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## Mutations and Polymorphisms in the Gene Encoding Regulatory Subunit Type 1-alpha of Protein Kinase A (*PRKAR1A*): An Update

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### Abstract

*PRKARIA* encodes the regulatory subunit type 1-alpha ( $RI\alpha$ ), of the main mediator of the cAMP effects in the eukaryotic cells – cAMP dependant Protein Kinase A (PKA). Inactivating *PRKARIA* mutations are known to be responsible for the multiple neoplasia and lentiginosis syndrome Carney complex (CNC). To date, at least 117 pathogenic variants in *PRKARIA* have been identified. The majority of them are subject to non-sense mediated mRNA decay (NMD), leading to *PRKARIA* haploinsufficiency and, as a result, activated cAMP signaling in the affected tissues. In recent years it became apparent that CNC may be caused not only by  $RI\alpha$  haploinsufficiency, but also by the expression of altered *PRKARIA* protein, as proven by analysis of the relatively small proportion of expressed mutations in the gene, consisting of aminoacid substitutions and in-frame genetic

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alterations. In addition, a new subgroup of mutations that potentially escape NMD and result in CNC through altered rather than missing protein has been analyzed – frame-shifts in the 3' end of the coding sequence that shift the stop codon downstream of the regular one and result in the expression of longer than the wild type protein. The *PRKARIA* mutation detection rate in CNC patients is recently estimated at above 60%; *PRKARIA* mutation negative CNC patients are characterized by significant phenotypic heterogeneity. In this paper, we present a comprehensive analysis of all 117 known to date pathogenic *PRKARIA* sequence variations, and discuss their molecular context and clinical relevance.

## Keywords

PRKARIA; PKA; mutations; polymorphisms; Carney complex

## Introduction

Cyclic AMP (cAMP) dependent Protein Kinase A (PKA, EC 2.7.1.37) is the major mediator of the cAMP effects in the eukaryotic cells. In its inactive state, the PKA holoenzyme is a tetramer comprised of dimer of two regulatory subunits bound to two catalytic subunits (Kinderman, et al., 2006; Tasken, et al., 1997). Upon elevated cellular cAMP concentrations, two molecules of cAMP bind each of the regulatory subunits, which leads to the dissociation of the tetramer into regulatory dimers and two free catalytic subunits (Tasken, et al., 1997). The free catalytic subunits represent active serine threonine kinases which in turn phosphorylate series of cellular targets that regulate downstream effector enzymes, ion channels, and transcription of specific genes that mediate cell growth and differentiation (Shabb, 2001).

In the human, 4 different regulatory (PRKAR1A, PRKAR1B, PRKAR2A, and PRKAR2B) and 3 catalytic subunits (PRKACA; PRKACB, PRKACG) have been identified so far. It is now assumed that the multiple combinations of homo- or heterodimers of regulatory subunits with associated catalytic subunits may, at least in part, account for the specificity of the transduction of the signal generated by numerous different hormones, neurotransmitters, and other signaling through one sole second messenger, cAMP.

The only PKA subunit in which mutations have been found to lead to human disease is PRKAR1A: inactivating *PRKARIA* mutations cause the multiple neoplasia and lentiginosis syndrome Carney complex (CNC) (Casey, et al., 1998; Kirschner, et al., 2000a). CNC (MIM# 160980) is an autosomal dominant disorder associated with skin lesions, cardiac and other myxomas, and different types of endocrine tumors including primary pigmented nodular adrenocortical disease (PPNAD), growth hormone-secreting pituitary tumors, gonadal and thyroid neoplasias; rarely, malignant psammomatous melanotic schwannomas (PMS) may also be present (Carney, 1995; Carney, et al., 1986; Stratakis, et al., 1998). The vast majority of the reported *PRKARIA* mutations lead to premature stop codons and subsequent degradation of the mutant mRNAs species by NMD (Kirschner, et al., 2000b). Inactivating *PRKARIA* mutations lead to excess PKA signaling in affected tissues (Robinson-White, et al., 2006a).

*PRKARIA* is located on chromosome 17q23-q24 and extends to a total genomic length of approximately 21 kB. The gene is composed of 11 exons, 10 of which coding, with a coding region of 1143 bp starting from exon 2. The RI $\alpha$  protein consists of 384 AA organized in a dimerization/docking domain at the amino-terminal, followed by a PKA inhibitor site, two tandem binding domains for cAMP at the carboxyl terminus (cAMP:A and cAMP:B), and a linker region that contains the main docking site for the C subunit (Zawadzki and Taylor, 2004). Until recently, it was widely accepted that, in contrast to RII $\alpha$ , which typically is docked

at discrete cellular locations through A Kinase Anchoring Proteins (AKAPs), RI $\alpha$  tends to diffuse in the cytoplasm (Kinderman, et al., 2006; Skalhegg, et al., 1994; Wong and Scott, 2004). In the past several years, however, evidence has accumulated that RI $\alpha$  can be dynamically recruited as well localized at certain compartments of the cell such as the plasma membrane, mitochondria, cytoskeleton, and centrosomes, and AKAPs capable of binding it have been described (Huang, et al., 1997a; Huang, et al., 1997b).

*PRKARIA* orthologues have been characterized in many species, and high level of preservation can be tracked back to invertebrates (*Aedes aegypti*, 73% homology) and protozoa (*Ciona intestinalis*, 63% homology). The high degree of amino acid sequence conservation underlines the significance of PRKAR1A, the functional “receptor” for cAMP. Indeed, both cloning and gene knockout studies on the individual regulatory subunits have confirmed the essential role of PRKAR1A in maintaining cAMP control of PKA activity through compensatory mechanisms assuring balanced regulation of the free catalytic subunits (Amieux and McKnight, 2002).

Since the identification of *PRKARIA* as the gene causing CNC, more than a hundred different pathogenic mutations have been reported, and their molecular and functional analysis has been investigated in several major studies (Bertherat, et al., 2009; Greene, et al., 2008; Groussin, et al., 2002; Kirschner, et al., 2000a; Veugelers, et al., 2004). Herein we review all described to date *PRKARIA* sequence variations, including 17 that have not been published before, in an attempt for better understanding of their molecular context and clinical relevance.

## Mutation spectrum

To date, a total of 117 different *PRKARIA* mutations have been identified in 387 unrelated families of various ethnic origin; they are summarized in Table 1. The molecular changes involve single base substitutions and small (up to 15bp) deletions, insertions or combined rearrangements that are spread along the whole open reading frame of the gene; in addition, several relatively large deletions are reported (Blyth, et al., 2008; Horvath, et al., 2008a). A schematic representation of the *PRKARIA* mutations’ type and location is shown on Figure 1.

The mutations in *PRKARIA* are spread along the whole coding sequence, without significant preference for an exon or a domain. Most of them are unique – they are identified in single families only (Bertherat, et al., 2009). To date, only 3 mutations have been found in more than three unrelated pedigrees: c.82C>T, c.491\_492delTG and c.709(-7-2)del6; these mutations have been seen in kindreds with different racial and ethnic background, suggesting that they are likely to result from more than one independent mutation events.

A graph representing the relationship between the molecular type of the *PRKARIA* mutations’ and their functional effect is shown on Figure 2. Relatively small proportion (20/117, 17.1%) of the unique mutations result in the expression of an altered protein; these group convenes the 11 missense substitutions, 6 frameshift mutations affecting the last exon of the gene and thus escaping NMD, one in-frame deletion that eliminates exon 3 in frame, and one splice variant that leads to exon 7 skipping. The vast majority of mutations (97/117, 82.9%) result in premature stop codon (PSC) generation caused by nonsense and frameshift changes upstream of the last gene exon; the mutant mRNAs are degraded through NMD. In this second group fall also the majority of the splice variants – disrupted donor or acceptor sequences lead to exon skip and/or retaining of part of the intron, and, thus, direct or by frame-shift incorporation of PSC. The different mutations’ type functional effect and relation to the phenotype are discussed below.

## Mutations generating premature stop codon

As mentioned, PSC are caused by nonsense, frame shift and splice variants located before the last exon of *PRKARIA*. The expression of the shortened protein is abolished by the NMD, as shown by our experiments (Kirschner, et al., 2000b); thus, the overall effect of this type of mutation is a 50% reduction in cellular *PRKARIA* levels. The resulting higher proportion of catalytic to regulatory subunits causes increased signaling and activation of the downstream cellular processes such as proliferation and differentiation, (Robinson-White, et al., 2006b). Hence, NMD mutations cause disease in the affected tissues through a mechanism of haploinsufficiency. This type of mutations is found in the majority of the CNC patients and the resulting phenotypes vary in a very wide range both as number of manifestation and severity of their expression. The factors to affect this variability, besides age, gender and environmental agents are likely to include other genetic determinants with modifying effects. The later is supported by family clustering of particular sets of manifestations as well as our recent studies showing influence of genetic variants in other cAMP-related molecules on the severity of the CNC phenotype (manuscript in preparation). Although the uniqueness of the most of the mutations limits genotype/phenotype analysis, it is now accepted that phenotype/genotype correlation is rare among carriers of NMD mutations in *PRKARIA* - they all share haploinsufficiency at the protein level.

## Expressed mutations

The expressed missense substitutions in *PRKARIA* are listed in Table 2. While the pathogenic effect of the NMD mutations is almost certain to destroy the *PRKARIA* function, the expressed mutations that lead to altered protein clearly require estimation of their pathogenic potential. Eight of these mutations – six aminoacid substitutions (S9N, R74C, R146S, D183Y, A213D and G289W), and the two in-frame deletions of exons 3 and 7 - have been subject of detailed investigation of their effect *in vitro* (Greene, et al., 2008; Meoli, et al., 2008). The expressed mutations were spread over all functional *PRKARIA* domains and every one of them led to variable extends of decrease of the cAMP binding and increase in the cAMP-specific PKA activation (Greene, et al., 2008; Meoli, et al., 2008). As expected, the highest impact on the protein function was measured for the in-frame deletion that eliminates exon 3, and, respectively, the primary binding site for the catalytic subunit (Greene, et al., 2008); this mutation leads to significant elevation in the PKA activity even in the absence of cAMP. Similar, although milder effect on the protein is caused by the R146S, which disrupts the secondary binding site for the catalytic subunit (residues 138-148 and 232-247 within the cAMP binding domain A (Greene, et al., 2008). Expressed mutations affecting the cAMP binding domains (A183Y, A213D and G289W), are shown, in line with their position, to significantly decrease the cAMP binding; the proposed mechanism of action is through conformational changes that alter the enzymatic function. Conformational changes are also proposed to take place for the last two of the studied expressed mutations: S9N, located in the dimerisation/docking domain, and R74C, which is positioned in the linker region, outside any known functional sequence.

Notably, substantial correlation between the *in vitro* studies and the *in silico* modeling of the effect of the expressed missense substitutions on the protein function, along with the homology data, was observed (See Table 2). *In silico* prediction of the effect of the missense substitutions on the protein function (<http://coot.embl.de/PolyPhen/>) has estimated highest potential to impact the protein function generally for mutations residing in the cAMP binding domains (G168S, D183Y, A213D and G289D, Table 2).

Separate attention requires the missense substitution affecting the first coding aminoacid - M1V. *In silico* modeling predicted very severe effect on the protein function (See Table 2). Consistent with the above, the *in vitro* studies have shown significant effect of the mutation

on both cAMP binding and cAMP specific PKA activation. However, although the mutant mRNA is found to be expressed in equal to the wt levels in carriers of the mutation, it is not clear if the mutant protein is expressed and stable *in vivo* (Kirschner, et al., 2000b). The possibility of alternate expression from a surrogate initiation site has been explored – an in-frame ATG in the context of a relatively good match for Kozak sequence is located 141 bp downstream from the original initiation codon; however, Western blot did not detect shorter protein forms.

Analysis of the phenotype characteristics in the carriers of expressed *PRKARIA* mutations revealed severe CNC phenotype in the carrier of the in-frame deletion of ex3 and the c.708 +1G>T mutation, expressed both as number of manifestations and severity of their expression. Apart from this finding, no genotype-phenotype connection could be characterized within this relatively small group, which is expected taken the limited number of affected patients and the variable number CNC manifestations. Nevertheless, the finding of expressed *PRKARIA* mutant variations that cause disease related changes *in vitro*, confirms that altered *PRKARIA* function, not only haploinsufficiency, is enough to lead to the disease phenotype.

### Frameshift mutations leading to elongated *PRKARIA* protein

Six of the frameshift mutations (c.1055\_1058delGACC, c.1067\_1070delAACGins5, c.1076\_1077delTTins13, c.1083delA, c.1131\_1132delTG, and c.1142\_1145delTCTG) are located in the last exon of *PRKARIA* and are predicted to escape NMD. Each of the mutations causes a frame shift that abolishes the wild type termination codon and generates a new one further downstream. Compared to the wild type *PRKARIA*, the predicted mutant proteins are longer and carry a different 3' AA sequence, resulting from codon rearrangement. The functional significance of the elongated proteins versus the wild-type R1 $\alpha$  is subject of another report under submission.

### Large deletions

Several relatively large deletions in the region of *PRKARIA* have been described so far (Blyth, et al., 2008; Horvath, et al., 2008a), including the above discussed expressed deletion that eliminates exon 3 in frame (c.178\_348del171/p.E60\_K116del). This mutation has been shown to result to the *in vivo* expression of shorter *PRKARIA* protein lacking part of the linker region connecting the dimerisation/docking domain and the first cAMP binding domain; the deleted region includes the binding site for the catalytic subunit/inhibitor (Horvath, et al., 2008a). Only one patient was found to carry this mutation, and he presented with very severe phenotype, both as number of CNC manifestation and level of their expression (Horvath, et al., 2008a). *In vitro* studies with expression vectors harboring *PRKARIA* ORF lacking exon 3 have shown extreme effect on the protein function (Greene, et al., 2008). The second identified deletion was located in the upstream regulatory region of *PRKARIA* and did not affect the ORF of the gene; this mutation is expected to lead to decreased *PRKARIA* mRNA levels but no other effects on the protein; the molecular phenotype is predicted to be *PRKARIA* haploinsufficiency, consistent with the majority of *PRKARIA* mutations causing CNC. The third noteworthy deletion encompasses significantly larger genomic region from 17q24.2-q24.3 and eliminates, along with *PRKARIA*, 13 more genes (Blyth, et al., 2008). This mutation was identified as a *de novo* genomic rearrangement in a 12 years old patient presenting with posterior laryngeal cleft, moderate growth and mental retardation, microcephaly, and, as the only CNC related manifestation - multiple freckles and lentigines. However, other CNC manifestations can be underrepresented due to the severity of the main phenotype, or, more likely may occur later in the development. Although larger rearrangements involving *PRKARIA* region are reported in the literature, in most of the cases they led to extremely severe phenotype and premature death that prevented comprehensive analysis of relatively mild CNC manifestation with later onset (Bridge, et al., 1985; Levin, et al., 1995; Olney, et al., 1999).



## Splice mutations

Twenty-eight splice variants are identified to date in the close proximity of the exon-intron junctions of *PRKARIA* (Table 3). As mentioned, the vast majority of them lead to a frame-shift and subsequent incorporation of PMS. Notably, for the most of the splice mutations (23, 82%), the *in silico* modeling predicted complete abrogation of the junction formation; in 2 of them accompanied by a shift of the predicted junction with 1 base (see Table 3). For the remaining five variants (c440+4delG, c.502G>A, c.550G>A, c.763delAT, and 891+3A>G) a significant decrease in the probability score to form junction compared to the wt was estimated (see Table 3). It is noteworthy that three of these last variants affected directly exonic sequences and their effect on the splice-site was complemented by a change in the approximate coding sequence, which additionally increased their pathogenic impact. Combined with the family data that show segregation of these variants with the CNC phenotype, all described 28 splice changes were classified as pathogenic.

Interestingly, some splice-site alleles may express both mutant and wild type mRNA molecules in a proportion depending on the type of the mutation and its location relevant to the junction. Thus, the relative decrease of the cellular wt *PRKARIA* for these mutations is expected to be lower, and, accordingly, to lead to a milder phenotype. This assumption is in line with a recent analysis of a “mild” splice variant (c.709(-7-2)del6), which is one of the very few mutations where incomplete penetrance of *PRKARIA* mutation is seen, and, in the affected individuals, only PPNAD and lentiginosis – the most common disease manifestations - have been diagnosed (Groussin, et al., 2006).

## Polymorphisms

Four nonpathogenic unique synonymous substitutions in the third base of the codon have been described so far in *PRKARIA*: c.87G>A/p.A29A, c.204G>A/p.L68L, c.318G>C/p.T106T, and c.492G>A/p.V164V. From them, the first one has been seen in at least five unrelated kindreds, and the remaining three are detected in single families only. Multiple intronic variants in close proximity of the junctions have been described, from them the most frequent being c.349-5dup5 (detected in approximately 15% of the studied alleles), followed by c.769-24A>G and c.891-34G>T (in approximately 12% and 10% of the studied alleles, respectively). However, the extend to which different research groups analyze the exon flanking sequences widely vary and the frequency of these variants may therefore be significantly underreported; furthermore, both missense variants and splice changes may be as well underreported. All these polymorphic variants are seen in healthy control individuals and in both CNC families, and in the later, they do not show segregation with the disease status.

## Mutation detection rate of *PRKARIA* mutations in CNC

Since the first description of the disease, numerous CNC patients have been reported from all ethnic groups and presenting with various number, combination and severity of manifestations. The most recently re-evaluated diagnostic criteria for CNC are listed in Table 4; a definite diagnosis is given when two or more major manifestations are present. Soon after the identification of *PRKARIA* and the initial patient screenings, the mutation detection rate was estimated to approximately 50% (Figure 3, A). After a decade of comprehensive phenotype analysis and re-evaluation of the diagnostic criteria, it is now assumed that mutations in *PRKARIA* are responsible for the disease in more than 60% of the CNC patients (Figure 2B). The largest to date study of 185 families, detects *PRKARIA* mutations in 114 (62%) (Bertherat, et al., 2009). Interestingly, this percent rises up to 80 among CNC patients presenting with Cushing syndrome due to PPNAD (Cazabat, et al., 2007). Among the mutation carriers, Cushing syndrome due to PPNAD is seen in 70% of the female patients before 45 years, but in only 45% of the male patients, likely reflecting the generally higher frequency of Cushing

syndrome in females (Grumbach, et al., 2003). These numbers, however, may represent an underestimate of the true incidence of Cushing syndrome, since autopsy of CNC patients detects PPNAD in almost 100% of the cases (Stratakis, et al., 2001).

When opposed to the *PRKARIA* mutation carriers, CNC patients with no genetic defect in *PRKARIA* presented with disease overall later in life and more frequently in a sporadic fashion suggesting implication of different genetic factors (Bertherat, et al., 2009). Further, several phenotype subgroups could be distinguished among the *PRKARIA* mutation negative individuals: with typical CNC manifestations (9%); with PPNAD and lentiginos only (11%); with isolated bilateral micronodular hyperplasia (12%); (see Figure 3, B). Recently, a genome wide based study on the last group identified inactivating mutations in two of the human genes encoding phosphodiesterases – *PDE11A* and *PDE8B* (Gunther, et al., 2004; Horvath, et al., 2006a; Horvath, et al., 2008b). Another CNC locus on region 2p16 has been suggested by linkage analysis, however, so far no other gene has been identified, and the genetic searches for additional molecular factors continues (Stratakis, et al., 1996; Stratakis, et al., 2001).

### Penetrance of CNC mutations

The overall penetrance of CNC among *PRKARIA* mutations' carriers is above 95% by the age of 50; only few *PRKARIA* carriers are reported who do not meet the diagnostic criteria. Only two mutations so far have been found to result in incomplete penetrance of CNC – the splice variant c.709(-7-2)del6 and the initiation alternating substitution p.M1Vp. Even more interesting is the observation that, when expressed, these two mutations lead to relatively mild CNC phenotype restricted mostly to PPNAD, in some cases accompanied by lentiginos. These findings were confirmed in all 12 unrelated families affected by c.709(-7-2)del6 and in the two unrelated kindreds where c.1A>G/p.M1V was seen (Groussin, et al., 2006). The explanation of this mild, and, in some cases, missing phenotype is seek in the light molecular effect of these two mutations – as a splice variant, c.709(-7-2)del6 does not affect 100% of the molecules, and, respectively, leads to lower level of *PRKARIA* insufficiency. The molecular effect of M1V substitution is not clear, but the same substitution in the first codon is suggested to be a mild mutation for other genetic disorders such as Smith-Lemli-Opitz syndrome (Witsch-Baumgartner, et al., 2005). Taking into account the particular sensitivity of the adrenal cells to even minor changes in the PKA activity and/or *PRKARIA* levels, it is suggested that adrenal will be the first tissue to react on these *PRKARIA* mutations.

### *PRKAR1A* in unselected population

Sequencing of the entire coding region of *PRKAR1A* has not identified alterations in at least 200 unrelated chromosomes. In addition, genotyping of 745 unrelated healthy individuals enrolled in New York Cancer Prospective study (Mitchell, et al., 2004) for 51 unique *PRKAR1A* mutations (Supplementary Table 1) did not detect the presence of any of them. Thus, *PRKAR1A* mutations are concluded to be very rare in general population. It is an essential parallel to be made with the gene encoding phosphodiesterase type 11A (*PDE11A*) – despite the proven association between pathogenic mutations, including null alleles, with adrenal neoplasias, such mutations are seen in general population with lower frequency (Horvath, et al., 2006a; Horvath, et al., 2006b).

### Genotype-phenotype correlations

Until recently, genotype-phenotype correlation studies were limited by the high variability of the CNC phenotype, both as number and combinations of manifestations, and the relatively limited series of patients. After more than 20 years of analysis and studying of CNC patients, as well as the fusion of the major databases from the National Institutes of Health, Bethesda, MD, and the Hospital Cochin, Paris, France, more than 380 patients are genotypically

characterized and clinically evaluated. An overall phenotypic segregation could be seen when the *PRKARIA* mutation carriers were opposed to the mutation negative CNC patients: the former presented more frequently and earlier in life with pigmented skin lesions, myxomas, thyroid and gonadal tumors (Bertherat, et al., 2009). Further, subgroups of patients presenting with particular genotype-phenotype correlation could be outlined. The first such a group was presented by patients with isolated PPNAD, in some cases accompanied with lentiginosis. Among these patients, the following tendencies were observed: (1) patients diagnosed before 8 years of age were rarely carriers of *PRKARIA* mutation; (2) most of the patients with isolated PPNAD and presence of *PRKARIA* mutation were carriers of either the c.709 (-7-2) del6(ttttta) mutation ( $p < 0.0001$ ) or the c.1A>G/p.M1V substitution affecting the initiation codon of the protein. These observations are in line with the discussed above mild, mainly adrenal or, in some cases absent phenotype associated with these two *PRKARIA* mutations.

The second group of CNC patients with particular genotype-phenotype correlation was comprised by individuals with myxomas (affecting all locations - skin, heart, and breast), PMS, thyroid tumors, and LCCSCT. In these patients, *PRKARIA* mutations were seen substantially more often. Related to this is the acknowledgment that certain tumors presented at significantly younger age in the *PRKARIA* mutation carriers: cardiac myxomas ( $p=0.02$ ), thyroid tumors ( $p=0.03$ ) and LCCSCTs ( $p=0.04$ ) (Bertherat et al., 2009). Within the group of *PRKARIA* carriers the mutations located in exons associated more frequently with lentiginosis, PMS, acromegaly and cardiac myxomas, compared to the intronic ones ( $p = 0.04$ ); this observation is in line with the above discussion of a milder phenotype associated with splice variants. And, finally, the “hot spot” c.491-492delTG mutation was most significantly associated with lentiginosis, cardiac myxoma, and thyroid tumors when opposed to all other *PRKARIA* defects combined ( $p = 0.03$ ).

## Diagnostic relevance

Clinical and genetic studies of CNC patients have revealed considerable heterogeneity both in mutational spectrum and in phenotypic variation. Patients with two or more diagnostic criteria from Table 4 or with isolated PPNAD are referred to genetic analysis of *PRKARIA*. Once a mutation is identified, genotyping for the particular variant is recommended for all at-risk relatives. The proportion of *de novo* mutations in *PRKARIA* is estimated to be approximately 20% (our unpublished results). Germline mosaicism has not been seen so far and may not be frequent, and thus not of diagnostic concern currently.

## Conclusions and future prospects

To date, most *PRKARIA* studies are focused on its coding regions and on screening for point mutations and small rearrangements detectable by PCR-based techniques. The identification of large deletions in the gene, and the significant proportion of patients with characteristic CNC phenotype and not detectable *PRKARIA* mutations have triggered the application of alternative technologies for detection of non-coding variations and larger re-arrangements that may account for the disease phenotype (our on-going project).

Apart from *PRKARIA*, three other genes from the cAMP signaling pathway are known to date to cause related or overlapping to CNC phenotype. Germline mutations in *PDE11A* and *PDE8B* associate with iMAD (Horvath, et al., 2006a; Horvath, et al., 2008b). Somatic activation mutations in G-alpha stimulatory subunit of the G-protein coupled receptor (*GNAS*) are known to result in McCune Albright syndrome – a postzygotic somatic multiple neoplasia characterized by polyostotic fibrous dysplasia (POFD), cafe-au-lait skin pigmentation, and peripheral precocious puberty (Weinstein, et al., 1991). Identification of genetic defects in cAMP-signaling molecules in patients with similar and/or overlapping



phenotype underlines the importance of this signaling in the normal functioning of the endocrine and other cells. Identification of genetic defects in cAMP-signaling molecules in patients with similar and/or overlapping with CNC features underscores the importance of this signaling pathway in endocrine and other tumorigenesis; on-going studies aim at characterizing this involvement better.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

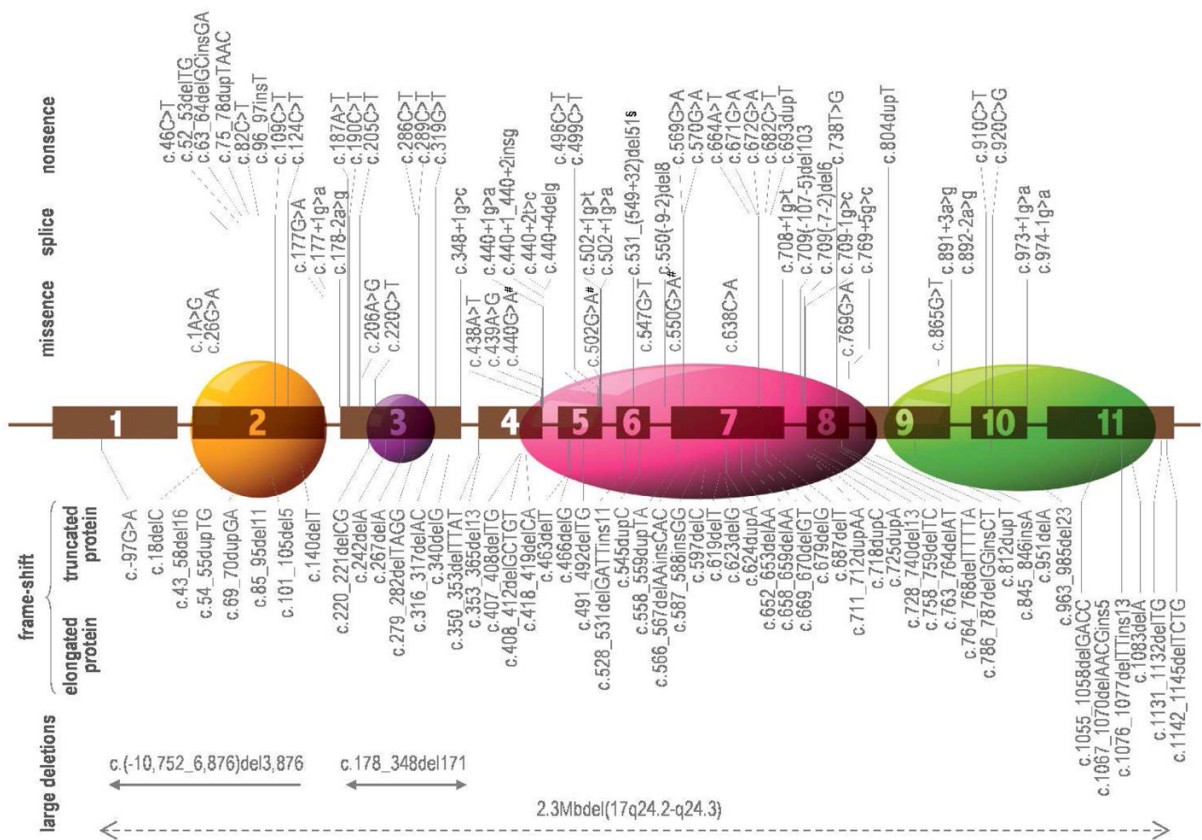
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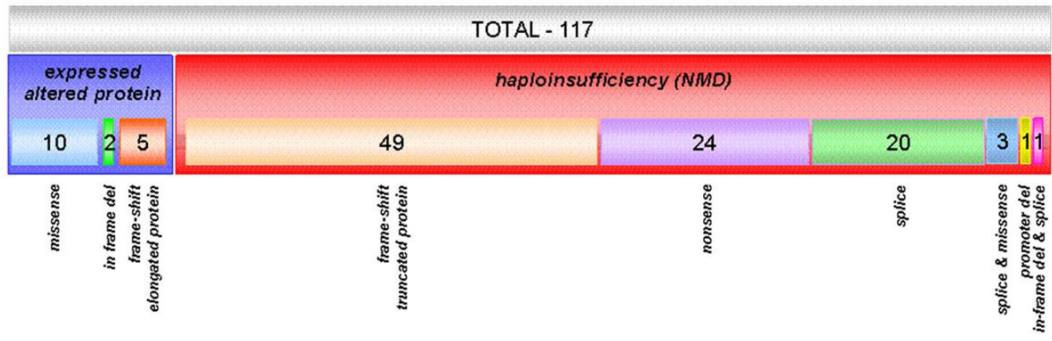
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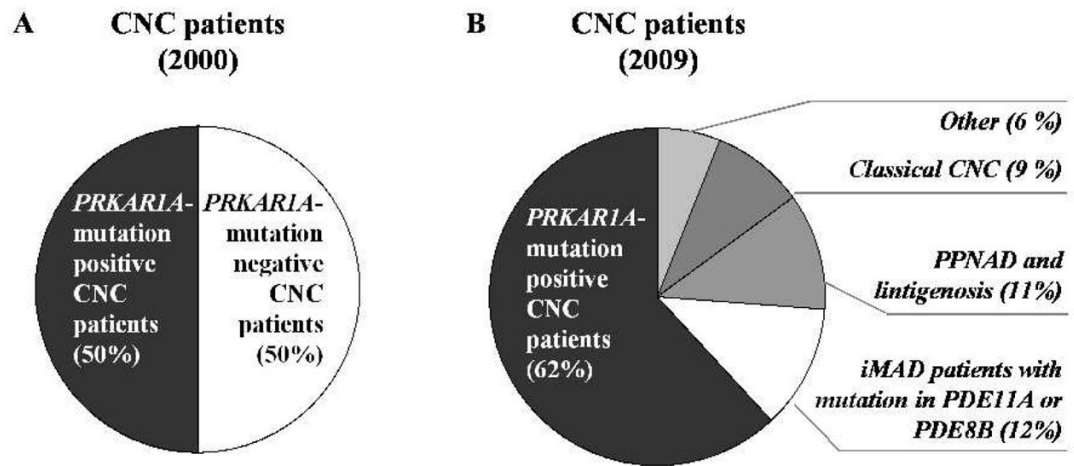


**Figure 1.** Schematic presentation of the type and the approximate location of the 117 identified to date mutations in *PRKARIA*; a large deletion eliminating the whole *PRKARIA* together with 13 more genes (2.3Mb del 17q24.2-q24.3) is also shown. The docking/dimerisation, the linker and the two cAMP functional domains (from left to right) of the protein are denoted with ovals.





**Figure 2.**  
Quantitative schematic representation of the expressed vs NMD mutations in PRKAR1A.



**Figure 3.**

Mutation detection rate among CNC patients A) soon after the identification of *PRKARIA* as a gene responsible for the disease phenotype, and, B) after the most recent analyses. The mutation detection rate increased from ~50 (2000) to above 62% (2009), and, in addition, genetic defect in *PDE11A* and *PDE8B* were identified in a phenotypic subgroup of initially recognized as CNC patients.

Table 1

List of known mutations in the PRKARIA (sorted by their position along the gene).

#	Position	DNA Change	Protein/RNA Change	Type	Effect on the protein	Phenotype	Reference
1	exon 1B	c.-97G>A	Pseudo ATG	frame-shift	NMD, no protein	CNC	(Groussin, et al., 2002)
2	exon 2	c.1A>G	p.M1V	missense	abolished initiation	CNC	(Kirschner, et al., 2000b)
3	exon 2	c.18delC	p.A7PfsX122	frame-shift	NMD, no protein	CNC	(Bertherat, et al., 2009)
4	exon 2	c.26G>A	p.S9N	missense	altered protein	CNC	(Greene, et al., 2008)
5	exon 2	c.43_58del16	p.L15SfsX109	frame-shift	NMD, no protein	CNC	(Bertherat, et al., 2009)
6	exon 2	c.46C>T	p.R16X	nonsense	NMD, no protein	CNC	(Bertherat, et al., 2009)
7	exon 2	c.52_53del1TG	p.C18X	nonsense	NMD, no protein	CNC	(Veugelers, et al., 2004)
8	exon 2	c.54_55dupTG	p.E19VfsX111	frame-shift	NMD, no protein	CNC	This study
9	exon 2	c.63_64delCCGinsGA	p.Y21X	nonsense	NMD, no protein	CNC	(Almeida, et al., 2008)
10	exon 2	c.69_70dup1GA	p.K24RfsX106	frame-shift	NMD, no protein	CNC	(Bertherat, et al., 2009)
11	exon 2	c.75_78dupTAAC	p.127X	nonsense	NMD, no protein	CNC	(Bertherat, et al., 2009)
12	exon 2	c.82C>T	p.Q28X	nonsense	NMD, no protein	CNC	(Kirschner, et al., 2000b)
13	exon 2	c.85_95del11	p.A29RfsX12	frame-shift	NMD, no protein	CNC	(Bertherat, et al., 2009)
14	exon 2	c.96_97insT	p.D33X	nonsense	NMD, no protein	CNC	This study
15	exon 2	c.101_105del5	p.S34CfsX9	frame-shift	NMD, no protein	CNC	(Kirschner, et al., 2000b)
16	exon 2	c.109C>T	p.Q37X	nonsense	NMD, no protein	CNC	(Bertherat, et al., 2009)
17	exon 2	c.124C>T	p.R42X	nonsense	NMD, no protein	CNC	(Kirschner, et al., 2000b)
18	exon 2	c.140delT	p.M46RfsX82	frame-shift	NMD, no protein	CNC	(Imai, et al., 2005)
19	exon 2	c.177G>A	retains part of intr 2	splice	NMD, no protein	CNC	(Bertherat, et al., 2009)
20	intron 2	c.177+1_g>a	retains part of intr 2	splice	NMD, no protein	CNC	(Bertherat, et al., 2009)
21	intron 2	c.178-2 a>g	skips ex 3	splice	shorter prot. (324AA)	CNC	(Kirschner, et al., 2000b)
22	intron 2	c.178_348del171	p.E60_K116del	In-frame del	shorter prot. (328AA)	CNC	(Horvath, et al., 2008)
23	exon 3	c.187A>T	p.K63X	nonsense	NMD, no protein	CNC	(Bertherat, et al., 2009)
24	exon 3	c.190C>T	p.Q64X	nonsense	NMD, no protein	CNC	(Bertherat, et al., 2009)
25	exon 3	c.205C>T	p.Q69X	nonsense	NMD, no protein	CNC	(Bertherat, et al., 2009)
26	exon 3	c.206A>G	p.Q69R	missense	altered protein	CNC	This study
27	exon 3	c.220_221delCG	p.R74YfsX7	frame-shift	NMD, no protein	CNC	(Bertherat, et al., 2009)
28	exon 3	c.220C>T	p.R74C	missense	altered protein	CNC	(Veugelers, et al., 2004)
29	exon 3	c.242delA	p.E81GfsX48	frame-shift	NMD, no protein	CNC	This study

#	Position	DNA Change	Protein/RNA Change	Type	Effect on the protein	Phenotype	Reference
30	exon 3	c.267delA	p.V90WfsX39	frame-shift	NMD, no protein	CNC	(Bertherat, et al., 2009)
31	exon 3	c.279_282delTTAGG	p.R94GfsX34	frame-shift	NMD, no protein	CNC	(Bertherat, et al., 2009)
32	exon 3	c.286C>T	p.R96X	nonsense	NMD, no protein	CNC	(Bertherat, et al., 2009)
33	exon 3	c.289C>T	p.R97X	nonsense	NMD, no protein	CNC	(Veugelers, et al., 2004)
34	exon 3	c.316_317delAC	p.T106GfsX9	frame-shift	NMD, no protein	CNC	(Skamrov, et al., 2003)
35	exon 3	c.319G>T	p.Q107X	nonsense	NMD, no protein	CNC	(Bertherat, et al., 2009)
36	exon 3	c.340delG	p.V114LfsX15	frame-shift	NMD, no protein	CNC	(Bertherat, et al., 2009)
37	Intron 3	c.348+1 g>c	retains intron 3	splice	NMD, no protein	CNC	(Kirschner, et al., 2000b)
38	exon 4	c.350_353delTTAT	p.V117DfsX11	frame-shift	NMD, no protein	CNC	(Kirschner, et al., 2000b)
39	exon 4	c.353_365del13	p.I118TfsX6	frame-shift	NMD, no protein	CNC	(Bertherat, et al., 2009)
40	exon 4	c.407_408delTG	p.V136AfsX6	frame-shift	NMD, no protein	CNC	(Mabuchi, et al., 2005)
41	exon 4	c.408_412delGCTGT	p.L137FfsX4	frame-shift	NMD, no protein	CNC	(Bertherat, et al., 2009)
42	exon 4	c.418_419delCA	p.H140SfsX1	frame-shift	NMD, no protein	CNC	This study
43	exon 4	c.438A>T	p.R146S	missense	altered protein	CNC	(Greene, et al., 2008)
44	exon 4	c.439A>G	p.S147G	missense	altered protein	CNC	(Bertherat, et al., 2009)
45	exon 4	c.440G>A	p.S147N	missense, splice	altered protein	CNC	(Gu, et al., 2004)
46	intron 4	c.440+1g>a	retains part of intron 4	splice	NMD, no protein	CNC	This study
47	intron 4	c.440+1_440+2insg	retains part of intron 4	splice	NMD, no protein	CNC	(Bertherat, et al., 2009)
48	intron 4	c.440+2t>c	retains part of intron 4	splice	NMD, no protein	CNC	(Bertherat, et al., 2009)
49	intron 4	c.440+4del g	retains part of intron 4	splice	NMD, no protein	CNC	(Bertherat, et al., 2009)
50	exon 5	c.463delT	p.S155RfsX10	frame-shift	NMD, no protein	CNC	This study
51	exon 5	c.466delG	p.V156SfsX9	frame-shift	NMD, no protein	CNC	(Vargas-Alarcon, et al., 2008)
52	exon 5	c.491_492delITG	p.V164DfsX5	frame-shift	NMD, no protein	CNC	(Kirschner, et al., 2000a)
53	exon 5	c.496C>T	p.Q166X	nonsense	NMD, no protein	CNC	This study
54	exon 5	c.499C>T*	p.Q167X	nonsense	NMD, no protein	UDC	(Sandrini, et al., 2002)
55	exon 5	c.502G>A	p.G168S	missense, splice	altered protein	CNC	(Bertherat, et al., 2009)
56	intron 5	c.502+1g>t	retains part of intron 5	splice	NMD, no protein	CNC	(Groussin, et al., 2002)
57	intron 5	c.502+1g>a	retains part of intron 5	splice	NMD, no protein	CNC	(Gennari, et al., 2008)
58	exon 6	c.528_531delGATTins11	p.I177MfsX13	frame-shift	NMD, no protein	CNC	(Veugelers, et al., 2004)
59	exon 6	c.531_(549+32)del51	p.I177MfsX1	frame-shift, splice	NMD, no protein	CNC	(Bertherat, et al., 2009)
60	exon 6	c.545dupC	p.D182GfsX5	frame-shift	NMD, no protein	CNC	(Veugelers, et al., 2004)

#	Position	DNA Change	Protein/RNA Change	Type	Effect on the protein	Phenotype	Reference
61	exon 6	c.547G>T	p.D183Y	missense	altered protein	CNC	(Greene, et al., 2008)
62	intron 6	c.550(-9-2)de18	Skip ex 7	splice	shorter prot. (328AA)	CNC	(Kirschner, et al., 2000b)
63	exon 7	c.550G>A	p.V184I	missense, splice	altered protein	CNC	(Bertherat, et al., 2009)
64	exon 7	c.558_559dupTA	p.N187IfsX20	frame-shift	NMD, no protein	CNC	(Bertherat, et al., 2009)
65	exon 7	c.566_567delAAinsCAC	p.E189AfsX44	frame-shift	NMD, no protein	CNC	(Kirschner, et al., 2000b)
66	exon 7	c.569G>A	p.W190X	nonsense	NMD, no protein	CNC	(Bertherat, et al., 2009)
67	exon 7	c.570G>A	p.W190X	nonsense	NMD, no protein	CNC	(Bertherat, et al., 2009)
68	exon 7	c.587_588insGG	p.G198EfsX10	frame-shift	NMD, no protein	CNC	(Veugelers, et al., 2004)
69	exon 7	c.597delC	p.F200LfsX6	frame-shift	NMD, no protein	CNC	(Sasaki, et al., 2008)
70	exon 7	c.619delIT	p.Y207MfsX15	frame-shift	NMD, no protein	CNC	(Veugelers, et al., 2004)
71	exon 7	c.623delIG	p.G208EfsX14	frame-shift	NMD, no protein	CNC	(Casey, et al., 2000)
72	exon 7	c.624dupA	p.T209NfsX24	frame-shift	NMD, no protein	CNC	(Veugelers, et al., 2004)
73	exon 7	c.638C>A*	p.A213D	missense	altered protein	ODM	(Perdigao, et al., 2005)
74	exon 7	c.652_653delAA	p.K218DfsX14	frame-shift	NMD, no protein	CNC	This study
75	exon 7	c.658_659delAA	p.N220CfsX12	frame-shift	NMD, no protein	CNC	(Veugelers, et al., 2004)
76	exon 7	c.664A>T*	p.L222X	nonsense	NMD, no protein	ACA	(Bertherat, et al., 2003)
77	exon 7	c.669_670delIGT	p.W224GfsX8	frame-shift	NMD, no protein	CNC	This study
78	exon 7	c.671G>A	p.W224X	nonsense	NMD, no protein	CNC	(Bertherat, et al., 2009)
79	exon 7	c.672G>A	p.W224X	nonsense	NMD, no protein	CNC	(Bertherat, et al., 2009)
80	exon 7	c.679delIG	p.D227TfsX14	frame-shift	NMD, no protein	CNC	(Bertherat, et al., 2009)
81	exon 7	c.682C>T	p.R228X	nonsense	NMD, no protein	CNC	(Kirschner, et al., 2000b)
82	exon 7	c.687delIT*	p.D229EfsX12	frame-shift	NMD, no protein	ODM	(Perdigao, et al., 2005)
83	exon 7	c.693dupT	p.R232X	nonsense	NMD, no protein	CNC	(Kirschner, et al., 2000b)
84	intron 7	c.708+1g>t	Skip ex 7	splice	shorter prot. (328AA)	CNC	(Groussin, et al., 2002)
85	intron 7	c.709(-5-107)del1103	skips ex 8	splice	NMD, no protein	CNC	(Bertherat, et al., 2009)
86	intron 7	c.709(-7-2)del6	skips ex 8	splice	NMD, no protein	CNC**	(Groussin, et al., 2002)
87	intron 7	c.709-1g>c	skips ex 8	splice	NMD, no protein	CNC	(Bertherat, et al., 2009)
88	exon 8	c.711_712dupAA	p.S238KfsX4	frame-shift	NMD, no protein	CNC	(Kirschner, et al., 2000b)
89	exon 8	c.718dupC	p.L240PfsX8	frame-shift	NMD, no protein	CNC	(Bertherat, et al., 2009)
90	exon 8	c.725dupA	p.R243AfsX5	frame-shift	NMD, no protein	CNC	(Groussin, et al., 2002)
91	exon 8	c.728_740del113	p.K244NfsX9	frame-shift	NMD, no protein	CNC	(Bertherat, et al., 2009)



#	Position	DNA Change	Protein/RNA Change	Type	Effect on the protein	Phenotype	Reference
92	exon 8	c.738T>G	p.Y246X	nonsense	NMD, no protein	CNC	(Bertherat, et al., 2009)
93	exon 8	c.758_759delTC	p.S254YfsX15	frame-shift	NMD, no protein	CNC	(Casey, et al., 2000)
94	exon 8	c.763_764delAT	p.I255PfsX14	frame-shift	NMD, no protein	CNC	(Groussin, et al., 2002)
95	exon 8	c.764_768delTTTTTA	p.I255RfsX12	frame-shift	NMD, no protein	CNC	This study
96	exon 8	c.769G>A	p.E257K	missense, splice	NMD, no protein	CNC	This study
97	intron 8	c.769+5g>c	Retains part of intr 8	splice	NMD, no protein	CNC	(Bertherat, et al., 2009)
98	exon 9	c.786_787delGGmsCT	p.W262CfsX2	frame-shift	NMD, no protein	CNC	(Kirschner, et al., 2000a)
99	exon 9	c.804dupT	p.D269X	nonsense	NMD, no protein	CNC	(Veugelers, et al., 2004)
100	exon 9	c.812dupT	p.L271FfsX7	frame-shift	NMD, no protein	CNC	(Bertherat, et al., 2009)
101	exon 9	c.845_846msA	p.V283CfsX9	frame-shift	NMD, no protein	CNC	(Bertherat, et al., 2009)
102	exon 9	c.865G>T	p.G289W	missense	altered protein	CNC	(Greene, et al., 2008)
103	intron 9	c.891+3a>g	Retains part of intr 9	splice	skips ex 10	CNC	(Kirschner, et al., 2000a)
104	intron 9	c.892-2a>g	skips ex 10	splice	skips ex 10	CNC	This study
105	exon 10	c.910C>T	p.Q304X	nonsense	NMD, no protein	CNC	(Veugelers, et al., 2004)
106	exon 10	c.920C>G	p.S307X	nonsense	NMD, no protein	CNC	This study
107	exon 10	c.951delA	p.R317SfsX14	frame-shift	NMD, no protein	CNC	(Veugelers, et al., 2004)
108	exon 10	c.963_985del23 *	p.D322TfsX20	frame-shift	NMD, no protein	ACA	(Bertherat, et al., 2003)
109	inton 10	c.973+1g>a	Retains part of intr 10	splice	NMD, no protein	CNC	This study
110	exon 11	c.974-1g>a *	skips ex 11	splice	NMD, no protein	ACA	(Bertherat, et al., 2003)
111	exon 11	c.1055_1058delGACC	p.R352LfsX88	frame-shift	elongated prot. (438AA)	CNC	(Bertherat, et al., 2009)
112	exon 11	c.1067_1070delAACGms5	p.E356GfsX71	frame-shift	elongated prot. (425AA)	CNC	(Bertherat, et al., 2009)
113	exon 11	c.1076_1077delTTms13	p.L359QfsX86	frame-shift	elongated prot. (443AA)	CNC	(Bertherat, et al., 2009)
114	exon 11	c.1083delA	p.C362AfsX79	frame-shift	elongated prot. (439AA)	CNC	This study
115	exon 11	c.1113_1132delITG	p.S378TfsX48	frame-shift	elongated prot. (425AA)	CNC	(Bertherat, et al., 2009)
116	exon 11	c.1142_1145delTCTG	p.V381EfsX59	frame-shift	elongated prot. (439AA)	CNC	(Bertherat, et al., 2009)
117	promoter?	c.(-10,752_6,876)del3,876	Before ex 1	deletion of part of the promoter	decreased prot. expression	CNC	(Horvath, et al., 2008)

\* somatic mutation (the presence of the mutation was ruled out in lymphocytes)

\*\* In the carriers of mutation c.709(-7-2)de16 and c.63\_64delCGmsGA CNC phenotype was restricted to PPNAD, and, in some cases, skin manifestations

\*\*\* CNC+ phenotype is defined by the authors as „posterior laryngeal cleft and the presence of numerous freckles and lentiginos, growth restriction, microcephaly and moderate mental retardation“.

CNC – Carney complex; ACA – adenocortical adenoma; UDC – undifferentiated thyroid carcinoma; ODM – odontogenic myxoma; NMD – Non-sense mediated m-RNA decay

Table 2

*In silico* modeling and *in vitro* analysis of the effect of *PRKAR1A* missense substitutions on the protein function

#	DNA Change	Protein Change	Domain	<i>In silico</i> modeling		<i>In vitro</i> analysis		Inter-species alignment					
				prediction	score*	cAMP binding**	cAMP-specific PKA activation**	<i>Mus musculus</i>	<i>Gallus gallus</i>	<i>Anolis carolinensis</i>	<i>Xenopus laevis</i>	<i>Danio regio</i>	<i>Plasmodium falciparum</i>
1	c.1A>G	p.M1V	Initiation codon	Probably damaging	2.055	0.31***	2.46***	M	M	M	M	M	M
2	c.26G>A	p.S9N	Dimerization/docking	Benign	0.243	0.28***	2.14**	S	S	S	S	S	S
3	c.206A>G	p.Q69R	Linker region	Probably damaging	2.013	na	na	Q	Q	Q	Q	Q	Q
4	c.220C>T	p.R74C	Linker region	Possibly damaging	1.686	0.69***	2.77	R	R	R	R	R	R
5	c.438A>T	p.R146S	cAMP binding A	Benign	0.733	0.49***	2.12***	R	R	R	R	R	R
6	c.439A>G	p.S147G	cAMP binding A	Benign	1.033	0.84***	2.16***	S	S	S	S	S	S
7	c.440G>A	p.S147N	cAMP binding A	Benign	0.145	na	na	S	S	S	S	S	S
8	c.502G>A	p.G168S	cAMP binding A	Probably damaging	2.250	na	na	G	G	G	G	G	G
9	c.547G>T	p.D183Y	cAMP binding A	Probably damaging	2.521	0.24***	1.63	D	D	D	D	D	D
10	c.550G>A	p.V184I	cAMP binding A	Benign	0.052	na	na	V	V	V	V	V	V
11	c.638C>A	p.A213D	cAMP binding A	Probably damaging	2.507	0.17***	2.06	A	A	A	A	A	A
12	c.865G>T	p.G289W	cAMP binding B	Probably damaging	2.592	0.43***	2.92***	G	G	G	G	G	G

\* greater score indicates higher probability to impair the protein function; the factors taken into account for the calculation of the score are

(1) difference in the thermo-physical properties of the wt and mutant protein, and

(2) evolutionary preservation of the residue in the corresponding position

\*\* both cAMP binding and PKA activity changes in response to cAMP are presented as ratio to the wt *PRKAR1A*

\*\*\* P<0.05

**Table 3***In silico* modeling of the effect of *PRKARIA* splice variants

#	DNA Change	Exon affected	<i>Spliceport</i> WT Score	<i>Spliceport</i> mutation Score
1	c.177G>A	Incorporates intron2	0.679639	0.00
2	c.177+1g>a	Incorporates intron2	0.679639	0.00
3	c.178-2a>g	Skip exon 2	0.894923	0.00
5	c.348 +1 g>c	Incorporates intron3	1.14417	0.00
6	c.438A>T	Incorporates intron4	0.820566	0.00
7	c.439A>G	Incorporates intron4	0.820566	0.00
8	c.440G>A	Incorporates intron4	0.820566	0.00
9	c.440+ 1 insG	Incorporates intron4	0.820566	0.268882 (+1 base)
10	c.440+ 4del g	Incorporates intron4	0.820566	0.142182
11	c.440+2 t>c	Incorporates intron4	0.820566	0.00
13	c.502 +1g>t	Incorporates intron5	1.69211	0.00
14	c.502+1g>a	Incorporates intron5	1.69211	0.00
15	c.502G>A	Incorporates intron5	1.69211	0.705629
17	c.550(-8-2) del 7	Skip exon 7	0.802186	0.00
18	c.550G>A	Skip exon 7 (+ -)	0.802186	0.478702
19	c.708 +1g>t	Skip exon 7	1.74672	0.00
20	c.709 (-7-2)del6(ttttta)	Skip exon 8	0.502791	0.0
21	c.709 (-5-107)del103	Skip exon 8	0.502791	0.0
22	c.709-1G>C	Skip exon 8	0.502791	0.0
24	c.763delAT	Incorporates intron8	1.02912	0.778435
25	c.769+5g>c	Incorporates intron8	1.02912	0.00
26	c.891+3A>G	Incorporates intron9	1.41361	0.158643
27	c.892-2 a>g	Skip exon 10	1.64415	0.0
28	c.974-1g>a	Skip exon 11	1.2552	0.112813 (+1 base)

**Table 4**

## Diagnostic criteria for CNC

<i>Major Diagnostic criteria for CNC</i>
1. Spotty skin pigmentation with typical distribution (lips, conjunctiva and inner or outer canthi, vaginal and penile mucosal)
2. Myxoma* (cutaneous and mucosal)
3. Cardiac myxoma*
4. Breast myxomatosis* or fat-suppressed magnetic resonance imaging findings suggestive of this diagnosis
5. PPNAD* or paradoxical positive response of urinary glucocorticosteroid excretion to dexamethasone administration during Liddle's test
6. Acromegaly due to GH-producing adenoma*
7. LCCST* or characteristic calcification on testicular ultrasound
8. Thyroid carcinoma* or multiple, hypoechoic nodules on thyroid ultrasound in a young patient
9. Psammomatous melanotic schwannomas*
10. Blue nevus, epithelioid blue nevus*
11. Breast ductal adenoma*
12. Osteochondromyxoma*
<i>Supplementary criteria</i>
1. Affected first-degree relative
2. Inactivating mutation of the <i>PRKARIA</i> gene
<i>Findings suggestive of or possibly associated with CNC, but not diagnostic for the disease</i>
1. Intense freckling (without darkly pigmented spots or typical distribution)
2. Blue nevus, common type (if multiple)
3. Café-au-lait spots or other "birthmarks"
4. Elevated IGF-I levels, abnormal GTT, or paradoxical GH response to TRH testing in the absence of clinical acromegaly
5. Cardiomyopathy
6. Pilonidal sinus
7. History of Cushing's syndrome, acromegaly, or sudden death in extended family
8. Multiple skin tags or other skin lesions; lipomas
9. Colonic polyps (usually in association with acromegaly)
10. Hyperprolactinemia (usually mild and almost always combined with clinical or subclinical acromegaly)
11. Single, benign thyroid nodule in a young patient; multiple thyroid nodules in an older patient (detected on ultrasound)
12. Family history of carcinoma, in particular of the thyroid, colon, pancreas, and ovary; other multiple benign or malignant tumors

\* After histological confirmation