Expression of progesterone and estradiol receptors in normal adrenal cortex, adrenocortical tumors, and primary pigmented nodular adrenocortical disease

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Abstract

Adrenal tumors occur more frequently in women and are the leading cause of Cushing’s syndrome during pregnancy. We aimed to evaluate the potential role of sex steroids in the susceptibility of women to adrenocortical tumors. We evaluated the presence of the progesterone receptor (PR), estradiol receptors (ERs), and aromatase in 5 patients with primary pigmented nodular adrenal disease (PPNAD), 15 adrenocortical adenomas (ACAs) and adjacent normal tissues, 12 adrenocortical carcinomas (ACCs), and 3 normal adrenal glands (NA). The expression of PR and ERs was evaluated by enzyme immunoassays, real-time RT-PCR, immunohistochemistry, and cytosol-based ligand-binding assays. ERβ and aromatase levels were evaluated by real-time RT-PCR. ERα concentrations were low in NA, in adrenal tissues adjacent to ACA (51 ± 33), in ACC (53 ± 78), and lower in ACA (11 ± 11 fmol/mg DNA). Conversely, PR concentrations were high in NA and adrenal tissues adjacent to ACA, at 307 ± 216 fmol/mg DNA, and were even higher in tumors – 726 ± 706 fmol/mg DNA in ACA and 1154 ± 1586 fmol/mg DNA in ACC – and in isolated PPNAD nodules. Binding study results in four tumors were compatible with binding to a steroid receptor. In patients with PPNAD, a strong positive immunohistochemical signal was associated with the sole isolated nodular regions. ERβ transcript levels were very high in all samples except those for two ACCs, whereas aromatase levels were low. PR and ERβ are clearly present in normal adrenal glands and adrenal tumors. Further studies may shed light on the possible pathogenic role of these receptors in adrenal proliferation.

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Introduction

Unilateral tumors account for most cases of adrenocorticotrophin (ACTH)-independent hypercortisolism and may be classified into benign adrenal adenomas (ACAs) and malignant adrenocortical carcinomas (ACCs). ACCs have an extremely poor prognosis, with a survival rate of only about 20% at 5 years (Luton et al. 1990). Macronodular adrenal hyperplasia and primary pigmented nodular adrenocortical disease (PPNAD) result in tumoral proliferation affecting the entire gland. There is some clinical evidence to suggest that adrenocortical tumors are more prevalent in women than in men. For example, a sex ratio of 4.2 was reported for a series of 199 adrenal tumors (Luton et al. 1990). Moreover, ACC generally affects women at a younger age than men (Luton et al. 1990). The prevalence of adrenocortical tumors is particularly high during pregnancy, accounting for 70% of all cases of Cushing’s syndrome (CS) diagnosed in pregnant women (Guilhaume et al. 1992, Lindsay & Nieman 2006), versus only about 25% of cases of CS diagnosed in non-pregnant patients. A few cases of pregnancy-dependent CS have been reported elsewhere (Caticha et al. 1993, Close et al. 1993, Wallace et al. 1996, Kasperlik-Zaluska et al. 2000). We recently observed a worsening of hypercortisolism during pregnancy in a patient with PPNAD, followed by a clear improvement in this condition after spontaneous abortion.

PPNAD is characterized by adrenal glands of the normal size containing small cortical pigmented nodules. It may occur in isolation or be diagnosed as a component of Carney complex (CNC) – a multiple neoplasia syndrome with spotty skin pigmentation, myxomas, and endocrine overactivity. PPNAD, like CNC, is transmitted in an autosomal dominant manner. Germline inactivating mutations of the gene encoding the protein kinase A (PKA) regulatory subunit type I (PRKAR1A) are found in some patients (Kirschner et al. 2000, Groussin et al. 2002c, Libe & Bertherat 2005), whereas other kindreds bear mutations in the gene encoding the phosphodiesterase (PDE11A4; Horvath et al. 2006). Like adrenal tumors, PPNAD occurs more frequently in female gene carriers than in male gene carriers: of the 64 patients diagnosed with PPNAD at the Department of Endocrinology and/or the Oncogenetics Laboratory of Cochin Hospital, 50 were females and 14 were males (sex ratio > 3/1; unpublished results). The nature of the link between being female and adrenal proliferation and secretion is unknown. The ectopic expression of hormone receptors (including receptors for gastric inhibitory polypeptide, catecholamines, serotonin, and vasopressin type 1A) has already been implicated in cortisol production by adrenal tumors and the macronodular adrenal hyperplasia responsible for CS. Such expression is responsible for food-dependent cortisol secretion and unexpected responses to posture or pharmacological agents (Groussin et al. 2002b, Bertagna et al. 2003). Luteinizing hormone (LH) receptor is present in normal adrenal glands, but is found mostly in the zona reticularis (Pabon et al. 1996). Overexpression of the LH receptor has recently been reported in some Conn adenomas (Saner-Amigh et al. 2006). A few cases of gonadotropin-dependent hypercortisolism have been reported (Bertherat et al. 2005). The stimulation of cortisol production by estradiol in cultured adrenal cells from one patient with pregnancy-induced CS has also been reported (Caticha et al. 1993).

Estrogen and progesterone receptors (PRs) belong to the steroid nuclear receptor superfamily and commonly function as ligand-regulated transcription factors. They are involved in the normal developmental and reproductive functions and in disease processes, such as the development of cancers of the breast and other female reproductive organs (Graham & Clarke 1997, Rossouw et al. 2002). Estrogen signals through two subtypes of estradiol receptor (ER), ERα and ERβ encoded by two different genes. The expression of PR and ER has been demonstrated in tissues known to be responsive to gonadal steroids: the uterus and the ovary, normal and neoplastic breast tissues, the brain, the pituitary gland, and the hypothalamus. It has also been described in tissues, in which the action of these hormones is less well established, including the vascular endothelium and osteoblast-like cells. ER is even more widely expressed. ERβ and ERα seem to have different distributions in normal human tissues, but are frequently coexpressed in breast cancers, with ERβ mRNA frequently less abundant than that for ERα. However, the respective roles of these receptors in cellular responses to estrogen remain unclear. ER expression has been demonstrated in the adrenal glands of rodents (Kuiper et al. 1997) and monkeys (Hirst et al. 1992), whereas PR was not detected in these species. During fetal development, large amounts of ERβ mRNA are present in the definitive zone of the human adrenal cortex. In these tissues, ERβ mRNA levels have been shown to be higher than those of ERα mRNA levels (Brandenberger et al. 1997). However, the expression of steroid receptors in normal human adult or tumoral adrenocortical tissues has not been investigated.

We investigated the possible role of steroid sex hormones in favoring the development of adrenocortical neoplasms and/or controlling secretion from these tumors, by evaluating the presence of estradiol and PRs and of aromatase in benign and malignant
adrenocortical tumors and in PPNAD. We demonstrated the presence of high levels of PR in normal and tumoral adrenal tissues, and in isolated nodes from patients with PPNAD. ERβ was strongly expressed in all samples tested, whereas ERα and aromatase were detected only in small amounts.

Patients and methods

Patients and tissue collection

In this study, 5 patients with PPNAD and 27 patients with sporadic adrenocortical tumors were included. The hormonal investigations were performed as described elsewhere (Luton et al. 2000, Bertherat et al. 2005). For patient PPNAD 5, whose disease was discovered during pregnancy, paradoxical responses to LH and other hormones were sought 1 month after delivery. Adrenal tumors were classified by the same pathologist in all cases, according to the McFarlane classification, Weiss criteria, and molecular markers (Macfarlane 1958, Weiss et al. 1989, Gicquel et al. 2001). Three normal adrenal samples, consisting of tissue adjacent to an adrenal cyst or another non-secreting benign tumor, were also studied. Adrenal tissue was obtained during surgery and immediately frozen in liquid nitrogen. Informed consent was obtained for analysis of the PRKARIA gene and adrenal tissue collection, as part of a COMETE network protocol approved by the Institutional Review Board and the Ethics Committee of Cochin Hospital.

The NCIH295R cell line

The H295R human steroid-producing adrenocortical tumor cell line, which was derived from a malignant tumor, was cultured as described previously (Groussin et al. 2002a).

RNA extraction and cDNA synthesis

Adrenal tissue collection, DNA and total RNA extraction, and reverse transcription were performed as described previously (Gicquel et al. 2001, de Cremoux et al. 2002). The cDNA Cycle kit (Invitrogen, Groningen, The Netherlands) was used for reverse transcription (RT). For mutation analysis, the 12 exons and the flanking intronic sequences of the PRKARIA gene were amplified separately by PCR and directly sequenced on an automated sequencer, as described previously (Groussin et al. 2002c).

Real-time PCR

Real-time PCR amplification was carried out with the ABI Prism 7700 Sequence Detection System (PE Applied Biosystem, Foster City, CA, USA). PR, ERα, ERβ and aromatase primers, probe, and PCR conditions were as described previously (de Cremoux et al. 2002, 2004). RPLPO transcripts were also quantified as an endogenous reference RNA (Gicquel et al. 2001, de Cremoux et al. 2004). Accurate quantification was achieved by generating calibration curves, by serial dilutions of cDNA from the T47D and MDA-MB 231 human breast cancer cell lines, making it possible to quantify PCR efficiency for each run. Quantitative values were obtained from the cycle threshold (Ct) number, as described previously, using Applied Biosystems analysis software. The ΔCt values of the sample were determined by subtracting the average of duplicate Ct values for the target gene from the average of duplicate Ct values for the reference gene. The relative gene expression level was also normalized with respect to a positive calibrator, consisting of one of the samples used to generate the calibration curve (T47D dilutions) for the assay. Results are expressed as ‘N target’ values, determined as follows: Ntarget = 2ΔCt calibrator

Breast tumors were considered ‘positive’ for PR and ERα (i.e., high probability of response to hormonal treatment) when ‘N target’ values exceeded 50 arbitrary units (AU) and 250 AU respectively. They were considered positive for ERβ when ‘N target’ values exceeded 4.5 AU (corresponding to the ERβ content of normal breast tissue; de Cremoux et al. 2004).

ERα and PR protein assays

ER and PR protein assays were routinely performed on frozen samples, using an EIA (ER EIA, PR EIA; Abbott Laboratories), as described previously for breast tumor samples (Bernoux et al. 1998). The EIA kit for ER can detect Erα, but not the ERβ subtype. The EIA kit for PR can detect both the PRα and PRβ isoforms. ER and PR levels are expressed with respect to tissue DNA status (fmol/mg DNA). The presence of significant amounts of ERα or PR in adrenal tissue was arbitrarily defined using a cutoff value determined for breast samples, 250 fmol/mg DNA. If this threshold is exceeded, breast tumors are considered ‘positive’ for ER or PR. This cutoff point is higher than the detection limit of the assay and, for breast cancer, corresponds to a clinically relevant threshold below which responses to endocrine treatment are poor (Bernoux et al. 1998).

Immunohistochemistry for ER and PR

Immunostaining for ER and PR was performed on paraffin-embedded adrenal tissue sections. The techniques and antibodies used (Nichols Institute...
Diagnosis, San Juan Capistrano, CA, USA) are routinely used for breast tumor analysis (Balaton et al. 1996). Antibodies against ER recognized ERα whereas antibodies against PR reacted with both isoforms of PR.

**Cytosol-based ligand-binding assays for ER and PR**

Scatchard curves were generated with tritiated [(16alpha-ethyl-21-hydroxy-19-nor-preg-4-ene-3,20-dione)-6,7-3H] (ORG-2058) for PR and tritiated estradiol for ER, as described previously (Goussard et al. 1989).

**Statistical analysis**

Clinical and biological data were input into a Microsoft Access 98 computerized database.

Data are reported as means ± 1s.d. Non-parametric statistical methods were used (Wilcoxon and Mann–Whitney tests) due to the non-normal distribution of variables and the small number of subjects in certain groups of interest.

**Results**

**Clinical and biological features**

The clinical and pathological characteristics of the patients are shown in Table 1. All five patients with PPNAD were female and were referred for CS. Two had isolated adrenal disease and three were diagnosed with CNC on the basis of family history or typical clinical features (lentigine, heart myxoma, café-au-lait spots).

In one patient (PPNAD 5), CS was discovered during pregnancy, due to the onset of central obesity, edema, Melanosis, and acne.

**Table 1 Clinical and biological features at presentation in the patients with primary pigmented nodular adrenocortical disease (PPNAD) and sporadic adrenocortical tumors**

<table>
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<th></th>
<th>Sex</th>
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ACA, adrenocortical adenoma; ACC, adrenocortical carcinoma; Adj Adr, tissue adjacent to ACA; CNC, carney complex; and i t, isolated tumor. Genetic data (i.e., mutations found in the coding sequence or splice sites of the cAMP-dependent protein kinase A (PKA) regulatory subunit 1A (PRKAR1A) gene) are also given for PPNAD.
diabetes, and high blood pressure. Only plasma cortisol determinations were available and these confirmed the high levels of cortisol hypersecretion. At 19 weeks of pregnancy, plasma cortisol concentration at 0800 h was 42 μg/dl (normal values for this term: 25 ± 2.1 μg/dl), and plasma cortisol concentration at 2000 h was 44 μg/dl. At 23 weeks of pregnancy, plasma cortisol concentration at 0800 h was 55 μg/dl (normal values for this term: 35 ± 1.9 μg/dl). The patient suffered a spontaneous abortion at 24 weeks of pregnancy. After 6 weeks, morning plasma cortisol concentration was within the normal range, whereas urinary cortisol excretion (UC) demonstrated the persistence of mild ACTH-independent hypercortisolism: UC = 109 μg/24 h (normal range: 25–90 μg/24 h), ACTH concentration at 0800 h < 5 pg/ml (normal range: 20–60 pg/ml). No abnormal response of cortisol secretion to food intake, human chorionic gonadotrophin (hCG), gonadotrophin-releasing hormone, or thyrotrophin-releasing hormone administration was observed. Abdominal CT scan showed adrenal glands of normal size. Bilateral adrenalectomy was performed a few weeks later, and histological features confirmed the diagnosis of PPNAD.

The sporadic adrenocortical tumors studied consisted of 15 ACAs (10 in women, 5 in men, with a mean age of 51.9 ± 13.9 years) and 12 ACCs (7 in women and 5 in men, with a mean age of 52.7 ± 18.8 years). The hypersecretion of cortisol, cortisol plus androgens, or mineralocorticoid was observed for all but three ACAs and three ACCs. In one patient (ACA5), ACA was diagnosed during pregnancy and the tumor was removed by surgical resection of the adrenal tumor at the time of Cesarean section.

Detection of ERα and PR protein

We used EIA to assess levels of the ERα subtype and PR in 5 cases of PPNAD, 11 ACAs and adjacent normal tissues, 8 ACCs, and 3 normal adrenal gland samples (NA). ERα was detectable, but present at only low concentrations in NA and adrenal tissue adjacent to ACA (51 ± 33). The concentration of this receptor was also low in ACC (53 ± 78) and was significantly lower in the ACA itself (11 ± 11 fmol/mg DNA (mean ± s.d.; P = 0.012; Fig. 1). Conversely, PR concentrations were high in NA and adrenal tissue adjacent to ACA: 307 ± 216 fmol/mg DNA, and were even higher in benign tumors, 726 ± 706 fmol/mg DNA (P = 0.036). PR expression was also very strong in malignant ACC, at 1154 ± 1586 fmol/mg DNA, but this level of expression was not significantly different from that in the normal tissues (P = 0.12; Fig. 2). We investigated PR expression in tumor cells further by determining PR protein levels in the H295R cell line: 2713 and 1455 fmol/mg DNA in two samples from two different batches.

We investigated the site of PR expression in the adrenal glands of PPNAD patients, by comparing PR expression in the total adrenal gland with samples isolated from the pigmented nodules of two patients (PPNAD 4 and PPNAD 5), in which the size of some
nodules allowed separate sampling. The PR concentration in the isolated nodules was high, at 1583 and 564 fmol/mg DNA, whereas much lower levels were found in adjacent micronodular/atrophic adrenal tissues, 112 and 140 fmol/mg DNA. The PR expression was also assessed by immunohistochemistry in the same tissues: immunostaining for PR was strong in the nodular regions with a nuclear and cytoplasmic localization, whereas no signal was detected in the adjacent atrophic tissue (Fig. 3A).

Immunohistochemistry was performed in adrenal tissues from patients with high PR levels. Cytoplasmic and nuclear immunostaining of PR was observed in adrenal cells, but not in vessels (Fig. 3B).

**ERα, ERβ and PR transcript levels**

Consistent with the data obtained for the corresponding protein, ERα mRNA was detectable, but present at only low levels in NA (84, 111) and adrenal tissue adjacent to ACA (262 ± 108; n = 5). The concentration of this mRNA was also low in ACC (149 ± 244; n = 14) and was significantly lower in the ACA samples (60 ± 37 AU; n = 11; P = 0.043; Fig. 4).

ERβ mRNA levels were very high in all samples, with the exception of three ACC samples. Mean ERβ mRNA levels seemed to be higher in ACA (31.4 ± 11.8 AU; n = 14) than in adjacent tissues (19.6 ± 4.5; n = 5), but this difference was not statistically significant (P = 0.07). ERβ mRNA levels were also high in ACC (35.43 ± 44.4 AU; n = 11) and were not significantly different from those in the two other groups (Fig. 5). For comparison, ERβ mRNA levels assayed with the same technique were much lower in normal breast tissue (4.91 ± 4.77; n = 22) and even lower in breast tumors (0.89 ± 0.70; n = 52; personal data).

An RT-PCR quantification of PR mRNA confirmed PR expression to be strong in most tissues. The Ntarget was 18 and 19 AU in two normal adrenal glands, 76.7 ± 60.1 and 45.5 ± 17.5 AU in ACA (n = 14) and adjacent tissue (n = 5) respectively, 117.7 ± 90.9 AU in 11 ACCs. By comparison, breast tumors are considered ‘positive’ for PR when Ntarget exceeds 50 AU. The difference between tumoral and non-tumoral tissues was not statistically significant (Fig. 6).

The concentrations of mRNA for these receptors did not differ in men and women. The very high levels of ER-β and PR in some tumors from male patients were striking. However, it is difficult to draw a conclusion because of the large dispersion of values.

Aromatase levels were low in ACA (1.8 ± 1.1 AU; n = 10) and adjacent normal tissues 2.7 ± 2.6 AU (n = 5), and in ACC (2.9 ± 3.1AU (n = 8)). One normal
adrenal sample had a high aromatase content (17.4 AU), whereas the other did not (2.85 AU).

Cytosol-based ligand-binding assays for ER and PR

Scatchard plots, used to assess PR binding in four tumors and ER binding in one tumor, yielded $k_d$ values compatible with a steroid receptor (0.35–1.99 nM) for all but one tumor (6.66 nM). The human malignant adrenocortical cell line H295R, which strongly expresses PR, was also studied for progesterone binding. The calculated $k_d$ for this line was 0.35 nM.

Discussion

The particular susceptibility of women to adrenocortical tumors is striking and remains unexplained. The high frequency of these tumors among hypercortisolic patients during pregnancy (Guilhaume et al. 1992) and the description of pregnancy-dependent ACTH-independent CS (Caticha et al. 1993, Close et al. 1993, Wallace et al. 1996, Kasperlik-Zaluska et al. 2000) suggest that adrenocortical proliferation and/or secretion may be sensitive to female hormones.

We demonstrate strong PR expression in normal adrenal tissues, adrenal tumors, and pigmented nodules from PPNAD. The ER$\alpha$ was weakly detected in normal adrenal tissues and its expression was downregulated in benign tumors. In contrast, ER$\beta$ transcript levels were higher than those in normal breast tissues and breast carcinoma (de Cremoux et al. 2002, 2004), in both normal adrenal tissues and adrenal tumors. This is the first demonstration of ER$\beta$ expression in human adult adrenal tissues. The ER$\beta$ does not seem to be downregulated in ACC, in contrast to what has been reported in the breast, in which ER$\beta$ levels are consistently higher in the normal tissue than in tumoral tissue. Few studies have investigated the expression of ER and PR in the adrenal gland. A previous study based on immunohistochemistry and gradient shift assays assessed the levels of steroid receptors in adult rhesus monkey adrenal gland and concluded that ER was present in adult adrenal gland whereas PR was absent (Hirst et al. 1992). A recent RT-PCR-based study showed that ER$\alpha$ was expressed in the rat adrenal gland, whereas ER$\beta$ was not detected and PR was not studied (Kuiper et al. 1997). The difference between the strong expression of PR in normal and tumoral adrenal tissues observed here and the results obtained by Hirst et al. (1992) in normal monkey adrenal glands may be due to differences in the species studied or in the techniques used.

Immunohistochemistry and studies of the H295R adrenocortical cell line showed that, in tumors, PR is expressed in the tumoral cells themselves, rather than in the stroma or the vessels. Cytoplasmic and nuclear localization observed here may suggest the presence of the isoform B of PR, although the quantitative techniques used here do not allow to distinguish between the two isoforms. Antibody against PR also specifically stained cortical cells in PPNAD nodules – the site of adrenal proliferation in this condition. In the surrounding cortex,
PR staining was weak in normal adrenal tissue. These findings were confirmed by EIA on separate samples from isolated PPNAD nodules and adjacent atrophic/micronodular zones.

Various factors may influence the regulation of PR expression in adrenal tissues. In reproductive organs, the induction of PR gene expression by estradiol involves mostly the ERβ subtype. However, in some regions of the central nervous system in male rodents, the induction of PR by estradiol depends on ERα (Kudwa et al. 2004). This may be the case in adrenal cells. PR may also be upregulated through activation of the cAMP/PKA pathway, as observed in preovulatory ovarian granulosa cells. In these cells, LH via the production of cAMP, but not estradiol, induces PR mRNA expression (Clemens et al. 1998). In patients with PPNAD, inactivating mutations of PDE11A4, which encodes a phosphodiesterase, and of PRKARIA, which encodes the RI subunit of the cAMP-dependent PKA, are frequently identified (Cazabat et al. 2006). Somatic mutations of this gene are also observed in a small number of cortisol-secreting ACA, unrelated to CNC. In those tissues, cAMP-stimulated kinase activity is increased (Bertherat et al. 2003). Given the strong and specific expression of PR observed in nodules from PPNAD, in which the PKA pathway is activated, the PR expression may be induced by cAMP stimulation in tumoral tissue.

The physiopathological relevance of steroid receptors in adrenal cell proliferation is unknown. By comparison with the ERα protein levels determined with the same tools in normal and tumoral mammary glands, we considered the level of expression of this receptor in normal adrenal tissues to be low. The even lower level of expression observed in adrenal tumors suggests that this receptor plays a more minor role in adrenal tumorigenesis. In contrast, ERβ levels were high, suggesting a more probable role. Montanaro et al. (2005) demonstrated that the H295R adrenal carcinoma cell line produces more mRNA for ERβ than for ERα. They also showed that 4-OH-tamoxifen (a selective estrogen receptor modulation (SERM)) and ICI 182 780 (a pure anti-estrogen) upregulate ERβ and inhibit cell proliferation.

The physiopathological relevance of the high levels of PR expression in adrenal cells remains unclear. However, as progesterone is a precursor of most of the steroids synthesized in adrenal glands, local production may activate PR in an autocrine/paracrine manner. The PR may also be activated by intracellular signaling pathways: the production of cAMP following PKA activation can induce the ligand-independent activation of chicken PR, enhancing its steroid-dependent activation (Beck et al. 1992). Could PR activation favor adrenal tumorigenesis. Transgenic mice expressing the simian virus 40 T antigen under control of the murine inhibin-α subunit promoter develop gonadal tumors. If early gonadectomy is performed, adrenal tumors appear. In these mice, adrenal tumorigenesis is dependent on steroidogenesis: the proliferation of C α1 cells (derived from these murine adrenal tumors) is stimulated by progesterone at physiological concentrations (Rilianawati et al. 1998). Progestin activation of Src/MAPK may occur outside the nucleus with the B isoform of PR, which is distributed between the cytoplasm and nucleus, and induce the transcription of target genes involved in cell cycle progression (Boonyaratanakornkit et al. 2007). Functional studies are required to evaluate the role of PR in human adrenal gland. Although the most convincing evidence would come from neoplasms isolated from human patients, the H295 cell line could be a useful tool to address the biological effect of progesterone on adrenal tumors. However, available data on animal models together with the presence of the high levels of ERβ and PR in human adrenal tumors reported here provide new insight into the potential therapeutic benefit of anti-estrogens and/or anti-progesterone for adrenal neoplasms.

We conclude that PR is present in human adrenal gland and specifically overexpressed in some adrenocortical tumors and in the nodules of PPNAD patients. This overexpression occurs in a context of very low levels of ERα expression and high levels of ERβ expression. These findings raise questions about the molecular mechanisms underlying steroid receptor regulation in adrenal tumors and their potential role in adrenal tumorigenesis.

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