A PRKAR1A Mutation Associated with Primary Pigmented Nodular Adrenocortical Disease in 12 Kindreds


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Context: Primary pigmented nodular adrenocortical disease (PPNAD), a rare cause of corticotropin-independent Cushing syndrome, can be part of Carney complex (CNC), an autosomal dominant multiple neoplasia syndrome characterized by spotty skin pigmentation, cardiac myxomas, and endocrine tumors or be isolated (i). Germline PRKAR1A-inactivating mutations have been observed in both CNC and iPPNAD, but with no apparent genotype-phenotype correlation.

Objective: The objectives of the study were a detailed phenotyping for CNC manifestations in 12 kindreds bearing the same PRKAR1A mutation and a study of the consequences of the mutation and a potential founder effect.

Design: The study consisted of descriptive case reports.

Setting: The study was conducted at two referral centers.

Results: We describe a 6-bp polypyrimidine tract deletion [exon 7 IVS del (−7→−2)] in 12 unrelated kindreds that were referred for Cushing syndrome due to PPNAD. Nine of the patients had no family history, in two, there was a family history of iPPNAD. Only one patient met the criteria for CNC. Relatives carrying the same mutation had no manifestations of CNC or PPNAD, suggesting a low penetrance of this PRKAR1A defect. A founder effect was excluded by extensive genotyping of chromosome 17 markers.

Conclusions: In conclusion, a small intronic deletion of the PRKAR1A gene is a low-penetrance cause of mainly iPPNAD; it is the first PRKAR1A genetic defect to have an association with a specific phenotype. (J Clin Endocrinol Metab 91: 1943–1949, 2006)

Primary Pigmented Nodular Adrenocortical Disease (PPNAD) is a rare form of primary bilateral adrenal disease that is often associated with ACTH-independent Cushing’s syndrome (CS). The adrenal glands contain multiple typical small cortical pigmented nodules and are most often of normal weight and size (1). Patients with PPNAD present usually in late childhood or young adulthood (2). Most patients with PPNAD also have a multiple neoplasia syndrome, the Carney complex (CNC; MIM 160980). CNC patients, in addition to PPNAD, which is the most common endocrine manifestation, have other tumors such as pituitary and thyroid adenomas, ovarian cysts, and large-cell calcifying Sertoli cell tumors as well as cardiac myxomas, spotty skin pigmentation, and a predisposition to develop a variety of carcinomas, including thyroid and ovarian cancer (3). Establishing the diagnosis of PPNAD can be challenging, in particular when PPNAD is the only manifestation of the disease and when there are no other members affected in the family. In addition, patients with PPNAD not only may present atypical forms of CS such as cyclical or episodic CS, but also radiologic imaging can be normal or show only subtle nodularity.

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* C.A.S. and J.B. contributed equally on this work, and they are thus sharing last authorship.

Abbreviations: CNC, Carney complex; CS, Cushing’s syndrome; GR, glucocorticoid receptor; iPPNAD, isolated PPNAD; NMD, nonsense-mediated mRNA decay; PKA, protein kinase A; PPNAD, primary pigmented nodular adrenocortical disease; PTB, polypyrimidine tract-binding protein; SNP, single-nucleotide polymorphism.

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Genetic testing is a potentially useful tool in the diagnosis of this difficult form of CS that has become available in the last 5 yr. Despite the known genetic heterogeneity in CNC, in most cases, PPNA in its sporadic or isolated forms (iPPNAD) is caused by inactivating heterozygous mutations of the PRKARIA gene, encoding the activating subunit type Iα of the cAMP-dependent protein kinase A (PKA) (2, 4, 5). In most PRKARIA mutations identified to date, the sequence change results in a functionally null allele (5–8). This appears to be the explanation for the apparent lack of a genotype-phenotype correlation in patients with PRKARIA mutations and their high penetrance, which has been estimated to be close to 100% by age 40 yr (2).

We first identified a germline mutation in intron 6 [exon 7 IVS del (−7→2)] of the PRKARIA gene in one of five patients referred with ACTH-independent CS and found to be secondary to iPPNAD (4); the present study reports a total of 12 apparently unrelated kindreds referred for CS due to iPPNAD, all carriers of this specific mutation. This splice site deletion seems to be a low penetrance mutation because some relatives were carriers of the deletion without clinical manifestation of PPNA or the CNC, even after complete hormonal and radiological investigations. A founder effect was eliminated where this could be tested. This is the first PRKARIA mutation with a specific phenotype, and these data have implications for both counseling and the molecular investigations of this gene.

**Patients and Methods**

**Patient population**

The index patients described in this study were referred for PRKARIA gene mutation analysis because of a diagnosis of apparently iPPNAD, CNC, or a familial history of CNC. Informed consent was obtained for genetic analysis of PRKARIA and adrenal tissue collection as part of a protocol approved by the Institutional Review Board of the Cochin Hospital. Twelve index cases had the same acceptor splice site deletion of intron 6. Three of these kindreds (pedigrees 6, 9, and 12 of Fig. 1) were previously reported (4, 5, 9). Sporadic cases of the disease were considered to be any patients who had no known affected family member. The patients and the available first-degree relatives were evaluated by a thorough history and physical exam as previously reported (5). Briefly, the patients were studied for clinical signs of CNC, including dermatological examination and thyroid palpation. Ovarian or testicular, thyroid, and cardiac ultrasound scans and pituitary magnetic resonance imaging were performed. Plasma concentrations of GH, prolactin, and IGF-I were determined. In all patients with the mutation, including relatives, we screened for CS caused by PPNA. We obtained computed tomography of the adrenal glands, 24-h urinary free cortisol, plasma ACTH, and circadian plasma cortisol variation levels. A paradoxical increase in urinary cortisol after oral high-dose dexamethasone was searched for and not found for the father and the brother of the proband in family 9. Affection status was determined based on the diagnostic criteria proposed by Stratakis et al. (2).

**Preparation of DNA and sequence analysis**

DNA was extracted from peripheral blood leukocytes using the Wizard Genomic DNA purification kit (Promega, Madison, WI), and the 12 exons and the flanking intronic sequences of the PRKARIA gene were separately PCR amplified using the primers and conditions described previously (5). Both strands of the amplified products were directly sequenced on an automated sequencer (ABI 3700; PerkinElmer Corp., Wellesley, MA). Nucleotides were numbered in accordance with the reference sequence for PRKARIA (GenBank accession no. NM_002734) used by Kirschner et al. (8).

Each sequence alteration also was tested on a panel of 200 DNA samples from unrelated, normal controls, using the same methods as above.

**Lymphocytes culture and cycloheximide treatment**

Primary cell cultures from the PPNA of two individuals were cultured as previously described (10). Lymphocyte cell lines from individual patients or control subjects were established by Epstein Barr virus transformation. PPNA or lymphocytes were treated with 100 μg/ml cycloheximide or vehicle for 6 h. Total RNA was extracted using RANABle (Eurobio, Courtaboeuf, France). cDNA was synthesized by Moloney murine leukemia virus-reverse transcriptase (Invitrogen, Groningen, The Netherlands). PRKARIA cDNA from lymphocytes was amplified using primers flanking exon 7: sense primer 5′-AATGCCCGCCTTACGGCCAAGCC-3′ (exon 4A) and antisense primer 5′-ATCTTCAAACTGCAGCTGTTCC-3′ (exon 8). The PCR fragments were elec-
trophoresed on polyacrylamide gel, purified, and analyzed by direct sequencing.

Genotyping to investigate a founder effect

For the determination of disease haplotypes in the PRKARIA locus, intragenic single-nucleotide polymorphisms (SNPs) were PCR amplified and directly sequenced. Common SNPs in the general population detected during previous investigations were studied. Three SNPs were located in intronic sequences: exon 1A 109 A/C, exon 8 IVS-24 A/G (8) and exon 4 IVS-9 insT (7). Additional SNPs were selected from the public SNP database (http://www.ncbi.nlm.nih.gov/SNP) and genotyped after two different amplifications; SNPs rs3785906 and rs3785905 located in intron 6 were amplified with the primers 5'-ATTGGGTGTAAATGACC-3' and 5'-GCTTTCAGTCGTCCTC-3'; SNPs rs2302232, rs2302233 located in intron 9 were amplified with the primers 5'-AGGACGTGGTTTGGTITAG-3' and 5'-AGGAGGCATTCCATCAG-3'. Genotype of SNP rs2302232 and other SNPs described in the database are not presented in Table 2 because there were noninformative for our patients.

In addition to the GA repeat in the promoter of the gene [PRKARIA(GA)n] (6), two flanking microsatellite markers were selected within a 1 cm interval around the PRKARIA locus: marker D17S590 (a CA repeat) amplified with primers 5’-CCTTTCAGACCCACAC-3’ and 5’-CCACACAGGCACCAATG-3’ and marker D17S795 (also a CA repeat), which was amplified with primers 5’-GGTACGAAAGTG-GAAAGGC-3’ and 5’-TGATGCTGTGTTCC-3’.

One oligomer for each repeat target was 5’ end labeled with -D4-PA WellRED (Proligo France, Paris). PCRs were done in 50 μl final [200 μM dethionucleotide triphosphates, 1.5 mM MgCl2, 100 μM primers (labeled and unlabeled), 250 ng of DNA and 2.5 U Taq Gold and 1X buffer]. The PCR conditions were: 40 cycles, denaturation 30 sec at 95°C, annealing 30 sec at 60°C, elongation 30 sec at 72°C. An aliquot of the PCR (0.4 l) was added to 40 μl standard buffer. The PCR products were loaded on a polyacrylamide gel, purified, and analyzed by direct sequencing.

Results

Families and genotyping results of the 12 kindreds

The 12 kindreds with the same germline mutation in intron 6 [exon 7 IVS del (−7→−2)] of the PRKARIA gene were identified from a cohort of more than 56 mutations in more than 120 kindreds from all over the world that have been collected through an international consortium (data not shown). In this study, 10 kindreds originated from France (no. 1–10), one from Spain (no. 11), and one from the United States (no. 12) (Fig. 1).

The 6-bp deletion (TTTTTA) is located in the polypyrimidine tract of the 3’ splicing site of intron 6 and involves a TTTTTA repeat region. The deletion preserves the invariant dinucleotide 3’-AG (Fig. 2). Screening of 200 unrelated controls did not reveal the presence of the same alteration; this genetic defect did not appear to be a common polymorphism in the general population.

Phenotypic analysis

Of note, all patients were referred for CS due to apparent iPPNAD. Investigations (Table 1) demonstrated a relatively homogenous phenotype for the adrenal disease. The majority of the patients were female (10 of 12). Age at diagnosis was between 13 and 39 yr. Computed tomography scan and histopathological examination revealed normally sized adrenergals with pigmented nodules of less than 1 cm, as described in at least one of these families (no. 12) (9).

The delay between the first reported symptom and the diagnosis of CS was less than 5 yr for 10 of the patients; in index case 9, CS was diagnosed 10 yr after the onset of the disease. One patient presented with cyclical CS.

In patients with PPNAD, a paradoxical response to dexamethasone leading to increased glucocorticoid excretion could be observed (11). Among six patients (2, 7–11) in whom data were available, three showed this response, a number that is lower than the approximately 70% reported by Stratakis et al. (11).

All 12 patients were carefully investigated for other manifestations of CNC. No lesions suggestive of this diagnosis could be found in all of them; for three patients there were minor findings that could be seen in the context of CNC but that are also common in the general population such an ovarian cyst (2), multiple thyroid nodules at a young age (1), and freckling (1). Another patient had a past history of a neck meningioma; no other tumors or cancers were recorded in these patients. The maximum follow-up interval was 29 yr, with four patients followed for at least 15 yr.

In 10 kindreds there was no family history suggestive of CNC. In the Spanish family (no. 11), two other members (sister and paternal aunt) presented also with CS due to iPPNAD. In the U.S. family (no. 12), the sister of the proband (and perhaps the brother and mother) were also diagnosed with iPPNAD.

We also identified the same deletion in seven relatives (Fig. 1). Parental DNA was studied in five cases, and in each case, one parent carried the same mutation. We applied a rigorous screening protocol for manifestations of CNC for these patients with a PRKARIA mutation. Four parents did not meet the criteria for CNC. Age-related penetrance of CNC phenotypes is probably not the explanation of this low penetrance mutation because the median age at detection of the first component among CNC patients is 20 yr.

The fifth parent (mother of index case no. 7) had history of thyroid cancer and according to the criteria proposed by Stratakis et al. (2) should be considered a CNC patient. The histology was consistent with bifocal follicular variant of papillary thyroid carcinoma and one follicular adenoma. The two last mutations were found in a sibling of index case no.
9 and in another paternal aunt of index case no. 11. Again there was no manifestation suggesting a CNC, even after complete clinical, hormonal, and radiological investigations as previously reported (4, 5).

Effects of the mutation on PRKAR1A mRNA

RT-PCR was performed using primers flanking exon 7; the splice site mutation was predicted to lead to an exon 7 skipping with a frameshift. Because mutant mRNAs bearing a premature stop codon are unstable, as a result of nonsense-mediated mRNA decay (NMD) (8), we treated transformed lymphocytes from two patients and an unaffected subject with cycloheximide to abrogate NMD. The treatment led to the stabilization of an abnormal mRNA containing an exon 7 skipping with a premature stop codon that was not detectable in lymphocytes from control subject (Fig. 3A). The frameshift occurs after codon 236 and leads to a stop codon after 11 missense residues.

To try to understand why this mutation was mainly associated with an adrenal phenotype, we conducted similar experiment on PPNAD cells in primary culture and on lymphocytes of another patient as a control (Fig. 3B): the abnormal mRNA was present only after cycloheximidine treatment.

Analysis of a possible founder effect

The 12 apparently unrelated families included in our studies live in France (10), Spain (1), and the United States (1). A founder effect could be excluded for the American and Spanish families by analyzing the GA repeat in the promoter of the PRKAR1A gene (data not shown). We did a complete analysis of a possible founder effect among the 10 French families.

For most of the index cases, family history gave no indication of a common ancestor. A common last name was present in two kindreds, but we were unable by examining pedigrees back to four generations to find a common ancestor. To determine whether the different families bearing the same mutation had a common founder, we generated microsatellite markers and SNPs to construct a disease haplotype for the 10 French index cases (Table 2). Three microsatellite markers, including the GA repeat in the promoter of the PRKAR1A gene and six intragenic single nucleotide polymorphisms, are shown. The analysis was limited by the size of the families and the availability of relatives. Alleles associated with exon 7 IVS del(−7−2) provided strong evidence to eliminate a common founder for all the families. At least three different haplotypes show association with this mutation: one haplotype (A) is common to index cases 1–3 and could be the same for patients 4–6. These six French index cases could descend from a common French founder. Index cases of families 2–5 were referred from the same center in northern France. Compared with these six first patients, index case no. 7 had a different haplotype (B) for three markers: the GA repeat in the promoter, one CA repeat (D17S795), and one SNP. A patient from family no. 8 could share the same haplotype B with family no. 7. Finally, index case from family no. 9 appeared to have a third disease haplotype (C) because of the difference in SNPs of exon 1A

### Table 1. Clinical, endocrinological, and radiological investigations in the 12 index cases at last follow-up

<table>
<thead>
<tr>
<th>Sex</th>
<th>Age at first reported symptom (yr)</th>
<th>Age at diagnosis of PPNAD (yr)</th>
<th>TC (mmol/liter; n = 276–552)</th>
<th>Plasma ACTH (pmol/liter; n = 0.4)</th>
<th>Plasma ACH (pmol/liter; n &lt; 13.2)</th>
<th>Plasma ACH cortisol (nmol/liter; n &lt; 416)</th>
<th>Plasma ACH cortisol (nmol/liter; n &lt; 360)</th>
<th>Plasma ACH cortisol (nmol/liter; n &lt; 360)</th>
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</table>

F, female; M, male; UC, urinary free cortisol; DXM, dexamethasone; CT, computed tomography; NA, not available.
and exon 8. A different disease haplotype could not be excluded for families no. 9 and 10. In conclusion, a common ancestor for all index cases could be excluded, although an ancient founder could exist for some, but not all, of these families.

**Discussion**

The present report describes a natural heterozygous deletion of six nucleotides within the polypyrimidine tract of intron 6 of human RI

\[H11002\] pre-mRNA. This genetic defect was found in 12 apparently unrelated kindreds. Compared with others mutations described for the \textit{PRKAR1A} gene, exon 7 IVS del(\textbackslash H110027\textbackslash H110023\textbackslash H110022) has a low penetrance and is almost exclusively associated with iPPNAD.

Most of the \textit{PRKAR1A} mutations described to date are functionally null heterozygous mutations (2, 5, 8, 12). \textit{PRKAR1A} splice-site mutations are frequent (approximately 25% of the total) (8). Splicing of pre-mRNA introns occurs within a multicomponent complex termed the spliceosome. This process requires \textit{cis}-acting sequences and \textit{trans}-acting factors. The two dinucleotides that define the 5' and 3' boundaries of introns are invariant and when mutated, splicing is greatly reduced or completely abolished due to exon skipping or activation of cryptic splice sites. For the \textit{PRKAR1A}, such mutations usually lead to frameshifts and premature stop codons, which activate nonsense mRNA degradation. Compared with other \textit{PRKAR1A} splice site mutations the exon 7 IVS del(\textbackslash H110027\textbackslash H110023\textbackslash H110022) is different because the splice acceptor consensus sequence is preserved. But two other components are important for the splicing: the branch point sequence and an adjacent pyrimidine rich tract of varying length, referred to as the polypyrimidine tract. Its length and composition are important in the 3' splice site recognition. Mammalian intron studies have shown that the polypyrimidine tract must be CU rich and have a minimum distance of 20 nucleotides between the A of the lariat branch site and the start of the following exon (13). The mutation described here shortens the polypyrimidine tract from six nucleotides, five of which are uridine. To investigate the consequences of the mutation on the splicing process, \textit{in vitro} experiments using lymphocyte and primary adrenal cell culture were performed. We were able to demonstrate \textit{in vitro} a reduction in splicing efficiency (skipping of exon 7) of the mutant pre-mRNA, compared with the wild-type control. Both PPNAD cells and lymphocytes expressed a nonsense mRNA that was actively destroyed as shown by the inhibi-

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<th>SNP and MS</th>
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<th>7</th>
<th>8</th>
<th>9</th>
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<td>541</td>
<td>541</td>
<td>541/545?</td>
<td>541</td>
<td>541/547?</td>
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<td>+T</td>
<td>+T</td>
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<td>T/T?</td>
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<td>G/A?</td>
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<tr>
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<td>T</td>
<td>A</td>
<td>T/A?</td>
<td>A</td>
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Name of haplotype


For marker details, see Patients and Methods. MS, Microsatellite; ?, disease allele unknown because the marker was uninformative in the family.
tion of this process by cycloheximide. Because it is not feasible to study the splicing process in vivo, we could not demonstrate whether this instability was adrenal specific, thus accounting for the isolated PPNAD phenotype.

Our experiments provide no insight as to how this mutation is responsible of a different phenotype, but we can speculate that this polypyrimidine tract deletion has a minor effect on the level of the Ria protein. A functional analysis of a variety of systematically mutated polypyrimidine tracts suggests that decreased numbers of consecutive uridines can still function to promote branch point selection, depending on the tract (14). This kind of deletion may have subtle consequences on the splicing. Mild disease related to a polypyrimidine tract mutation (4-bp deletion) has been reported for the factor IX gene, the low clinical severity reflecting the fraction of normal splicing (15).

The above could be an explanation for the low penetrance of this 6-bp deletion and its incomplete clinical expression: iPPNAD was the only manifestation for most of the patients, even after a long period of follow-up. Compared with PPND seen in other patients with CNC, this was similar clinically and histologically. It is noteworthy that most patients were females (13 of 15), but this is also true for other PPND and CNC patients. Adrenal imaging was also normal or with minor abnormalities (one patient), as is the case in other patients with CNC and PPNAD (16). Finally, only one patient presented with cyclic CS, and in another the disease became clinically evident in older years. In sum, this PPND was not substantially different from any other PPND, with the exception of its lack of association with any other component of CNC.

One hypothesis for this specific adrenal phenotype for the identified mutation could be a particular sensitivity of the adrenal gland to even subtle alterations in PKA activity. Indeed, PPND is the most frequent endocrine manifestation of CNC, occurring in about one quarter of patients. Furthermore, to develop CS, it may be necessary that susceptibility genes or factors interacting with the cAMP pathway shall be active. Other steroid hormones could be such an interacting factor as it has been proposed before for estradiol (17). Glucocorticoid receptor (GR) is involved in PPNAD’s clinical expression, but it would most likely not be involved in tissue specificity. Overexpression of GR was not studied on the PPND tissue of these kindreds because GR overexpression is present in PPND nodules of patients with and without PRKAR1A mutations and does not appear to be specific to the presence of a PKA-signaling abnormality (18). Another hypothesis could be a tissue-specific variation of the abnormal pre-mRNA splicing and particularly in tissues in which the cAMP pathway plays a role in that function. Indeed the PKA can phosphorylate the polypyrimidine tract-binding protein (PTB). PTB is an important regulator of pre-mRNA splicing. Phosphorylation modulates the nucleocytoplasmic distribution of PTB (19).

Because of the low penetrance of this mutation and its discovery in 12 apparently unrelated kindreds, it was important to determine whether these families had a common ancestor. Approximately half of PRKAR1A mutations are sporadic mutations (8). The mutation was transmitted in the five kindreds for which parental DNA could be analyzed. By genotyping different markers located in the PRKAR1A region, we could exclude a common ancestor for at least the 10 French families. Three different disease haplotypes were found. However, in six families a common founder in northern part of France could not be excluded. The haplotype analysis was in agreement with the geographical origin of the patients.

For the PRKAR1A gene, most of the mutations are private. However, hot spot mutation has been described like the 578delTG mutation located in exon 4B (8). The exon 7 IVS del(−7→−2) is the first hot spot splice site mutation reported. The presence of a TTTTA repeat at the deletion site may be why it is created frequently: a common feature of short deletions (<20) is the presence of direct repeats of between 2 and 8 bp within the immediate vicinity of the deletion (20).

In summary, we describe a polypyrimidine tract mutation of the PRKAR1A gene leading to a probable mild alteration of PRKAR1A mRNA splicing. The exon 7 IVS del(−7→−2) is responsible for a mild phenotype, almost exclusively CS due to iPPNAD. The low penetrance of this genetic defect could explain why apparently unrelated index cases share a common ancestor. Our data suggested that this PRKAR1A gene mutation may be the result of a 6-bp repeat at the deletion site. To our knowledge this is the first strong genotype-phenotype correlation in CNC and/or PPNAD for a PRKAR1A mutation. This observation has important consequences for clinical management, genetic counseling, and the molecular investigations of the PRKAR1A gene.

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