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The transcriptome that mediates increased cyclic adenosine monophosphate signaling in *PRKAR1A* defects and other settings

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Abstract

Objective—To review current knowledge on the involvement of cyclic adenosine monophosphate (cAMP) and interacting signaling pathways in predisposition to tumor formation in primary pigmented nodular adrenocortical disease (PPNAD), a type of bilateral adrenal hyperplasia (BAH) related to the multiple endocrine neoplasia Carney complex, and also in isolated PPNAD and other BAHs.

Methods—We review the pertinent literature and discuss genetic defects associated with various endocrine and nonendocrine tumors.

Results—A decade ago, we discovered that PPNAD and the Carney complex are caused by *PRKAR1A* mutations. *PRKAR1A* encodes the protein kinase A (PKA) regulatory subunit type IA, an important regulator of cAMP signaling in most cells. Recently, we described PKA or *PRKAR1A* abnormalities in a variety of other BAHs; in some of these cases, mutations in additional genes of the cAMP signaling pathway, the phosphodiesterases, were identified. Transcriptomic analyses of human lesions or animal models showed that abnormal cAMP/PKA signaling in the adrenal glands, and also in other tissues such as bone, leads to proliferation of tissue-specific pluripotent cells through activation of *Wnt* signaling.

Conclusion—Recent findings indicate the relevance of cAMP signaling in the pathogenesis of adrenocortical disease and point to the *Wnt* signaling pathway as a potential important mediator of tumorigenesis related to increased cAMP or PKA signaling (or both).

Introduction

The multiple endocrine neoplasia (MEN) syndrome named Carney complex (CNC), first described in the mid-1980s, is characterized by several types of skin tumors and pigmented lesions, myxomas, schwannomas, and liver and other cancers, in association with endocrine neoplasms, most frequently affecting the adrenal cortex, the pituitary gland, and the thyroid gland (1,2). The inheritance pattern of CNC is autosomal dominant, and inactivating

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Conflict of Interest

The authors have nothing to disclose.

mutations in the protein kinase A (PKA) type Ia regulatory (R) subunit gene (*PRKARIA*) are responsible for the disease in about two-thirds of the patients with CNC (3). The disorder is distinct from MEN type 1 and MEN type 2, caused by mutations in the *MEN1* gene and the *RET* gene, respectively (4).

Primary pigmented nodular adrenocortical disease (PPNAD), a micronodular form of bilateral adrenal hyperplasia (BAH) typically observed in patients with CNC, can also manifest as an isolated primary pigmented nodular adrenocortical disease (iPPNAD) that is caused by *PRKARIA* mutations in some cases. Moreover, genetic defects in cyclic adenosine monophosphate (cAMP)-binding phosphodiesterases (PDEs) were recently described in patients with isolated micronodular adrenocortical hyperplasia (iMAH) and iPPNAD, in which no *PRKARIA* mutations were found (5). Thus, the involvement of the cAMP/PKA signaling pathway in the development of adrenal hyperplasias and tumors has been clearly demonstrated during the past few years, and a better comprehension of the molecular mechanisms that underlie these processes may provide clues not only for diagnosis but also especially for treatment and prevention of various endocrine and nonendocrine diseases. In the present report, we discuss the current knowledge about the abnormalities of the cAMP signaling pathway in the predisposition to development of endocrine tumors, and the possible interactions with the *Wnt* signaling pathway, in the context of CNC as well as other settings of increased PKA activity.

cAMP/PKA signaling in adrenal hyperplasias and tumors

The association between cAMP signaling and endocrine tumors was initially observed in the McCune-Albright syndrome, which is caused by activating mutations in *Gsa*, the product of the *GNAS* gene (the *Gsp* oncogene). *GNAS*-activating mutations lead to constitutive stimulation of adenylate cyclase and, consequently, PKA activation, resulting in a variety of clinical manifestations, including the classic triad of polyostotic fibrous dysplasia, café au lait skin pigmentation, and autonomous endocrine hyperfunction (6). The most frequently affected endocrine tissues are the pituitary gland, the ovaries, and the thyroid gland, but bilateral macronodular adrenocortical hyperplasia can also be seen in a subgroup of patients with McCune-Albright syndrome (7,8).

Further evidence for the biologic relevance of PKA involvement in human disease was provided by the demonstration of inactivating mutations in the *PRKARIA* gene in patients with CNC (3,9). *PRKARIA* encodes the regulatory subunit Ia (RIa) of cAMP-dependent PKA. PKA has a major role in eukaryotic cell signaling. In its inactive state, the holoenzyme consists of a tetramer of 2 homodimers or heterodimers of R subunits (RIa, RIb, RIIf, and RIIfb), each bound to 1 of 4 catalytic (C) subunits (Ca, Cb, Cg, and Prkx) (10). Each R subunit contains a dimerization/docking domain at the amino terminus, 2 tandem binding domains for cAMP at the carboxyl terminus, and a linker region that contains the main docking site for the C subunit (11). Elevation in cellular cAMP levels (after activation of adenylate cyclase downstream of G protein-coupled receptors) leads to binding of the cAMP molecules to the R subunits and their subsequent activation, which in turn promotes dissolution of the holoenzymes and release of the C subunits. The free C subunits can then phosphorylate a series of targets that regulate downstream effector enzymes, ion channels, and transcription of specific genes that mediate cell growth and differentiation (12,13). Thus, functionally, inactivation of *PRKARIA* is associated with excess PKA signaling in affected tissues (14,15).

Since the identification of the involvement of *PRKARIA* in CNC, several disease-causing mutations have been reported, and most of these sequence changes are unique because they have been identified in primarily single families (2,16). In a recent review of 353 patients with CNC from 185 families, patients from all ethnicities and with a wide spectrum of

clinical manifestations were described (5). More than 60% of patients with CNC harbored pathogenic *PRKARIA* mutations, and PPNAD was the most frequent endocrine manifestation of the syndrome (3,9,17,18).

More recently, genetic defects in cAMP-binding PDEs were described in patients with another form of BAH, iMAH (or isolated micronodular adrenocortical disease) with no evidence of *PRKARIA* mutations (19). In a genome-wide study of 10 kindreds negative for *PRKARIA* mutations, a strong association between adrenocortical hyperplasia and inactivating mutations in the *PDE11A* gene, located on chromosome 2q31.2, was observed (20). Five different *PDE11A* mutations have been described thus far in patients with iMAH or iPPNAD, 3 of them resulting in a premature stop codon and the other 2 demonstrating single base substitutions that significantly affected the in vitro ability of *PDE11A* to decrease cAMP levels (20,21). Thus, cAMP levels in adrenocortical tumors from the patients harboring inactivating *PDE11A* mutations were significantly elevated in comparison with those in control samples. A role for *PDE11A* in the development of adrenal and testicular tumors has also been suggested by the recent demonstration of a high frequency of *PDE11A* sequence variants in a large cohort of patients with CNC (22). In that study, patients with CNC and PPNAD were significantly more frequently carriers of *PDE11A* variants than patients without PPNAD. The frequency of *PDE11A* variants was also higher among patients with CNC who had large-cell calcifying Sertoli cell tumors in comparison with those who did not, an indication that *PDE11A* may act as a genetic modifying factor for the development of these tumors (22).

Additionally, the chromosomal locus harboring the gene encoding *PDE8B* was the second most likely region to be associated with predisposition to iMAH (20), and sequencing of the *PDE8B*-coding regions revealed a missense mutation (c.914A>T, p.H305P) in a young girl with Cushing syndrome. The *PDE8B* mutation impaired the ability of the mutant protein to decrease cAMP levels (23). *PDE8B* was also shown to have significantly higher expression in the adrenal gland in comparison with all other PDEs (20,24).

Moreover, common adrenal lesions (that is, adrenal adenomas) that did not harbor germline or somatic *GNAS*, *PRKARIA*, *PDE11A*, and *PDE8B* mutations and were associated with adrenocorticotrophic hormone-independent Cushing syndrome were demonstrated to have functional abnormalities of the cAMP signaling pathway, as shown by increased cAMP levels, decreased total PDE activity, increased PKA activity, or some combination of these findings (25). The cAMP signaling pathway, its genetic defects, and involvement in some endocrine diseases are illustrated in Figure 1.

RI α *in vitro* studies and mouse models

Important clues about the function of RI α domains have been provided by recent studies. The first *PRKARIA* mutation associated with CNC leading to an expressed protein, RI α Δ 184-236, was found to increase cAMP response element-binding protein (CREB) phosphorylation in transfected cells. This result was due to the lack of binding of the C subunit because an important sequence for this function was missing from the mutant RI α (26,27). In a study that examined 6 expressed *PRKARIA* variants resulting from missense substitutions in the coding region for the functional domains of the protein, together with the previously reported exon 3-skipping expressed *PRKARIA* isoform, the expressed mutations showed increased PKA activity, which was attributed to decreased binding to cAMP or the C subunit (or both) (28). A correlation among the localization of the mutation, domain function, and in vitro effects was observed. A significant decrease in the cAMP binding ability was measured for the mutations located in the cAMP-binding domains of the RI α protein (Asp183Tyr, Ala213Asp, and Gly289Trp), suggesting the importance of this region for normal PKA holoenzyme conformation and function (28). In contrast to previous studies

reporting only partial kinase activation for R1a subunits defective in cAMP binding domains, this study showed increased PKA activity, PKA-specific activation, and phosphorylated CREB/CREB ratios for these 3 naturally occurring mutations. Interestingly, patients with these expressed mutant R1a proteins generally had a more severe and aggressive CNC phenotype (5,16,26-28).

Most human *PRKARIA* mutations result in premature stop codons, with unstable messenger RNAs that undergo nonsense-mediated decay (9,29). Human and mouse studies have shown that R1a haploinsufficiency predisposes to tumor development (3,30). R1a-haploinsufficient cells showed high basal and cAMP-stimulated PKA activity as well as increased cell proliferation and survival (29,31).

Prkar1a haploinsufficiency in mice leads to the development of tumors arising in cAMP-responsive tissues, including nonpigmented schwannomas, fibro-osseous bone lesions, and benign and malignant thyroid neoplasms (30). The spectrum of tumors overlapped with what is seen in patients with CNC but lacked some of the most frequent CNC-related tumors, such as PPNAD, skin and heart myxomas, and pituitary adenomas (30). A different mouse model, carrying an antisense transgene for *Prkar1a* exon 2 (X2AS), led to significantly higher *Prkar1a* downregulation with consequently augmented cAMP signaling and produced a more severe phenotype that included thyroid follicular hyperplasia and adenomas as well as adrenocortical hyperplasia, especially of the X-zone (32,33). More recently, adrenal cortex-specific *Prkar1a* knockout mice also developed autonomous adrenal hyperactivity and bilateral hyperplasia, centered around the X-zone and caused by proliferation of cortisol-producing fetal adrenocortical cells induced by PKA dysregulation (34).

Collectively, these studies not only demonstrated the significance of increased cAMP or PKA signaling (or both) in causing tumors but also indicated that additional factors may be acting synergistically with *Prkar1a* haploinsufficiency to produce these lesions. This finding was not unexpected: *Trp53*^{+/-} mice and other animal models for diseases similar to CNC, such as neurofibromatosis type 1 and Peutz-Jeghers syndrome, failed to reproduce the exact respective human phenotypes until the mouse gene was knocked out in the background of one or more other tumor suppressor genes (35-37). *Prkar1a* haploinsufficiency in the background of *Trp53* or *Rb1* haploinsufficiency led to significantly decreased survival and development of significantly more tumors than in *Trp53*^{+/-} or *Rb1*^{+/-} mice (38). In endocrine tissues, *Rb1*^{+/-} *Prkar1a*^{+/-} mice exhibited more pituitary tumors and medullary thyroid carcinomas (38) than did *Rb1*^{+/-} mice (39). Moreover, *Prkar1a*^{+/-} mice, which did not develop any skin lesions spontaneously, developed more skin papillomas than wild-type animals after treatment with a skin carcinogenesis protocol (38). These results suggested that *Prkar1a* haploinsufficiency may act as a generic but relatively weak tumorigenic signal that can cooperate with other tumor suppressors, tissue-specific factors, or even chemicals to induce tumors (40).

cAMP/PKA signaling and bone lesions

Osteochondromyxomas are benign tumors observed in patients with CNC (18), and *Prkar1a*^{+/-} mice also develop bone lesions that are derived from cAMP-responsive osteogenic cells (30,41). In a recent study, we showed that *Prkaca*^{+/-} haploinsufficiency abrogated the development of thyroid tumors and schwannomas in *Prkar1a*^{+/-} mice but, interestingly, the *Prkar1a*^{+/-} *Prkaca*^{+/-} animals developed more bone tumors, starting at earlier ages and with a more aggressive behavior (42). Osteochondromyxomas that were histologically similar to the bone tumors associated with CNC were present in 13% of *Prkar1a*^{+/-} *Prkaca*^{+/-} mice, whereas cartilaginous metaplasia, chondromas, and osteochondrodysplasia were seen in a large number of the animals (42). Tumor cells from *Prkar1a*^{+/-} *Prkaca*^{+/-} mice expressed early osteogenic and bone stromal cell markers and showed higher cAMP levels and PKA

activity (42). In addition, increased PKA led to inflammation-independent activation of caspase-1 by means of overexpression of the proto-oncogene (and early osteoblast factor) Ets-1 (43). High prostaglandin E₂ levels in cells with defective PKA regulation increased cAMP levels and, in turn, activated *Wnt* signaling, as in other states of inappropriate PKA activity (43). Recently, *Prkar1a* gene deletion was identified as a recurrent genetic trait for a molecularly distinct subclass of mouse osteosarcoma (44). Thus, a pivotal role has been attributed to PKA in bone tumor formation, and in at least 2 tissues (bone and the adrenal cortex), *Prkar1a* deficiency leads to the proliferation of cells of fetal origin (34,42,44).

cAMP/PKA and the *Wnt* signaling pathway

The *Wnt* signaling pathway regulates many important cellular and developmental processes, including proliferation, cell-to-cell adhesion, and differentiation (45). A serial analysis of gene expression showed the involvement of the *Wnt* pathway in PPNAD; additional gene expression studies revealed involvement of the *Wnt* pathway in macronodular hyperplasia and other BAHs (46,47). Genes such as catenin-like 1 (*CTNNAL1*), catenin-β1 (*CTNNB1*), WNT1-inducible signaling pathway protein 2 (*WISP2*), and glycogen synthase kinase-3b (*GSK3B*) were all found overexpressed (46,47). Furthermore, somatic-activating mutations in *CTNNB1* are present in as high as approximately 30% of adrenal adenomas and carcinomas (48) and have also been found in adrenal tumors from patients with PPNAD, CNC, and germline *PRKARIA*-inactivating mutations (49,50). Recently, a microRNA profile analysis showed that cAMP or PKA (or both) through microRNA regulation affects the *Wnt* signaling pathway in both PPNAD (51) and adrenocorticotrophic hormone-independent macronodular adrenal hyperplasia (52).

A whole-genome transcriptome profiling of tumors produced by mouse models of *Prkar1a* haploinsufficiency identified *Wnt* signaling as the main pathway activated by abnormal cAMP signaling, along with cell cycle abnormalities (38). Upregulation of *Wnt3*, *Wnt3a*, *Wnt7a*, *Wnt8a*, and *Wnt8b* was also identified in bone tumors from *Prkar1a*^{+/-} *Prkaca*^{+/-} mice (42), and in another mouse model associated with cAMP/PKA dysregulation, overexpression of *Wnt* and mitogen-activated protein kinase pathway target genes was induced by the *GNAS*R201C mutation (53).

Conclusions

The pathogenesis of adrenocortical disease involves defects in several key molecules of the cAMP pathway. Abnormal cAMP/PKA signaling (increased/alternate) in the adrenal glands, and also in other tissues such as bone, leads to proliferation of tissue-specific pluripotent cells through activation of *Wnt* signaling. This process involves various molecules that control pluripotentiality in different tissues, such as Ets-1 in bone. Aberrant cAMP/PKA signaling also predisposes to tumor formation through upregulation of *E2F1*, cyclin D1 (*CCND1*), and other cell cycle genes.

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Figure 1.

The cyclic adenosine monophosphate (cAMP) signaling pathway and its genetic defects causing endocrine diseases. ATP = adenosine triphosphate; C = catalytic subunit of PKA; CNC = Carney complex; GDP = guanosine diphosphate; GTP = guanosine triphosphate; MAS = McCune-Albright syndrome; PDE = phosphodiesterase; PKA = protein kinase A; R = regulatory subunit of PKA; α , β , γ = subunits of G protein.