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Unravelling the molecular basis of micronodular adrenal hyperplasia

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Abstract

Purpose of review—This review discusses the molecular basis of micronodular adrenal hyperplasia (MAH). It focuses on the role of genetic defects in cAMP signaling related molecules, namely *PRKARIA*, *GNAS*, *PDE11A* and *PDE8B* in the predisposition for tumor formation. It also discusses the involvement of cAMP signaling and related pathways, and their impact on the adrenocortical phenotype formation.

Recent findings—Molecular defects in the phosphodiesterases (PDE) family are the most recently discovered genetic defects predisposing to various adrenocortical tumor formation. In contrast to *GNAS* and *PRKARIA*, defects in PDE genes are associated more frequently with incomplete penetrance.

Summary—Recent findings indicate the importance of cAMP signaling for normal adrenocortical functioning and the sensitivity of the adrenal gland to subtle alterations in cAMP levels. Identifying low-penetrance mutations in more than one *PDEs* in patients with MAH is suggestive for a complementary role of the different PDEs in the adrenal gland, and possible involvement of other members of this gene family in adrenocortical signaling

Keywords

adrenocortical tumors; cAMP signaling; phosphodiesterases; PKA

Introduction

Embryologically, the adrenal cortex is derived from the mesoderm, and is composed of 3 layers: in the outer region *zona glomerulosa*, in the middle *zona fasciculata*, and in the inner part of the gland *zona reticularis*. Adrenocortical lesions include hyperplasia (which may be diffuse, or nodular) adenomas and carcinomas. The frequency of benign adrenocortical lesions is very high; it has been estimated that more than 1 in 10 adults has at least one adrenocortical nodule up to 1 cm on autopsy; these benign tumors may contribute to metabolic syndrome, hypertension, obesity, and abnormalities of the hypothalamic-pituitary-adrenal axis that can be linked to other serious disorders such as osteoporosis, depression, and late-onset diabetes mellitus [1•]. In contrast, adrenocortical cancer is a very rare disease (with an incidence of less than 1 per million), which, unless identified at an early stage, is almost invariably fatal. Clonal composition analyses and comparative genomic hybridization experiments have established that adrenocortical tumorigenesis is a multi-step process resulting from sequential genetic

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alterations that lead to progression from normal to adenomatous and, eventually, malignant phenotypes [2,3]. Despite intensive investigations, the remarkable discrepancy between the frequencies of benign and malignant tumor formation in this tissue remains obscure. Better understanding of the molecular processes that underlie the benign and malignant adrenocortical transformation is expected to provide clues not only for diagnosis, but also for improved treatment and prevention.

The present report discusses the existing knowledge on the molecular basis of micronodular bilateral adrenocortical hyperplasias (BAH) and reviews current concepts on the genetic factors involved in its predisposition and progress.

Adrenocortical hyperplasias – nomenclature and features

A recent article reviews and classifies the types of benign and malignant adrenocortical lesions; it also proposes a nomenclature to be used in both scientific and clinical application fields [4•]. In brief, corticotrophin independent BAH can be classified as macro- and micronodular on the basis of the size of their nodules (>1 cm, or <1 cm in diameter, respectively). Nodules less than 1 cm in diameter can be seen in macronodular adrenocortical disease, especially in the form associated with McCune-Albright syndrome (MAS), and single large nodules can be associated with primary pigmented nodular adrenocortical disease (PPNAD) in older individuals, however, the vast majority of patients with BAH present with either a macronodular or a micronodular form. Micronodular adrenocortical hyperplasia (MAH), which is in the focus of the current review, can be further subdivided in 3 groups: PPNAD as part of Carney Complex (CNC) - a multiple endocrine neoplasia syndrome (CNC-PPNAD or c-PPNAD), isolated micronodular adrenocortical disease (i-MAD), and its pigmented variant, isolated PPNAD (i-PPNAD) [4•–6]. Two additional criteria are used in the classification of MAHs: the status of the internodular cortex – hyperplastic or atrophic; and the presence of pigment, most often lipofuscin. Lipofuscin is thought to be derived from lysosomal residual bodies containing end products of oxidative damage to lipid molecules [7]. In PPNAD (and other cortisol-producing tumors), lipofuscin appears microscopically as light-to brown dark, or, sometimes, black stained spots. Lipofuscin accumulates also with age in both human and other mammalian adrenocortical tissue [6–8].

PPNAD has a bimodal age of presentation – although in most cases, and, particularly the ones associated with CNC, the disease is diagnosed in the second and third decade of life: a group of patients presents during early childhood (2–3 yr) [5]. Non-pigmented, early onset BAH, or iMAD may be a distinct entity, which, although very similar to PPNAD, displays some specific features especially with regards to the presence or absence of hyperplasia in the surrounding cortex [5,9]. Patients with iMAD may present with classical Cushing syndrome (CS), as well as with a variant, called “atypical” (ACS) [10]. ACS is characterized by an asthenic, rather than obese, body habitus that is caused by severe osteoporosis, short stature, and severe muscle and skin wasting. Patients with ACS tend to have normal or near-normal 24-hour urinary free cortisol (UFC) production, with, however, disturbed normal circadian rhythmicity [11,12]. Occasionally, normal cortisol production is interrupted by days or weeks of hypercortisolism which gives rise to a yet another variant called “periodic CS” (PCS). PCS is frequently found in children and adolescents with PPNAD [12]. In both ACS and PCS, as well as in classic CS, caused by PPNAD, paradoxical increase of UFC and/or 17-hydroxy-corticosteroids is seen during the second phase (high dose dexamethasone administration) of the Liddle’s test [13]. This feature may be useful diagnostically for PPNAD, iMAD and related abnormalities of the adrenal cortex; it probably reflects a tendency that these nodules have for increased responsiveness to other steroids [14,15].

Genetics of micronodular adrenocortical disease

PPNAD associated with CNC

Most commonly PPNAD is seen in the context of CNC – c-PPNAD accounts for more than 90% of all reported cases [16]. CNC is associated with several other lesions, including cardiac myxomas and other cutaneous tumors, breast myxomatosis, spotty skin pigmentation and other lesions, pituitary adenomas and acromegaly, large-cell calcifying Sertoli cell tumors (among the rarest of testicular neoplasms), adrenocortical lesions, and Leydig cell tumors, psammomatous melanotic schwannoma, epithelioid blue nevus, and ductal adenoma of the breast and thyroid follicular neoplasms, both benign and malignant [17]. PPNAD is the most frequent endocrine manifestation of CNC [6].

CNC is transmitted as an autosomal dominant trait, and a genome-wide screen of CNC families was able to demonstrate linkage to genetic loci at chromosomes 2p16 and 17q22-24 [18–20]. Further studies have led to the identification of the responsible gene on 17q22-24:

PRKARIA, the gene that encodes the type 1 α regulatory subunit (RI α) of cAMP-dependent protein kinase A (PKA) [21]. To date, more than 60% of CNC patients are identified to harbor pathogenic *PRKARIA* mutations [21–23]. The gene on chromosome 2p16 is yet to be revealed.

Protein kinase A (PKA) plays a major role in eukaryotic cell signaling. In its inactive state, the holoenzyme consists of a tetramer of two homo- or heterodimers of regulatory subunits (*PRKARIA*, *PRKAR1B*, *PRKAR2A*, *PRKAR2B*) and two catalytic subunits (*PRKACA*, *PRKACB*) [24]. Each regulatory monomer contains a dimerization/docking domain at the amino-terminal, two tandem binding domains for adenosine 3',5'-cyclic monophosphate (cAMP) at the carboxyl terminus (cAMP:A and cAMP:B), and a linker region that contains the main docking site for the C subunit [25]. Elevation in the cellular cAMP levels, and, consequently, binding of cAMP molecules to the regulatory subunits leads to activation of the regulatory subunits, dissolution of the holoenzyme and release of the catalytic subunits [24]. The free catalytic subunits, which are serine-threonine kinases, can then go on to phosphorylate a series of targets that regulate downstream effector enzymes, ion channels, and transcription of specific genes that mediate cell growth and differentiation [26]. Thus, functionally, inactivation of *PRKARIA* is associated with excess PKA signaling in affected tissues [27,28].

PRKARIA extends to a total genomic length of approximately 21 kb and is composed of 11 exons with a coding region of 1,143 bp, starting from exon 2. Since the identification of *PRKARIA* mutations in CNC, more than 50 disease-causing pathogenic sequence changes have been reported; they are spread all over the coding length of the gene, without a preference for a region or exon. Most of the mutations are unique – they are identified in single families only. Only two mutations (c.491_492delTG/p.Val164fsX4, and c.709 (-7-2) del6(ttttta)) in the gene have been seen so far in more than 3 unrelated CNC families [22,29].

The vast majority of the mutations consist of base substitutions, small deletions and insertions or combined rearrangements, involving up to 15 bp [21]; although rare, large *PRKARIA* deletions have been reported [30•]. In the majority of the cases, the sequence change results in a premature stop codon; this leads to degradation of mutant mRNAs by nonsense mediated mRNA decay and, consequentially, the absence of the predicted mutant protein product [22]. Loss of the normal 17q22-23 allele in CNC lesions (loss-of-heterozygosity, LOH) has also been noted, indicating that *PRKARIA* may act as a tumor-suppressor gene [21,31•]; oncogenesis in CNC tumors was due to the complete lack of a functional *PRKARIA* [5]. A strong genotype-phenotype correlation appears to be absent in premature stop-codon generating mutations, since NMD leads to *PRKARIA* haploinsufficiency for all these mutations.

Rarely, mutations that escape NMD and lead to the expression of an abnormal, defective PRKAR1A protein have been identified [29,30•,32••]: these “expressed” mutations appear to lead to different phenotypes. A splice-site variant that eliminates “in frame” exon 7 is seen mostly with isolated PPNAD, presumably due to adrenal-specific regulation of expression or partial splicing defect and an increased sensitivity of the adrenal gland to even subtle alterations in cAMP signaling [29]. In contrast, germline “in frame” deletion of exon 3 results in severe expression of the majority of the CNC manifestations – this phenotype likely reflects the importance of exon 3, linking the dimerisation/docking domain and the first cAMP binding domain for the overall functional conformation of PRKAR1A [30•].

Important clues on the function of the different PRKAR1A domains is provided by a recent study that investigates the *in vitro* effect of naturally occurring expressed mutations in the gene [32••]. The study examined six expressed variants resulting from missense substitutions in the coding region for the functional domains of the protein (Ser9Asn, Arg74Cys, Arg146Ser, Asp183Tyr, Ala213Asp, Gly289Trp), together with the previously reported exon 3-skipping expressed PRKAR1A isoform [30•,32••]. Transfection studies with mutant constructs bearing the expressed mutations showed increased PKA activity, which was attributed to decreased binding to cAMP and/or the catalytic subunit. A correlation between the localization of the mutation, domain function, and *in vitro* effects was observed. Three of the investigated mutations (Asp183Tyr, Ala213Asp, and Gly289Trp) were located in the cAMP-binding domains of PRKAR1A. These domains contain the Phosphate Binding Cassettes (PBCs), where the residues essential for cAMP binding are positioned; these regions are the most conserved portions of these domains [25,33]. Significant decrease in the cAMP binding ability was measured for all three mutations, consistent with their proximity to or placement in a PBC and suggesting their possible importance for normal PKA conformation and function [25]. Furthermore, in contrast to previous studies reporting only partial kinase activation for PRKAR1A subunits defective in cAMP binding domains, this study showed increased PKA activity, PKA specific activation, and pCREB/CREB ratios for these three naturally occurring mutations.

Perhaps even more interesting results were revealed by mutations that affect the catalytic subunit binding sites. In the absence of cAMP, the two regulatory subunits bind the two catalytic subunits, rendering the PKA holoenzyme inactive. Experimental data have shown that binding of the regulatory to the catalytic subunit requires at least two sites to achieve high affinity binding. The exon 3 skipping mutation, which lacks residues 60–117, spans the primary site (residues 94–98, the inhibitor sequence), while the Arg146Ser mutation disrupts the secondary site (residues 138–148, 232–247) [33]. Increased PKA specific activation and extremely high PKA activity even without cAMP were measured for these mutations due to the inability of the mutated PRKAR1A to bind the catalytic subunit, as supported by co-immunoprecipitation studies [33]. As expected, levels of unbound cAMP declined even as cAMP binding affinity fell; since the catalytic subunit is coupled to both the inhibitor sequence and cAMP binding domain, it is not surprising that a mutation that affects the catalytic subunit binding would also affect cAMP binding, likely by altering the arrangement of amino acids important for these functions.

The remaining two expressed mutations (Ser9Asn and Arg74Cys) were located in the dimerization/docking domain, and in the linker region, outside of any known functional domain, respectively; it has been hypothesized that they likely affect higher order protein structure and/or RI α interaction with partner proteins outside of the PKA tetramer [33,34]. Indeed, increased PKA activity and PKA-specific activation and decreased binding of both cAMP and C α . were observed [33]. These studies support previous suggestions that alteration of PRKAR1A function alone (not only its complete loss) is sufficient for increasing PKA

activity leading to tumorigenic changes in the adrenal cortex and other cAMP-sensitive tissues, and to CNC.

Isolated PPNAD

Although rare, familial cases of isolated PPNAD have also been reported [29]. Like CNC, PPNAD is inherited in an autosomal dominant manner. Usually no other clinical features associated with CNC are seen in these families; however, subtle disease manifestations may have been missed [35,36]. Isolated, non-familial PPNAD has been very rarely reported, and, in most of the cases, like familial PPNAD, the genetic defect is a germline mutation in *PRKARIA* [29].

Isolated MAD

The genetic basis of isolated corticotropin-independent MAD is supported by its invariably bilateral appearance and very early disease onset. In a recent study, employing a genome wide screen of 10 kindreds negative for *PRKARIA* mutations, a strong association between the disease and inactivating mutations in phosphodiesterase 11A (*PDE11A*), was identified [37, 38]. Most of the individuals included in this study presented with an overall normal adrenoglandular size and weight; adrenocortical samples from these patients featured multiple small yellow-to-brown nodules surrounded by a cortex with a uniform appearance. Microscopy showed moderate diffuse cortical hyperplasia with multiple capsular deficits and massive circumscribed and infiltrating extra-adrenal cortical excrescences with micronodules; in other patients, histology was indistinguishable from that in PPNAD.

PDE11A is located on chromosome 2q31.2 and belongs to the large family of human phosphodiesterases (PDE) comprised by 21 so far identified genes that are classified in 11 different subfamilies based on structural similarity such as sequence homology, protein domains, and enzymatic properties including substrate specificity, kinetic properties, and sensitivity to endogenous regulators and inhibitors [39,40•]. All PDE genes are transcribed in multiple splice isoforms that have mostly unique tissue-expression patterns, kinetic properties, regulation, subcellular localization, and interaction with associated proteins. *PDE11A* is a dual specificity phosphodiesterase that degrades the second messengers cAMP and cGMP with little preference to each nucleotide; *PDE11A* is expressed in several endocrine tissues, including the adrenal cortex [41•,42]. It is composed from 2 GAF and a catalytic domain; two N-terminal phosphorylation sites for PKA and PKG are identified. The gene spans approximately 0.5MB and is composed by 23 exons which alternative splicing results in the expression of four different isoforms *PDE11A1*, *PDE11A2*, *PDE11A3*, and *PDE11A4* [39]. Of the four, only the full length (*PDE11A4*, ~2.8KB) shows high adrenal specific expression.

Five different *PDE11A* mutations were identified so far among the patients with isolated PPNAD – 3 of them resulted in premature stop codon generation; the remaining 2 were single base substitutions in the catalytic domain of the protein and were shown to significantly affect the ability of *PDE11A* to degrade cAMP *in vitro* [37,38]. Cyclic AMP and cGMP levels in adrenocortical tumors from individuals with inactivating *PDE11A* mutations were significantly elevated compared to control samples. This observation suggested that the pathophysiological mechanism by which mutations in *PDE11A* predispose to adrenocortical tumor formation seems to be linked to activation or abnormal cAMP signaling. Investigation of the different *PDE11A* isoforms showed adrenal expression of only one of the four known splice variants – *PDE11A4*. Decreased *PDE11A4* mRNA and protein expression levels were measured in adrenocortical tumors of patients carrying inactivating mutations in the gene [37]. Further, fluorescent *in situ* hybridization analysis (FISH) indicated loss-of-heterozygosity (LOH), with retention of the mutant allele in some of these patients with inactivating *PDE11A* mutations [37].

The above described genome wide association study suggested a number of other chromosomal loci as potentially linked to the development of iMAD; the second most favored such locus was 5q13 containing the gene for a cAMP-specific PDE, *PDE8B* [37]. *PDE8B* was also shown to have significantly higher expression in the adrenal gland compared to all other cAMP-specific PDEs [37]. Sequencing of the *PDE8B*-coding regions identified a single base substitution (c.914A>T, p.H305P) in a young girl with CS; the patient inherited the mutation from her father, who presented with very mild to indistinguishable adrenocortical phenotype [43••]. The substitution affects an evolutionary conserved residue (H305) and was not seen among extended cohorts of healthy unrelated control subjects. *In vitro* studies indicated an impaired ability of the protein to degrade cAMP [43••].

Adrenocortical hyperplasia associated with MAS

Another type of BAH leading to Cushing syndrome – macronodular adrenocortical hyperplasia – is associated with somatic mutations in the stimulatory ($G\alpha$) gene of the G protein cascade (*GNAS*) in MAS [44]. The members of the G protein family of signal transducers form heterotrimers (subunits α , β , γ), most clearly distinguished by their different alpha chains. The α -stimulating subunit encoded by *GNAS*, located on chromosome 20q13.3 [45] has intrinsic GTPase activity and after activation stimulates the adenylyl cyclase-mediated cAMP production and interacts with other cell-specific intracellular effectors [45].

Expressed genes and pathways in PPNAD

Serial Analysis of Gene Expression (SAGE) of a human adrenal affected by PPNAD was completed to compare gene expression with that of a normal adrenal and previous studies in macronodular hyperplasias [46,47]. An interesting finding was the involvement of the Wnt signaling pathway in both types of hyperplasia: the expression levels of several members of this pathway were found elevated. Genes such as catenin (cadherin-associated protein)-like 1 (*CTNNAL1*), disheveled, dsh homolog 2 (Drosophila; *DVL2*), casein kinase 1 (*CSNK1E*), axin 1 (*AXIN1*), catenin- β 1 (*CTNNB1*), WNT1-inducible signaling pathway protein 2 (*WISP2*), and glycogen synthase kinase-3 β (*GSK3B*) were all overexpressed. The Wnt-signaling pathway regulates many important cellular and developmental processes, including proliferation, cell-to-cell adhesion, cell fate decisions, and differentiation. The involvement of this pathway in other adrenocortical tumors was recently supported by the finding of somatic activating mutations in β -catenin in as high as approximately 30% of adrenal adenomas and carcinomas [48]. Another finding in common for the two hyperplasias was increased expression of *PRKAR2B* one of the other PKA subunits that seem to counteract *PRKAR1A* actions [21,49] in human tissues bearing *PRKAR1A* inactivating mutations, as well as in *Prkar1a*-deficient mice [50–52]. An important group of genes that were found inversely regulated in PPNAD and macronodular hyperplasia were those involved in steroidogenesis, and particularly, in cortisol production. In PPNAD, as expected from a high cortisol-producing tissue, *CYP11B1*, *CYP17A1*, *CYP21A2*, and *HSD3B2*, were overexpressed; in contrast, in macronodular hyperplasia, expression levels of *CYP11B1*, *CYP17A1*, and *CYP21A2* were decreased [53], a finding that reflects the relatively low efficiency with which macronodular cells produce cortisol compared to the volume of the tissue and the degree of their hypertrophy [54].

Conclusions

Defects in several key molecules of the cAMP pathway predispose nodular adrenocortical disease: *GNAS*, *PRKAR1A*, *PDE11A* and *PDE8B*. PDE-inactivating mutations appear to be a novel cause of inherited predisposition to mostly micronodular forms of hyperplasia. Incomplete penetrance of functionally harmful sequence variations in these genes requires further investigation. In the adrenal cortex, factors that affect tumorigenicity are likely to be developmental, hormonal, and possibly gender-related. Adrenocortical tumors and CS are

generally more frequent in females [37,38,43••]; on the other hand, in all cases where inheritance of a *PDE11A* mutation could be shown, the asymptomatic carrier was the father [37,38,43••]. The presence of allelic losses of the corresponding normal allele in adrenal tissues seems to be a determining factor in the development of an adrenal tumor as it is suggested by the *PDE11A*-associated tumor genetic studies [37]. Although the cause of all forms of nodular adrenocortical disease studied to date seems to be linked to increased cAMP signaling, 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. *PRKARIA* mutations are associated with PPNAD, whereas *GNAS* mutations are associated with the mostly macronodular and clearly nonpigmented form of BAH that one sees in McCune-Albright syndrome. *PDE11A* and *PDE8B* mutations seem to predispose to a variety of lesions from isolated (without any other associated tumors) PPNAD to non-pigmented iMAD and other forms of BAH. The importance of PDEs for normal adrenocortical function is currently being explored.

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