Mutations in Regulatory Subunit Type 1A of Cyclic Adenosine 5′-Monophosphate-Dependent Protein Kinase (*PRKAR1A*): Phenotype Analysis in 353 Patients and 80 Different Genotypes

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Background: The "complex of myxomas, spotty skin pigmentation, and endocrine overactivity," or "Carney complex" (CNC), is caused by inactivating mutations of the regulatory subunit type 1A of the cAMP-dependent protein kinase (*PRKAR1A*) gene and as yet unknown defect(s) in other gene(s). Delineation of a genotype-phenotype correlation for CNC patients is essential for understanding PRKAR1A function and providing counseling and preventive care.

Methods: A transatlantic consortium studied the molecular genotype and clinical phenotype of 353 patients (221 females and 132 males, age 34 $\pm$ 19 yr) who carried a germline *PRKAR1A* mutation or were diagnosed with CNC and/or primary pigmented nodular adrenocortical disease.

Results: A total of 258 patients (73%) carried 80 different *PRKAR1A* mutations; 114 (62%) of the index cases had a *PRKAR1A* mutation. Most *PRKAR1A* mutations (82%) led to lack of detectable mutant protein (nonexpressed mutations) because of nonsense mRNA mediated decay. Patients with a *PRKAR1A* mutation were more likely to have pigmented skin lesions, myxomas, and thyroid and gonadal tumors; they also presented earlier with these tumors. Primary pigmented nodular adrenocortical disease occurred earlier, was more frequent in females, and was the only manifestation of CNC with a gender predilection. Mutations located in exons were more often associated with acromegaly, myxomas, lentigines, and schwannomas, whereas the frequent c.491-492delTG mutation was commonly associated with lentigines, cardiac myxomas, and thyroid tumors. Overall, nonexpressed *PRKAR1A* mutations were associated with less severe disease.

Conclusion: CNC is genetically and clinically heterogeneous. Certain tumors are more frequent, with specific mutations providing some genotype-phenotype correlation for *PRKAR1A* mutations. **(*J Clin Endocrinol Metab* 94: 2085–2091, 2009)**

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Abbreviations: CNC, Carney complex; IQR, interquartile range; LCCSCT, large-cell calcifying Sertoli cell tumor(s); NMD, nonsense-mediated mRNA decay; NR, not reached; PKA, protein kinase A; PMS, psammomatous melanotic schwannomas; PPNAD, primary pigmented nodular adrenocortical disease; RIα, regulatory subunit type 1A.

Since the first description of the "complex of myxomas, spotty skin pigmentation, and endocrine overactivity" in 1985 (1, 2), a disorder that is now known as "Carney complex" (CNC) (3–6), numerous patients have been reported from all ethnicities and with a variety of manifestations (7–11). The identification of inactivating germline mutations of the regulatory subunit type 1A (RIα) of cAMP-dependent protein kinase (*PRKAR1A*) gene in the majority of CNC patients (12, 13) led to an estimate of its penetrance, the validation of diagnostic criteria for the disease (14), and the suggestion that there was no obvious genotype-phenotype correlation (13). The proposed explanation for the apparent lack of clinical differences between kindreds with different sequence defects was that all of the first identified *PRKAR1A* mutations in CNC patients led to nonsense mRNA that was not translated into protein through the process known as nonsense-mediated mRNA decay (NMD) (12, 13). Thus, at the molecular level, all mutations had the same effect: lack of a detectable mutant protein product and an overall reduction of the total RIα protein level by 50% because only the wild-type allele was being translated (13, 15).

Soon thereafter, the first *PRKAR1A* mutation with an expressed RIα variant was described (16). The kindred had severe disease with at least one member dying from pancreatic cancer; the mutant RIα appeared to be associated with increased protein kinase A (PKA) activity, as evidenced from increased cAMP-responsive element activity in transfected cells (16). Increased PKA activity was also the mechanism that had been proposed for tumorigenesis caused by *PRKAR1A* mutations that were subject to NMD (12, 13, 15). Indeed, reduction of RIα by 50% could lead to increased PKA activity (caused by unrestricted PKA catalytic subunit activity) and cell cycle abnormalities in both human and mouse cells (17, 18). Recently, additional *PRKAR1A* mutations leading to an expressed mutant RIα protein have been described; these mutations were also associated with more aggressive disease and increased PKA activity *in vitro* (19).

In addition, over the last few years, it has become apparent that even among mutations that led to NMD there might be some phenotypic differences. For example, a 6-bp polypyrimidine tract (TTTTTA) deletion in intron 7 of the *PRKAR1A* gene (leading to skipping of the exon 7 and production of mRNA with a frameshift that was subject to NMD) resulted in a mild CNC phenotype; in 12 unrelated families with this mutation, affected individuals exhibited mostly isolated primary pigmented nodular adrenocortical disease (PPNAD) and Cushing syndrome without any other clinically significant lesions (20).

The establishment of a consortium for studies on CNC that was run in common by the National Institute of Child Health & Human Development (NICHD), National Institutes of Health (NIH), Bethesda, Maryland, and the Hospital Cochin, Paris, France, allowed the investigation of 353 patients with CNC whose clinical phenotype and genotype were studied over the last 7 yr, providing data allowing for genotype-phenotype correlation. A small number of *PRKAR1A* mutations in this cohort have been published (12, 13, 16, 19–21), including those that were included in the first reports of *PRKAR1A*'s role in CNC (12, 13). This is the first time, however, that all *PRKAR1A* mutations found to date (more than half of them entirely novel) were analyzed against CNC phenotypes that had been studied longitudinally in patients who, in at least some cases, were included in the original reports of the syndrome in 1985 (1, 2). The data are useful for evaluation of prognosis and genetic counseling of patients with CNC and/or *PRKAR1A* mutations (22), and also for understanding PKA's involvement in endocrine and other tumorigenesis (23).

Patients and Methods

Patients

The institutional review boards of the NICHD, NIH, the Mayo Clinic, and the Cochin Hospital approved contact of the families and participation of their members in clinical protocols run by the respective institutions. All patients signed informed consent for genetic analysis. Patients selected for inclusion in this study were those who met the diagnostic criteria for CNC (14) and/or PPNAD or those that had been found to have a pathogenic *PRKAR1A* mutation (the latter most often discovered in the course of a family study after detection of a mutation in a proband). Clinical investigations were done as previously recommended to screen for the main CNC manifestations (14). Patients who did not meet diagnostic criteria and did not have a *PRKAR1A* mutation, or those that were found to have a mutation in another gene of the cAMP signaling pathway associated with endocrine and other tumors and pigmentation defects (*GNAS*, *PDE11A*, or *PDE8B*) (24–26) were excluded from this analysis.

A datasheet was completed for each patient at the respective institutions and data were merged for this analysis. In addition to molecular genetics analysis, information collected included age at diagnosis and at last follow-up, current status, and data related to the following manifestations of CNC: PPNAD, cardiac myxomas (including data on recurrences), cutaneous myxomas, breast myxomatosis, lentiginosis and other pigmented lesions (blue, Spitz, and compound nevi and café-au-lait spots), acromegaly, large-cell calcifying Sertoli cell tumors (LCCSCT) and other testicular lesions, ovarian tumors, benign and malignant psammomatous melanotic schwannomas (PMS), benign thyroid disease, thyroid cancer, and other cancers.

DNA, RNA, and NMD studies

DNA and RNA were extracted from whole blood and cell cultures using standard procedures. After separation of B-lymphocytes and their transformation by Epstein-Barr virus, for mutations predicted to lead to NMD, cycloheximide treatment of transformed lymphocyte was done as previously reported to demonstrate this mechanism of *PRKAR1A* inactivation (12, 13, 16, 20). For mutations that escape NMD, the presence of the mutant mRNA or protein was demonstrated on transformed leukocytes or tumor tissues as previously reported (16, 27). Sequencing of the 12 exons and proximal intronic regions of the *PRKAR1A* gene was completed at the NIH, the Cochin laboratories, and at GeneDx (Gaithersburg, MD; www.genedx.com) by methods that have been reported elsewhere (12, 13, 16). DNA samples from CNC patients that were negative for sequencing defects underwent Southern hybridization analysis for the identification of large intra- or perigenic deletions and/or other rearrangements (19).

Statistical analysis

Chi-square or (when necessary) Fisher exact tests were used to test for association between categories. Kaplan-Meier survival curves were used to analyze the cumulative percentage of diagnosis of each manifestation by age. Log-rank test was used to compare the survival curves. All tests were two-sided, and P values <0.05 were considered to denote statistical significance. Statistical analyses were done with SAS software package version 9.01 (SAS Institute, Cary, NC).

Results

Epidemiology and clinical manifestations

A total of 353 patients from 185 families met the criteria of inclusion in the investigation and were studied. A total of 221 patients (63%) were female, and 132 were male. The mean age at last follow-up was 34 ± 19 yr (mean ± SD) (range, 1 to 81 yr). A total of 113 patients (32%) had no family history consistent with CNC, and none of their screened family members carried the *PRKAR1A* mutation of the index case; these patients were classified as "sporadic"; the remaining were "familial" cases.

PPNAD was diagnosed in 212 patients (60%), making it the most common tumor among all patients. In 44 patients (12%), PPNAD was the only (isolated) finding, after extensive screening for other CNC-associated lesions. The median age at diagnosis of PPNAD was 34 yr [interquartile range (IQR), 19–60]. PPNAD was significantly more frequent in females (n = 150; 71%) than in males (n = 62; 29%) ($P = 0.0001$), and was also diagnosed at a significantly younger age in females; the median age was 30 yr in females (IQR, 18–42) *vs.* 46 yr in males [IQR, 22-not reached (NR); $P = 0.0004$] (Fig. 1). Interestingly, this gender difference became evident only after patients had reached puberty.

GH-producing pituitary adenomas were diagnosed in 42 cases (12%). Thyroid tumors were present in 88 patients (25%); thyroid cancer (papillary or follicular or both) was found in nine cases (2.5%). Testicular tumors (LCCSCT) were diagnosed in 54 patients (41% of the males) and ovarian lesions in 31 patients (14% of the females). Among the nonendocrine components of CNC, lentiginosis was observed in 248 patients (70%); other pigmented lesions (blue, Spitz, and compound nevi and café-au-lait spots) were seen in 177 patients (50%). Pigmentary lesions were the most frequent nonendocrine manifestation of CNC.

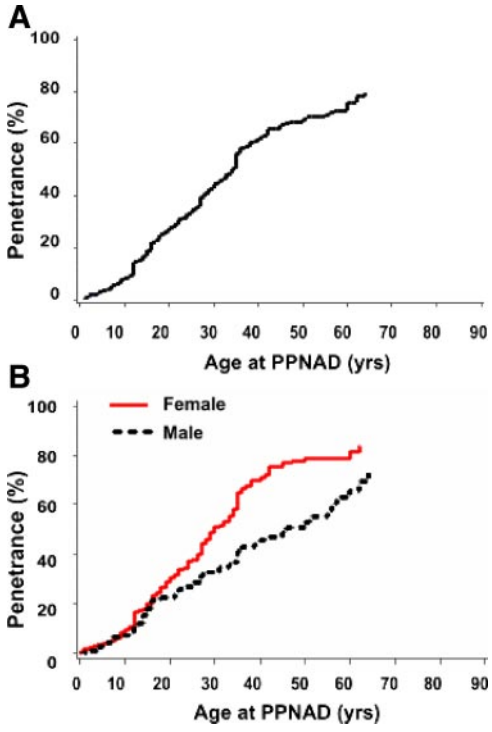

FIG. 1. Age of diagnosis of PPNAD. A, Age of diagnosis of PPNAD for all the patients. The estimated median age of diagnosis was 34 yr (IQR, 19–60). B, Age of diagnosis of PPNAD by gender (females in *red*, n = 221 patients, of whom 150 were diagnosed with PPNAD; males in *black*, n = 152 patients, of whom 62 were diagnosed with PPNAD).

Cardiac myxomas occurred in 112 patients (32%). The median age at diagnosis of the first cardiac tumor was 50 yr (IQR, 32-NR), but there was a large range in the age at diagnosis; the tumor was detected as early as 3 yr of age and as late as 67 yr. In 62 patients (55% of the total number of patients with myxomas), the tumors were multiple (there were two lesions in 33 patients (30%), three lesions in 21 patients (19%), and multiple and/or recurrent up to 10 tumors in two patients.

Skin and breast myxomas occurred in 20% of the total and in 20% of the female patients, respectively; these percentages are likely to underestimate the true incidence of these lesions because not every skin lesion in every patient was biopsied, and breast magnetic resonance imaging (14) was obtained in only a small number of women. Among the other significant nonendocrine tumors was PMS; this rare type of schwannoma was found in 28 patients (8%).

At the time of this analysis, 25 of the 353 patients had died from CNC-related causes. Four succumbed to metastatic PMS, one to thyroid carcinoma and, interestingly, 14 patients (56% of all deaths) to metastatic cancer, including five from pancreatic cancer.

PRKAR1A molecular genetic analysis

Of 185 kindreds affected by CNC, 114 (62%) had a detectable *PRKAR1A* mutation or a deletion (19); among the total of 80 *PRKAR1A* defects, six were missense mutations; 33 led to a frame shift through an insertion, deletion, or another change involving a few pairs of bases; 19 were nonsense mutations; and 20 mutations involved a splice site. Two deletions were found by Southern blot analysis and involved large intronic and exonic regions of the gene (19).

In total, 258 of the 353 patients (73%) carried a *PRKAR1A* defect (Fig. 2) that could be anywhere in the *PRKAR1A* gene (Fig. 2), although mutations involving exons 2, 3, 5, 7, and 8 were more frequent ($P < 0.05$); 16 mutations (20%) were found in intronic sequences and affected splicing (13, 16, 21). Two mutations were more prevalent and occurred independently in several unrelated families of various ethnic backgrounds: the c.709-7del6 in intron 7 (20) and the c.491-492delTG in exon 5 (formerly known as exon 4B) (12, 13) that were observed in 14 and 11 families, respectively; these were the two single "hot spots" of the *PRKAR1A* gene. The c.491-492delTG was also reported by Veugelers *et al.* (28) in keeping with a hot spot mutation. For the two hot spot mutations (c.709-7del6 and c.491-492delTG), a founder effect was excluded in most families as we have previously reported (12, 13). In at least one case (where parental DNA was available), the c.491-492delTG was proven to be a *de novo* mutation. When parental DNA was available for genetic analysis, it appeared that more than 85% of the sporadic cases with a *PRKAR1A* mutation had a *de novo* mutation.

Sixty-three (79%) mutations were shown to be subject to NMD (Refs. 12, 13, 16, and 19–21, and this report); these mutations were present in 82% of the 258 patients with *PRKAR1A*

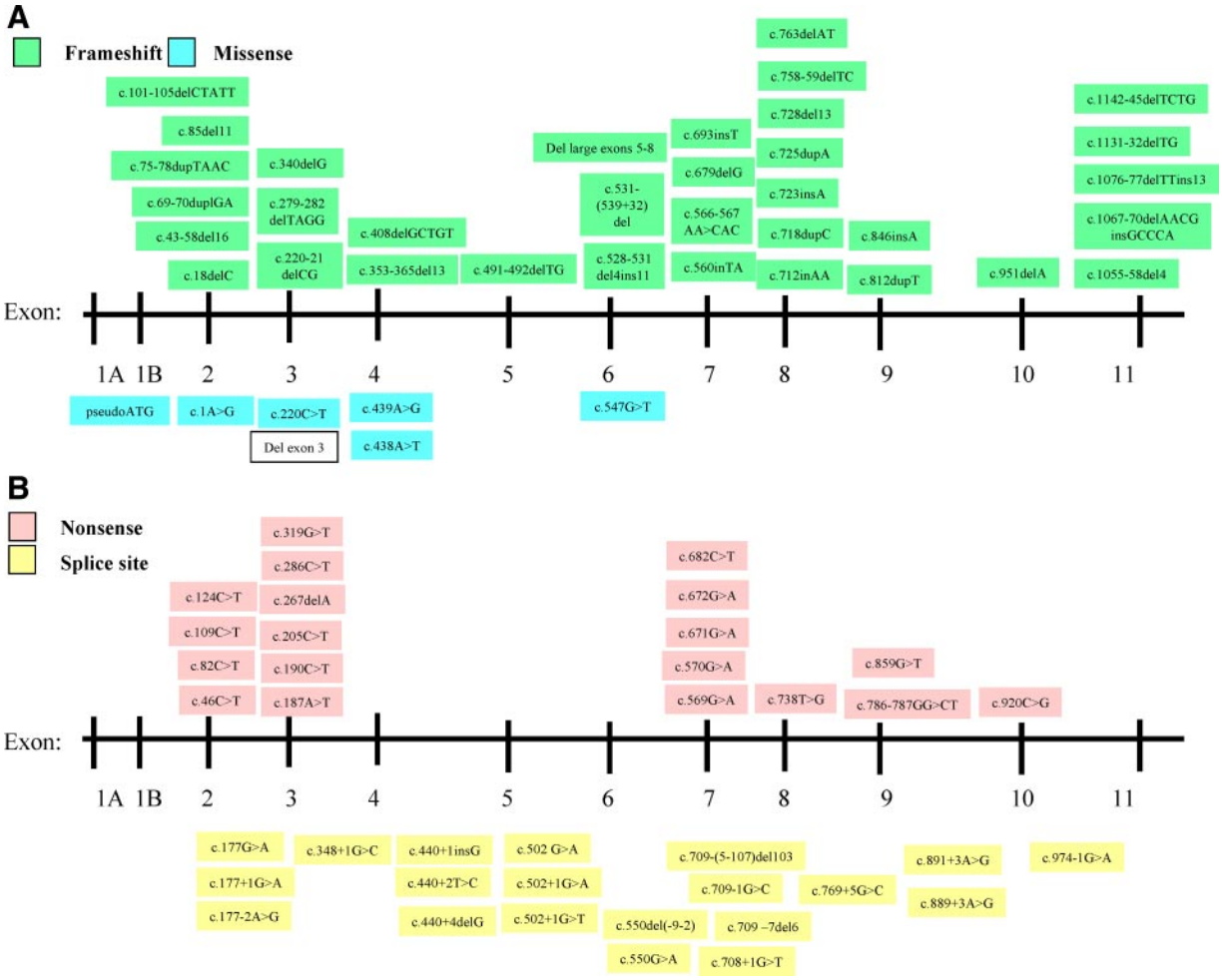

FIG. 2. Mutations and large deletions of the *PRKAR1A* gene. Gene localization of 80 *PRKAR1A* mutations or large deletions found in 258 patients with *PRKAR1A* genetic defects; mutations are marked below the schematic representation of the *PRKAR1A* gene that contains 11 exons. A, Mutations or deletions leading to a frameshift (*green*) or mutations creating a missense substitutions (*blue*). B, Mutations leading to nonsense changes (*pink*) or mutations or deletions altering splice sites (*yellow*).

defects. The remaining 17 mutations are expressed at the mRNA level (16, 19, 21).

PRKAR1A mutations and phenotypic correlation

The overall penetrance of CNC among *PRKAR1A* mutation carriers was 97.5%; only two of the 258 patients did not meet the reported (14) diagnostic criteria. In total, *PRKAR1A* mutations were observed in only 37% of patients classified as "sporadic"; in contrast, 80% of the patients with family history of CNC had a *PRKAR1A* defect ($P < 0.0001$).

Subsets of patients were identified that pointed to specific genotype-phenotype correlation: First, among patients presenting with isolated PPNAD, those that were younger than 8 yr were only rarely *PRKAR1A* mutation carriers (Figs. 1 and 3 and Table 1). Furthermore, most *PRKAR1A* mutation carriers with isolated PPNAD had a germline c.709-7del6 mutation ($P < 0.0001$), consistent with our previously published observation (20). Most of the remaining isolated PPNAD patients with a *PRKAR1A* genetic defect carried the Met1Val mutation (13). Second, myxomas in all locations (heart, skin, and breast), thyroid tumors, PMS, and LCCSCT were more frequent in patients with *PRKAR1A* defects than in those without (Table 1). In addition, cardiac myxomas ($P = 0.02$), thyroid tumors ($P = 0.03$), and LCCSCT ($P = 0.04$) presented at a significantly younger age in *PRKAR1A* mutation carriers (Fig. 3).

Other significant observations were: 1) mutations located in exons (*vs.* those in introns) were more often associated with acromegaly, cardiac myxomas, lentigines, and PMS ($P = 0.04$); 2) the hot spot c.491-492delTG mutation was most significantly associated with cardiac myxoma, lentigines, and thyroid tumors when compared against all other *PRKAR1A* defects ($P = 0.03$); and 3) mutations that escaped NMD and led to an alternate, usually shorter, protein were associated with an overall higher total number of CNC manifestations ($P = 0.04$).

Discussion

The present investigation is the first attempt to identify a genotype-phenotype correlation for *PRKAR1A* sequence defects using the largest population of patients with CNC available. The study led to several, significant observations.

First, several lines of evidence supported the notion that CNC is a clinically and molecularly heterogeneous disorder. The study

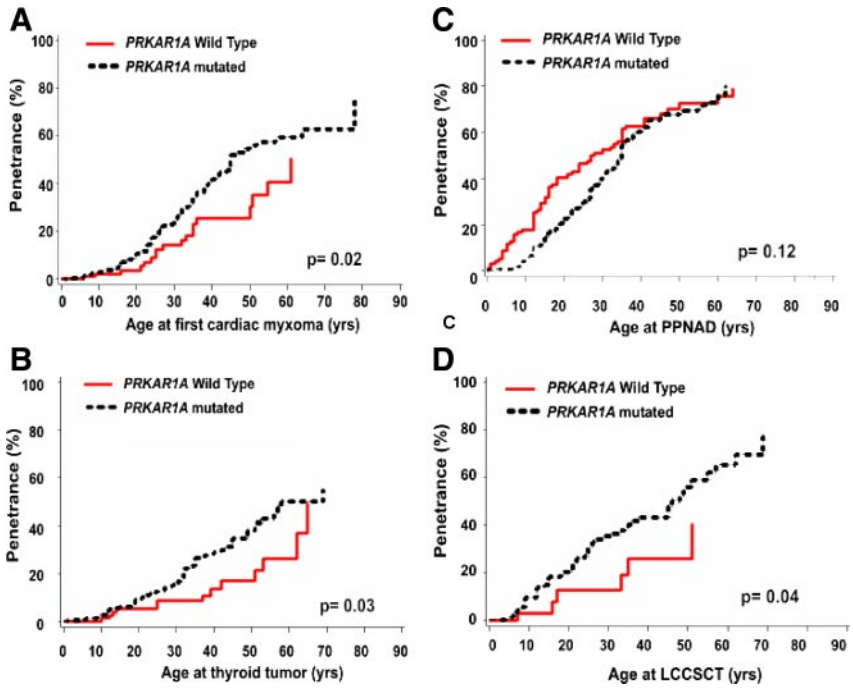

FIG. 3. Age of diagnosis of cardiac myxoma, thyroid tumor, PPNAD, and LCCSCT according to *PRKAR1A* genotype. The figure shows the estimated age of diagnosis in patients without (*red line*, n = 95 patients) or with *PRKAR1A* germline mutation (*dotted black line*, n = 258 patients), with the respective *P* value of the log-rank test comparing the two groups. A, Cardiac myxoma: for patients with a *PRKAR1A* mutation, the estimated median age of diagnosis of the first tumor was 45 yr (IQR, 30–78). B, Thyroid tumors: for patients with a *PRKAR1A* mutation, the estimated median age of diagnosis of the first thyroid tumor was 69 yr (IQR, 40-NR). C, PPNAD: for patients with a *PRKAR1A* mutation, the estimated median age of diagnosis of PPNAD was 35 yr (IQR, 22–60). D, LCCSCT: for male patients with a *PRKAR1A* mutation, the estimated median age of diagnosis of LCCSCT was 46 yr (IQR, 22–69).

showed that within the population of patients currently diagnosed with CNC there are at least three groups: 1) patients who are *PRKAR1A* mutation carriers and who have at least two of the manifestations of the originally described triad of "myxomas, spotty skin pigmentation, and endocrine overactivity"; within this population of patients, there is some genotype-phenotype correlation that is important for both clinical prognosis and *PRKAR1A* molecular and cell biological investigations; 2) another group of patients who do not have *PRKAR1A* sequence defects, deletions, or *PRKAR1A* locus-specific genomic abnormalities appeared to present later, usually in a sporadic fashion, and with a slightly different phenotype, but still meets diagnostic criteria for CNC; and 3) patients with isolated PPNAD.

PRKAR1A mutation carriers with classic CNC manifestations usually came from families that included other affected individuals; they presented with clinically significant disease at a younger age and had more myxomas, schwannomas, and thyroid and gonadal tumors than patients without *PRKAR1A* mutations. They were also more likely to be severely affected, if the *PRKAR1A* mutations were expressed at the protein level. This is consistent with earlier reports on a smaller number of patients (16, 19, 21). It is unclear why this happens because both expressed (16, 19, 21) and nonexpressed mutants (12, 13, 16) result in increased PKA activity without apparent quantitative differences between the two (21). A dominant-negative effect of the expressed mutant protein could explain these differences. Indeed allelic losses, which are frequent in CNC tumors, are not required for mutants that escape NMD (16). One other hypothetical explanation is that the RIα protein may also have PKA-independent effects. For example, a direct involvement with the mammalian target of rapamycin (mTOR) was recently proposed (29) along with the previously suggested interaction with the PAP7 protein (30). A mutant RIα might have different protein-protein interactions that are associated with apparently increased tumorigenic potential and are not limited to inadequate control of PKA catalytic subunits.

Within this group of individuals, there were certain mutations that were associated more frequently with certain phenotypes.

TABLE 1. CNC manifestations in patients according to *PRKAR1A* genotype

Manifestation	*PRKAR1A* wild type, n (%)	*PRKAR1A* mutated, n (%)	OR univariate	95% CI	*P*[a]
Lentiginosis	47 (50)	201 (78)	3.60	2.19–5.93	<0.0001
PPNAD	63 (66)	149 (58)	0.69	0.43–1.14	0.145
Isolated PPNAD	22 (23)	22 (9)	0.31	0.16–0.59	0.0002
Cardiac myxoma	19 (20)	93 (36)	2.26	1.28–3.96	0.004
Acromegaly	12 (13)	30 (12)	0.91	0.44–1.86	0.796
Thyroid tumors	15 (16)	73 (28)	2.11	1.14–3.89	0.016
Psammomatous melanotic schwannoma	3 (3)	25 (10)	3.29	0.97–11.16	0.044
LCCSCT	6 (18)	48 (49)	3.39	1.40–8.21	0.002
Ovarian lesions	6 (10)	25 (16)	1.59	0.63–4.01	0.244
Breast myxomas	4 (6)	38 (24)	4.05	1.41–11.67	0.003
Skin myxomas	5 (5)	64 (25)	5.94	2.31–15.26	<0.0001
Cutaneous other pigmented lesions	36 (38)	141 (55)	1.98	1.22–3.20	0.0052
Sporadic presentation	60 (63)	53 (20)	0.15	0.09–0.25	<0.0001

The table gives the number (frequency expressed as pecentage) of patients without *PRKAR1A* germline mutation (*PRKAR1A* wild type) and with *PRKAR1A* mutation for each CNC manifestation. OR univariate, Odds ratio computed by univariate logistic regression; CI, confidence interval.

[a] χ^2 or Fisher tests.

The hot spot mutation c.709-7del6 was associated mostly with PPNAD. The other hot spot mutation, c.491-492delTG, was seen more frequently in association with cardiac myxomas, lentigines, and thyroid tumors. Because both of these hot spot mutations lead to NMD and produce no mutant RIα protein (at least in lymphocytes, fibroblasts, and adrenal cells) (12, 13, 16, 20), it is difficult to envisage on what molecular mechanisms underlie these phenotypic differences. One suggestion is that small amounts of mutant RIα protein are in fact made, at least in certain tissues. If this is the case, then it is possible that we were able to identify the phenotypes associated with these two mutations only because they were so frequent, and that additional phenotypic differences between the various *PRKAR1A* mutations leading to NMD will emerge as the number of observations increases.

The penetrance of CNC manifestation in *PRKAR1A* mutation carriers is almost complete, but one should keep in mind that mean age at last follow-up in this cohort is 34 yr. As shown by the Kaplan-Meier survival curves, median age at diagnosis is 35 and 50 yr for PPNAD and cardiac myxomas, respectively. This clearly illustrates that penetrance of CNC manifestations increases during adulthood.

The second group of patients (n = 95) met the diagnostic criteria for CNC or PPNAD but had no abnormalities of the *PRKAR1A* gene or its genomic locus at chromosome 17q22–24. Although most of these patients in the current series had sporadic disease (only 37% of sporadic cases carried a *PRKAR1A* defect), this group included families that collectively were mapped to chromosome 2, the *CNC2* locus on 2p16 (5, 6, 13). Analysis of the associated clinical phenotype revealed for the first time some phenotypic differences for *CNC2* patients: they presented later in life, were unlikely to have family history of the disease, and were also less likely to develop myxomas, thyroid tumors, PMS, and LCCSCT. There were no differences in the prevalence of lentigines and other pigmented skin lesions between this group and the patients with *PRKAR1A* mutations. Very few CNC2 patients with three or more manifestations had clinically proven PPNAD but acromegaly was equally present in the CNC1 and CNC2 groups. It appears that CNC2 patients exhibit a hamartomatosis/lentiginosis syndrome with fewer and later presenting endocrine and other tumors. Because PKA abnormalities exist in tumor tissues that exhibit chromosome 2p16 dosage changes (31), it is tempting to speculate that the responsible genetic defect(s) in these patients are *PRKAR1A* molecular partners or regulators.

The third distinct group of patients in this study had PPNAD only. It now appears clear that very young patients (before the age of 8 yr) with ACTH-independent, micronodular adrenocortical disease and no other tumors or lentigines very rarely, if ever, have CNC or *PRKAR1A* mutations. Because only a few of these patients have *GNAS* or other known defects (*PDE11A* or *PDE8B*) (24–26), there are as yet unidentified molecular cause(s) of isolated micronodular adrenocortical disease. Given the involvement of the cAMP-signaling pathway in all forms of benign adrenocortical tumors, and the cortisol-producing hyperplasias in particular (32–34), one can hypothesize that the defects in these patients are in related molecules.

Patients with *PRKAR1A* defects presented with PPNAD later. In addition, after the teenage years, female patients with PPNAD exceeded males; by the age of 40 yr, more than 70% of female carriers of *PRKAR1A* defects had manifested with PPNAD, whereas only 45% of males with CNC had clinical evidence of this disease. Cushing syndrome and adrenocortical tumors, in particular, are more frequent in females (35). *PRKAR1A* expression is not known to be dependent on or affected by gender in humans or mice (36–38), and there were no sex differences in tumor development in the related transgenic rodent models (37–39). The observed differences in the prevalence of PPNAD between female and male carriers of *PRKAR1A* defects may therefore be an example of *PRKAR1A*'s tumorigenic potential modified by tissue-specific microenvironmental factors.

In conclusion, the largest analysis to date of *PRKAR1A* mutations in patients with CNC confirmed prior suggestions of molecular heterogeneity (13) and also provided important data for *PRKAR1A*'s apparent versatile role in causing specific phenotypes. These data are valuable for genetic counseling of CNC patients and their families, for treating CNC and its various manifestations, PPNAD in particular, and for directing molecular investigations of PKA signaling pathway in the future.

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