Objective: Primary pigmented nodular adrenocortical disease (PPNAD) is the main endocrine manifestation of Carney complex, a multiple neoplasia syndrome caused by *PRKAR1A* gene mutations. The presence of *PRKAR1A* loss of heterozygosity (LOH) in adrenocortical tumorigenesis remains controversial. The aim of the present study is to investigate the presence of *PRKAR1A* LOH in adrenocortical cells in a patient with Carney complex. **Methods:** The LOH was investigated using a *PRKAR1A* informative intragenic marker by GeneScan software analysis in DNA obtained from laser-captured microdissected cells of several adrenal nodules. **Patients:** A young adult male patient with Carney complex and his family were studied. **Results:** A novel heterozygous mutation (p. Y21X) was identified at *PRKAR1A* in blood DNA of the male proband and his relatives. No *PRKAR1A* LOH was evidenced in the laser-captured microdissected cells from PPNAD tissue by different methodologies. **Conclusion:** We identified a new *PRKAR1A* nonsense mutation and in addition we did not evidence *PRKAR1A* LOH in laser-captured nodules cells, suggesting that adrenocortical tumorigenesis in PPNAD may occurs apart from the second hit. (Arq Bras Endocrinol Metab 2008; 52/8:1257-1263)

**Keywords:** *PRKAR1A*; PPNAD; LOH; Laser-captured microdissection

**RESUMO**

Ausência da Perda de Heterozigose do *PRKAR1A* em Células Capturadas por Microdissecção a Laser de Tecido de Nódulo Pigmentoso Adrenocortical de um Paciente com Complexo de Carney Causado por uma Nova Mutação Nonsense.

**Objetivo:** A doença adrenocortical nodular pigmentosa primária (PPNAD) é uma das manifestações do complexo de Carney, uma neoplasia endócrina múltipla causada por mutações no *PRKAR1A*. A perda de heterozigose (LOH) do *PRKAR1A* na tumorigênese adrenal permanece controversa dada à possibilidade de contaminação com o tecido normal. Nosso objetivo foi investigar a presença de LOH no *PRKAR1A* a partir de células do nódulo adrenal de um paciente com complexo de Carney. **Métodos:** A pesquisa da LOH do *PRKAR1A* foi realizada através do estudo de um marcador intragênico em DNA de células do nódulo adrenal microdissecadas a laser, evitando contaminação com o tecido normal. Pacientes: Um paciente com PPNAD e cinco familiares foram estudados. **Resultados:** A nova mutação (p. Y21X) foi identificada no *PRKAR1A* sem evidência de LOH no tecido adrenal. **Conclusão:** Identificamos uma nova mutação no *PRKAR1A* e não evidenciamos LOH nas células dos nódulos adrenocorticais, sugerindo que a PPNAD possa ocorrer na ausência de um segundo evento molecular. (Arq Bras Endocrinol Metab 2008; 52/8:1257-1263)

**Descritores:** *PRKAR1A*; PPNAD; LOH; Microdissecção; Captura a laser
INTRODUCTION

Carney complex is a multiple endocrine neoplasia syndrome characterized by spotty skin pigmentation, cardiac and extracardiac myxomas, schwannomas, and endocrine tumours (1). Primary pigmented nodular adrenocortical disease (PPNAD), a rare cause of ACTH-independent Cushing syndrome, is the main endocrine manifestation of Carney complex (1,2). Mutations of the gene encoding protein kinase A regulatory subunit 1α (PRKAR1A) represent the molecular aetiology of approximately 41% of the kindreds affected by Carney complex (3-6). Additionally, genetic linkage analysis has identified other genetic loci on chromosome 2p in families with Carney complex (7,8).

PRKAR1A appears to function as a classic tumour suppressor gene (3). To date, PRKAR1A loss of heterozygosity (LOH) studies were reported in 16 lesions caused by Carney complex (3,4,9-12). In several of these cases, tumours displayed 17q22-24 LOH or allelic loss, particularly in GH-producing pituitary adenomas, thyroid and testicular tumours (3,10). In contrast, subsequent studies demonstrated that cardiac and other myxomas exhibited PRKAR1A LOH inconsistently (4,9). In addition, retention of heterozygosity was also demonstrated by direct sequencing of PRKAR1A in adrenocortical tissue from three patients with isolated PPNAD (11). LOH analysis can often be misdiagnosing due to significant contamination with normal cells that surround tumours. The laser-captured microdissection is currently considered the best way to overcome surrounding tissue admixture (13,14). Very recently, Mavrakis and cols. (15) demonstrated the presence of LOH exclusively in nodular areas of laser-captured microdissected PPNAD tissue from a patient with Carney complex. However, PRKAR1A LOH was based only on the segregation of a single polymorphic marker (GATAE12) located 3 cM upstream the PRKAR1A gene, which makes this finding very inconsistent (15). Therefore, additional LOH studies in laser-captured microdissected adrenal nodules of PPNAD tissue would be relevant to clarify the controversial aspects involving PRKAR1A LOH occurrence. Herein, we carried out an LOH analysis for an intragenic PRKAR1A marker in laser-captured microdissected cells of PPNAD tissue from a patient with familial Carney complex, caused by a novel PRKAR1A mutation.

SUBJECTS AND METHODS

The protocol was approved by the Ethical Committee of Hospital das Clínicas, School of Medicine of the University of São Paulo. Informed and written consent was obtained from the proband and his relatives.

A 17-year-old male patient was referred for endocrine evaluation of suspected Cushing’s syndrome. He presented central obesity (BMI 32 kg/m²), facial plethora, abdominal purple striae, spotty skin pigmentation (lentiginoses) and high blood pressure (160/100 mmHg). Hormonal investigation revealed ACTH-independent Cushing syndrome (Table 1). Magnetic resonance imaging (MRI) revealed multiple left and right adrenocortical micronodules (Figure 1A). Testicular...
thyroid and cardiac ultrasound and pituitary MRI were normal. Serum concentrations of GH, prolactin and IGF1 were within the normal range.

He underwent laparoscopic bilateral adrenalectomy. Both adrenal glands had an overall normal size and weight and featured multiple small yellow to dark brown nodules. Microscopically, both adrenal glands showed several multiple pigmented nodules (1-5 mm) surrounded by apparently normal adjacent adrenal cortex; Fontana-Masson staining revealed intracellular lipofuscin pigments (Figure 1B).

Four first-degree relatives were evaluated through history and physical examination. Hormonal and imaging screenings for diagnosis of hypercortisolism were performed in his father and two younger sisters without Cushing’s syndrome features. Bone densitometry was also performed in the male proband and his youngest sister.

**Hormonal assays**

ACTH was measured by an immunoradiometric kit (ELSA-ACTH, CIS bio international) with intra- and interassay CVs <14% and <20%, respectively, which are appropriate for an ACTH range of 18-2,420 pg/mL. Serum and urinary cortisol were measured by fluorometric assay in an AutoDelfia System (Wallac Oy, Turku, Finland), with intra- and interassay coefficients of variation (CVs) <10% and <12%, respectively.

**DNA analysis**

Genomic DNA was extracted from peripheral blood leukocytes and frozen tumour tissue of the proband using standard procedures. Molecular studies were also performed in six relatives (his parents, two sisters, one paternal aunt and a female cousin). The 12 exons and the flanking intronic sequences of the PRKAR1A gene.
were amplified using the previously described primers and conditions (5). The PCR products were pretreated with an enzymatic combination of exonuclease I and shrimp alkaline phosphatase (United States Biochemical Corp., Cleveland, OH) and directly sequenced using the BigDyeTM terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Foster City, CA) in an ABI PRISM 310 automatic sequencer (Perkin Elmer Cetus).

**Laser-captured microdissection and DNA extraction of PPNAD cells**

Adrenal frozen tissue was cut into 7-μm sections and subsequently hydrated in 70% ethanol solution for 30 seconds, followed by staining with 100 μl of Toluidine blue for 20 seconds after rinsing with distilled water. The sections were dehydrated in graded 75%, 95% and 100% ethanol for 30 seconds each and dipped in xylene for 5 minutes. After air-drying, laser-captured microdissection was performed in selected regions onto a thermoplastic film mounted on optically transparent LCM cap (CapSure Macro LCM Cap, Arcturus). A Pix Cell II Laser capture instrument (Arcturus, Mountain View, CA) was used to microdissect several PPNAD nodules (>10,000 cells) by focal melting of the membrane through laser activation. A cap with captured cells was inserted into a 0.5 ml microcentrifuge tube and DNA extraction was performed according to Pico pure DNA Extraction Kit (Arcturus, Mountain View, CA). The exon 2 of \textit{PRKAR1A} was amplified and sequenced as previously described (5).

**PRKAR1A LOH analysis**

Primer for the dinucleotide repeats (CA)$_n$ intragenic marker for \textit{PRKAR1A} gene have been published elsewhere (5). The forward primer was labelled with fluorescent dye (FAM). Genomic DNA (100 ng), extracted from blood leukocytes, PPNAD tissue and laser-captured microdissected tumour cells was then added to a 50-μl reaction mixture of 1x PCR buffer (50 mM KCl, 1.5 mM MgCl$_2$, and 10 mM Tris/HCl, pH 9.0), 200 μM of each dNTP, 20 pmol of each primer, and 2.5 U Taq DNA polymerase (Amersham Pharmacia, Uppsala, Sweden). The PCR mixture was denatured for 5 min at 94 °C and cycled 35 times (94 °C for 30 sec, 50–62 °C for 30 sec, and 72 °C for 30 sec), followed by a 30-min extension at 72 °C. Internal size standard TAMRA 350 (Applied Biosystems, Foster City, CA) was added to 2 μl of PCR products and 24 μl of formamide, and those were submitted to capillary electrophoresis in an automatic sequencer (ABI Prism 310 genetic analyser) followed by GeneScan fragment analysis (Applied Biosystems, Foster City, CA). We applied a criterion for LOH scoring using the ratio of the heights of the allele peaks obtained from the tumour and the blood samples. An allelic imbalance ratio less than 0.5 or more than 2.0 was defined as LOH (16,17).

**RESULTS**

Automated sequencing revealed a novel germline \textit{PRKAR1A} mutation in exon 2 at codon 21 (TAC→TAA), encoding a substitution of a tyrosine by a stop codon (p. Y21X), in heterozygous state in the proband as well as in his father, two younger sisters, one paternal aunt and a female cousin (Figure 1C and 1D). All of them exhibited lentiginoses. Even though only the proband had overt Cushing syndrome, his father and the two sisters who carried the same mutation had evidence of hormonal abnormalities (Table 1) (18). Although the serum cortisol levels after overnight 1 mg dexamethasone test were borderline, the suppressed ACTH levels, as well as the slightly elevated 24-h urinary non-extracted cortisol indicated the diagnosis of subclinical cortisol hypersecretion in the father. Similarly, both sisters also had suppressed ACTH levels and non-suppressed serum cortisol levels after the overnight low-dose dexamethasone suppression test. The male proband and his youngest sister also presented low bone mass (Z score < -2 SD).

The p. Y21X mutation was also identified in heterozygous state in PPNAD tissue from the proband. The sequencing analysis of the entire coding region of the \textit{PRKAR1A} ruled out the possibility of a somatic defect in compound heterozygosity with the p. Y21X mutation in this patient. Two distinct alleles of the intragenic marker for \textit{PRKAR1A} were identified in DNA from blood lymphocytes as well as in adrenocortical tumour tissue, demonstrating no allelic loss involving \textit{PRKAR1A} gene in PPNAD tissue. Absence of \textit{PRKAR1A} LOH for the p. Y21X mutation as well as for the dinucleotide repeat (CA)$_n$ intragenic marker were confirmed in DNA obtained from PPNAD cells after laser-captured microdissection, indicating that wild-type (WT) \textit{PRKAR1A} allele retention is not a consequence of normal tissue contamination (Figure 2).
DISCUSSION

Tumour-suppressor genes generally act in a recessive way, requiring loss of both copies to induce tumorigenesis, whereas haploinsufficiency only leads to tumour development predisposition. However, PRKAR1A, a potential tumour suppressor gene, has not been consistently associated with LOH in adrenocortical tumour tissues of patients with Carney complex. This might be ascribed to the fact that the exclusion of potential normal tissue contamination was not performed (4,12).

A recent LOH study using a single polymorphic marker GATA1E12, located 3 cM upstream the PRKAR1A gene, showed LOH occurrence only in microdissected PPNAD tissue, suggesting that the absence of LOH in previous analyses could occur due to contamination with surrounding cortex (15). In our study, both the p. Y21X mutation and the intragenic marker for PRKAR1A were identified in heterozygous state in the laser-captured microdissected cells using two different molecular approaches: automated sequencing and Genescan analysis, respectively. In summary, we demonstrated that the laser-captured PPNAD cells can preserve the WT PRKAR1A allele, indicating that this allele retention is not a consequence of contamination with normal surrounding cortex.

PRKAR1A haploinsufficiency has been hypothesized to cause tumours in patients with Carney complex (4). The most likely mechanism by which a truncated R1α protein could lead to cAMP signalling alterations is a dominant negative effect on R1α-mediated inhibition of the PKA catalytic subunit (12). The heterozygous p. Y21X mutation encodes a substitution of a highly conserved tyrosine by a stop codon, predicted to lead to a truncated protein in this young Brazilian male patient with PPNAD. Most of the PRKAR1A defects described to date are functionally null heterozygous mutations (5,12,19). Interestingly, two large families that share the 578delTG PRKAR1A mutation have different phenotypes, suggesting failure of PRKAR1A genotype-phenotype correlation (4,20). Similarly, a variable clinical expression ranging from subclinical to overt Cushing syndrome was associated with the p. Y21X mutation of
PRKAR1A in the family described here. This phenotypic variability is likely to be a consequence of as-yet-unidentified modifying genetic and/or environmental factors.

Recently, Horvath and cols. (8) identified germline mutations of the phosphodiesterase gene in 3 kindreds with Cushing syndrome and micronodular hyperplasia that were not caused by known defects in other genes. The finding of inactivating mutations of the phosphodiesterase gene in micronodular hyperplasia raises the possibility of yet-unknown abnormalities in signal transduction proteins that interact with R1α and cAMP-dependent PKA, and eventually contribute to tumorigenesis in patients whose tumours do not display PRKAR1A LOH.

Similarities and dissimilarities between human Carney complex phenotypes and murine Prkar1a haploinsufficiency have been observed (19,21). Two hallmarks of Carney complex, spotty pigmentation and cardiac myxomas are not evident in Prkar1a−/− mice. Absence of cardiac myxomas in prkar1a−/− mice suggests that Prkar1a haploinsufficiency is insufficient to produce cardiac tumorigenesis. Nevertheless, tumours such as hemangio- sarcomas and myxoid fibrosarcomas that arise in prkar1a−/− mice do not display prkar1a LOH (19). As demonstrated in a mouse model for the Carney complex tumour syndrome, we showed that human tumour cells can similarly preserve the WT PRKAR1A allele in affected tissues of patients with Carney complex.

In conclusion, we identified a new PRKAR1A nonsense mutation (p. Y21X) associated with a variable clinical expression of the PPNAD phenotype. In addition, we demonstrated absence of PRKAR1A LOH in laser-captured tumour cells, indicating that adrenocortical tumorigenesis in Carney complex occurs apart from the second hit.

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