Genetics of micronodular adrenal hyperplasia and Carney complex

Amit Tiros#1, Nuria Valdés3, Constantine A. Stratakis1

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1. Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Section on Endocrinology and Genetics, Bethesda, MD 20892, USA
2. Tel-Aviv University, Sackler Faculty of Medicine, 6997801 Tel Aviv, Israel
3. Hospital Universitario Central de Asturias, Department of Endocrinology and Nutrition, Avenida de Roma s/n, 33011 Oviedo, Asturias, Spain

Correspondence:
Constantine A. Stratakis, Section on Genetics & Endocrinology (SEGEN) Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD), National Institutes of Health (NIH), 10, Center Drive, MSC 1103, CRC, Rm 1E-3216, 20892-1862 Bethesda, USA.
stratak@mail.nih.gov

Summary

Micronodular bilateral adrenal hyperplasia (MiBAH) is a rare cause of adrenal Cushing syndrome (CS). The investigations carried out on this disorder during the last two decades suggested that it could be divided into at least two entities: primary pigmented nodular adrenocortical disease (PPNAD) and isolated micronodular adrenocortical disease (i-MAD). The most common presentation of MiBAH is familial PPNAD as part of Carney complex (CNC) (cPPNAD). CNC, associated with multiple endocrine and non-endocrine neoplasias, was first described in 1985 in 40 patients, 10 of whom were familial cases. In 2000, we identified inactivating germline mutations of the PRKAR1A gene, encoding the regulatory subunit type 1α (R1α) of protein kinase A (PKA), in the majority of patients with CNC and PPNAD. PRKAR1A mutations causing CNC lead to increased PKA activity. Since then, additional genetic alterations in the cAMP/PKA signaling pathway leading to increased PKA activity have been described in association with MiBAH. This review summarizes older and recent findings on the genetics and pathophysiology of MiBAH, PPNAD, and related disorders.

Introduction

Micronodular bilateral adrenal hyperplasia (MiBAH) is a rare cause of adrenal Cushing syndrome (CS) [1] that may be divided into at least two main distinct entities [2]: primary pigmented nodular adrenocortical disease (PPNAD) and isolated micronodular adrenocortical disease (i-MAD). Although both of these conditions can present as isolated conditions, sporadic or familial, the more common presentation is familial PPNAD, as part of Carney complex (CNC) (cPPNAD). CNC was first described in 1985 [3] as a disease that could be sporadic or inherited as an autosomal...
dominant trait. In the year 2000, our laboratory identified inactivating germline mutations in the *PRKAR1A* gene, encoding the regulatory subunit type 1α (R1α) of protein kinase A (PKA), causing the disease in the majority of CNC patients [4,5]. These *PRKAR1A* mutations led to increased PKA activity. Research during the following years identified additional genetic alterations in the cAMP/PKA signaling pathway [6-8] in patients with various forms of MiBAH.

The identification of MiBAH and its subclassification into separate diagnostic entities, all in the last 20 years, are the result of meticulous familial history taking and examination of physical signs as stated by Sir William Osler, the father of modern medicine: "Just listen to your patient, he is telling you the diagnosis" [9]. In the current report, we go back in time to when Dr. J. Aidan Carney of Mayo Clinic first discovered PPNAD and described what is known today as CNC and, then, go over all new developments on the pathophysiology, clinical and molecular genetics, of the known forms of MiBAH.

**Background—Cushing syndrome (CS)**

**Diagnosis of CS**

CS is a rare disease, while many of its manifestations, such as hypertension, hyperglycemia and obesity, have high prevalence in the general population [10,11]. Hence, attempting to diagnose CS in a patient with suggestive signs should be reserved for those with high clinical suspicion of CS [11-15]. The steps for the diagnosis of CS are divided into separate but consecutive stages. First, an endogenous and autonomous cause for hypercortisolism is confirmed, based on hypercortisoluria, abnormal response of the hypothalamus-pituitary-adrenal (HPA) axis to the administration of dexamethasone and/or blunted diurnal cortisol variation [11]. Only after demonstrating the presence of endogenous CS, the cause of hypercortisolism should be sought. The discrimination between the different etiologies for CS is often challenging due to some overlap between the biochemical profiles of the different CS etiologies [16]. The pivotal test at this stage is plasma corticotropin (ACTH) levels: suppressed or low ACTH suggests primary adrenal CS, whereas levels above 30 pg/mL suggest ACTH-dependent disease [11]. In patients with suspected ACTH-dependent CS, high dexamethasone suppression [17] and/or CRH stimulation test [18] may be used for identifying the cause, followed by pituitary magnetic resonance imaging (MRI), inferior petrosal sinus sampling (IPSs) [19] and/or other imaging modalities, as necessary. On the other hand, for patients with suppressed or low ACTH levels, computerized tomography (CT) of the adrenal glands should be obtained. To maximize the information from adrenal CT, thin slices should be obtained, and both early and delayed (15 minutes) scans following contrast media injection should be taken. Interpretation by an experienced radiologist and/or by an experienced endocrinologist is essential. One should look for the overall adrenal gland size, the presence of solitary and/or multiple nodules unilaterally vs. bilaterally; the size of the lesion (s), their attenuation measured by Hounsfield units (HU), homogeneity and borders [20]; calculating contrast media washout is always useful for the functionality and/or malignant potential of the lesion(s) [20]. Radiological criteria are used to exclude the possibility of adrenocortical carcinoma based on these parameters [20], with small (<4 cm), homogenous and low attenuated lesions (<10 HU) predicting very low-risk for malignancy [21].

Several features on CT of the adrenal glands may suggest adrenocortical hyperplasia. The presence of multiple nodules and/or enlarged adrenal glands bilaterally confirms the diagnosis [7]. In cases where one adrenal gland has larger lesion(s), given the low or suppressed ACTH levels, if the contralateral gland is not atrophied (or has some but smaller lesions) bilateral disease is again the diagnosis. The diagnosis of patients with MiBAH is even more challenging as they may have cyclical (see below) or atypical CS, and the adrenal glands can be normal on imaging studies [1,12,13].

**Subtypes of CS**

Classical CS results from steady (endogenous) hypercortisolism due to autonomous secretion of one of the HPA axis hormones (CRH, ACTH and/or cortisol). There are instances in which CS presents with variable symptoms and signs, such as when excess cortisol secretion is paroxysmal, or cyclical, or occurs during pregnancy.

**Cyclical CS**

In cyclical CS the biochemical abnormalities fluctuate, usually together with the clinical manifestations of the disease [24]. The length of the cycles of hypercortisolism has been reported to range from 12 hours to 85 days. The main risk to these patients is to erroneously rule out CS because they have a normal biochemical evaluation by the time they get evaluated for their symptoms. Thus, cyclical CS, albeit rare, should be suspected in patients with highly suggestive clinical manifestations of CS, but with a negative biochemical evaluation. Since the evaluation for cyclical CS requires multiple tests at unpredictable times, one proposed method is to perform ambulatory measurement of early morning urinary cortisol-to-creatinine ratio: this test showed high stability in room temperature, and strong correlation with 24 hour urinary free cortisol (UFC) levels [25]. However, a better alternative appears to be mid-night salivary cortisol that has been shown to correlate well with UFC [26], and is easier to perform. Cases of cyclical CS have been reported in patients with CD, but also (rarely) in a few cases of ectopic ACTH- or CRH-secreting neuroendocrine tumors, and more frequently in patients with PPNAD [24].

**CS in pregnancy**

The first two reports of CS during pregnancy featured women with classic clinical manifestations of CS that resolved completely following labor; in the first case, testing showed ACTH-independent CS, and a solitary adrenal tumor was resected.
[27], whereas in the other, the etiology was not found but the symptoms did not recur[28].

About 30 years after these two reports, a woman with recurrent miscarriages due to pregnancy-dependent CS was reported [29]. Analysis of the adrenal tissue revealed high expression of mRNA of both luteinizing hormone (LH) and progesterone receptors, but low expression of the estrogen receptor. This aberrant receptor expression explained the paroxysmal CS flaring with each pregnancy, as the high human-chorionic gonadotropin and progesterone levels stimulated the LH and progesterone receptors, respectively, that were abnormally expressed in the adrenal. Further connection between the gonadotroph axis and hypercortisolism was found later. Indeed, estrogen receptor signaling cross-talks with the beta-catenin pathway [30]. At least two of 18 patients with PPNAD were found to have somatic beta-catenin (CTNNB1) mutations [31]; at least one of them developed CS during pregnancy. We also know of several other cases of women with PPNAD that developed CS-related symptomatology during pregnancy. Since the glucocorticoid receptor is highly expressed in PPNAD compared to normal adrenal tissue, and increased cortisol secretion in PPNAD may be caused by exposure to estrogen, we may speculate that high estrogen in pregnancy induces PPNAD to secrete even more cortisol causing transient CS that resolves post-partum except in these cases where a new adenoma forms in the background of hyperplasia and due to a secondary, somatic CTNNB1 mutations [32].

**Classification of bilateral adrenocortical disease**

In 2007, we proposed [2] a new nomenclature for bilateral adrenal hyperplasias associated with CS, incorporating epidemiological, histological and genetic characteristics of each entity. Bilateral adrenocortical hyperplasias are divided into two groups according to the size of adrenocortical nodules: macronodular and micronodular hyperplasias or MiBAH. By definition, in macronodular hyperplasias, the size of the nodules exceeds 1 cm; if the size of each nodule is less than 1 cm, the hyperplasia is micronodular. Adenomas larger than 1 cm may occur in MiBAH, just like nodules smaller than 1 cm may be present in macronodular diseases. Although somewhat counter-intuitive, micro- and macronodular adrenocortical hyperplasias do not constitute different stages of the same disease, but are distinct from each other in their genetic causes, mechanism, biochemical profiles and clinical implications. Two additional basic characteristics are used in the classification of MiBAH: the status—hyperplasia or atrophy—of the internodular cortex; and the presence of pigment within the lesion or surrounding cortex. The pigment, named lipofuscin, is the end product of lipids oxidation [33]. It appears macroscopically as light brown to dark brown or even black discoloration of the tumorous or hyperplastic lesions, and microscopically can be seen as dark granular pigment. Electron microscopy provides the ultimate proof of its existence. Based on these criterion MiBAH is subdivided in 3 groups (*Table 1*): Primary Pigmented Nodular Adrenocortical Disease (PPNAD) as part of the multiple endocrine neoplasia syndrome Carney complex (CNC) or c-PPNAD, PPNAD not associated with CNC or isolated PPNAD (i-PPNAD), and isolated micronodular adrenal disease (i-MAD) which does not have the characteristic pigmentation of PPNAD and is not associated with CNC; i-MAD, like i-PPNAD or cPPNAD can be sporadic or familial.

**Clinical characteristics of MiBAH**

CNC is a rare multiple neoplasia syndrome, characterized by pigmented lesions of skin and/or mucosa; myxomas of the

**Table 1**

<table>
<thead>
<tr>
<th>Adrenal lesions</th>
<th>Aged-group affected</th>
<th>Histopathology</th>
<th>Associated disease and inheritance</th>
<th>Gene or locus affected</th>
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| c-PPNAD         | Children, young and middle aged adults | Hyperplasia, with (mostly) internodular atrophy and (mainly nodular) pigment | CNC (AD) | P6KAR1A  
|                  |                     |                |                                     | PDE8B  
|                  |                     |                |                                     | 2p16 locus |
| i-PPNAD         | Children and young adults | Hyperplasia, with (mostly) internodular atrophy and (mainly nodular) pigment | Isolated (AD) | P6KAR1A  
|                  |                     |                |                                     | PRKACA  
|                  |                     |                |                                     | PDE11A  
|                  |                     |                |                                     | 2p16 locus |
| i-MAD           | Children and young adults | Microadenomatous with hyperplasia of the surrounding zona fasciculata and limited or absent pigment | Isolated (AD); occasionally part of other developmental defects | PDE11A  
|                  |                     |                |                                     | PRKACA  
|                  |                     |                |                                     | 2p16  
|                  |                     |                |                                     | 5q |

AD: autosomal dominant; c-PPNAD: CNC-associated PPNAD; CNC: Carney complex; i-MAD: isolated micronodular adrenocortical disease; i-PPNAD: isolated PPNAD; PDE8B: phosphodiesterase 8B gene; PDE11A: phosphodiesterase 11A gene; PPNAD: primary pigmented nodular adrenocortical disease; P6KAR1A: protein kinase A regulatory subunit 6x gene; PRKACA: protein kinase A catalytic subunit α gene.
heart, skin and other tissues; benign/malignant tumors of the thyroid, breast, testicles (large-cell calcifying Sertoli cell tumors (LCST) and Leydig cell tumors) and pancreas; pituitary adenomas and acromegaly, psammomatous melanotic schwannoma (PMS), and other manifestations [23,34]. PPNAD is the most common endocrine manifestation of CNC, occurring in 60% of the CNC patients [35]. PPNAD can manifest as an isolated disease (i-PPNAD), but in nearly 90% of cases presents with other manifestations of CNC; we call it, then, c-PPNAD [35] (see above).

Patients with PPNAD present with CS when they are young adults typically, but there is variability: most cases are diagnosed in the second and third decade of life, but a few patients present in early childhood (as early as at 3 years of age) [23]. In a recent study of 353 patients, the median age of diagnosis with PPNAD was 34 years. It was more common in females than in males (71% vs. 29%); in addition, the age related penetrance was higher in females than in males [35].

PPNAD has been reported in 25 to 60% of CNC patients, but autopsies report constant histological evidence of PPNAD in all patients with CNC that have been examined [22,35]. It is not clear what causes CS in most patient and what protects some patients from developing the condition. It is possible that some of the discrepancy is due to atypical manifestations of CS: although it may cause “classic” CS, PPNAD often presents with atypical, subclinical or cyclical CS that makes the diagnosis of CS more difficult [1,23]. Periodic/cyclical CS has been reported in 26% of patients with PPNAD or i-MAD [1]. Patients with PPNAD some times present with another variant of CS characterized by short stature (pointing to the chronicity of the condition), an asthenic body habitus, severe osteoporosis and muscle and skin wasting [1]. In patients that develop classical CS, clinical signs are quite similar to those of patients with other causes of CS, so central obesity and weight gain are common. Some of the milder symptomatology may also be due to simultaneous growth hormone dysregulation due to somatotroph hyperplasia or a pituitary adenoma in patients with CNC [22]. Although most cases of PPNAD are benign, there are two cases of adrenal cancer developing in the context of a PRKAR1A mutation and pre-existing PPNAD or PPNAD and CNC running in the family. In those cases, co-secretion of androgen and cortisol were observed with rapid occurrence of metastasis; at least one of these patients died from complications of her disease [36,37].

**Hormonal investigations**

The diagnosis of CS is challenging due to the cyclical or atypical presentation that is sometimes associated with normal or near normal 24-hour urinary free cortisol (UFC). However, PPNAD has a feature which is very useful for its diagnosis: a paradoxical increase in UFC and/or 17-hydroxy-corticosteroids following the administration of high dose dexamethasone, with a 50% increase of UFC levels over baseline on day 6 of the Liddle’s test [38]. One study showed that not all patients with i-MAD respond to dexamethasone as patients with PPNAD [39]. We showed that the dexamethasone-stimulated cortisol release can be replicated in vitro and it is due to glucocorticoid receptor upregulation of expression [40].

**Imaging studies**

In PPNAD, the affected adrenal glands are usually of normal or slightly enlarged size with a weight between 4 to 17 g; the pigmented nodules usually measure < 10 mm. That is one of the reasons why in 1 out of 3 patients the adrenal glands can appear as normal on computed tomography scan (CT) [41]. This emphasizes the need to use thin slices while inspecting adrenal imaging of patients with suspected PPNAD. The pigmented micronodules can be visible as round, well delineated and hypodense (in the pre- and post-contrast studies) compared to the rest of the adrenal parenchyma glands [41].

**Treatment**

The therapy of all forms of PPNAD and i-MAD is bilateral adrenalctomy. Therefore, it is important to establish the diagnosis preoperatively to avoid the need for re-operation after unilateral resection. Furthermore, once the diagnosis is suspected it is crucial to rule out CNC and especially cardiac myxomas in order to avoid severe complications during adrenalectomy [23]. In the few patients, in whom overt CS did not recur after unilateral adrenalectomy, abnormalities in the adrenocortical function could be observed on long-term follow-up [42], demonstrating that despite apparent cure, the disease is indeed bilateral.

**Genetic and molecular pathogenesis**

**Adrenal cAMP/Protein Kinase A signaling pathway**

The cAMP-PKA pathway has a fundamental role in controlling the development, proliferation and function of adrenocortical cells. Alterations in this pathway are responsible for development of all micronodular adrenal hyperplasias associated with CS that have been molecularly elucidated to date.

PKA plays a major role in eukaryotic cell signalling. In its inactive state, the PKA holoenzyme is a heterotetramer compromised of dimer of two molecules of regulatory (R) subunits (PRKAR1α, PRKAR1β), PRKAR2α, PRKAR2β) bound to two molecules of catalytic (C) subunits (PRKACα, PRKACβ, PRKACγ and PRKX) [43] (figure 1A). Briefly, ACTH stimulates its G-coupled receptors in the adrenocortical cells, leading to the dissociation of G-protein alpha subunit (encoded by the GNAS gene) from the heterotrimeric G-protein that activates the adenylate cyclase (AC) enzyme. Activated AC produced cAMP from ATP [43]. cAMP binds to the regulatory subunits and leads to their dissociation from the catalytic subunits, which, then, phosphorylate many enzymes and transcription factors downstream that mediate cell growth and differentiation, such as the cAMP-response
element-binding protein (CREB) [44]. Intracellular phosphodiesterases (PDEs) degrade cAMP to AMP in a negative feedback loop that controls cAMP levels and responses. Thus, PKA pathological over-activation can stem from increased intracellular cAMP due to decreased PDE activity (figure 1B), from abnormally cAMP-independent PKA activity due to mutated PKA regulatory subunits (figure 1C), or by an increased number of PKA catalytic subunits, exceeding the regulatory subunits inhibition capacity (figure 1D). These alterations in

the cAMP-PKA pathway that result in increased PKA activity has been described in CS associated with i-MAD and PPNAD [45-47].

**PPNAD and Carney Complex**

When Dr. J. Aidan Carney first described CNC in 1985 [3], he suggested its genetic origin and proposed that it is inherited as an autosomal dominant trait. However, CNC appears to be a genetically heterogeneous disorder. Linkage analysis showed
that at least 2 loci are involved in CNC: 2p16 and 17q22-24 [48,49]. In 2000, our group identified the gene located at the 17q22-24 locus (CNC1 locus) as the regulatory subunit α (R1α) of PKA (PRKAR1A gene) [50]. PRKAR1A gene encodes for the most widely expressed regulatory subunit of the PKA enzyme. PRKAR1A defects associated with CNC lead to PRKAR1A haploinsufficiency and, consequently, to the loss of this regulatory subunit's function: "unrestrained" catalytic subunit activity leads to increased cell proliferation in cAMP-responsive tissues [4,51].

Heterozygous inactivated PRKAR1A mutations have been detected in 70% of patients diagnosed with CNC and this percentage increases to 80% for those with CS due to PPANAD [35,52]. Approximately 70% of the cases are familiar, whereas the remaining cases carry de novo germline mutations. The penetrance of CNC in patients with PRKAR1A mutations was almost complete (97.5%).

To date approximately 135 pathogenic mutations have been reported (https://prkar1a.nichd.nih.gov/hmdb/mutations.html). Most of the germline mutations are located in exons, especially the exons 2,3,5,7 and 8, while a minority (20%) are located in intronic sequences and affected splicing [35,52]. Most of the mutations are unique, while only a few pathogenic variants (c.82C>T, c.491_492delT6 and c.709–2_709-7delATTITTT) have been identified in more than three unrelated pedigrees [5,35,53]. The vast majority of the mutations consists of base substitutions, small deletions and insertions or combined rearrangements, involving up to 15 bp [4]. Although rare, large chromosomal deletions involving the PRKAR1A gene have been recently identified [54,55].

In more than 90% of the mutations, the sequence change results in a premature stop codon; this leads to degradation of mutant mRNAs by nonsense mediated mRNA decay (NMD) and, consequently, the absence of the predicted mutant protein product and a reduction of R1α protein levels by 50% [4,47,56]. Rarely, missense mutations of the PRKAR1A gene, short in-frame insertions/deletions and splice variants that are expressed at the protein level lead to the disease due to a defective protein that fails to respond appropriately to cAMP or does not bind effectively to the PKA catalytic subunits [56,57].

Loss of the normal 17q22-23 allele in CNC lesions (loss-of-heterozygosity, LOH) has also been noted, implicating PRKAR1A as a classic tumor-suppressor gene [4]. The observation in these studies that neither the normal PRKAR1A protein nor the mutant allele was present in tumors from CNC patients suggested that the oncogenesis in CNC tumors was due to the complete lack of a functional PRKAR1A gene. However, inactivation of the remaining wild-type allele by genetic alteration seems not a constant step in PPANAD and CNC tumor development [51].

Some families without germline mutations in PRKAR1A gene have been mapped to a second locus (the CN2 locus) [49]; CN2 is a 10Mb region on chromosome 2p16 with an unknown gene or genes that may be responsible. Most of the cases mapped at CNC2 locus have been diagnosed with CNC later in life. Interestingly, somatic alterations of the 2p16 region in CNC tumors have been reported, even in patients with a germ-line mutation of the PRKAR1A gene. These alterations are usually gene amplifications, suggesting a potential oncogene at 2p16 [58].

Our group recently found that genomic amplification of the PRKACB locus might lead to CNC without any PRKAR1A mutations. In 2014, Forlino et al. [59] described a 19-year-old-female presenting with acromegaly, pigmented spots, and myxomas, but no CS, who harbored a germline 1.6-Mb triplication of chromosome 1p31.1, including the PRKACB gene that codes for the PKA catalytic subunit beta (Cβ). Levels of Cβ, but not of α, were increased in the patient’s lymphocytes and fibroblasts. In her lymphocytes, cAMP had increased kinase activity, comparable to patients with CNC caused by PRKAR1A mutations. However, for the majority of the PRKAR1A-negative CNC cases the genetic cause remains unknown.

**Genotype-phenotype association**

CNC is a clinically and molecularly heterogeneous disorder: for example, the severity of PPANAD, the most frequent endocrine manifestation of CNC, varies considerably among patients with the same mutation, even between members of the same family [1]. The results of an extensive phenotype analysis including 353 patients from 185 unrelated kindreds with CNC reported in 2009 [35], suggested that there are at least 3 groups of patients within the patients diagnosed with CNC:

- subjects with PRKAR1A mutations and at least two of the manifestations of the originally described triad of “myxomas, spotty skin pigmentation, and endocrine overactivity”. These patients had higher frequency of myxomas, PMS and LCCSCT and thyroid lesions than patients without PRKAR1A mutation. Furthermore, LCCSCT, cardiac myxomas and thyroid tumors presented at an earlier age than in patients without PRKAR1A mutations. These patients usually were familial cases. Within this group of patients, some PRKAR1A mutations were associated more frequently with some phenotypes. The hot spot mutation c.709-7del6 was associated with PPANAD, while the other hot spot mutation c.491-492delT6 showed an association with cardiac myxomas, lentigines and thyroid tumors. Both of these mutations result in NMD and no mutant protein R1α protein (at least in lymphocytes, fibroblasts and adrenal cells), so it is difficult to explain the molecular origin of these phenotypic differences. Patients with PRKAR1A mutations that escaped NMD and led to an alternate protein had an overall higher total number of CNC manifestations than patients with PRKAR1A mutations that were subject to NMD. It is unclear why this happens because both types of mutations increase PKA activity in a similar quantity. It has been speculated that this alternate protein might have PKA-independent effects and interact with other proteins that increase tumorigenic
potential. Finally, mutations located in exons were more often associated with acromegaly, cardiac myxomas, lentigines and schwanomas;

- patients with the diagnostic criteria of CNC or PPANAD who did not harbor PRKAR1A mutations or abnormalities of its genomic locus 17q22-24. Most of them were sporadic cases but also included families that were mapped to CNC2 locus on 2q16. These patients were diagnosed later in life, most of them had no family history of the disease and they were less likely to develop cardiac myxomas, thyroid tumors, PMS and LCCSCT. It appears that they exhibit a hamartomatosis/lentiginosis syndrome with lower frequency and later presentation of endocrine and other tumors than patients carrying PRKAR1A mutations. The tumors of these patients had PKA abnormalities so it is thought that the genetic cause in these patients relates to cAMP signaling.

- the third group of patients showed isolated PPANAD (i-PPANAD). Most of these patients carried the germline c.709-7del6 mutation, whereas most of the others harbored the Met17Val mutation. Patients with i-PPANAD and PRKAR1A mutations present a gender predilection. The penetrance of PPANAD by the age of 40 years was approximately 45% in males and reached more than 70% in females. Patients younger than 8 years diagnosed with only PPANAD had very rarely CNC; a few had germline mutations in genes related with bilateral adrenal nodular hyperplasia like PRKAR1A, GNAS, or PDE11A or PDE8B.

In 2014, our group identified large deletions at 17q24.2-q24.3 including the PRKAR1A gene in 1 out of 5 patients with CNC and without PRKAR1A mutations [55]. We found that the phenotype of these patients varied but was generally severe and included manifestations that are not commonly associated with CNC, such as developmental and skeletal abnormalities. We assume that the cause for these additional abnormalities is haplinsufficiency of other genes in the 17q24.2- q24.3 region in addition to PRKAR1A gene.

**Isolated micronodular adrenal disease**
The genetic basis of i-MAD is supported by its very early onset and bilateral appearance but only in few patients a molecular defect has been identified so far.

**Phosphodiesterase 11A mutations**
Phosphodiesterase 11A belongs to the large family of human phosphodiesterases (PDEs) [60–63], which are enzymes that catalyze the hydrolysis of the 3’ cyclic phosphate bond of cyclic nucleotide. Different PDEs can share the same catalytic function, but may differ in tissue expression and intracellular localization. The PDE11A gene is located on chromosome 2q31.2, encodes a dual-specificity PDE that degrades both cAMP and cGMP. There are four different transcript variants but only PDE11A4 has been identified in adrenal tissues [64].

In 2006, our group performed a genome wide single-nucleotide polymorphism (SNP) study in ten individuals with CS secondary to adrenocortical hyperplasia and no known genetic defect (PRKAR1A or GNAS) [6]; we found a strong association between the disease and inactivating mutations in phosphodiesterase 11A (PDE11A). Most of the individuals included in this study presented with an overall normal adrenal size and weight, and 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; although in other patients, histology was indistinguishable from that of PPANAD.

Five different PDE11A mutations were identified so far [6,7], two among patients with i-PPANAD and the remaining in patients with i-MAD; 3 out of 5 mutations 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 [65]. 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 are linked to abnormally activated cAMP-dependent signaling. Adrenocortical tumors of patients carrying inactivating mutations in the gene showed decreased PDE11A4 mRNA and protein expression levels [65]. Furthermore, fluorescent in situ hybridization analysis indicated LOH, with retention of the mutant allele in some of these patients with inactivating PDE11A mutations.

**Phosphodiesterase 8B mutations**
The above described genome wide study suggested a number of other chromosomal loci as potentially linked to the development of i-MAD; the second most favored such locus was 5q13 containing the gene for a cAMP-specific PDE, PDE8B [6]. PDE8B was also shown to have significantly higher expression in the adrenal gland compared to all other cAMP-specific PDEs. Sequencing of the PDE8B-coding regions identified a single base substitution (c.914A > T, p.H305P) in a 2-year-old girl with CS and i-MAD; the patient inherited the mutation from her father, who presented with very mild to indistinguishable adrenocortical phenotype [7]. This pattern of an unaffected male passing on the disease to an affected female was also seen in PDE11A mutations. Another recent study studying 84 patients with adrenal tumors without PRKAR1A, PDE11A or GNAS mutations found a missense substitution PDE8B, at p.H391A [66]. Functional studies have demonstrated that mutant PDE8B is altered in its capacity to degrade cAMP leading to an increased PKA activity.

**PRKACA gene copy-number gain**
Comparative genomic hybridization study in 35 patients with cortisol-producing bilateral adrenal hyperplasia and overt CS
without PRKAR1A and GNAS mutations identified five patients (3 with i-MAD and 2 with bilateral macronodular adrenocortical hyperplasia) with germline copy-number gains of the genomic region on chromosome 19p that includes the PRKACA gene, which encodes the catalytic subunit alpha (αc) of PKA [8]. PRKACA overexpression presumably overwhelms the inhibiting capacity of the regulatory subunits, thus causing constitutive and cAMP-independent activation of PKA, and eventually, autonomous overproduction of cortisol.

The study observed both duplications and triplications encompassing PRKACA and after analyzing clinical and genomic data [67] it has been suggested that there might be a genomic dosage-dependent phenotype because patients with triplications presented at a younger age. The number of patients included in the study was very small so more studies are necessary before definitive conclusions may be drawn.

Given the ubiquitous expression of PRKACA it was surprising that germline amplification of the PRKACA gene resulted in adrenocortical tumors only [67]. Today, it is not known why the loss of R1α leads to the full phenotype of CNC, whereas the gain of function in C0α leads to adrenal tumors and CS only and the amplification of Cβ is associated (at least in the only patient that it has been demonstrated so far) with mostly non-adrenal manifestations of CNC.

**Genetic Modifiers**

The fact that the severity of PPNAD varied considerably among patients with the same mutation, even among members of the same family suggested that modifier genes might modulate the phenotype in CNC.

We identified PDE11A as a modifier gene of the phenotype in CNC patients with PRKAR1A mutations [68], at least with regards to the incidence of PPNAD and testicular tumors (LCCSCSs). It was also noteworthy that men with PDE11A sequence variants had higher prevalence of PPNAD than women, whereas overall PPNAD and CS are more prevalent in women than in men [35]. Several studies have suggested that Wnt signaling pathway is an important mediator in the tumorigenesis of PPNAD with germ-line PRKAR1A inactivating mutations [31,69–72]. Tadjine et al. [31] described somatic β-catenin gene mutations in 2 of 18 (11%) patients with PPNAD with PRKAR1A mutations. Two different point mutation of codons 41 (T41A) and 45 (S45P) were identified, both in exon 3 of the β-catenin gene (CTNNB1) affect serine and threonine residues, which are normally involved in β-catenin degradation. Interestingly, CTNNB1 mutations were detected in an adenoma formed within the background of PPNAD but not in the adjacent nodular tissue, suggesting that CTNNB1 mutations can be secondary genetic events participating in the nodular development of PPNAD. Another study by Gaujoux et al. [70] also showed somatic CTNNB1 mutations in 2 of 9 (22%) of PPNAD with PRKAR1A mutations.

Transcriptome studies also demonstrated over expression of genes that regulate (or are part of) the Wnt signaling pathway [71]. In addition, a whole-genome expression profile of benign adrenal lesions from patients with germ-line PRKAR1A mutations showed overexpression of Wnt related genes, whereas adrenal nodules harboring somatic GNAS mutations increased expression of other genes, such as NFKB and others [72].

In 2016, Bram et al. [73] described a pathogenic mechanism that could also contribute to an explanation on why there is so much phenotypic variability in PPNAD: apparently, in PPNAD, there is an autocrine/paracrine serotonergic aberrant regulatory loop, that activates cortisol production, and therefore participates in the pathogenesis of hypercortisolism.

**Conclusions**

The research carried out on PPNAD and i-MAD and related disorders during the last two decades has confirmed the cAMP-PKA signaling pathway as the main one involved in its molecular pathogenesis. Although inactivating mutations in inhibitors of this pathway or activating mutations in effectors of this pathway lead to cortisol excess, the histopathological changes in the adrenal glands and its association with CNC and/or CS differ significantly and overlap only partially; PRKAR1A mutations are associated with c-PPNAD, whereas PDE11A mutations are mostly associated with i-MAD. Recently, other genes and/or molecules have been described as modifiers or modulators after the increase of cAMP/PKA signaling. This knowledge about genetic pathogenesis has identified several molecules, which might serve as treatment targets that might be a substitute for adrenalectomy in the future, and highlight the importance of genetic testing for earlier diagnosis and better management of these diseases.

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References


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