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Genetic heterogeneity and spectrum of mutations of the *PRKAR1A* gene in patients with the Carney complex

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Carney complex (CNC) is an autosomal dominant multiple neoplasia syndrome, which has been linked to loci on 2p16 and 17q22–24. We recently reported that *PRKAR1A*, which codes for the type 1A regulatory subunit of protein kinase A (PKA), is a tumor suppressor gene on chromosome 17 that is mutated in some CNC families. To evaluate the spectrum of *PRKAR1A* mutations, we identified its genomic structure and screened for mutations in 54 CNC kindreds (34 families and 20 patients with sporadic disease). Fourteen families were informative for linkage analysis: four of four families that mapped to 17q had *PRKAR1A* mutations, whereas there were no mutations found in seven families exhibiting at least one recombination with 17q. In six of the latter, CNC mapped to 2p16. *PRKAR1A* mutations were also found in 12 of 20 non-informative families and 7 of 20 sporadic cases. Altogether, 15 distinct *PRKAR1A* mutations were identified in 22 of 54 kindreds (40.7%). In 14 mutations, the sequence change was predicted to lead to a premature stop codon; one altered the initiator ATG codon. Mutant mRNAs containing a premature stop codon were unstable, as a result of nonsense-mediated mRNA decay. Accordingly, the predicted truncated PRKAR1A protein products were absent in these cells. We conclude that (i) genetic heterogeneity exists in CNC; and (ii) all of the CNC alleles on 17q are functionally null mutations of *PRKAR1A*. CNC is the first human disease recognized to be caused by mutations of the PKA holoenzyme, a critical component of cellular signaling.

INTRODUCTION

Carney complex (CNC; MIM 160980) (1) is a multiple neoplasia syndrome that is inherited in an autosomal dominant manner (2). It was initially described in 1985 as a complex of 'myxomas, spotty skin pigmentation and endocrine overactivity' (3). Patients with some components of the complex, in particular cardiac myxomas and pigmentation anomalies, had previously been described under the acronyms NAME (nevi, atrial myxomas and ephelides) or LAMB (lentigines, atrial myxomas and blue nevi) (4,5). Today, it is accepted that most, if not all, of these patients had CNC (6). CNC has recently been recognized as a form of multiple endocrine neoplasia (MEN) (7,8), because patients often have synchronous tumors of two or more endocrine organs, including primary pigmented nodular adrenocortical disease (PPNAD), growth hormone and prolactin-producing pituitary adenoma, thyroid nodules or carcinoma, testicular neoplasms [mainly large-cell calcifying Sertoli cell tumor (LCCSCT)] and ovarian cysts (9–15). Additional manifestations of CNC include psammomatous melanotic schwannoma (PMS), epithelioid blue nevus, breast ductal adenoma and a rare bone tumor, osteochondromyxoma (16–20).

Linkage analysis of families with CNC initially demonstrated a genetic locus on 2p16 with an aggregate LOD score of 5.97 ($\theta = 0.03$), although no single family in that study had a LOD score of >1.8 (21). Additional genetic studies identified families in whom CNC did not segregate with 2p16 markers (22,23). A genome-wide screen with these families demonstrated linkage to a locus on 17q22–24 (24). Most recently, following loss-of heterozygosity (LOH) studies in CNC tumors and genetic linkage analysis in newly collected kindreds, we identified mutations of the *PRKAR1A* gene, encoding the type 1A regulatory subunit of protein kinase A (PKA), in 17q22–24-mapping families and several sporadic cases (25). To further these observations, we now report the complete genomic structure of the *PRKAR1A* tumor suppressor gene and the results of its analysis in our entire cohort of 54 kindreds collected over the past 20 years at the National Institutes of Health (Bethesda, MD) and the Mayo Clinic (Rochester, MN). These data confirm genetic heterogeneity in CNC and indicate that, despite the large number of *PRKAR1A* mutations, all of these mutations are functionally equivalent to

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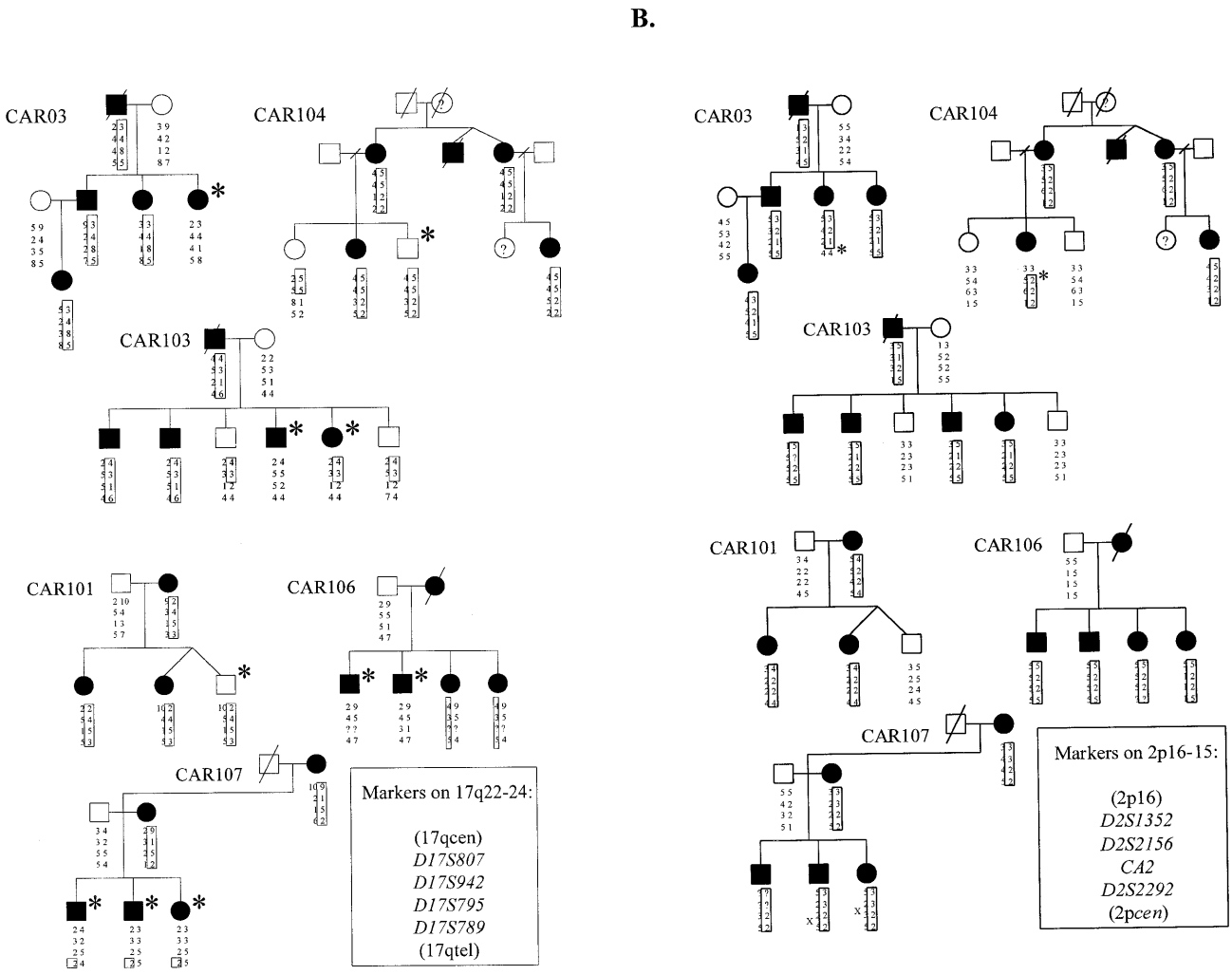

Figure 1. (**A**) Haplotypes for four markers encompassing the *PRKAR1A* locus on chromosome 17 (area 17q22–24) of six families with CNC that have been described (21). Haplotypes were constructed based on the minimum number of recombinations that would be required to produce each chromosome. The allelotype most likely to segregate with the disease chromosome is boxed. Accordingly, the asterisk indicates recombinations. (**B**) Haplotypes for four markers encompassing the CNC locus previously identified on 2p16 (21) were generated as above. The asterisk indicates an informative recombinant in this chromosome. X, recombination in the other chromosome.

null mutations, leading to a single molecular phenotype causing the disease in chromosome 17-mapping families.

RESULTS

Genetic linkage analysis

Of the 34 kindreds in whom the disease was familial, 14 were informative for linkage analysis. The remaining 20 families were either too small to provide meaningful linkage data or had too many phenotypic uncertainties. To perform linkage studies, each family was analyzed for segregation using markers from 2p16 and 17q22–24 that had previously been shown to lie around the respective disease loci (21,24,25). The location of most of these markers has been confirmed on physical maps of the two regions (26–28). In most cases, genetic phase had to be inferred; haplotypes were constructed based on the minimum number of recombinations that would be required to produce each chromosome (Fig. 1).

This analysis generated four types of family: (i) those that mapped to 17q with recombinations with 2p (chromosome 17 families, $n = 4$) (see below); (ii) those that mapped to 2p with recombinations with 17q (chromosome 2 families, $n = 6$) (Fig. 1); (iii) those that had no recombinations with either locus ($n = 3$) (data not shown); and (iv) those that had recombinations with both loci ($n = 1$) (data not shown). One family previously thought to map to chromosome 2 was reassigned to the chromosome 17 group after the detection of a *PRKAR1A* mutation in the family and reassessment of the clinical phenotypes [this is family CAR108 (21,25)]. The misassignment in this kindred

Table 1. Two-point LOD scores between the disease and 2p16 and 17q22–24 markers for kindreds with Carney complex that have at least one recombination with the 17q22–24 *PRKARIA* locus

Chromosome	marker	θ					θ				
		0	0.1	0.2	0.3	0.4	0	0.1	0.2	0.3	0.4
		CAR03					CAR101				
2	*D2S1352*	0.9	0.72	0.52	0.32	0.13	0.36	0.35	0.28	0.17	0.06
	D2S2156	0.9	0.72	0.52	0.32	0.13	0.6	0.47	0.32	0.17	0.05
	CA2	0.9	0.72	0.52	0.32	0.13	0.6	0.47	0.32	0.17	0.05
	D2S2292	0	0	0	0	0	0.3	0.21	0.13	0.06	0.02
	D2S2153	–3.1	–0.19	0.01	0.07	0.06	–∞	0.02	0.12	0.09	0.03
17	*D17S807*	–3.1	–0.19	0.01	0.07	0.06	–∞	–0.19	0.01	0.07	0.06
	D17S942	0	0	0	0	0	–∞	–0.44	–0.19	–0.08	0.02
	D17S795	–3.1	–0.19	0.01	0.07	0.06	–∞	–0.89	–0.39	–0.15	–0.04
	D17S789	0.3	0.26	0.2	0.15	0.08	–∞	–0.48	–0.23	–0.10	–0.03
		CAR103					CAR106				
2	*D2S1352*	1.2	0.93	0.64	0.34	0.1	0.4	0.29	0.19	0.09	0.03
	D2S2156	1.2	0.93	0.64	0.34	0.1	0.58	0.43	0.28	0.14	0.04
	CA2	1.2	0.93	0.64	0.1	0.1	0.43	0.32	0.21	0.1	0.03
	D2S2292	1.2	0.94	0.66	0.37	0.12	0.35	0.25	0.16	0.08	0.02
	D2S2153	1.51	1.23	0.92	0.58	0.21	–3.1	–0.44	–0.19	–0.08	–0.02
17	*D17S807*	–0.11	–0.08	–0.04	–0.02	0	–3.1	–0.44	–0.19	–0.08	–0.02
	D17S942	–∞	–0.44	–0.19	–0.08	–0.02	–3.1	–0.44	–0.19	–0.08	–0.02
	D17S795	–∞	–0.14	–0.01	0.01	0.01	–3.1	–0.44	–0.19	–0.08	–0.02
	D17S789	–3.1	–0.44	–0.19	–0.08	–0.02	–3.1	–0.44	–0.19	–0.08	–0.02
		CAR104					CAR107				
2	*D2S1352*	–∞	–0.09	0.02	0.03	0.01	0	0	0	0	0
	D2S2156	0.84	0.63	0.42	0.22	0.06	–∞	–0.19	0.01	0.07	0.06
	CA2	0.84	0.63	0.42	0.22	0.06	0.6	0.47	0.32	0.17	0.05
	D2S2292	0.51	0.36	0.22	0.11	0.03	0.6	0.47	0.32	0.17	0.05
	D2S2153	–3.77	–0.58	–0.24	–0.08	–0.02	0.9	0.77	0.61	0.44	0.24
17	*D17S807*	–∞	–0.26	–0.08	–0.02	0	–∞	–2.1	–1.19	–0.67	–0.29
	D17S942	–0.23	–0.08	–0.03	–0.01	0	–∞	–2.1	–1.19	–0.67	–0.29
	D17S795	–∞	–0.06	0.04	0.11	0.01	–∞	–2.1	–1.19	–0.67	–0.29
	D17S789	0.25	0.18	0.11	0.05	0.01	–∞	–2.1	–1.19	–0.67	–0.29

arose from the inclusion of an apparently unaffected individual with only pigmentation abnormalities from among the various manifestations of CNC; this patient, who also had two unaffected children, was phenotyped as 'unaffected' according to the CNC diagnostic criteria (see Materials and Methods). On analysis, the patient was found to have the familial mutation, although his children did not (25).

Owing to the fact that no chromosome 2-mapping family with a LOD score of >3 has been detected to date, there is debate about the existence of a CNC gene in this location. To re-examine this question, LOD score analysis of the six families who exhibited neither full segregation with chromosome 17 markers (Fig. 1A) nor *PRKAR1A* mutations was performed (Table 1). These families not only excluded the 17q22–24 locus, but also generated a positive two-point LOD score for markers on 2p16. Cumulative two-point LOD scores for the six families yielded a maximum two-point LOD score of 3.97 at θ for the marker *CA-2* at 2p16 (Table 2). Haplotype (Fig. 1B) and multipoint LOD score (MLS) analyses also suggested linkage with 2p16 (maximum MLS of 4.5 for *CA-2*) (data not shown).

Although the above data confirm genetic heterogeneity in CNC, they cannot be conclusive about the existence of a 2p16 CNC locus. The finding of a large CNC family (maximum simulated two-point LOD score of 1.8 with hypothesis of linkage to a putative locus) with at least one recombination with 2p16 markers, in whom no *PRKAR1A* mutation could be found and the 17q22–24 locus could be firmly excluded

Table 2. Aggregate two–point LOD scores between Carney complex and markers on 2p16 for 6 families with at least one recombination with 17q22–24

Marker (2p*tel*–2p*cen*)	θ			
	0	0.1	0.2	0.3
D2S1352	–∞	2.2	1.65	0.95
D2S2156	–∞	2.99	2.19	1.26
CA2	3.97	3.07	2.11	1.15
D2S2292	2.96	2.23	1.49	0.79
D2S2153	–∞	1.02	1.38	1.09

Table 3. Revised gene structure of *PRKAR1A*

Exon	Splice acceptor	Size (bp)	Splice donor
Exon 1A		144	CAG gta
Exon 1B		102	CAG gtg
Exon 2	cag AGA	183	AAG gta
Exon 3	cag GAG	171	AAG gta
Exon 4A	**tag GTT**	**92**	**AAG gta**
Exon 4B	**cag TGA**	**62**	**AAG gta**
Exon 5	tag GTG	47	**GAT gta**
Exon 6	**tag TCT**	159	ATG gta
Exon 7	tag GGA	61	**TAG gtg**
Exon 8	**cag AGT**	122	GAG gta
Exon 9	tag GGG	82	TTG gta
Exon 10	cag GTG		

Bold text indicates changes from the intron–exon structure of Solberg *et al.* (30). Note that exon 4 in that paper has been divided into two smaller exons (4A and 4B).

[family CAR102 (21)], confirms heterogeneity but does not support the existence of a 2p16 locus. On the other hand, the findings of CNC tumor genetic changes involving genomic material from 2p16 (29) is supportive of a second CNC gene located in that region.

As part of the genetic analysis of CNC kindreds, we also examined the possibility of a founder effect, especially in those families that shared a common *PRKAR1A* mutation (see below) or those that came from geographically proximate regions. Haplotype analysis did not show a common origin of the linking chromosome (data not shown). This observation suggests that, at least for the *PRKAR1A* gene, each family represents a *de novo* mutational event, and is consistent with the relatively large number of sporadic cases of CNC (2,3,6).

Structure of the *PRKAR1A* gene

The previously reported genomic structure of the *PRKAR1A* gene (30) was revised (Table 3), based on the comparison of this gene's cDNA (30,31) to available online sequences of genomic clones from the area (32). A bacterial artificial chromosome (BAC) clone (RPCI 62-F-10) containing exons 3–10 of the gene was identified from the GenBank database and compared with the *PRKAR1A* cDNA sequence. Firstly, what had previously been considered a single exon (exon 4) actually appeared to be two small exons separated by a 199 bp intron; these exons we termed exons 4A and 4B (Table 3). Secondly, there were apparent shifts in some of the previously suggested intron–exon junctions (30); the new boundaries are in compliance with conventional GT-AG rules (33).

To generate the sequence surrounding the remaining coding exon of the gene (exon 2), an additional BAC (CITB 321-G-8) containing this region of the gene was isolated. Furthermore, despite the previous report that intron 2 of this gene was large (30), the entire *PRKAR1A* gene was contained on this single BAC (data not shown). Direct sequencing of the clone identified intronic sequence around the second exon. Based on available sequences and those generated during this study, oligonucleotide primers were designed to amplify each exon of the *PRKAR1A* gene specifically (Table 4).

Identification of the *PRKAR1A* mutations in CNC kindreds

Recently, we reported the identification of CNC-associated mutations in seven kindreds, six families and one patient with sporadic disease (25). One of these families (designated MYX.01) had familial cardiac myxomas only, without other clinical manifestations of CNC (25,34). In this report, we include these seven kindreds for the purposes of completeness and statistical analyses.

All 54 kindreds were screened for mutations in the *PRKAR1A* gene using the primers listed in Table 4. The samples were first analyzed for heterozygosity using denaturing high performance liquid chromatography (DHPLC). Heterozygote samples were then sequenced. This analysis detected mutations in 15 kindreds, in addition to the original 7. In 10 of the newly identified kindreds, the *PRKAR1A* mutation segregated with CNC in more than one family member; in five kindreds the mutation was found only in the proband (sporadic cases). Table 5 contains a comprehensive list of all *PRKAR1A* mutations identified to date in our kindreds and their relative frequency; the location of these genetic defects is shown in Figure 2.

In our earlier report (25), four kindreds shared the same *PRKAR1A* mutation, a deletion of two nucleotides (TG) following base pair 578 of the cDNA sequence (del578TG). It appears that, in the entire cohort of CNC families, two additional kindreds shared this mutation in exon 4B without sharing common 17q22–24 allelotypes (see above). Thus, 578delTG is the most common *PRKAR1A* mutation and has arisen independently in at least six kindreds. We have suggested that this 'hot spot' in the *PRKAR1A* gene may be the result of a 'TGTG' sequence pattern that was present at the deletion site (25,35).

The only other *PRKAR1A* mutations that appeared to have arisen *de novo* in more than one kindred with CNC were: (i) a nonsense point mutation in exon 2 (211C→T) that was found in two kindreds; and (ii) a G→C transversion in the 5′ splice site of intron 3 of the gene (exon 3 IVS+1G→C) that was also found in two kindreds (Table 5).

Of the remaining 12 mutations, three that were previously described (25) and nine newly reported here, each represents a unique mutation (Table 5). The mutations have been detected throughout the coding region of the *PRKAR1A* cDNA and

Table 4. Primers used for amplification of *PRKAR1A* exons

Exon	Left primer	Size (bp)	Right primer
5′ $(CA)_n$	CCCCCACTGTACTGAACACC	167	CATGGCCACACAGCTAACAT
Exon 1A	AGTCGCCCACCTGTCATCT	284	CACTTCTCCTTTCCGCAGTC
Exon 1B	CATTGACGTCAGTAGCCGAA	254	ATCTTGGATCGGTCCAGCTC
Exon 2	CCTAGTCCCCACTTCCCTGT	364	ATCACCTCATCATCTCCCCA
Exon 3	CATGCCGAAGGATCTCATTT	327	ATGGATGAAGTTCCACCCTG
Exon 4A	CAGGTTGCAAACGTGAAATG	397	CTGCGATAAAGGAGACCGAA
Exon 4B	AGCCAAAGCCATTGAAAAGA	362	GCCTCCTCTCCCGTAACAAT
Exon 5	TTGCTTGATTTTCTTTCCCC	270	ATTCTTATTGCTCGGAAGCG
Exon 6	TCATTTAACTCGTCAGAAATCACC	370	TTCTAAATCACACTCTCAAACACCA
Exon 7	GGCATAATATTGGCGGAAAA	363	AAGGCTTTTCCCAAGTCCAT
Exon 8	AGAATGTTGAATGGGCATGG	331	TTAGCCCACTCTTTCCCTCTT
Exon 9	CACCCTGGGTTTGAGAGTGT	272	TTCCCTCTCAGAGCCAAAAA
Exon 10	CCCATCTTTGCTTTCTCCAG	314	AACAGACAGGAAGCTGCGAT

Table 5. Mutations of the *PRKAR1A* gene in patients with Carney complex

Mutations	Exon[a]	No. of alleles	Effect	Frequency
Point				
88A→G	2	1	Abolishes initiator ATG	4.5
169C→T	2	1	Nonsense	4.5
211C→T	2	2	Nonsense	9.1
769C→T	6	1	Nonsense	4.5
873GG→CT[b]	8	1	Nonsense	4.5
Frameshift				
188delCTATT	2	1	Frameshift/stop	4.5
578delTG[b]	4B	6	Frameshift/stop	27.3
618delTGAT	5	1	Frameshift/stop	4.5
653AA→CAC	6	1	Frameshift/stop	4.5
781insT	6	1	Frameshift/stop	4.5
799insAA	7	1	Frameshift/stop	4.5
Splice site				
Exon 2 IVS–2A→G		1	Unsure	4.5
Exon 3 IVS+1G→C		2	Cryptic splice	9.1
Exon 6 IVS del(–9→–2)		1	Exon skipping	4.5
Exon 8 IVS+3G→A[b]		1	Cryptic splice	4.5
Total		22		100

[a]Exons are numbered according to Table 3.
[b]This mutation was included in ref. 25. For the 578delTG mutations, four kindreds were reported previously and two new kindreds in this report.

involve all exons except 4A, 9 and 10. The mutations tend to cluster in certain exons, with the majority occurring in exons 2 (n = 5) and 6 (n = 4). On the other hand, 4B is the most commonly mutated exon, albeit with a single genetic defect (578delTG) accounting for all of its mutations identified to date (n = 6). It is not surprising, perhaps, that exons 2 and 6 harbor many of the mutations, as these are the largest and third-largest exons of *PRKAR1A*, respectively. On the other hand, the second largest exon, exon 3, has been found mutated in only two cases.

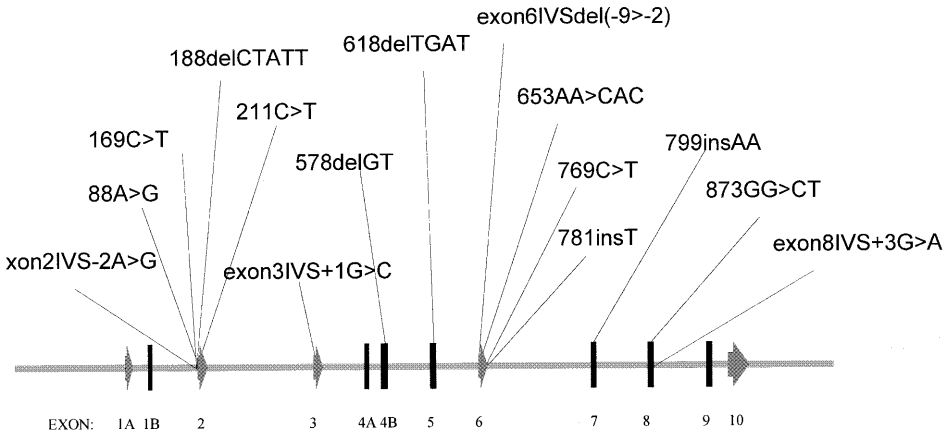

Figure 2. Structure of the *PRKAR1A* gene and locations of all *PRKAR1A* mutations identified to date.

Table 6. Location of polymorphic sites within the *PRKAR1A* gene[a]

Exon	Sequence change	Effect
1A	109A/C	5′-UTR
2	174G/A	Ala (no change)
8	IVS –27G/A	Intronic

[a]Exons are numbered according to Table 3. For exon 1A, sequence numbering is relative to the beginning of exon 1A, which is absent in the reference sequence for *PRKAR1A* (GenBank accession no. NM_002734, to which all other base pair designations refer).

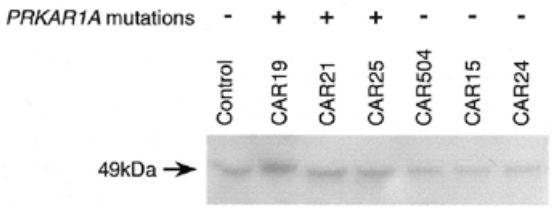

Figure 3. Western blotting of PRKARIA in protein lysates from transformed lymphocytes of a control cell line and CNC patients with (CAR19, CAR21 and CAR25) or without (CAR504, CAR15 and CAR24) mutations in *PRKAR1A*. Only the normal PRKAR1A is detectable, independently of *PRKAR1A* mutation status.

With the exception of the 88A→G mutation, which changes the initiator ATG to a GTG codon, each of the mutations detected in this study is predicted to lead to a premature termination codon. Each of the five point mutations (including the 2 bp change in CAR13) encodes a nonsense mutation. The frameshift mutations also lead to the generation of missense residues, followed by an early termination codon. The effects of the four splice site mutations on the protein coding potential of their respective messages have similar effects (L.S. Kirschner, unpublished data).

During this investigation, we also detected the presence of three *PRKAR1A* polymorphisms, which did not appear to segregate with CNC in the families in whom the disease mapped to 17q22–24 (Table 6). These single nucleotide polymorphisms (SNPs), which either were located in intronic sequences (109A→C and exon 8 IVS –27G→A) or did not change the coded amino acid (174G→A), can be used for linkage studies of CNC families, because they appear to be common in the general population (data not shown). Thus, analysis of the intron 7 G/A polymorphism in family members from the CAR03 and CAR103 kindreds (that are included in Table 1 as potentially mapping to 2p16) confirmed the lack of segregation of the *PRKAR1A* gene with the CNC phenotype in these families (data not shown). The other families included in Table 1 were uninformative for the *PRKAR1A* SNPs.

It should be mentioned that the presence of a *PRKAR1A* pseudogene on chromosome 1 (36) did not interfere with mutational analysis of the functional *PRKAR1A* gene on chromosome 17, because its sequence could not be detected by the methods used above (data not shown).

Overall, four families fully mapped to the chromosome 17 locus and exhibited recombinations with the 2p locus. Each of these families was found to have a mutation of the *PRKAR1A* gene. In contrast, none of the families exhibiting recombination with 17q was found to have a mutation, suggesting that genetic linkage analysis was able to correctly identify the families in whom a mutation search was worthwhile. This observation suggests that genetic linkage will be helpful in identifying families to screen for *PRKAR1A*, such as the large family described by Casey *et al.* (24).

In total, 34 familial cases of CNC were evaluated and 15 were found to carry *PRKAR1A* mutations (44.1%). Also, 7 of 20 (35%) sporadic CNC patients had mutations in that gene. Assuming that 5–10% of mutations may not be detected by our current screening methods, this would suggest that *PRKAR1A*

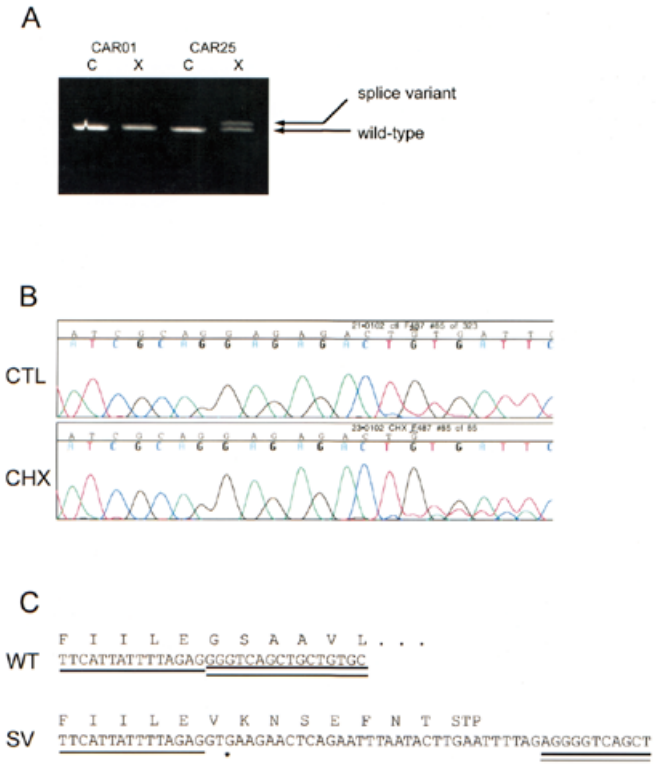

Figure 4. (**A**) RT–PCR products from transformed lymphocytes from patients CAR01.08 (578delTG mutation) and CAR25.03 (exon8 IVS+3A→G mutation) generated after 4 h treatment with vehicle (C) or 100 µg/ml cycloheximide (X). No obvious change is induced in CAR01.08, whereas CAR25.03 exhibits a novel splice product. (**B**) Direct sequencing of RT–PCR products from CAR01.08 shown in (A). The CTL and CHX traces correspond to the sequencing reactions of lanes C and X, respectively. The disruption in the sequence traces exhibited in the CHX lane occurs after base pair 578. (**C**) Sequence analysis of the CHX-induced splice variant seen in CAR25.03, determined after cloning and sequencing of RT–PCR products. The top sequence shows the normal mRNA sequence, whereas the bottom sequence shows the 38 bp insertion containing mRNA detected only after CHX treatment of the cells. Protein translations are indicated above each sequence.

mutations would account for ~50% of patients with CNC, although the actual value in this study is 40.7%.

Penetrance of CNC in kindreds carrying *PRKAR1A* mutations appears to be >97%, because only 1 of 48 mutation carriers (2.08%) (one individual in family CAR108) did not fully meet the diagnostic criteria. However, penetrance of the disease per age group remains to be accurately estimated, an analysis that can be performed after extended family members are screened for *PRKAR1A* mutations.

Effect of the mutation on *PRKAR1A* mRNA levels

Western blot analysis of samples from patients with known truncating mutations did not detect shortened forms of the PRKAR1A protein (Fig. 3). To investigate this phenomenon, we analyzed mRNA in transformed lymphocyte and other tissue cell lines established from CNC patients. Amplification of full-length or shorter forms of the *PRKAR1A* cDNA from kindreds with the 578delTG and exon 8 IVS+3G→A mutations and subsequent sequence analysis demonstrated the presence of normal *PRKAR1A* mRNA only. This suggested that the mutant mRNA was not widely present in the lymphocytes and other cells of these patients, as a result of either decreased transcription or enhanced degradation.

Nonsense-mediated mRNA decay (NMD) is a mechanism by which cells can degrade mRNAs containing premature stop codons before they reach the translational machinery (37,38). This mechanism has been shown to be operational in a variety of human diseases, including Ehlers–Danlos syndrome (39) and Stickler syndrome (40). Treatment of cells with translation inhibitors such as cycloheximide (CHX) or puromycin can abrogate this phenomenon (37,38).

Indeed, treatment of transformed lymphocytes from a patient (CAR01.08) carrying the 578delTG mutation with CHX followed by RT–PCR and sequencing revealed that this treatment led to the stabilization of mRNA containing the expected 2 bp deletion, as evidenced by the disruption of the sequence trace (Fig. 4A and B). Similarly, treatment of transformed lymphocytes from CAR25.03 (carrying the exon 8 IVS+3G→A mutation) with CHX led to the induction of a readily visible splice variant cDNA (Fig. 4A), which was the result of a cryptic splice donor site produced by mutation of the cognate site. Subsequent sequence analysis also showed that this led to the presence of a premature stop codon in the mRNA (Fig. 4C).

In contrast to this, the 88A→G mutation, which abolishes the translational start codon (Table 5) but does not introduce a premature stop codon, would not be predicted to be subject to NMD. Analysis of mRNA from a patient carrying this mutation (family CAR19) demonstrated that the mRNA population either with or without CHX treatment was a 50:50 mixture of wild-type and mutant mRNA (data not shown). In western blotting studies from this patient, no shortened forms of PRKAR1A were detected (Fig. 3). Analysis of the primary sequence of the mRNA revealed that after the cognate ATG, there are two out-of-frame ATG sequences, neither of which is part of a Kozak initiation consensus (41). There was an in-frame ATG what was located within a reasonably good match sequence (at least equivalent to the cognate), which would be predicted to produce a protein lacking the 46 N-terminal amino acids of the protein (data not shown). This protein was not detected in patient's cells, although the mechanism of suppression of translation of this particular transcript has not yet been elucidated.

DISCUSSION

Is there a genotype–phenotype correlation?

Because all of the CNC mutations are predicted to lead to premature peptide chain termination and NMD was shown to operate in these cells, all CNC mutations detected to date should be functionally equivalent to null alleles. Therefore, it follows that any patient with a *PRKAR1A* mutation would be expected to exhibit the same defect at the molecular level. This analysis suggests that differences in patient presentations may be due to contributions from disease-modifying loci located outside the PRKAR1A locus. Convincing evidence for this hypothesis comes from a comparison of CAR01 and CAR20, two large families that share the 578delTG *PRKAR1A* mutation but have dramatically different clinical phenotypes (25,42). All of the affected members of family CAR01 had PPNAD, a disorder completely absent from CAR20. Acromegaly

and cardiac myxomas are strongly featured in CAR20, but are almost absent from CAR01. Many members of CAR20 (although not all) are highly pigmented, whereas this feature is much less prominent in CAR01. Thus, the protean manifestations of CNC appear to be present or absent regardless of genotype, suggesting that a genotype–phenotype correlation does not exist. Until the gene from chromosome 2 is identified and each family or individual definitively genotyped, it will be difficult to make authoritative statements comparing 'chromosome 2' cases with 'chromosome 17' (i.e. *PRKAR1A*) cases. However, in our analysis of the phenotypes of patients with and without *PRKAR1A* mutations, we have not been able to detect any significant clinical differences between these subgroups (L.S. Kirschner, J.A. Carney. and C.A. Stratakis, unpublished data).

Finally, the absence of expression of the abnormal PRKAR1A protein in CNC cells, together with the loss of the normal *PRKARIA* allele in CNC tumors (25), suggests that, in tissues affected by CNC, tumorigenesis is caused by abolition of type I PKA activity. However, the precise mechanisms leading to neoplastic growth and proliferation remain unclear and are the subject of ongoing experiments in our laboratory.

MATERIALS AND METHODS

Patient population, preparation of DNA and linkage analysis

All of the patients described in this study were participants in NIH protocol 95-CH-0059, 'Definition of the genotype and clinical phenotype of primary pigmented nodular adrenocortical disease (PPNAD) and its associated conditions (Carney complex)', initiated in 1995. The clinical history and histopathological specimens from each patient were reviewed by two of the authors (J.A.C. and C.A.S.) to determine diagnosis. Affectation status for familial cases was determined based on the diagnostic criteria proposed by Stratakis *et al.* (8,21). Briefly, 'affected' included all individuals who exhibited two of the following manifestations: lentiginosis and other pigmented skin lesions with characteristic distribution, myxoma (cardiac, breast or other), PPNAD, acromegaly, multiple thyroid nodules, LCCSCT or psammomatous melanotic schwannoma. In the analysis of the familial cases, an individual was also considered affected if he had one of the foregoing manifestations and at least one affected first degree relative. Sporadic cases of the disease were considered to be all patients that had no affected family members; the available first degree relatives of these patients were examined for CNC stigmata.

DNA was prepared from collected blood samples as previously described (21). Linkage analysis was performed with polymorphic microsatellite markers from the 2p16 and 17q22–24 loci (Table 1 and Fig. 1). Additional markers were typed around loci that could not be read with accuracy, using only the informative members of each family (data not shown). The sequences of these primers and genomic order of their loci were derived from the publicly available genomic databases (http://gdbwww.gdb.org/ and http://www-genome.wi.mit.edu) (43,44) and the physical map of the 2p15–16 area that we recently reported (27). Primers for the CA-2 marker have been published elsewhere (22). In several cases, radiation hybrid mapping was also performed to establish the order of individual STSs, as previously described (26,44).

Two-point and multi-point LOD scores were calculated using the LINKAGE (version 5.1) computer software, a dominant model of inheritance, 100% penetrance in both sexes and a gene frequency of 0.001, as previously described (45). All marker allele frequencies were calculated using ILINK, also from the LINKAGE suite of programs, as previously described (21).

DHPLC and sequence analysis

DHPLC was performed using the HELIX system (Varian, Walnut Creek, CA) as described (25). Runs were performed at temperatures recommended by the DHPLC Melt program (http://insertion.stanford.edu/melt.html). Samples that produced aberrant DHPLC traces were selected for further study. After purification of the PCR products (GFX; Amersham Pharmacia Biotech, Piscataway, NJ), samples were subject to sequencing using the BigDye terminator kit (PE Biosystems, Foster City, CA) and analyzed on an ABI 377 sequencer (PE Biosystems). Traces were analyzed with Sequencher 3.0 (Genecodes, Ann Arbor, MI). When the sequence was unclear (e.g. as a result of a frameshift), samples were subject to TA cloning (Invitrogen, Carlsbad, CA) and analysis of individual clones, as described (25).

Cell culture and cycloheximide treatment

Lymphocyte cell lines from individual patients had been established by EBV transformation, as described (21) and maintained in culture in RPMI 1640 with 10% fetal bovine serum. For cycloheximide (CHX) treatment, individual flasks were split into two. One flask was treated with 100 µg/ml CHX (Calbiochem, La Jolla, CA) and the other was treated with vehicle only. Cells were grown for 4 h and then RNA was prepared using the RNAEasy kit (Qiagen, Valencia, CA). First strand cDNA was prepared using an oligo(dT) primer (cDNA synthesis kit; Roche Biochemicals, Indianapolis, IN), which was then used directly for PCR analysis.

Preparation of proteins and western blotting

To prepare total cellular protein extracts, cultured lymphocytes were harvested and pelleted. After washing with phosphate-buffered saline, the cells were resuspended as in 3 vol M-PER reagent (Pierce, Rockford, IL) supplemented with complete protease inhibitor cocktail (Roche Biochemicals). Protein concentrations were determined by the BCA assay kit (Pierce) and 20 µg of protein was resolved in a 4–12% NuPage gel in MES buffer (Invitrogen) before transfer to PVDF membranes. Western blots were performed using the western Breeze kit for murine antibodies as directed by the manufacturer (Invitrogen). Monoclonal antibodies specific for PRKAR1A were obtained from BD Transduction Laboratories (Lexington, KY) and were used as directed by the manufacturer.

NOTE ADDED IN PROOF

In confirmation of our earlier report (25), another paper appeared recently reporting mutations of *PRKAR1A* in three of five CNC families (Casey *et al.*, J. Clin. Invest., 106, R31–R38). One of these families appears to have the 'common'

578delTG mutation, and the other two are unique frameshifts. These findings support our hypotheses that *PRKAR1A* is mutated in up to 50% of cases and that all disease-causing mutatons are functionally null.

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