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Phosphodiesterase 11A Expression in the Adrenal Cortex, Primary Pigmented Nodular Adrenocortical Disease, and other Corticotropin-independent Lesions

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Abstract

A variety of adrenal tumors and bilateral adrenocortical hyperplasias (BAH) leading to Cushing syndrome (CS) may be caused by aberrant cAMP signaling. We recently identified patients with a micronodular form of BAH that we have called “isolated micronodular adrenocortical disease” (iMAD) in whom CS was associated with inactivating mutations in phosphodiesterase (PDE) 11A (*PDE11A*). In the present study, we examined PDE11A expression in normal adrenocortical tissue, sporadic tumors, and hyperplasias without *PDE11A* mutations, and primary pigmented nodular adrenocortical disease (PPNAD) and adenomas from patients with *PRKAR1A* and a single tumor with a *GNAS* mutation. The total number of the tumor samples that we studied was 22. Normal human tissues showed consistent PDE11A expression. There was variable expression of PDE11A in sporadic adrenocortical hyperplasia or adenomas; PPNAD tissues from patients with *PRKAR1A* mutations expressed consistently high levels of PDE11A in contrast to adenomas caused by *GNAS* mutations. Phosphorylated CREB was the highest in tissues from patients with iMAD compared to all other forms of BAH and normal adrenal tissue. We conclude that PDE11A is expressed widely in adrenal cortex. Its expression appears to be increased in PPNAD but varies widely among other adrenocortical tumors. PRKAR1A expression appears to be higher in tissues with *PDE11A* defects. Finally, sequencing defects in *PDE11A* are associated with a high state of CREB phosphorylation, just like *PRKAR1A* mutations. These preliminary data suggest that these two molecules are perhaps regulated in a reverse manner in their control of cAMP signaling in adrenocortical tissues.

Keywords

adrenal tumors; Cushing syndrome; protein kinase A; cyclic AMP; phosphodiesterases

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Introduction

Aberrant cyclic adenosine monophosphate (AMP) (or cAMP) signaling has been linked to genetic forms of cortisol excess that lead to Cushing syndrome (CS) [1,2]. Macronodular bilateral adrenocortical hyperplasia (BAH) may be due to *GNAS* mutations associated with, either McCune-Albright syndrome (MAS) or sporadic adrenal tumors [2]; massive macronodular adrenocortical disease or MMAD (also known as ACTH-independent macronodular adrenocortical hyperplasia or AIMAH) may also be caused by *GNAS* mutations or aberrant expression of cAMP-generating, G-protein coupled receptors [1,2]. Micronodular BAH and its better-known variant primary pigmented nodular adrenocortical disease (PPNAD) may be caused by germline inactivating mutations of the *PRKAR1A* gene [3–6]. Most patients with PPNAD also have Carney Complex (CNC), an autosomal dominant multiple neoplasia syndrome associated with skin lesions, cardiac myxomas, and other nonendocrine and endocrine tumors; in most patients with CNC, the disease is caused by *PRKAR1A* mutations [5]. Over the last several years, it has become apparent that there are several forms of micronodular BAH that are not caused by germline inactivating mutations of the *PRKAR1A* gene. We described one such case associated with an atypical episodic form of CS in a young child [7]. Her adrenal histology showed moderate diffuse cortical hyperplasia, multiple capsular deficits, and massive circumscribed and infiltrating extra-adrenal cortical excrescences that in many cases formed micronodules that were largely nonpigmented [7]. We called this disease isolated micronodular adrenocortical disease or iMAD [1,7,8] (Table 1), pending a more definite terminology.

Recently, we reported that inactivating mutations of the *PDE11A* gene may be found in increased frequency in a subgroup of patients with PPNAD, iMAD, and other forms of BAH [8,9]. Two frameshift mutations disrupting the PDE11A4 adrenal-specific isoform protein (c. 171Tdel/fs41X and c.1655_1657delTCTinsCC/fs15X) and a single base pair substitution (c. 919C>T p.R307X) were the first such *PDE11A* gene variants that were reported (Fig. 1A). In addition, two missense substitutions that are relatively frequent polymorphisms of the *PDE11A* gene (c.2411G>A, p.R804H and c.2599C>G, p.R867G) were found in increased frequency among patients with adrenal lesions, including one patient with MMAD [9] and the patient with iMAD reported by Gunther et al. [7]. Tumors bearing *PDE11A*-sequencing defects have an increased frequency of allelic losses of the 2q locus of this gene (Fig. 1B) [8,9].

PDE11A is a dual-specificity phosphodiesterase catalyzing the hydrolysis of both cAMP and cGMP; it is expressed in several endocrine tissues, including the adrenal cortex [9–13]. The *PDE11A* gene was mapped to the 2q31–35 chromosomal region and tumors from patients with PDE11A-inactivating mutations demonstrated 2q allelic losses [8]. The *PDE11A* locus, like that of other PDEs, has a complex genomic organization [13–18]. Of the four possible splice variants, only A4 appears to be expressed in the adrenal cortex, whereas A1 is ubiquitous, and A2 and A3 have a more limited expression pattern.

In the present study, we used a PDE11A-specific antibody to examine the expression of PDE11A in a variety of human adrenocortical adenomas and hyperplasias that did not carry *PDE11A*-inactivating mutations. We also looked at PDE11A expression in PPNAD with *PRKAR1A* mutations and at the expression and phosphorylation levels of cAMP-regulatory element binding protein (CREB). These data confirm PDE11A's wide expression in normal adrenocortical tissue; its expression is modified in a variety of tumors and appears to be increased in PPNAD with *PRKAR1A* mutations and its defects are associated with increased phosphorylation of CREB.

Methods

Clinical studies, tissue samples, and DNA sequencing

The institutional review boards of NICHD, NIH, the Mayo Clinic, and Hospital Cochin, Paris, France, approved the genetic investigation of patients with adrenocortical tumors, including BAH, under NICHD protocols 95-CH-0059 and 00-CH-0160 after giving informed consent. Patients with CNC or BAH were classified as "affected", as described previously [1,2,5,7]. Patients who were diagnosed with CS by standard clinical testing and criteria underwent adrenalectomy. Blood and tissue samples were collected from patients as previously described [8]. When possible, tissue was collected at surgery and processed for routine histopathology and immunohistochemistry (IHC) after being formalin-fixed and paraffin-embedded; additional fragments were frozen immediately at − 70 ° C for later use. DNA was extracted from patient and tissue samples and/or cell lines using standard methods (Qiagen). All adrenal samples were dissected, from their surrounding normal tissues and from parts of the adrenal that would not normally include medullary or other tissues; thus, mostly abnormal tissue was used for DNA, mRNA, and protein studies. All our patients have previously been screened for *PRKAR1A* and *MYH8* [19] mutations and tumor samples had been screened for *GNAS* mutations; one patient with BAH had a classic *GNAS* mutation (R204H); the most frequent *PRKAR1A* mutation was del478TG. For light microscopy and immunocytochemistry, tissue was paraffin-embedded; sections were then stained with hematoxylin and eosin (H & E) and synaptophysin, a marker for PPNAD and related adrenocortical tumors, as previously described [7]. All samples were stained for PDE11A, CREB, and phosphorylated CREB (P-CREB) as we have published elsewhere [8,9].

The samples that had *PDE11A* mutations were those that we have reported elsewhere [7,8] and were used here as controls and for examining *PRKAR1A* expression; they have been sequenced for *PRKAR1A* and no mutations were found [7,8]. The genomic sequence of human *PDE11A* was used to design intronic primers to amplify all exons and exon/intron junctions, as provided by Yuasa et al. [17], and published by Horvath et al. [8,9].

RNA studies

Total RNA was isolated from 30 to 50 mg of previously frozen human adrenocortical tumor (ACT) using the RNeasy RNA Midi-Prep kit (Qiagen). Tumors were prepared in a 4 ° C cold room, sliced into fine pieces using a sterile scalpel, and homogenized with 18- and 19-gauge needles in lysis buffer RLT (Qiagen) containing β-mercaptoethanol. The RNA was resuspended in diethyl pyrocarbonate-treated water, quantified by UV absorbance at 260/280 nm, and stored at − 80 ° C. RNA was reverse transcribed into cDNA using oligo (dT)s and the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen Catalog No. 11904–018). PCR primer sequences were designed using VectorNTI software (Informax) to amplify the 5′-regions of *PDE11A1*, *PDE11A2*, *PDE11A3*, and *PDE11A4* isoform-specific full-length cDNA using Accuprime Taq High-Fidelity Polymerase (Invitrogen). *PRKAR1A* and *PDE11A* genes were amplified by real-time PCR using the iQSybrGreen PCR amplification mix (Bio-Rad Laboratories; according to the manufacturer's instructions). *GAPDH* was also amplified as a loading control. Each normal adrenal and tumor sample was amplified in triplicate via separate PCR conditions and compared with *GAPDH* expression levels using the $\Delta\Delta C_t$ method.

Preparation of proteins, Western blotting and immunohistochemistry

Tissues and cell lines from patients and their tumors were maintained in RPMI-1640 or DMEM supplemented with 10–15 % fetal bovine serum. Total cellular protein extracts from frozen tissues or cultured cells were prepared using RIPA buffer (20 mM Hepes, 250 mM NaCl, 10 % glycerol, 1 % NP-40, 0.5 % deoxycholate, 2 mM DTT, and protease inhibitor). Twenty μg from cell lysates (and 50 μg from tissue lysates) of total protein was subjected to SDS/PAGE

using a 4–20 % gradient gel. The proteins were transferred to nitrocellulose membranes and PDE11A4 was detected using a rabbit polyclonal antibody specific for PDE11A as directed by the manufacturer (Abcam Catalog No. ab14624) at 1:500, and 1:100 dilutions, as described previously [11]. The same antibody was used for immunohistochemistry (IHC) of paraffin-embedded tissue slides. Similar methods were used for the CREB and p-CREB immunostaining (Western blot and IHC) [8,9]; the antibodies are commercially available (Upstate).

Statistical analysis

All data are expressed as the mean ± SEM. Statistical analysis consisted of χ^2, Fisher's exact test, and *t*-test when appropriate. For all statistical comparisons, $p < 0.05$ was considered significant. Data were analyzed using the STATA 8.0 statistical software (STATA corp., College Station, TX).

Results

PDE11A and *PRKAR1A* mRNA expression

qRT-PCR demonstrated variable levels of *PRKAR1A* levels among patients with *PDE11A* mutations (Fig. 2a, b) and consistent with the genetic defect levels of PDE11A expression (Fig. 2c). When we combined the expression levels for specimens from all different patients with adrenal hyperplasias, qRT-PCR demonstrated elevated levels of *PRKAR1A* in patients with *PDE11A* mutations in comparison with those without *PDE11A* mutation ($p < 0.05$) (Fig. 2b). PDE11A expression was decreased in adrenal tissue of a patient with a PDE11A protein truncating defect, as expected (Fig. 2c). *PDE11A* expression showed consistently higher levels in tissues with *PRKAR1A* mutations, but the difference was not statistically significant ($p = 0.1$) (Fig. 2d).

Immunostaining of iMAD and other adrenocortical lesions for PDE11A

Immunohistochemistry studies with a PDE11A-specific antibody showed that in normal adrenal and corticotropin-driven benign adrenocortical hyperplasia the PDE11A antibody stains the cortex homogenously but not the medulla (Fig. 3a, c). There was decreased PDE11A staining from a patient with iMAD who did not have a *PDE11A*-sequencing defect (Fig. 3d). The nodules [Nod] have lost staining completely, whereas the cortex [C] retains staining; (Fig. 3e). Tumor tissue from a patient with BAH due to the classic R204H *GNAS* mutation showed loss of PDE11A staining within the tumor (Fig. 3f). Low and high resolution images from a patient with the del478TG *PRKAR1A* mutation showed strong PDE11A staining within the hyperplastic cortex [C], and even stronger within the nodules [Nod]. Microadenomatous lesions from a patient with mainly androgen, but also glucocorticoid-producing BAH and no known genetic defects showed that the PDE11A protein was completely absent from within the tumors (Fig. 3i). In a common cortisol-producing adenoma from a patient with CS and no known genetic defects, the PDE11A protein was slightly decreased in tumor cells (Fig. 3o). Finally, an aldosterone-producing tumor, from a patient with no known genetic defects showed significantly decreased expression of PDE11A protein in the tumor [Fig. 3r].

CREB and p-CREB in adrenocortical lesions with and without PDE11A defects

We also examined the effect of *PDE11A* or *PRKAR1A* defects on CREB and its phosphorylation status (p-CREB) in tumors from the individuals with sequence abnormalities and compared it with normal adrenal and other types of adrenal hyperplasia (by immunoblotting) (Fig. 4). Protein blots of tissue lysates from normal adrenal cortex and from various affected individuals with iMAD, PPNAD, or MMAD showed different ratios of pCREB to CREB: Patients with iMAD carrying *PDE11A* defects (n = 4) had the highest

pCREB-to-CREB ratio from all other samples (6 normal adrenal samples, 4 PPNAD with *PRKAR1A*-inactivating mutations, and 2 from patients with MMAD) ($p < 0.001$); there were no statistically significant differences between the other diagnostic groups although the number of tissues used per group was small (for example, only 2 samples from patients with MMAD were studied).

Discussion

The data presented here suggest the possibility that *PDE11A* and *PRKAR1A* are coordinated in opposite directions in their regulation of cAMP signaling in adrenocortical tumor development (adrenal hyperplasias and/or adenomas). A mutant *PRKAR1A* that leads to excess cAMP-dependent protein kinase or protein kinase A (PKA) signaling and excess CREB phosphorylation [20] would need to lower cAMP levels in the tissue that bears this defect. One mechanism to decrease cAMP signaling in that tissue could be degradation of cAMP levels by increasing the expression of a PDE such as PDE11A. And vice versa: in a tissue with a PDE11A-inactivating defect, increased cAMP levels would lead not only to increased PKA signaling and increased p-CREB (as we show here), but possibly also to increased *PRKAR1A* expression: the *PRKAR1A* promoter harbors cAMP regulatory element (CRE) sequences and increased R1 α protein would ultimately bind more PKA catalytic subunit in the inactive PKA tetramer terminating responses to cAMP [21]. Of course the data presented here are only from a small number of tumors from these rare conditions, and further experiments need to confirm the above *in vitro* results. In addition, at least one of our presented experiments do not support this counter-regulation: tumor tissue from a patient with BAH due to the classic R204H *GNAS* mutation showed loss of PDE11A staining within the tumor (Fig. 3f). If our hypothesis above was correct, increased cAMP levels in this tumor should have led to the opposite, that is increased PDE11A expression. Nevertheless, it is possible that the regulation of PDE11A expression by cAMP is not direct (as that of PRKAR1A).

Although the cause of all forms of BAH studied to date [22] seems to be linked to increased cAMP signaling [23], the histopathological changes in the adrenal glands of patients with the various mutations or functional abnormalities of this pathway differ significantly and overlap only partially [1]. *PRKAR1A* mutations are associated with PPNAD, whereas *PDE11A* mutations seem to predispose to a variety of lesions (Fig. 5) from isolated (without any other associated tumors) PPNAD to nonpigmented micronodular hyperplasia [8,9]; *GNAS* mutations are associated with the mostly macronodular and clearly nonpigmented form of BAH [22, 23]. The identification of more mutations in *PDE11A* and possibly other PDEs, such as *PDE8B* [24], and other genes [25] will shed more light to this apparent lack of tight genotype-phenotype correlation which is somewhat unexpected since tissues affected by PPNAD are almost identical histologically.

We conclude that PDE11A's expression appears to be increased in PPNAD, but varies widely among other adrenocortical tumors, suggesting a possible counter-regulation with PRKAR1A. *In vitro* molecular studies need to address the relationship between cAMP levels, PDE11A and (other PDEs) and *PRKAR1A* expression, which will shed further light into the involvement of cAMP signaling in adrenocortical tumorigenesis.

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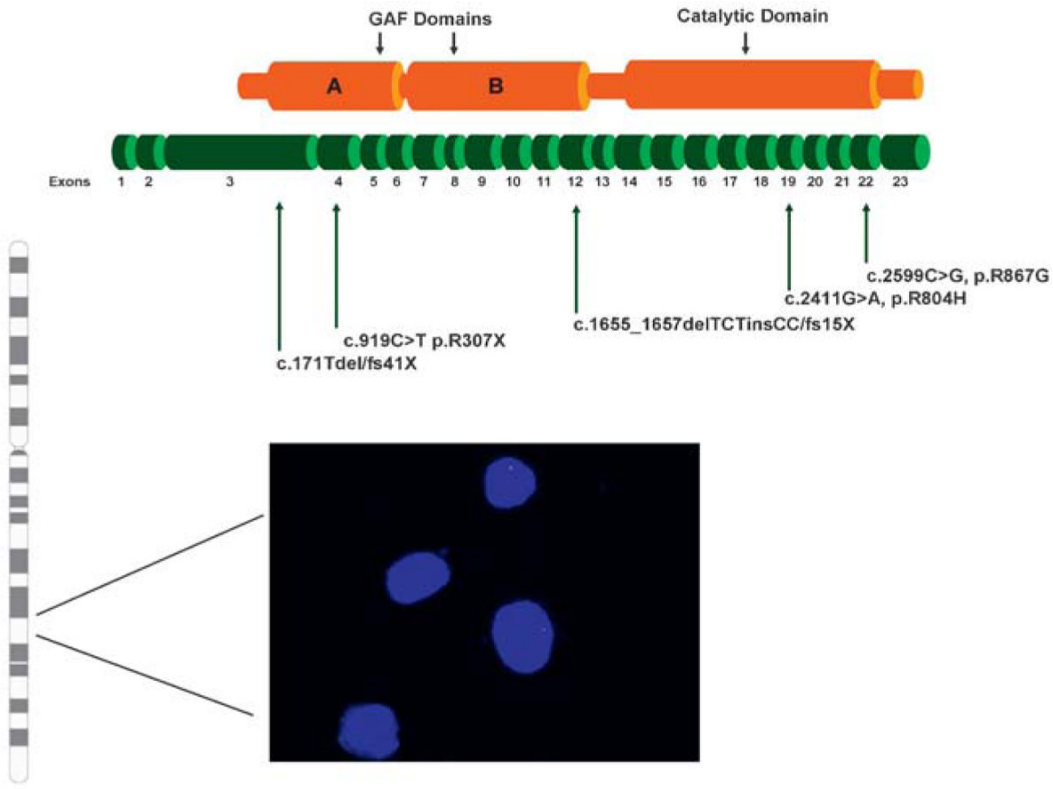

Fig. 1.

(**a**) *PDE11A* locus alterations in patients with adrenal disease (see also ref [8,9].); (**b**) FISH on tumor cells from individuals with adrenocortical hyperplasia and *PDE11A* mutation showed allelic loss (one signal) of the RP11–428I14 BAC probe containing the *PDE11A* gene.

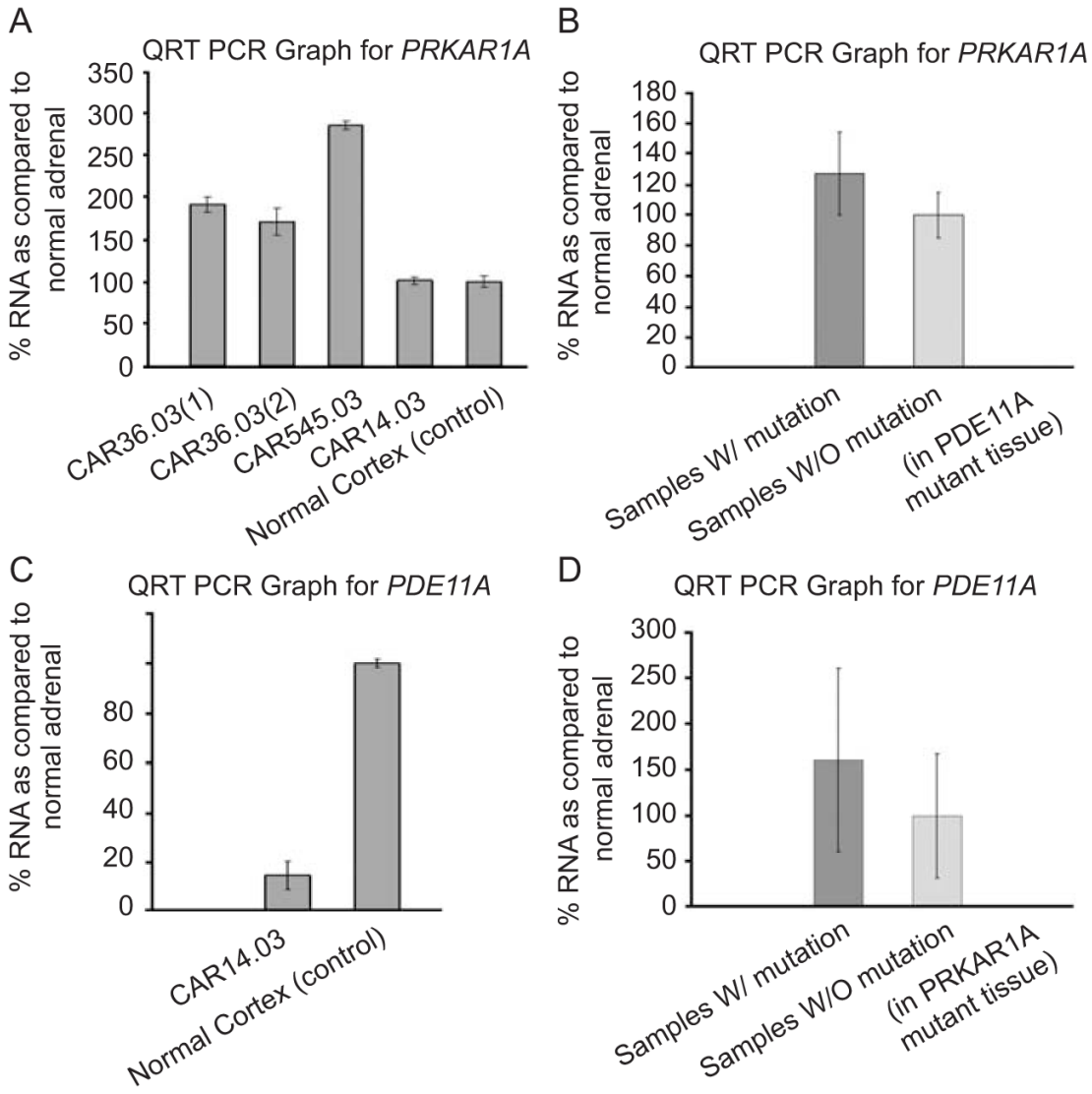

Fig. 2.

(**a**) *PRKAR1A* levels in patients with PDE11A sequencing defects and normal adrenal tissue from 6 glands; each sample has been measured in duplicate in at least two different experiments; (**b**) collectively, tissues with PDE11A sequencing defects (n = 4) have statistically higher *PRKAR1A* expression that those without (n = 8); (**c**) a tissue with a PDE11A-protein truncating defect has lower PDE11A expression than normal adrenal (n = 6), as expected; (**d**) PDE11A expression was not statistically higher in samples with PRKAR1A defects but there were wide expression differences and only 4 tissues available (p = 0.1).

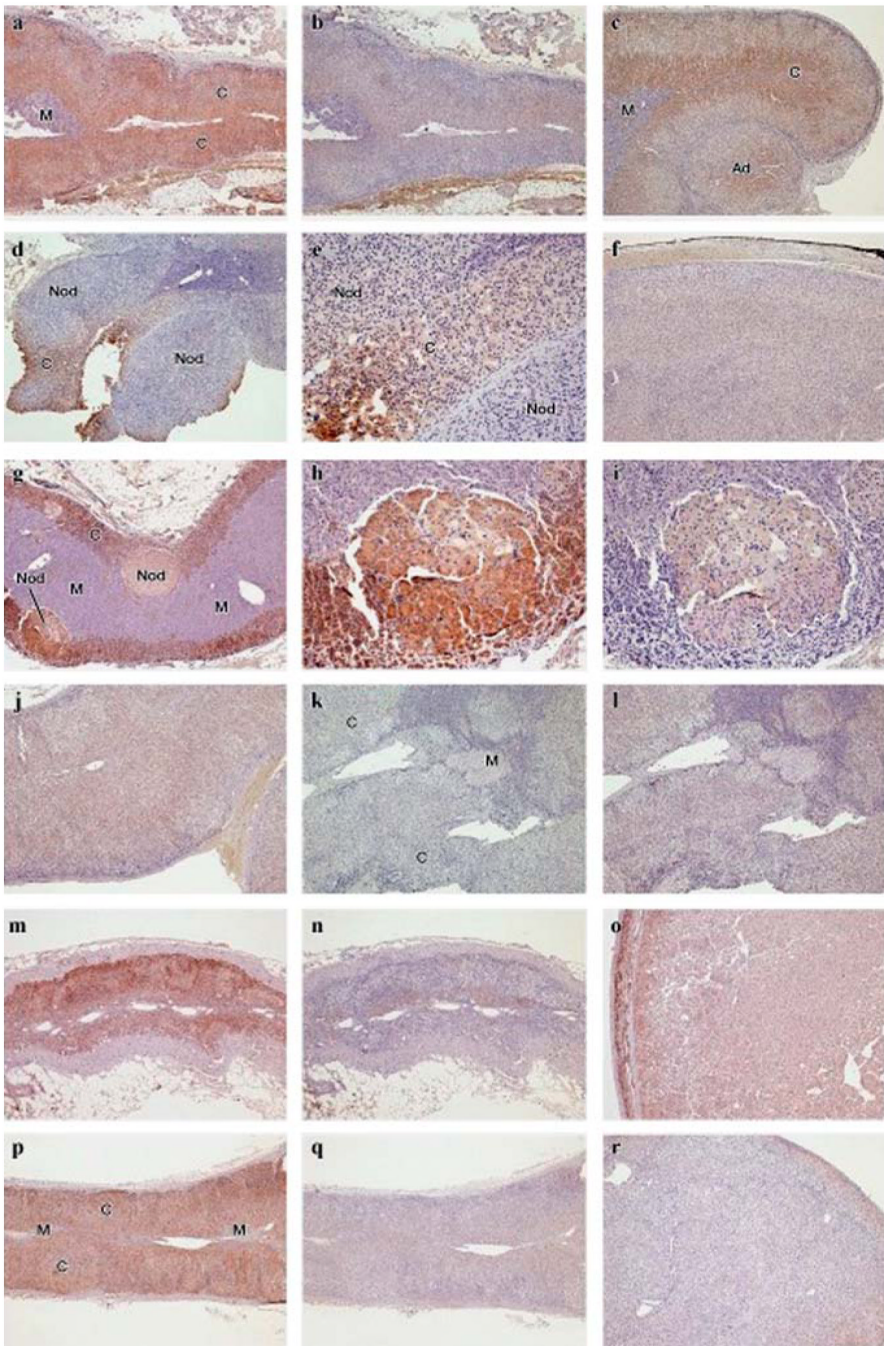

Fig. 3.

(**a**) Normal adrenal cortex [C] and medulla [M]. The PDE11A antibody stains the cortex homogenously but not the medulla; (**b**) negative control of the same tissue; (**c**) corticotropin-driven benign adrenocortical hyperplasia: again, PDE11A antibody stains the hyperplastic cortex homogenously [C] and the single adenomatous nodule [Ad], but not the medulla [M]; (**d**) decreased PDE11A4 staining from a patient with micronodular hyperplasia similar to the one we describe in our paper; the mutation in this patient has yet to be found despite complete sequencing of the coding region of the gene and presence of LOH in the tissue; nevertheless, the nodules [Nod] have lost staining completely, whereas the cortex [C] retains staining; (**e**) high-resolution image of the previous tissue showing the contrast in staining between non-

nodular cortex [C] and nodular cortex [Nod]; (**f**) tumor tissue from a patient with micro/macronodular hyperplasia due to the classic R204H *GNAS* mutation – there is loss of PDE11A staining within the tumor, in contrast to the normal staining of the surrounding cortex; (**g**), (**h**), and (**i**) low and high resolution images, and negative control, respectively, from a patient with the del478TG *PRKAR1A* mutation; PDE11A staining is strong within the hyperplastic cortex [C], and even stronger within the nodules [Nod] but remains negative in the medulla [M]; (**j**) normal cortex, (**k**) negative control, and (**l**) microadenomas, all from an androgen-producing hyperplasia in an older patient with no known genetic defects; the PDE11A protein is absent in most of these cortical lesions, but is present in the normal part of the gland; (**m**) normal cortex, (**n**) negative control, and (**o**) cortisol-producing tumor, all from an older patient with no known genetic defects; the PDE11A protein is present in the normal cortex, and is somewhat decreased in the tumor, (**p**) normal cortex, (**q**) negative control, and (**r**) aldosterone-producing tumor, all from an older patient with no known genetic defects; the PDE11A protein is present in the normal cortex, and is significantly decreased in the tumor.

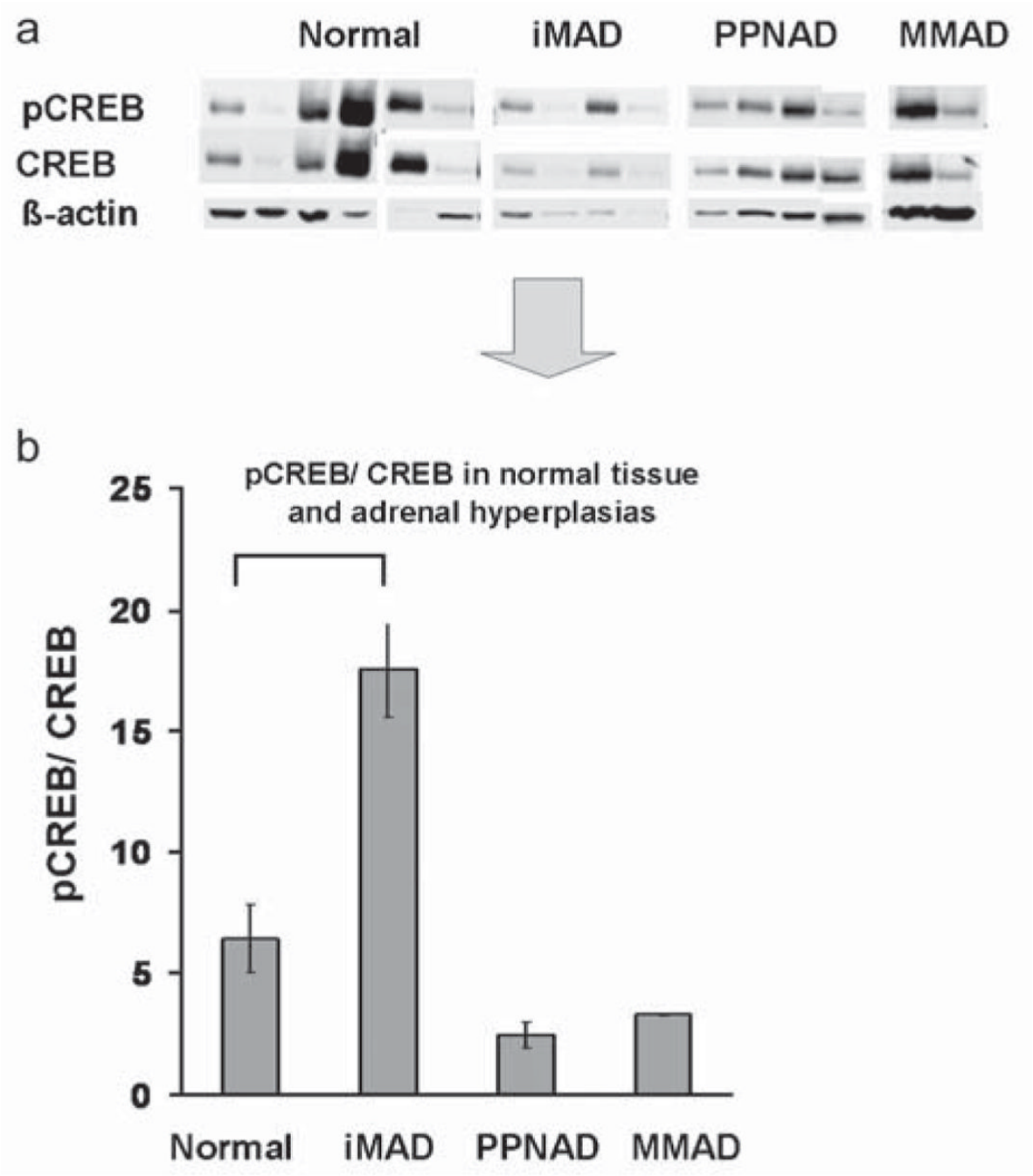

Fig. 4.

CREB and p-CREB levels in different adrenal tumors. Tissue lysates from normal adrenal glands (Normal), iMAD, PPNAD and MMAD. iMAD had the higher ratio in comparison with normal adrenal. The ratios were calculated after scanning the individual protein bands and correcting for β-actin absorbance; the y-axis measures random absorbance units. Each sample was tested at least twice, each time in duplicate.

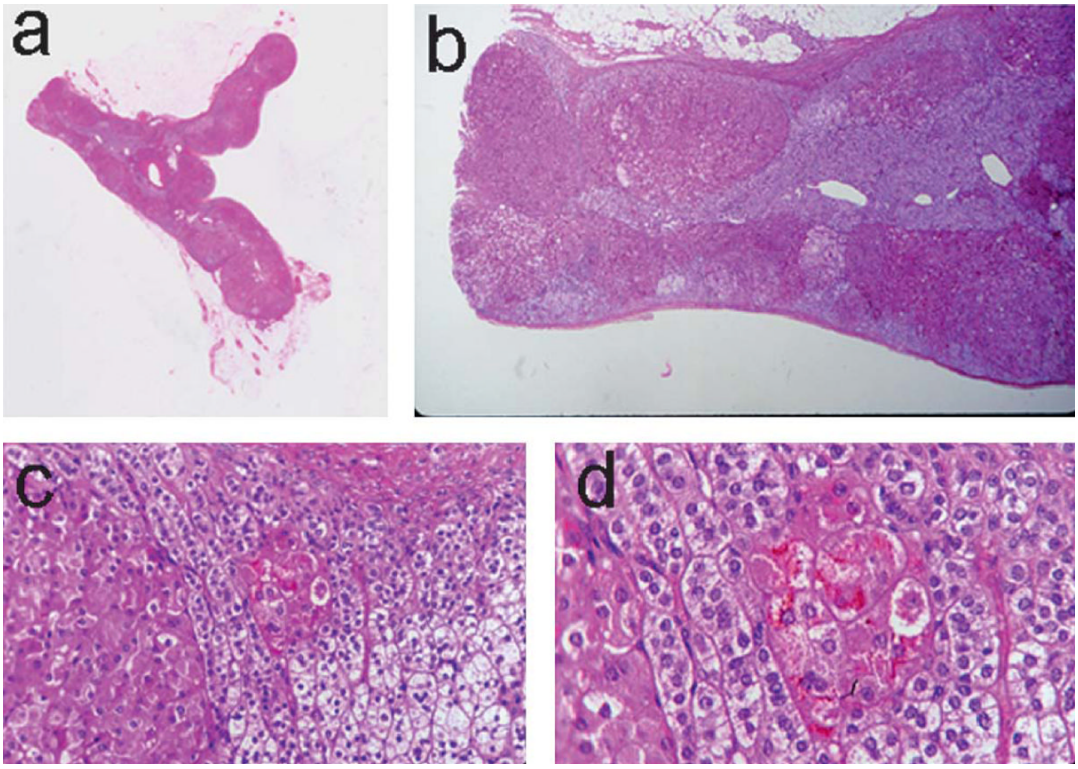

Fig. 5.

Histological sections of the excised adrenal glands from an individual with a PDE11A protein-truncating defect stained by hematoxylin and eosin. (**a**) Panoramic view of a cross-section of a sharply outlined adrenal gland. The normal cortex is replaced by a series of nodules many of which are confluent. Most of the nodules have cells with eosinophilic (pink) cytoplasm, a few have clear cytoplasm and a few show fatty metaplasia. (**b**) The cortex shows a nodule with large eosinophilic cells (left) and a small nodule with eosinophilic and pigmented cytoplasm (center). The cortical cells between the nodules are small and have lost their normal clear vacuolated appearance. (**c**) There is a roughly oval zone (very small nodule) in the center of the field composed of large cells with eosinophilic and pigmented (lipochrome) cytoplasm. The nuclei and nucleoli are slightly larger than those of the surrounding smaller atrophic cortical cells. Portion of a larger nodule (left) has similar eosinophilic cytoplasm. There is lipochrome pigment in one cell. (**d**) The cortex is occupied by juxtaposed and confluent nodules. Most of the nodules have eosinophilic cytoplasm, a few have clear cytoplasm and one has mixed cytoplasm.

Table 1
Bilateral adrenal hyperplasias

Type of adrenal hyperplasia	Histopathology
Macronodular hyperplasias (multiple nodules, mostly > 1 cm in diameter)	
BMAH	distinct adenomas (usually two or three), with internodular atrophy
c-BMAH	as for BMAH; occasional microadenomas
AIMAH	adenomatous hyperplasia (multiple), with internodular hyperplasia of the zona fasciculate
Micronodular hyperplasias (multiple nodules, mostly < 1 cm in diameter)	
i-PPNAD	microadenomatous hyperplasia, with (mostly) internodular atrophy and nodular pigment (lipofuscin)
c-PPNAD	microadenomatous hyperplasia, with (mostly) internodular atrophy and (mainly nodular) pigment (lipofuscin)
i-MAD	microadenomatous, with hyperplasia of the surrounding zona fasciculate and limited or absent pigment

Abbreviations: AIMAH: adrenocorticotropin-independent macronodular adrenocor-tical hyperplasia; BMAH: bilateral macroadenomatous hyperplasia; BWS: c-BMAH, childhood BMAH, usually seen in the context of McCune-Albright syndrome or with sporadic GNAS mutations; c-PPNAD; c-PPNAD: CNC-associated PPNAD; i-MAD: isolated micronodular adrenocortical disease; i-PPNAD: isolated PPNAD