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NEW GENES AND/OR MOLECULAR PATHWAYS ASSOCIATED WITH ADRENAL HYPERPLASIAS AND RELATED ADRENOCORTICAL TUMORS

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Abstract

Over the course of the last 10 years, we have studied the genetic and molecular mechanisms leading to disorders that affect the adrenal cortex, with emphasis on those that are developmental, hereditary and associated with adrenal hypoplasia or hyperplasia, multiple tumors and abnormalities in other endocrine glands. On the basis of this work, we propose an hypothesis on how adrenocortical tumors form and the importance of the cyclic AMP-dependent signaling pathway in this process. The regulatory subunit type 1- α (RI α) of protein kinase A (PKA), (the *PRKARIA* gene) is mutated in most patients with Carney complex and primary pigmented nodular adrenocortical disease (PPNAD). Phosphodiesterase-11A (the *PDE11A* gene) and -8B (the *PDE8B* gene) mutations were found in patients with isolated adrenal hyperplasia and Cushing syndrome, as well in patients with PPNAD. PKA effects on tumor suppression and/or development and the cell cycle are becoming clear: PKA and/or cAMP act as a coordinator of growth and proliferation in the adrenal cortex. Mouse models in which the respective genes have been knocked out seem to support this notion. Genome-wide searches for other genes responsible for adrenal tumors and related diseases are ongoing; recent evidence of the involvement of the mitochondrial oxidation pathway in adrenocortical tumorigenesis is derived from our study of rare associations such as those of disorders predisposing to adrenomedullary and related tumors (Carney triad, the dyad of paragangliomas and gastric stromal sarcomas or Carney-Stratakis syndrome, hereditary leiomyomatosis and renal cancer syndrome) which appear to be associated with adrenocortical lesions.

Keywords

Phosphodiesterases; multiple endocrine neoplasia; Carney Triad; Carney-Stratakis syndrome; fumarate hydratase

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1. Introduction: adrenocortical tumorigenesis

Significant progress has been made through studies from our laboratory and other investigators on the understanding of molecular genetics of endocrine tumors such as adrenocortical tumors (ADT) (Stratakis, 2003). What was limited to *TP53* gene's involvement in adrenocortical cancer (ACC) in the early 1990s extends now from cAMP- to the *Wnt*-signaling pathways; a number of alterations have been detected at the genomic, transcript or protein levels (Bourdeau et al., 2004; Horvath et al., 2006, Bornstein and Hornsby, 2005). The multi-step model of tumorigenesis that had been described in other tissues, and that we first proposed for ADTs (Stratakis, 2003), is now widely accepted (Bernard et al., 2003). The notion of studying, first, benign hyperplasias and “cloning genes and identifying molecular pathways involved in the first steps of ADT formation” that we suggested in the early 1990s has paid off with the identification of the cAMP/PKA-signaling pathway as the main molecular route that when defective leads to benign ADTs and hyperplasias; *Wnt*-signaling abnormalities (present also in some hyperplasias), growth factor expression (i.e. IGF-II, IGFBPs), and cell cycle gene defects (*TP53*, *CHEK2*) are associated with large adenomas and ACC (de Fraipont et al., 2005) (Figure 1).

In this model, hyperplasias of the adrenal cortex remain the cornerstone of the elucidation of the molecular phenomena that culminate in cancer formation. This process does not necessarily precede linearly other steps (this may still happen rarely): one example of non-linear phenomena are those that happen in parallel in different cell types, such as the stroma and tumor cells. It is noteworthy that a recent autopsy study identified adrenal hyperplasia in 36% of the subjects studied (saeger et al., 1998). It is for this reason, first, that we continue to focus on the clinical, genetic and molecular identification of bilateral adrenocortical hyperplasias (BAH): elucidation of all genetic defects leading to BAH will assist us in understanding the first (and apparently common) steps that adrenocortical tissue undertakes towards any pathology. The second reason is that unlike adenomas and cancer, BAHs are to a much larger extent inherited or more directly due to genetic causes (Bourdeau and Stratakis, 2002); this allows for the more efficient use of gene mapping tools that take advantage of positional approaches based on SNPs and other family data in addition to classic cancer genetic techniques such as loss-of-heterozygosity (LOH). And, finally, BAHs associated with Cushing syndrome have a distinct biochemical phenotype of hypercortisolemia that we and others have striven to define with the development of proper testing; recently, we presented a tentative classification of all BAHs helped by the new genetic discoveries (Table 1) (Stratakis and Boikos, 2007).

2. Phosphodiesterases: extending the role of cAMP-signaling in adrenocortical tumors

In the year 2000, we identified the molecular cause of the most common among the micronodular BAHs, primary pigmented nodular adrenocortical disease (PPNAD) (Kirschner et al., 2000). PPNAD is characterized by small, pigmented nodules that are surrounded by mostly atrophic cortex in an otherwise normal-sized gland (Carney et al., 1985). Most cases of PPNAD, inherited or sporadic, are associated with Carney complex (CNC), a syndrome that causes abnormal skin and mucosal pigmentation in addition to a variety of tumors [myxomas of the skin, heart, breast and other sites, psammomatous melanotic schwannoma, growth hormone (GH)-producing pituitary adenomas, testicular Sertoli and Leydig cell neoplasms, thyroid tumors, breast adenomas, nevi, and, perhaps, colon and other cancers] (Stratakis et al., 2001). CNC shares features with multiple endocrine neoplasia (MEN) and lentiginosis syndromes, such as MEN 1 and Peutz-Jeghers syndrome (PJS), and other hamartomatoses (MRash and Stratakis, 2001). By linkage analysis we and others identified two loci harboring genes for CNC on 2p16 (*CNC2*) and 17q22-24 (*CNC1*) (Stratakis et al., 2001). We then identified the *PRKARIA* gene from the *CNC1* 17q22-24 locus (Kirschner et al., 2000).

PRKARIA encodes the regulatory subunit type 1A (R1 α) of PKA, a molecule that is the main regulator of this cAMP-dependent protein kinase, one of the most important regulatory pathways in cellular signaling (Amieux, 2002).

Following the identification of *PRKARIA* gene mutations in PPNAD, we had, in addition to clinical and histopathological features, a molecular genetic marker that could be used to identify diagnostic subgroups of the BAH and CNC patients that we were working with (Table 1). This allowed us to identify significant genetic heterogeneity within patients that were previously thought to have one disease.

Before embarking on using the samples that were *PRKARIA*-mutation-negative to search for other genes, we wanted to eliminate the possibility that genetic defects that would not be identifiable by sequencing were present. Fluorescent in situ hybridization (FISH) with bacterial artificial chromosome (BAC) probes containing the *PRKARIA* gene did not show any large defects, but survey by restriction digestion and long-range PCR resulted in the identification of two chromosome 17q22-24 microdeletions among 36 *PRKARIA*-mutation-negative unrelated kindreds (Horvath et al., 2008). Based on this study, one would expect that about 6% of PPNAD and/or CNC patients that do not have a coding sequence mutation may still have a defect of the *PRKARIA* gene.

Among the remaining kindreds that did not have *PRKARIA* mutations, we then used the clinical and histopathological criteria that we developed and are listed in Table 1 to identify subgroups of patients/families. First, we identified patients (10 kindreds) with micronodular adrenocortical disease (MAD) (Gunther et al., 2004). Prior to the identification of the *PRKARIA* gene, MAD patients had been grouped together with those that had “classic” PPNAD, because even though pigment (lipofuscin) was not evident by regular microscopy, in some patients electron microscopy could identify pigment granules. It had been assumed, since most patients with MAD were very young (2 to 7 years old), that the lack of heavy pigmentation was a function of their age. However, following the identification of *PRKARIA*, it became clear that almost none of these patients had *PRKARIA* gene defects. Like PPNAD, MAD leads to corticotropin (ACTH)-independent hypercortisolism caused by small nodules randomly arising within the cortex of both adrenal glands of affected individuals. Most cases of non-pigmented MAD were isolated (iMAD – not associated with any other tumors or conditions), sporadic (patients did not have any family history) and occurred in very young children. Some patients had a presentation consistent with atypical Cushing syndrome; most patients were female; and, autosomal dominant inheritance could be documented in one kindred that had previously been mapped to the *CNC2* locus (CAR14).

Since most patients were sporadic, we teamed with investigators from a private bioinformatics enterprise to apply novel software (EXEMPLAR[®]) for a GWA study that took into account the prior information of linkage to chromosome 2. Tetrads of samples were genotyped: the two unaffected parents, peripheral blood DNA and tumor DNA microdissected from the adrenal lesions of the proband; the single family (CAR14) and their tumor tissue were also genotyped. The Affymetrix[®] 10K genechip was used (the study was done in 2006). By a combination of association analyses and the detection of gene dosage alterations, loss-of-heterozygosity (LOH) and amplification events, the 2q31-33 was identified as potentially harboring a susceptibility locus along with the previously identified 2p16-15 locus and other loci in the genome including regions on 5q, 8q, and others (these data are available on line at <http://www.nature.com/ng/journal/v38/n7/extref/ng1809-S8.pdf>). These studies culminated in the identification of the phosphodiesterase (PDE) genes, *PDE11A* (Horvath et al., 2006) and, more recently, *PDE8B* (Horvath et al., 2008b) from the 2q and 5q13 areas. Ongoing search includes the study of candidate genes from the locations that had the highest linkage *p* values.

These published data and ongoing work suggest that *PDE11A* inactivating defects are found in increased frequency in a subgroup of patients with PPNAD, iMAD and other forms of BAH and possibly other adrenal and endocrine tumors (Horvath et al., 2006b). Two frameshift mutations disrupting the *PDE11A4* adrenal-specific isoform protein (c.171Tdel/fs41X and c.1655_1657delTCTinsCC/fs15X) and a base pair substitution (c.919C>T p.R307X) were the first such *PDE11A* gene variants that were reported (Fig. 3); 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. We confirmed *PDE11A* gene mapping to the 2q31-35 by FISH and tumors from patients with *PDE11A*-inactivating mutations demonstrated 2q allelic losses (Libe et al., 2008) (Figure 2).

PDE11A is a dual-specificity PDE catalyzing the hydrolysis of both cAMP and cGMP; it is expressed in several endocrine tissues (Boikos et al. 2008): the *PDE11A* gene, like that of other PDEs, has a complex organization: only the A4 splice variant is expressed in adrenal, whereas A1 is ubiquitous, and A2 and A3 have a more limited expression pattern.

PDE8B codes for a PDE that has the highest affinity to cAMP among all known PDEs (Conti and Beavo, 2007). The locus harboring *PDE8B* on 5q13 was also among the most likely regions to be associated with MAD in our GWA study and *PDE8B* was highly expressed in the adrenal gland. We sequenced the *PDE8B*-coding regions in all remaining patients with MAD and identified a single base substitution (c.914A>T or H305P) in a female (CAR559.03) who had Cushing syndrome (Figure 3, A-C) (Horvath et al. 2008). The patient inherited the mutation from her father, who was obese and had hypertension, abnormal midnight cortisol levels, and on computed tomography mild hyperplasia (Fig. 3, B-D). The c.914A>T substitution was not present in more than 2,000 control chromosomes and the H305P mutation affects an evolutionary conserved residue (Fig. 3, E and F). *In vitro* studies on HEK293 cells showed significantly higher cAMP levels after transfection with the mutant *PDE8B* indicating an impaired ability of the protein to degrade cAMP (Fig. 3G).

These studies identified mutations in a new class of genes, those coding for cAMP-binding PDEs, as predisposing to BAHs and possibly other adrenal tumors. The discovery reinforced the notion that aberrant cAMP signaling is tightly linked to genetic forms of cortisol excess that lead to Cushing syndrome. Ongoing work is aimed at testing other PDEs that bind cAMP for mutations in our patients. An important aspect of the *PDE11A* studies was the recent identification of 3 *PDE11A*-inactivating (protein-truncating) mutations in a population of control patients that are being followed for the development of cancer (the New York Cancer Project, NYCP) with a combined frequency of 1.6%. Two functional *PDE11A* missense substitutions, R804H and R867G, were present at much higher rates of 2.4% and 3%, respectively (Horvath et al., 2006b). These findings raised the possibility that carrying *PDE11A* gene defects has wider implications that go beyond a predisposition to adrenocortical hyperplasia or tumors (Libe et al., 2008).

3. Clinical and molecular elucidation of other conditions associated with adrenal tumors

As part of our efforts to clinically and molecularly elucidate all genetic forms of adrenocortical adenomatosis (Table 1), we have not limited our studies to PPNAD and MAD. We have also studied the main form of BAH in adults, massive macronodular adrenocortical disease or ACTH-independent macronodular adrenocortical hyperplasia (MMAD/AIMAH). The clinical complexity and genetic heterogeneity of MMAD/AIMAH appears to be at least as extensive as that of PPNAD, MAD and related disorders, as our microarray studies indicated. Although the importance of the cAMP-signaling pathway is again undisputable, from the ectopic

expression of G-protein coupled receptors to the somatic mutations of the *GNAS* gene (coding for the G α Gs protein subunit) in McCune-Albright syndrome and sporadic cases of MMAD/AIMAH and the deletions of the *PRKARIA* locus in MMAD nodules (Bourdeau et al., 2006), we have also demonstrated for the first time the involvement of the mitochondrial oxidation pathway in adrenocortical tumor formation: patients with fumarate hydratase (*FH*) defects and the hereditary leiomyomatosis and renal cancer syndrome (HLRCS) develop adrenocortical adenomas (Matyakhina et al., 2005); direct involvement of the *FH* gene in MMAD was shown in a patient with HLRCS.

In addition, recently, we described five unrelated families that had gastrointestinal tumors (GIST), paragangliomas (PGL) and occasionally ADTs (mostly adenomas or MMAD/AIMAH); the trait was inherited in an autosomal dominant manner (with incomplete penetrance) (Carney and Stratakis, 2002). The condition has been referred to as the dyad of “paraganglioma and gastric stromal tumors” or the “Carney-Stratakis syndrome or dyad” (Passini et al., 2008; McWhinney et al., 2007) and is distinct from Carney Triad (PGL, GIST, pulmonary chondroma and other tumors, including ADTs) (Matyakhina et al., 2007). We found germline mutations of the genes encoding succinate dehydrogenase subunits B, C and D (*SDHB*, *SDHC* and *SDHD*) that were known to be involved in inherited PGL and pheochromocytoma but were not previously involved in familial GIST or in ADTs (Passini et al., 2007). The patients did not have mutations of the *KIT* or platelet-derived growth factor receptor- α (*PDGFRA*) genes that have been associated with GIST⁵⁵⁻⁵⁹. Ongoing studies by an international consortium (that our laboratory participates in) are aimed at identifying the remaining genes for patients with Carney Triad who also get ADTs (mostly MMAD/AIMAH) (Matyakhina et al., 2007).

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Figure 1.

Tumorigenesis in the adrenal cortex, like in other endocrine (i.e. pituitary) and non-endocrine tissues (i.e. colon) is subject to a multi-step process, although not all steps are detected in pathologic specimens, nor it is necessary for all steps to occur in all cell types. Both adenoma and cancer can form without first going through hyperplastic and adenomatous, respectively, transformation; the cAMP-signaling pathway leads to hyperplasias^{1, 2}; IGF-II overexpression (and that of other growth factors) is associated with adenomas and cancer; defects of cell cycle genes (*TP53*, *CHEK2*) are the hallmark of both sporadic and familial adrenal cancer⁸, but large benign adenomas can also harbor somatic *TP53* mutations⁹. If the first “hit” is in *TP53*, such as is the case in LFS, cancer forms without need for any precursor steps; if, on the other hand, the first defect is in a gene of the cAMP-signaling pathway (as in CNC or MAS), the most likely outcome is hyperplasia and adenomas, which only rarely (if ever) progress to cancer. In support of this hypothesis, we and others reported somatic *Wnt*-signaling pathway abnormalities in adenomas formed in the context of hyperplasias^{3, 10-12}; somatic *PRKARIA* mutations also occur in sporadic adenomas that behave like those in CNC. Finally, chromosomal abnormalities are common in all ADTs and their complexity progresses with the severity of the pathology^{13, 14}.

Abbreviations: APC = Familial polyposis coli; CNC = Carney complex; CAH = congenital adrenal hyperplasia; LFS = Li-Fraumeni syndrome; LFSv = LFS variant (*TP53*, *CHEK2* “mild” mutations); MAS = McCune-Albright syndrome; MEN 1 = menin - multiple endocrine neoplasia 1; PKA = protein kinase A.

Figure 2.

(a) *PDE11A* sequence defects in patients with adrenal disease⁴²: the upper (in red) bar shows the protein and its main PDE functional domains; the lower (in green) shows the location of the sequence defects in the 22-exon gene; R804H and R867G are relatively common polymorphisms (b) FISH on tumor cells from a single individual with iMAD and an inactivating *PDE11A*-sequence defect showed allelic loss (one signal) of the RP11-428I14 BAC probe containing the *PDE11A* gene in most cells (arrow); the ideogram of chromosome 2 and the location of the *PDE11A* gene on 2q31-35 are also shown to the left of the panel.

Figure 3.

A. Pictures of patient CAR559.03 with Cushing syndrome⁴³ B. Family CAR 559: the mutant allele (C) of the proband (CAR559.03) was inherited from her father (CAR559.01); her mother (CAR559.02) had normal *PDE8B* alleles; C. Midnight cortisol levels (in mg/dl) in patients with adrenocortical tumors (N=23) and control (N=7) individuals at the NIH and the levels of the proband and her father; D. Imaging and pathological findings in the proband: (1) Adrenal computed tomography (CT) of CAR 559.03; mild enlargement of both the left and right adrenal glands is present; (2) Hematoxylin and eosin staining (5x magnification) of the adrenal gland of the proband: the normal adrenocortical zonation pattern is disturbed; both nodular tissue and hyperplasia of the surrounding cortex are seen; (3) pigment in adrenocortical cells is seen by Fontana-Masson stain (40x); E. Adrenal CT of the father (CAR 559.01): (1) right and (2) left; F. The A>T substitution in patient CAR 559.03; G. Conservation of histidine (H) at position 305 of PDE8B across different species; H. *In vitro* expression of constructs employing the normal (wild-type or wt) and mutant (H305P) *PDE8B* in HEK293 cells leads to significant decrease of cAMP levels or no change, respectively, compared to the “mock” transfection; thus, the H305P mutation abolishes the ability of PDE8B to degrade cAMP.

Table 1

Bilateral adrenocortical hyperplasias leading to Cushing syndrome and molecular causes

DISEASE	Epidemiology	Description	Syndrome/inheritance	Genes
Macronodular hyperplasias (multiple nodules, most >1 cm diameter)				
BMAH (MMAD type I)	5th-6th decade, rarely young adults and middle age	Distinct adenomas with internodular atrophy	Isolated; MEN1, FAP, MAS, HLRCS, Carney Triad; Carney-Stratakis syndrome	Menin, APC, GNAS, FH, SDHx defects, aberrant GPCRs, 17q22-24, other
c-BMAH (childhood BMAH)	Infants, very young children	As for BMAH; bilateral macroadenomas	MAS	GNAS
AIMAH (MMAD type-II)	5th-6th decade, rarely young adults and middle age	Adenomatous hyperplasia with internodular hyperplasia	Mostly isolated	Aberrant GPCRs; 17q22-24, others
Micronodular hyperplasias (multiple nodules, most <1 cm diameter)				
iPPNAD	Children and young adults	Microadenomatous hyperplasia with at least some pigment	Isolated, (AD)	PRKARIA, PDE11A, PDE8B, 2p16; others
C-PPNAD (CNC-associated)	Children; young and middle aged adults	Microadenomatous hyperplasia with (mostly) internodular atrophy and pigment	CNC (AD)	PRKARIA, 2p16, others
MAD (iMAD when isolated)	Mostly children and young adults	Microadenomatous hyperplasia with internodular hyperplasia and limited or absent pigment	Often isolated (iMAD); some times associated with other defects; often sporadic, rarely AD	PDE11A, 2p12-p16, 5q, others

Abbreviations: 1. *Adrenocortical hyperplasias:* BMAH = Bilateral macronodular hyperplasia – also known as “massive macronodular adrenocortical disease” (MMAD) type-I; c-BMAH = childhood BMAH; AIMAH (or MMAD type-II) = ACTH-independent macronodular adrenocortical hyperplasia (AIMAH); i-PPNAD = isolated primary pigmented nodular adrenocortical disease (PPNAD); C-PPNAD=PPNAD associated with Carney complex; MAD = adrenocortical disease; iMAD = isolated MAD. 2. *Other abbreviations:* AD = autosomal dominant; APC = Familial polyposis coli; CNC = Carney complex; HLRCS = hereditary leiomyomatosis and renal cancer syndrome; MAS =McCune-Albright syndrome; MEN 1 = multiple endocrine neoplasia 1. AD= autosomal dominant; APC=Familial polyposis coli; CNC=Carney complex; FH=fumarate hydratase; GPCR=G-protein coupled receptors; GNAS= the G-protein stimulatory subunit (Gsα) gene; HLRCS=hereditary leiomyomatosis and renal cancer syndrome; MAS=McCune-Albright syndrome; MEN 1=multiple endocrine neoplasia 1; PDE=phosphodiesterase; PDE11A=the gene for PDE11A; PDE8B=the gene for PDE8B, PKA=protein kinase A; SDHx=succinate dehydrogenase subunit (-B, -C, and -D).