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Detection of somatic β -catenin mutations in primary pigmented nodular adrenocortical disease (PPNAD)

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Summary

Background—Primary pigmented nodular adrenocortical disease (PPNAD) leads to Cushing syndrome (CS) and is often associated with Carney complex (CNC). Genetic alterations of the type 1- α regulatory subunit of cAMP-dependent protein kinase A (*PRKARIA*) and phosphodiesterase 11A4 (*PDE11A*) genes have been found in PPNAD. Recent studies have demonstrated that β -catenin mutations are frequent in adrenocortical adenomas and carcinomas and that the Wnt-signalling pathway is involved in PPNAD tumorigenesis. We hypothesized that adrenocortical adenomas that form in the context of PPNAD may harbour β -catenin mutations.

Methods—We studied 18 patients with CS secondary to PPNAD who were screened for germline *PRKARIA* and *PDE11A* mutations. Tumor DNA was extracted from pigmented adrenocortical adenoma and nodular adrenal hyperplasia. Mutation analysis of exons 3 and 5 of β -catenin was performed using polymerase chain reaction and direct sequencing. Sections from formalin-fixed, paraffin-embedded tumour samples were studied by immunohistochemistry with an antibody against β -catenin.

Results—Nine patients were carrying germline *PRKARIA* mutations and one patient had a *PDE11A* mutation. We found somatic β -catenin mutations in 2 of 18 patients (11%). In both cases, the mutations occurred in relatively large adenomas that had formed in the background of PPNAD. Tumor DNA analysis revealed a heterozygous ACC-to-GCC missense mutation in codon 41 (T41A) and a TCT-to-CCT missense mutation in codon 45 (S45P) of exon 3 of the *β -catenin* gene that was confirmed at the cDNA level. There were no alterations in the DNA of PPNAD-adjacent tissues and lymphocytes from the patients, indicating somatic events. Immunohistochemistry showed nuclear accumulation of β -catenin in more than 90% of cells in adenomatous tissue whereas no nuclear immunoreactivity was detected in adjacent PPNAD nodular cells. Nuclear translocation of β -catenin protein in the PPNAD adenoma suggests activation of the Wnt- β -catenin pathway in PPNAD.

Conclusions—We report, for the first time, β -catenin mutations in adenomas associated with PPNAD, further implicating Wnt- β -catenin signalling in tumorigenesis linked to bilateral adrenal hyperplasias.

Introduction

Primary pigmented nodular adrenocortical disease (PPNAD) and nonpigmented micronodular adrenal hyperplasia lead to ACTH-independent CS.^{1–3} PPNAD is the most common endocrine manifestation of the Carney complex (CNC).⁴ CNC and isolated PPNAD may be caused by inactivating mutations in the *PRKARIA* gene localized on 17q22–24.⁵ More recently, mutations of the *PDE11A* gene have been found in adrenocortical hyperplasias of both the pigmented and nonpigmented forms.^{6–8} However, the genetic alterations remain largely unknown in a subgroup of patients with bilateral adrenal hyperplasias.

Recently, our group and others reported that β -catenin gene may be mutated in adult adrenocortical adenomas and carcinomas^{9,10} including cortisol and aldosterone-secreting tumours.⁹ These mutations affect serine and threonine residues localized in exon 3 of the gene that are essential for the targeted degradation of β -catenin. Thus, in the presence of mutations, β -catenin degradation will be decreased, resulting in cytoplasmic and nuclear β -catenin protein accumulation as well as transcription activation.^{11,12} In addition to adrenal tumours, β -catenin somatic mutations have been described in various neoplasms such as colorectal, endometrial, and hepatocellular cancers and medulloblastomas.^{13–16} Mutations in exon 5 of β -catenin have been described in cell lines established from human colorectal cancers.¹⁷

Wnt signalling is initiated by secreted Wnt proteins that bind to a complex containing *Frizzled* receptors and low-density lipoprotein receptor-associated proteins at the cell surface. This activation elicits the phosphorylation of *Dishevelled* protein which, in turn, inhibits glycogen synthase kinase-3 β (GSK-3 β) activity. GSK-3 β forms a complex with 2 β -catenin-binding proteins, APC, and AXIN. In the absence of Wnt signalling, β -catenin is recruited into the destruction complex that contains APC and AXIN, which facilitate its phosphorylation by casein kinase 1 and active GSK-3 β , evoking its degradation.¹⁶ In contrast, Wnt-signalling activation inhibits GSK-3 β , resulting in β -catenin accumulation in the nucleus which activates the transcription of target genes.^{18,19}

We hypothesized that β -catenin mutations may be present in adenomas formed in the context of PPNAD. Indeed, in this study, we identified somatic β -catenin mutations in two patients with PPNAD; these were found in the largest adenomas that formed in the background of bilateral adrenal hyperplasias. One of the patients had a germline *PRKARIA* mutation, indicating that *PRKARIA* initiated tumorigenesis can be associated with Wnt-signalling pathway mutations; the other had PPNAD due to a yet unknown defect.

Materials and methods

Patients and adrenocortical samples

All adrenal tissue samples were obtained from patients under research protocols approved by the Institutional Review Board of the National Institute of Child Health & Human Development, Bethesda, MD, USA (00-CH-160) and the Centre hospitalier de l'université de Montréal (CHUM), Montréal, Canada. Every patient signed informed consent. Tissue samples from 18 patients with PPNAD were available for analysis. All patients were diagnosed with ACTH-independent CS by standard diagnostic testing. The clinical profile of the investigated patients is given in Table 1.

Adrenal tissue specimens from the patients studied were obtained at the time of surgery, frozen immediately in liquid nitrogen and stored for later use. Tissue sections were fixed in formalin and embedded in paraffin for histopathological analysis. Preparations of samples

obtained at surgery from the adrenal glands and surrounding normal fibrous and fat tissue were processed for genetic analyses.

DNA and cDNA preparation

DNA was extracted from peripheral lymphocytes by standard methods. Tumor DNA was extracted from frozen tissues with TriZOL reagent® (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. This was followed by proteinase K treatment and phenol–chloroform extraction. Total RNA was extracted from frozen tissues, also with TriZOL reagent® (Invitrogen). The quantity and quality of RNA and DNA were evaluated by spectrophotometry at 260 nm. RNA (3 µg) was reverse-transcribed, using Moloney murine leukaemia virus transcriptase (Invitrogen) in a final volume of 20 µl with random hexamers (Invitrogen).⁹

Screening for the presence of *PRKAR1A*, *PDE11A* and *β-catenin* gene (*CTNNB1*) mutations

Patients were screened for germline *PRKARIA* and *PDE11A* mutations (Table 1). Sequencing methods have been described elsewhere.^{5,6,20} Exons 3 and 5 of the *β-catenin* coding region was studied as described previously.⁹ Briefly, primers for polymerase chain reaction (PCR) were designed to amplify a 534-bp fragment of exon 3 and a 567-bp fragment of exon 5 of *β-catenin* gene. DNA of 300 ng were applied in each reaction. PCR products were separated in 2% agarose gel. The amplicons were purified with a QIAquick gel extraction kit (Qiagen, Valencia, CA) and directly sequenced on an automated sequencer in both directions (SUCOF, Université Laval, Québec, QC, Canada).

Mutation analysis by reverse transcription (RT)-PCR

RT-PCR was undertaken with a specific primer set: forward 5'CCCTGAGGGTATTTGAAGTAT3' and reverse 5'ATAGCAGACACCATCTGAGG3'. They were designed to amplify a 650-bp product spanning exons 1–5. The PCR conditions were the same as those described above. PCR products were visualized on 2% agarose gel containing ethidium bromide. cDNA bands were extracted, purified and finally sequenced as above.

Immunohistochemical analysis of *β-catenin*

Sections from four paraffin-embedded PPNAD samples were available for immunohistochemistry: patient 13 carrying a *β-catenin* mutation and patients 15–17 without *β-catenin* mutations. A single adrenal gland from a normal individual was used as control. Tissue sections were deparaffinized with toluene, rehydrated through graded ethanol, treated with 3% H₂O₂ to eliminate endogenous peroxidase activity, and incubated for 17 min at 95 °C in citrate buffer (pH 6.0) for antigen retrieval. Nonspecific antigens were blocked with a protein-blocking, serum-free reagent (Dako Diagnostics Canada, Inc., Mississauga, ON, Canada). The slides were then incubated for 60 min in a humidified chamber with specific monoclonal anti-*β-catenin* antibody (1: 25, BD Transduction Laboratories, BD Biosciences, Mississauga, Canada). Twenty-minutes incubations with secondary biotinylated antibody and streptavidin-horseradish peroxidase (Dako Diagnostics Canada, Inc.) followed sequentially. Reaction products were developed with diaminobenzidine containing 0.3% H₂O₂ as substrate for peroxidase. Hematoxylin counterstaining was included for ease of analysis. The negative controls included substitution of the primary antibody by PBS and an isotype control, IgG1 (Cymbus Biotechnology, Hampshire, UK). Ten consecutive fields (400×) of each tumour studied were examined independently under standard light microscopy by two readers, including a pathologist. Staining of the nucleus, cytoplasm and membrane was described for tumour and adjacent tissues and normal adrenal gland. Staining was quantified as a percentage of cells

stained from 0 to 100% which represents a mean of the percentage found in 10 consecutive fields of each tissue.

Results

Mutational analysis of *CTNNB1* gene

Table 1 summarizes the results of mutational analysis of *CTNNB1* gene in 18 human PPNAD samples: we found mutations in 2 of 18 samples. All patients with PPNAD showed classic pigmented micronodular adrenal hyperplasia. However, patients 12 and 13 had in addition each a single large adenoma which was shown to harbour β -catenin mutations. Clinical details and a description of the mutations in these two patients appear below. We did not detect genetic alterations in exon 5 of β -catenin gene in any of the studied samples.

Patient 12

A 23-year-old woman was referred to the NIH for evaluation of CS and CNC. Her family history revealed a phenotype consistent with CNC in her mother and sister (lentigines, heart myxomas and thyroid adenomas). Echocardiography disclosed an atrial myxoma. The patient was also found to have a growth hormone-producing pituitary adenoma. In addition, she presented with continuous hypertension and mild glucose intolerance. ACTH-independent CS was diagnosed: a 3.5 cm right adrenal mass and a slightly irregular left adrenal gland were seen on imaging studies. The patient underwent bilateral adrenalectomy, and microscopical and histological evaluation showed PPNAD with a large adenoma on the right adrenal gland. Genetic testing for *PRKARIA* mutations identified an heterozygote germline deletion of 103 bp, eliminating nucleotides, (5–107) from the 3' end of intron 7 (c. 709-(5–107) del103). The mutation destroys the splice acceptor site of this intron and generates a shift in the reading frame, resulting in a nontranslated, nonsense mRNA that is decayed by nonsense mRNA-mediated decay (NMD). The patient's DNA was negative for *PDE11A* sequence alterations. In DNA extracted from the adenoma, an heterozygous ACC-to-GCC missense mutation was identified at codon 41 of exon 3 of the β -catenin gene leading to an amino acid change from threonine to alanine (T41A). No genetic modifications were observed in PPNAD micronodules and adipose tissue adjacent to the adenoma, indicating a somatic genetic alteration.

Patient 13

A 61-year-old man was referred for suspected CS during the last 5 years. His family history revealed a diagnosis of CS secondary to PPNAD in his daughter (at age 28 years) and in his grand-daughter (at the age of 8 years). ACTH-independent CS without CNC manifestations was confirmed; Liddle's test did not show a paradoxical response of cortisol to dexamethasone, a biochemical feature of PPNAD associated with CNC.^{21,22} Imaging showed a 2.4-cm right-sided adrenal adenoma; the left adrenal was irregular (Figure 1a). He underwent right adrenalectomy. Histology disclosed a cortical pigmented adenoma and multiple, small, pigmented nodules in adjacent tissue, consistent with PPNAD (Figure 1b). Sequencing analysis of the coding regions of *PRKARIA* failed to detect any mutations. DNA analysis of the cortical pigmented adenoma revealed an heterozygous TCT-to-CCT missense mutation in codon 45 in exon 3 of β -catenin (Figure 2). This is predicted to cause an amino acid change from serine to proline (S45P). There were no genetic alterations in the DNA of PPNAD-adjacent tissue and lymphocyte DNA from the patient, indicating a somatic mutation in adenomatous tissue *only*. The genetic change was confirmed expressed by cDNA sequencing of adrenal tissue RNA (Figure 2c).

Immunohistochemical analysis of β -catenin

Immunohistochemistry for the expression and localization of β -catenin protein was obtained in four PPNAD samples: patient 13 carrying the S45P β -catenin mutation and patients 15, 16 and 17 without any β -catenin mutations; control adrenal tissue was also studied. As described previously,⁹ we observed β -catenin staining of the outer cell membrane in 100% of the cells from the normal adrenal gland. In patient 13, immunohistochemistry of β -catenin of the PPNAD adenoma showed diffuse nuclear staining in more than 98% of the tumour cells; there was also cytoplasmic immunoreactivity in all cells (Figure 3A2) and membranous staining was decreased (36%) compared to adjacent tissue (Figure 3B2) and the control adrenal gland (data not shown). The smaller adjacent nodules showed positive membranous (77%) and cytoplasmic (92%) staining which sometimes was dot-like (in up to 77% of the cells) (Figure 3C2), in contrast to adjacent non-nodular tissue where there was only membranous β -catenin staining. In patients 15–17 harbouring no β -catenin mutation, membranous β -catenin staining was observed in 76% of the cells from PPNAD nodules. In addition, no significant nuclear staining was evident and 67% of cells presented cytoplasmic staining (data not shown).

Discussion

In recent years, the identification of mutations in *PRKARIA* and *PDE11A* genes led to better understanding of the mechanisms involved in the development of cortisol-secreting bilateral adrenal hyperplasias.⁸ However, genetic alterations that are involved in the secondary development of tumours within the hyperplasia background remain unknown. Gene expression profiling studies of adrenocortical adenomas, carcinomas²³ and ACTH-independent macronodular adrenal hyperplasias (AIMAH)²⁴ have shown abnormal expression of various genes related to the Wnt- β -catenin signalling. Recently, β -catenin mutations were found in two different cohorts of patients with adrenocortical adenomas^{9,10} and carcinomas¹⁰ but not in AIMAH.⁹ *CTNNB1* gene maps to 3p21, a region frequently affected by somatic alterations in a variety of tumours. Tissier *et al.* described mutations of β -catenin gene in 7 of 26 adrenocortical adenomas and in 4 of 13 carcinomas.¹⁰ We also identified β -catenin gene mutations in 5 of 33 adrenocortical adenomas, including nonsecreting, cortisol-secreting and aldosterone-secreting tumours.⁹ In the present study, we investigated the hypothesis that β -catenin mutations may be involved in secondary tumorigenesis within the context of primary hyperplasias as well.

Indeed, we found β -catenin gene mutations in 2 (11%) of 18 patients with PPNAD. Two different point mutation of codons 41 (T41A) and 45 (S45P) were identified. As described previously in adrenocortical tumours and various other cancers, these mutations in exon 3 of β -catenin gene affect serine and threonine residues, which are normally involved in β -catenin degradation.^{12,25,26} In both cases, the mutations were shown to be somatic.

The S45P mutation has been seen before in two nonsecreting adrenocortical adenomas, one cortisol-secreting adrenocortical adenoma and the human adrenocortical cancer cell line H295R.^{9,10} The T41A mutation has previously been found only in a nonsecreting adrenocortical carcinoma.¹⁰ Thus, it appears that there is no phenotype-genotype correlation with the type of β -catenin gene mutations in adrenocortical tumours; in addition, both mutations have been described previously in various cancers, including hepatoblastomas.¹¹ Tissier *et al.* reported that the S45P mutation which is found in the human adrenocortical cancer H295R cell line was associated with constitutive activity of the Wnt-signalling pathway.¹⁰ Interestingly, in both cases, β -catenin mutations were detected in an adenoma formed within the background of PPNAD but not in adjacent nodular tissue, suggesting that β -catenin mutations are secondary genetic events participating in the nodular development of PPNAD. As demonstrated previously, *CTNNB1* mutations at potential GSK3B

phosphorylation sites affect accumulation of β -catenin protein within cells and its nuclear translocation.²⁵ This was illustrated in our immunohistochemistry studies, too; there was no significant nuclear β -catenin staining in adjacent (nonmutant) micronodules and in the cells of adjacent non-nodular tissues.

Mutations in *PRKARIA* (CNC1) lead to an increase of cAMP-stimulated PKA activity.⁵ Recently, a link between the cAMP and Wnt pathways was demonstrated: adenylyl cyclase signalling via PKA and its target transcription factor CREB were required for Wnt-directed myogenic gene expression.²⁷

In summary, we describe for the first time, β -catenin mutations in PPNAD adenomas associated with CS. Decreased cytoplasmic degradation and nuclear translocation of β -catenin in adenomatous cells suggests that Wnt- β -catenin pathway may mediate tumorigenic signals initiated by *PRKARIA* (and other PPNAD genes) deficiency.

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Figure 1.

(a) Abdominal CT scan of patient 13 showing a 2.4-cm right adrenal adenoma (arrow). The left adrenal gland was of normal size but slightly irregular. (b) Macroscopic appearance of the removed right adrenal gland: a large 2-cm adenoma is evident (black arrow), and the adjacent adrenal gland presents pigmented micronodular adrenocortical hyperplasia (red arrow).

Figure 2. Nucleotide sequencing of exon 3 of β -catenin revealed a normal sequence in lymphocyte DNA of patient 13 and PPNAD adjacent tissue (a), and T $\rightarrow$ C transition (S45P) in the cortical pigmented adenoma at the tumour DNA (b) and cDNA levels (c).

Figure 3.

Histology and immunohistochemistry of the adenoma and adjacent micronodular tissue from patient 13 with PPNAD and β -catenin gene mutation. (A, A1) Light microscopy features of an H&E-stained PPNAD adenoma. (A2) Immunohistochemical staining of β -catenin showing diffuse nuclear (N→) and cytoplasmic immunoreactivity (C→) as well as decreased membranous staining in the PPNAD dominant adenoma (original magnifications: A: $\times 40$, A1,A2: $\times 400$). In contrast, the adjacent tissue of the adenoma (B, B1, B2) presents mainly membranous staining (original magnifications: B: $\times 40$, B1,B2: $\times 400$). (C) H&E-stained adjacent PPNAD small nodules and internodular tissue from patient 13. (C2) Small adjacent nodules display no nuclear staining but irregular cytoplasmic (C→) and membranous staining (M→). Adjacent non-nodular tissue shows mainly diffuse membranous (M) immunoreactivity of β -catenin (M→) (original magnifications: C: $\times 40$, C1,C2: $\times 400$).

Table 1

Description of clinical data and genetic screening results in patients with PPNAD

Patients	Sex	Age (year)	Diagnosis	Germline <i>PRKRA</i> /A mutations	Germline <i>PDE1A</i> mutations	Somatic β -catenin mutations
1	M	3	PPNAD	c.693insT/p.Arg232X	No	No
2	F	25	PPNAD	c.1 A > G/p.Met1Val	No	No
3	F	42	PPNAD	No	No	No
4	F	21	PPNAD	c.101-105delCTATT/p.Ser34fsX9	No	No
5	F	7	PPNAD	No	171delTfs41X	No
6	F	13	PPNAD	c.891 + 3 A > G	No	No
7	F	21	PPNAD	No	Not available	No
8	F	10	PPNAD	c.438 A > T/p.Arg146Ser	No	No
9	M	9	PPNAD	No	No	No
10	F	5	PPNAD	No	No	No
11	F	39	PPNAD	c.682C > T/p.Arg228X	No	No
12	F	23	PPNAD (3.5 cm-macronodule)	c.709-(5-107) del103	No	T41A
13	M	61	PPNAD (2 cm-macronodule)	No	Not available	S45P
14	F	8	PPNAD	No	N/A	No
15	F	15	PPNAD	c.502 + 5delG	N/A	No
16	F	19	PPNAD	c.491-492delTG/p.Val164fsX4	N/A	No
17	F	43	PPNAD	No	N/A	No
18	F	23	PPNAD	No	N/A	No