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A novel *PRKAR1A* mutation associated with hepatocellular carcinoma in a young patient and a variable Carney complex phenotype in affected subjects in older generations

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Summary

Context—Carney complex (CNC) is an autosomal dominant multiple endocrine neoplasia syndrome (OMIM 160980). About 70% of cases are familial; most have mutations of the *PRKARIA* gene on chromosome 17q22–24. There is little phenotype–genotype correlation known to date.

Objective—To study the genotype–phenotype correlation in a family with newly diagnosed CNC and three generations of subjects bearing the same *PRKARIA* mutation. The proband was diagnosed with hepatocellular carcinoma, a tumour that appears to be associated with CNC.

Design—The study consisted of clinical and genetic analysis of a total of 10 individuals belonging to a large Italian family.

Patients—The index case was referred for *PRKARIA* gene mutation analysis because he met the diagnostic criteria for a clinical diagnosis of CNC.

Results—The *PRKARIA*-inactivating mutation c.502 +1G > A in the intron 5 splice-donor site was detected after bidirectional sequencing of germline DNA. The mutation causes a frameshift in the transcribed sequence and a nonsense mRNA that was shown to be degraded; this leads to *PRKARIA* haploinsufficiency in all tissues. All available relatives were screened first by DNA testing and, if the latter was positive, by clinical, biochemical and imaging means.

Conclusions—A novel *PRKARIA* mutation with an apparently low penetrance and variable expression is reported; the same mutation is also associated with a hepatocellular carcinoma. This is the first time a *PRKARIA* mutation is reported in individuals who were diagnosed with CNC after retrospective family screening and following the identification of a proband; the finding has implications for genetic counselling on *PRKARIA* and/or CNC.

Introduction

Carney complex (CNC), is an autosomal dominant multiple neoplasia syndrome (OMIM 160980).¹ Patients may present with tumours in two or more endocrine glands such as in the

adrenal cortex (primary pigmented nodular adrenocortical disease or PPNAD), the pituitary (GH-producing pituitary adenoma), gonads (testicular tumours, primarily large-cell calcifying Sertoli cell tumour or LCCSCT, and ovarian lesions that range from simple cysts to cancer), thyroid (most frequently follicular adenomas, but also papillary or follicular thyroid carcinoma). Other, non-endocrine, lesions that these patients may suffer from include cardiac, skin, breast and other myxomas, psammomatous melanotic schwannoma (PMS), breast ductal adenoma and a rare bone tumour, osteochondromyxoma.^{2–5} A recent review⁶ identified 500 patients known as affected (registered by the NIH-Mayo Clinic-USA and the Cochin Center-France); 43% were males and 57% were females. Most cases were familial (70%), more frequently transmitted through an affected female and with a small number of affected members in each family.

Recently^{7,8} mutations were identified in the *PRKARIA* gene in CNC families that were genetically mapped to 17q22–24 and sporadic cases. The *PRKARIA* gene encodes the type 1 α regulatory subunit (R1 α) of the cAMP-dependent protein kinase (PKA).⁹ Absence or deficiency of R1 α and tumour-specific loss of heterozygosity (LOH) within the chromosomal regions harbouring *PRKARIA* have been associated with dysregulated PKA activity and tumorigenicity in CNC-affected tissue^{10,11} and mouse models of R1 α -down-regulation.^{12–15} To date there have been few if any genotype–phenotype correlations with the exception of a recently published mutation [exon 7 IVS del (–7 $\geq$ –2)] that appears to be uniquely associated with PPNAD.¹⁶

The present work describes a family with three generations of subjects (Figure 1) bearing the same, novel *PRKARIA*-inactivating mutation: c.502 +1G > A mutation in the intron 5 splice-donor site of the gene. The index case was referred for *PRKARIA* gene mutation analysis because of a clinical diagnosis of CNC.¹⁰ The patient presented lentigines and developed PPNAD, pituitary adenoma, thyroid nodules, fibrocystic breast disease in her first 30 years. To our surprise, there were several relatives carrying the same mutation; we were able to identify their various CNC manifestations after careful evaluation and extensive biochemical and imaging testing. In addition to bringing to attention the possible association of hepatocellular carcinoma with a novel *PRKARIA* mutation, the study underscores the variability of expression of CNC.

Methods

Clinical studies

Patients were evaluated and followed in the Paediatrics Department of the University of Bologna until adulthood and subsequently in the Department of General Medicine of Padua. The subjects were also enrolled in protocol 95CH-0059 of the National Institute of Health, Bethesda, MD, USA, after they gave an informed consent.

DNA analysis

DNA of all family members was extracted from peripheral blood leucocytes according to manufacturer instructions (Qiagen, Hilden, Germany). Exons 2–11 of the *PRKARIA* gene and the surrounding intron boundaries were amplified and the purified PCR products were directly sequenced as previously described.²

Lymphocytes culture and cyclohexamide treatment

Lymphocyte cell lines from the patients were established by Epstein Barr virus transformation as described.² Lymphocytes were treated with 100 μ g/ml cyclohexamide or vehicle for 6 h. Total RNA was isolated using TRIzol reagent (Invitrogen Life Technologies, Inc., Carlsbad, CA). 1 μ g of total RNA was reverse transcribed to cDNA by AMV reverse

transcriptase (Roche Applied Science, Indianapolis, IN) with Oligo dT primer according to the manufacturers' instructions. *PRKAR1A* cDNA from lymphocytes was amplified using the following primers (5'–3'): R1AF – TAACATTCAAGCGCTGCTCA and R1AR – TTCTCCAAAGCTCCCTCCTT. The RT-PCR fragments were agarose gel purified and analysed by direct sequencing.

Analysis for LOH

To examine for a possible LOH at 17q23–q24 in the liver neoplastic tissue, we carried out a high-resolution PCR-LOH study on three different tumour DNA samples and compared with three DNA samples isolated from normal liver tissue from the same patient. We analysed a total of six highly polymorphic microsatellite markers, including D17S1870, D17S694, D17S2193, D17S789, D17S795 and D17S940, which spanned a region of 1.5 Mb that centres *PRKAR1A* (positioned at region 64019705–64040503), Table 1. Each marker was amplified using fluorescently labelled PCR primers, and peaks were quantified on an automated sequencer (3130xl Genetic Analyser, Applied Biosystems, Foster City, CA).

Case reports

Patient 1: (CAR52.III.5, proband)

A 30-year-old white woman first presented when she was 6 years. Physical examination revealed lentigines on the face, lips, genitals, arms and back. She was born at term by spontaneous delivery of a pregnancy characterized by tendency of abortion and maternal hypertension (neonatal weight 2200 g) and had neonatal hepatitis of an undefined aetiology which gradually evolved into hepatic steatosis. Clinical and histological pattern improved followed by a complete recovery later. At 4 years of age, she started to experience recurrent cycles of hypercortisolism that were associated with childhood diseases, usually febrile episodes. The family was not given a definite diagnosis. At the age of 6 years, however, she finally presented with moon facies, a rapid weight gain, and a consistent hypercortisolism: loss of diurnal rhythm of plasma cortisol, low ACTH, and high urinary glucocorticoids both at baseline and following the administration of dexametasone for 2 days. Because computed tomography (CT) showed adrenal glands of normal size, an iodomethyl-19-norcholesterol scan was obtained that showed a mildly larger left gland with homogeneous uptake. She underwent left adrenalectomy for ACTH-independent Cushing's syndrome in 1983. The patient became normocortisolemic after the procedure, but the pathology was consistent with PPNAD.

Other clinical problems in her included: (i) the detection of hypothyroidism at 13 years of age and the presence, during the follow up, of multiple hypoechoic nodules at sonography (US). A needle biopsy was performed and histology was consistent with follicular adenomas. She was started on T4 replacement. (ii) At the age of 18 years, a pituitary microadenoma was diagnosed after gradual elevation of IGF-1 levels and eventually a nonsuppressible GH level on oral glucose tolerance test (OGTT) were detected. The patient was placed on low dose octreotide therapy. (iii) At the age of 21 years, mammary US showed multiple hypoechoic nodules: fibrocystic disease without solid nodules or myxoid tumours was confirmed during follow up. (iv) At the age of 19 years, expansive hepatic lesions began to appear, hypointense in T1, disomogeneous and hyperintense in T2 at MRI; at the same time, a pancreatic cyst (1.5 cm) was found at the pancreatic head. Arteriography discovered four intrahepatic focal lesions located in the right lobes, with irregular vascularization, hypercaptant, of moruliform (blackberry-like) aspect, with regular outline. Displastic lesions were suspected. The first hepatic biopsy showed hyperplastic hepatocellular nodules; she underwent partial hepatectomy and the pancreatic cyst was removed when she was 26 years old. Pathology report identified hepatocellular adenoma

with haemorrhagic areas and calcified areas in the wall of the pancreatic cyst. No other liver lesions appeared after the surgery.

Patient 2: (CAR52.III.4)

A 33-year-old man, the brother of patient 1, currently had presented with extreme tall stature for the first time when he was 16 years old. Physical examination revealed lentigines on the face, lips, genitals, arms and on his back. A GH-secreting pituitary adenoma was diagnosed, which was at first treated pharmacologically unsuccessfully. Transsphenoidal surgery was performed 2 years later; the patient also required maxillo-facial reconstruction to treat his significant prognathism. His adult stature is 201.8 cm. No other clinical features (cardiac, testicular, thyroid, liver, adrenal lesions) are present currently.

Patient 3: (CAR52.II.3)

The proband's mother presented with lentigines on the face, lips and arms. She had been diagnosed with hypothyroidism in the past and was on replacement. Thyroid US showed multiple hypoechoic nodules. She also had fibrocystic mastopathy and had undergone multiple skin surgeries for the excision of cutaneous nodules (not available histology) but had no other clinical problems.

Patient 4: (CAR52.I.2)

The maternal grandfather had lentigines. In his medical history, the excision of multiple skin tags and colonic polyps was notable. He had also undergone thyroidectomy for a nodule identified as a thyroid carcinoma but details were not available. No other clinical problems were known.

Genetic studies

Detection of a heterozygous c.501 +1G > A mutation

Sequencing of coding exons of *PRKARIA* gene in the index case revealed a single heterozygous substitution at the +1 position of the intron 5 donor site. This mutation affects the consensus 'GT' splice donor sequence and is expected to destroy the splice donor site of intron 5, causing all or part of intron 5 to be retained in the message. The family members were referred to genetic analysis and three additional carriers were identified.

Effects of the mutation on mRNA

Deletion of exon 5 results in frameshift and subsequent premature stop codon generation. As in other *PRKARIA* premature stop codon mutation, the mutant allele is predicted to be degraded by nonsense-mediated mRNA decay NMD. To prevent NMD, we treated transformed lymphocytes from two patients with cyclohexamide. RT-PCR was performed using primers flanking exon 5. The treatment led to stabilization of an abnormal, lacking exon 5 mRNA with a premature stop codon (Figure 2). The frameshift occurs at codon 147 changing it from AGA to AGG (R147R) and creates a stop codon immediately after that location.

LOH in tumour samples

To address whether genetic aberrations could contribute to the tumour formation, genomic imbalance of the *PRKARIA* locus was evaluated by analysing microsatellite markers in three tumour and three normal DNA tissue samples from the affected patient. A total of six polymorphic microsatellite markers (D17S1870, D17S694, D17S2193, D17S789, D17S795 and D17S940), spanning a region of 1.5 Mb that centres *PRKARIA* were analysed (Figure 3a). The results from the microsatellite analysis are presented on Figure 3; overall, three out

of the six studied markers were informative: D17S694, D17S789 and D17S795. The family analysis showed moderate decrease in the allele segregating with the disease status, thus suggesting involvement of LOH events in the liver tumour formation. The results are presented as an average score of the three informative markers; the mean value of the three tumour and the three normal DNA samples from the same tissue is also shown. The ratio between the wild type allele and the mutant allele was 1.55 (Figure 3b), however, this observation may also reflect a possible contamination with normal cells of the dissected tissue sections.

Discussion

The present report describes a novel heterozygous G > A substitution of the invariant 'GT' splice donor sequence of intron 5 of the human *PRKARIA* premRNA. This variant was identified in four individuals – the index case and three additional family members, referred to the genetic analysis after the identification of the mutation in the proband. Their phenotype was milder but met the diagnostic criteria for CNC (at least two major criteria).¹⁰ The c.502 +1G > A substitution appears to have therefore significantly variable expression.

To study the effect of c.502 +1G > A variant *in vitro*, we established transformed lymphocyte cell line from the affected family. After abrogation of NMD by cyclohexamide treatment, an additional mRNA variant lacking exon 5 was observed, signifying decreased splicing efficiency of the mutant premRNA compared with the wild-type control samples (see Figure 2).

The medical history of our index case suggests the possible association of hepatocellular carcinoma with this novel *PRKARIA* mutation and possibly CNC. In our most recent review on the subject we included this tumour as a possibility.¹⁷ Liver tumours were seen in almost all CNC mouse models studied to date;^{12–15} liver tissue showed a PKA activity pattern that was similar to that in other tissues that developed tumours in at least one of the mouse models.^{12,13} Veugelers *et al.*¹⁵ found that five *prka1a*^{+/-} mice (out of a total of 17) had hepatocellular carcinomas; the authors suggested that even though CNC's association with hepatocellular carcinoma was not clear, *PRKARIA* was initially identified as a locus that repressed transcription specially in hepatocellular carcinoma cells.^{18,19} In a recent update of pathology of our database of the tTA-X2AS *prkar1a*-under-expressing mice^{12,13} we identified 3 mice of 38 with hepatocellular hyperplastic lesions and/or adenomas (8%). Another case of a fibrolamellar hepatocellular carcinoma that was excised in a 14-year-old girl developing 5 years after the detection of an hepatocellular adenoma has been published.²⁰ This patient was studied by us recently and was found to have both PPNAD and acromegaly (data not shown). The LOH study in our patient seems to confirm that an involvement of this mechanism in the liver tumour formation could be possible also if the ratio between the wild type allele and the mutant allele was not so high (1.55) reflecting probably a possible contamination with normal cells. The neonatal hepatitis presented, unlikely not well documented, was completely recovered later and we think it was not related to the following liver lesions.

We conclude that the *PRKARIA*-inactivating mutation c.502 +1G > A has a variable phenotype in patients with Carney complex; an association between liver tumours and CNC appears to be probable.

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Figure 1. Family CAR52 tree. Patients I.2, II.1-2-3-4 and III.1-2-3-4-5 underwent PRKAR1A analysis. Arrow indicates the index case.

Figure 2.

RT-PCR on transformed lymphocytes from the affected family and control individuals. Lanes 1 and 2 – before cyclohexamide treatment; lanes 3 and 4, and, 6 and 7 – after 4 and 8 h, respectively, with cyclohexamide treatment – the abrogation of NMD allows expression of the shortened RNA, lacking exon 5 and containing premature stop codon. After gel extraction and purification, RT-PCR products were directly sequenced.

Figure 3.

Loss of heterozygosity (LOH) analysis on liver tumour samples. (a) Schematic representation of the microsatellite markers of located on chromosome 17, the informative markers are bolded. (b) Graph representation of the tumour and the normal tissues – comparisons of the signal intensity; all the valued are averaged.

Table 1

Macrosatellite markers, position on Chromosome 17 and primers for their amplification

Marker	Position on chromosome 17	Right PRIMER (52–32)	Left Primer (52–32)
D17S1870	63010430–63010663	TGTTGCCCCCTTAGATTCA	CGCCTGGCTAATTTTG
D17S694	63897627–63897828	TCACTTGAACCCGGAAGCA	CTTCTGGAACGAGCCCCCTGTG
D17S2193	64059357–64059498	TCTCTAATCTGCCCAAGCAC	ACACTTAGGTCATTGTGTGGC
D17S789	64140160–64140315	ACTCCAAATCAAGTTGTACTGAGA	CTGCATACGAAGGGTAGGAC
D17S795	64189802–64189894	GCTATCTCCAGCATTCTCA	GTTGTTAGCAATACTCGTAGGA
D17S940	64590012–64590150	AGTTTCTGCTCCACCTTCTT	TAGAGAGTGAAAGGGGTATG