immunohistochemically positive cells were assessed by the Mann–Whitney test. The level of significance was chosen as $P<0{\cdot}05$.

Results

Normal and hyperplastic adrenal glands

Inhibin α subunit mRNA was detected as a 1·6 kb species in all normal adrenal samples (Fig. 1). In diffuse and nodular hyperplasias the median α subunit mRNA levels were about 2- to 3-fold as high as in normal adrenals (Table 1). Immunohistochemically, normal adrenal cortex possessed strong immunoreactivity for inhibin α subunit in the zona reticularis. Weaker reactivity was seen in the zona fasciculata, whereas the zona glomerulosa was negative. Adrenal medullar cells were also negative (Fig. 2). The staining pattern seen in diffusely hyperplastic adrenals was similar to that in normal adrenals, although the fasciculata cells were more intensely stained than in normal adrenals. Nodularly hyperplastic adrenals had a different staining pattern, as no zonal architecture is present; 10–30% of the adrenocortical cells were positive for inhibin α in these adrenals.

Adrenocortical adenomas

All adrenocortical adenomas expressed inhibin α mRNA and 71% of them were positive for inhibin α subunit immunohistochemically. In non-functional, Conn's or Cushing's adenomas, median inhibin α mRNA levels were close to those detected in normal adrenals, whereas virilizing adenomas possessed 10-fold as high median inhibin α mRNA levels as the normal adrenals (Table 1, Fig. 1). Immunohistochemically inhibin α subunit expression reflected the hormonal secretion of the tumour. Seven of 15 non-functional adenomas, 8 of 23 Conn's adenomas and 4 of 21 Cushing's adenomas were completely negative for inhibin α subunit, but all 6 virilizing adenomas were strongly positive. The median percentage of inhibin α-positive cells was 10 in non-functional and Conn's adenomas, 30 in Cushing's adenomas and 75 in virilizing adenomas (Table 1, Figs 2 and 3)

Adrenocortical carcinomas

Inhibin α mRNA was detected in all adrenocortical carcinomas regardless of the functional status of the tumour. Virilizing carcinomas ($n=2$) had about 2-fold inhibin α mRNA levels compared with the other carcinomas and normal adrenals (Table 1, Fig. 1). Immunohistochemically, 76% of the carcinomas stained positively for inhibin α. The median percentage of inhibin α-positive cells was 20 in non-functional carcinomas ($n=12$), 30 in Conn's carcinomas ($n=4$), 65 in Cushing's carcinomas ($n=10$) and 75 in virilizing carcinomas ($n=4$) (Table 1, Figs 2 and 3).

Phaeochromocytomas

All phaeochromocytomas ($n=20$) were negative for inhibin α immunohistochemically. Northern blot analysis of inhibin α subunit was negative in all phaeochromocytoma samples ($n=6$) as well (Table 1, Fig. 2).

Discussion

Both inhibin α and β subunit mRNA species and peptides can be detected in adrenal cortex, but not in the medulla (Meunier *et al.* 1988, Voutilainen *et al.* 1991, Spencer *et al.* 1992, McCluggage *et al.* 1998). The first reports suggested all adrenocortical tumours to be inhibin α positive (Chivite *et al.* 1998, McCluggage *et al.* 1998). Later reports, however, showed equal amounts of

Table 1 Expression of inhibin α subunit in adrenal tissues. Inhibin α mRNA – the mRNA values of inhibin α subunit were calculated from scanned autoradiographic signals of Northern blots, as described in Materials and Methods. The filters were sequentially hybridized with inhibin α and 28S ribosomal RNA probes. The values for the inhibin α mRNA represent the 1·6 kb transcript. All inhibin α signals were normalized with the respective 28S ribosomal RNA values. Medians and ranges are shown. The means of the RNA values from normal adrenals were adjusted to 100. Inhibin α peptide – the percentage of positively staining cells in immunohistochemistry performed with the inhibin α antibody was calculated from one slide per tumour, as described in Materials and Methods. Medians and ranges in each group are shown

	Inhibin α mRNA		Inhibin α peptide	
	n	Value	*n*	Per cent
Normal adrenal	5	100 (73–127)	10	See text
Hyperplasia				
Diffuse	8	200 (46–828)	10	See text
Nodular	6	328 (19–493)	15	See text
Adrenocortical adenoma				
Non-functional	5	52 (25–140)	15	10 (0–10)
Conn's	7	71 (6–164)	23	10 (0–40)
Cushing's	9	65 (15–315)	21	30 (0–80)
Virilizing	3	1040 (447–2804)*	6	75 (60–100)
Adrenocortical carcinoma				
Non-functional	2	81 (70–91)	12	20 (0–90)
Conn's	1	98	4	30 (10–40)
Cushing's	3	107 (15–212)	10	65 (0–90)
Virilizing	2	221 (198–244)	4	75 (50–80)
Phaeochromocytomas	6	0*	20	0

*$P<0.05$, compared with normal adrenals.

immunonegative and immunopositive adrenocortical tumours (Renshaw & Granter 1998). In our large series of tumours, 27% of the adrenocortical neoplasms were immunohistochemically negative for inhibin α, confirming that negative inhibin α immunohistochemistry does not exclude the possibility of an adrenocortical tumour origin.

Many questions need to be answered concerning the role of locally produced inhibins in the growth and steroidogenesis of the adrenal gland. Very recently, Munro *et al.* (1999) suggested that loss of inhibin α immunopositivity in some adrenocortical carcinomas is an indicator of tumour progression. We did not find that inhibin α expression reflects the malignant potential of the tumour, as 29% of the adenomas and 23% of the carcinomas were negative for inhibin α. Although studies with inhibin-deficient mice suggested a tumour suppressor role of inhibin α in the adrenal gland (Matzuk *et al.* 1992) a very recent report demonstrated that a loss of genetic material from 2p16 was strongly associated with a malignant phenotype of adrenocortical tumours (Kjellman *et al.* 1999). This locus is different from that of α inhibin (Barton *et al.* 1989).

What is the function of adrenal inhibins? Although in early studies zona glomerulosa was suggested to be immunohistochemically positive for inhibin α (Spencer *et al.* 1992), most recent studies have suggested an inner zone-specific staining pattern (McCluggage *et al.* 1998). We also showed negative inhibin α immunostaining in the zona glomerulosa, weak staining in the zona fasciculata, and very strong staining in the zona reticularis. The slightly higher (though not significantly) inhibin α subunit gene expression detected in hyperplastic compared with normal adrenals could be explained by increased ACTH action on inhibin α subunit gene expression (Voutilainen *et al.* 1991) in hyperplastic adrenals. Alternatively, but less likely, this difference could be caused by a higher cortical to medullary tissue ratio in hyperplastic than in normal adrenals. The first alternative is supported by the impression that the fasciculata cells were more intensely stained in the diffusely hyperplastic than in normal adrenals.

Kananen *et al.* (1996) suggested that adrenal inhibins have a functional role in the inner zones of the adrenal cortex. Histopathological analysis of adrenocortical tumours in mice transgenic for the mouse inhibin α subunit promoter/simian virus 40 T-antigen fusion gene indicated an inner layer origin of tumorigenesis. However, in our study it was not only the androgen-producing adrenocortical cells that were expressing inhibin α. Inhibin α expression in adrenocortical tumours appears to be associated with the hormonal activity of the tumours. All our virilizing tumours were strongly positive for inhibin α both

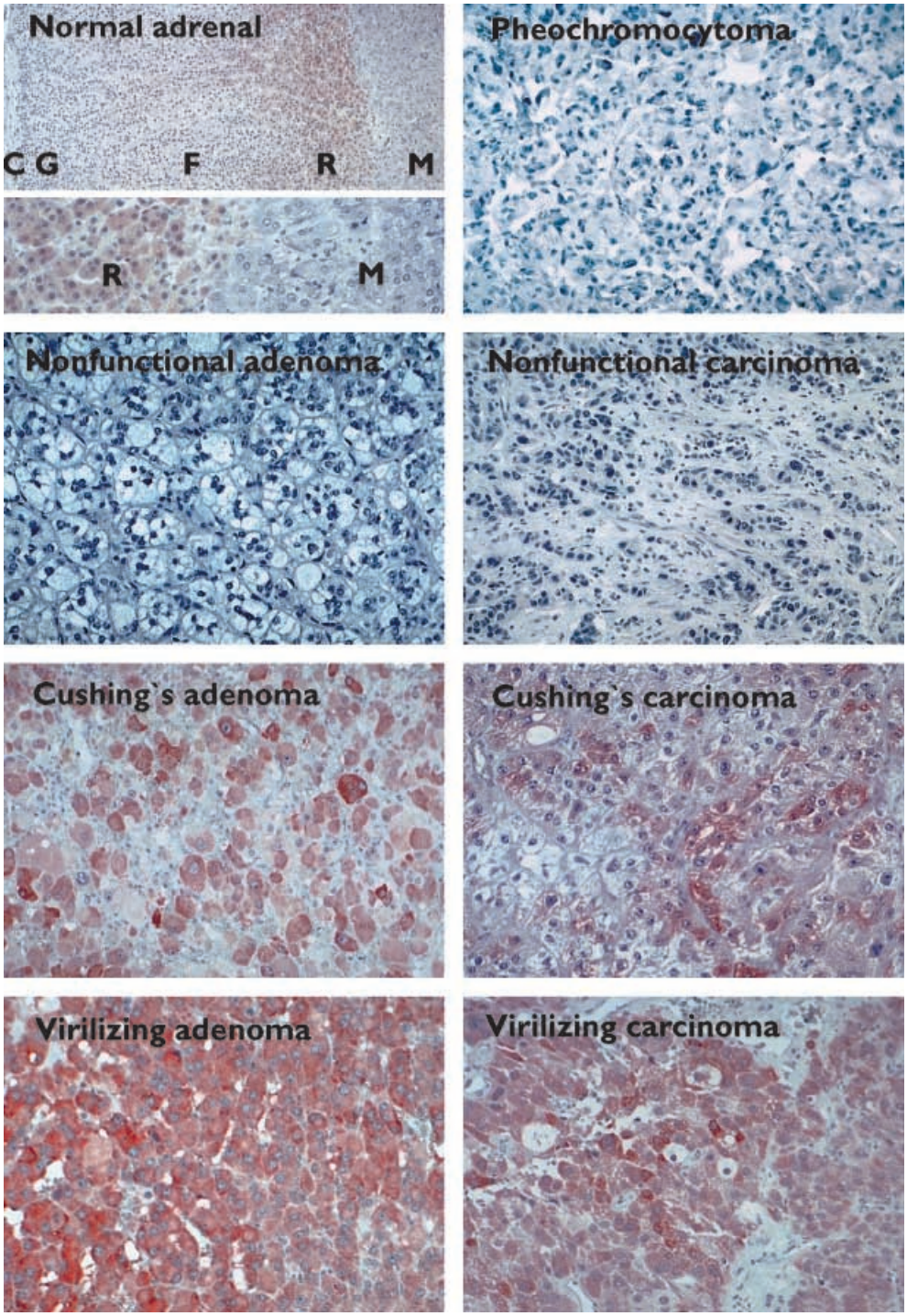

Figure 2 Expression of inhibin α subunit in normal adrenal gland and adrenal tumours. Immunohistochemical staining with inhibin α antibody was performed as described in Materials and Methods. C=capsule, G=zona glomerulosa, F=zona fasciculata, R=zona reticularis and M=medulla. Inset below normal adrenal illustrates higher magnification of zona reticularis and medulla.

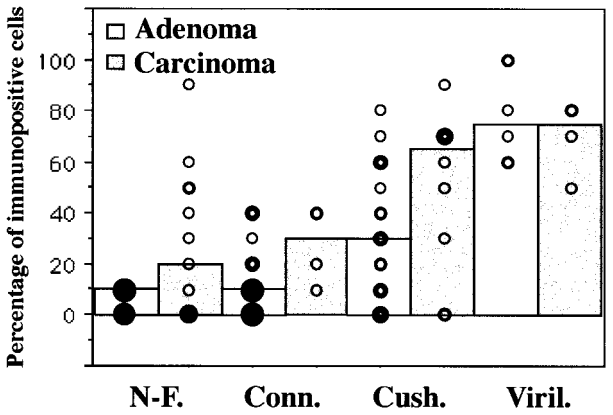

Figure 3 Expression of inhibin α subunit peptides in adrenocortical tumours. Immunohistochemical staining with inhibin α antibody was performed as described in Materials and Methods. The percentage of positively staining cells was counted from one slide per tumour. The scattergram demonstrates the percentage of positively staining tumour cells in each case. The thickness of the ring stands for the number of cases (n=1–8). The median bars are shown. N-F.=non-functional adenomas (n=15) and carcinoma (n=12), Conn.=Conn's adenomas (n=23) and carcinoma (n=4), Cush.=Cushing's adenomas (n=21) and carcinoma (n=10), and Viril.=virilizing adenomas (n=6) and carcinoma (n=4).

at the mRNA level and immunohistochemically. Most of the Cushing's tumours showed fairly strong immunostaining, although the mRNA expression was basically at the same level as in normal adrenals. Pelkey *et al.* (1998) also presented strong immunohistochemical staining of inhibin α in Cushing's and virilizing adrenocortical tumours. Very high expression of inhibin α in zona reticularis and in virilizing tumours supports a role of inhibin α in androgen production.

In summary, we observed a strong inhibin α expression in the zona reticularis, and a weak expression in the zona fasciculata, whereas the zona glomerulosa and adrenal medulla were negative. Inhibin α expression was about equal in adrenocortical adenomas and carcinomas. Virilizing tumours were strongly positive for inhibin α both at the mRNA level and immunohistochemically. Most glucocorticoid-producing tumours were moderately positive immunohistochemically, whereas non-functional and aldosterone-producing tumours were either negative or only weakly positive. No inhibin α expression was detected in any of the phaeochromocytomas. Our data suggest a steroidogenesis-related expression of inhibin α in normal adrenal gland and its tumours. Inhibin α expression does not differentiate malignant adrenocortical tumours from benign.

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