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Somatic *PRKACA* mutations: association with transition from pituitary-dependent to adrenal-dependent Cushing's syndrome

Authors

Guido Di Dalmazi[1], Henri J.L.M. Timmers[2], Giorgio Arnaldi[3], Benno Küsters[4], Marina Scarpelli[5], Kerstin Bathon[6], Davide Calebiro[6,7], Felix Beuschlein[8,9], Ad Hermus[2], Martin Reincke[9].

Affiliations

[1]Division of Endocrinology, Department of Medical and Surgical Sciences, Alma Mater University of Bologna, S. Orsola-Malpighi Hospital, Bologna, Italy. [2]Department of Internal Medicine, Radboud University Medical Center, Nijmegen, The Netherlands. [3]Division of Endocrinology, Department of Clinical and Molecular Sciences, Polytechnic University of Marche, Ancona, Italy. [4]Department of Pathology, Radboud University Medical Center, Nijmegen, The Netherlands. [5]Section of Pathological Anatomy, Polytechnic University of Marche, Ancona, Italy. [6]Institute of Pharmacology and Toxicology and Bio-Imaging Center, University of Würzburg, Würzburg, Germany. [7]Institute of Metabolism and Systems Research, University of Birmingham, Birmingham, UK. [8]Klinik für EndokrinologieDiabetologie und Klinische Ernährung, UniversitätsSpital Zürich, Zürich, Switzerland. [9]Medizinische Klinik und Poliklinik IV, Klinikum der Universität München, Munich, Germany.

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Corresponding author/Reprint requests: Dr. Guido Di Dalmazi, Division of Endocrinology, Department of Medical and Surgical Sciences, Alma Mater University of Bologna, S. Orsola-Malpighi Hospital, via Massarenti, 9 – 40138 Bologna (Italy). Tel. +39 051 2143009. Fax +39 051 2143080. Email: guido.didalmazi@unibo.it

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ABSTRACT

Context. Prolonged adrenal stimulation by corticotropin, as in long-standing Cushing´s disease (CD), leads to diffuse to nodular hyperplasia. Adrenal functional autonomy has been described in a subset of patients with CD, leading to the hypothesis of transition from ACTH-dependent to ACTH-independent hypercortisolism.

Objective. Considering that *PRKACA* somatic mutations are the most common finding in adrenal adenomas associated with ACTH-independent Cushing´s syndrome, our aim was to analyze *PRKACA* mutations in adrenals of patients with persistent/long-standing CD.

Design. Cross-sectional.

Setting. University hospital.

Patients. Two patients with long-standing CD and suspicion of coexistence of autonomous adrenal hyperfunction, according to pre- and postoperative evaluations, were selected for this study following intensive literature search and patient chart reviewing.

Intervention. Clinical data were analyzed. DNA was extracted from adrenal tissue for *PRKACA* sequencing. PKA activity was assayed.

Main outcome measure. *PRKACA* somatic mutations.

Results. Both patients showed mutations of *PRKACA* in macronodule in the context of micronodular adrenal hyperplasia. One patient harbored the previously described p.Leu206Arg substitution, whereas a p.Ser213Arg missense variation was detected in the adrenal nodule of the second patient. No mutations were detected in the adjacent adrenal cortex of the second patient. In silico analysis predicts that p.Ser213Arg can interfere with the interaction between the regulatory and catalytic subunits of PKA.

Conclusions. Our study shows that *PRKACA* somatic mutations can be found in adrenal nodules of patients with CD. These genetic alterations could represent a possible mechanism underlying adrenal nodule formation and autonomous cortisol hyperproduction in a subgroup of patients with long-standing CD.

Précis

Long-standing Cushing's disease may lead to transition from pituitary to adrenal-dependent hypercortisolism. In our patients, adrenal functional autonomy was caused by somatic mutations of *PRKACA*.

Introduction

Prolonged hyperstimulation by ACTH results in alterations of adrenal gland architecture that range from diffuse hyperplasia to micro- and macronodules (1, 2), driven by the trophic effects of corticotropin. Those morphological changes are a common feature of adrenals of patients with long-standing Cushing´s disease (CD). According to several previous reports (3-11), the occurrence of nodules in the context of ACTH-stimulated adrenal hyperplasia has been associated with variable degrees of functional adrenal autonomy, leading to the hypothesis that transition from ACTH-dependent to ACTH-independent hypercortisolism could indeed occur in a subgroup of patients with long duration of the disease.

In the last years, the molecular mechanisms of ACTH-independent hypercortisolism have been extensively investigated in several independent cohorts (12-19). The results of these studies showed that somatic mutations in the gene encoding the catalytic α (Cα) subunit of protein kinase A (PKA) (*PRKACA*), leading to constitutive PKA activation, are a common finding in patients with Cushing´s syndrome due to adrenal adenoma.

The aim of our study was to analyze *PRKACA* mutations in adrenal glands of patients with persistent/long-standing CD and suspected coexistence of adrenal autonomy, to test whether the gain of function by the adrenal nodules can be driven by constitutive PKA activation.

Materials and Methods

Patients and clinical data

We enrolled two patients who were referred for severe hypercortisolism to two European centers: Department of Internal Medicine, Nijmegen, The Netherlands (patient 1) and Endocrinology Division, Ancona, Italy (patient 2). Patients were selected based on the clinical history, according to the following inclusion criteria: (i) diagnosis of Cushing´s disease due to pituitary adenoma, (ii) suspected coexistence of autonomous adrenal hyperfunction, according to pre- and postoperative evaluation, (iii) trans-sphenoidal surgery, (iv) recurrence or persistence of the disease without evidence of pituitary remnant, and (v) histological evidence of adrenal hyperplasia with and without nodules. The medical

history of patient 1 has been already published elsewhere (4). Detailed clinical and preoperative hormonal data were collected for patient 2 by reviewing the medical charts.

All patients gave written informed consent for genetic analysis. The study was approved by the ethics committee of the individual institutions.

DNA extraction, sequencing, and in silico analysis

Formalin-Fixed Paraffin Embedded (FFPE) (patient 1) and fresh-frozen (patient 2) adrenal tissues were employed for DNA extraction. The DNA was selectively extracted from the macronodules after a careful microdissection of the tumoral area performed by experienced pathologists (B.K. for patient 1, and M.S. for patient 2). In patient 2, adrenal tissue adjacent to nodular area was also available for DNA extraction.

Genomic DNA was extracted with QIAmp® DNA FFPE tissue kit (Qiagen, Hilden, Germany) in FFPE tissue, and Maxwell® Blood DNA Kit (Promega Corp., Madison, WI) in frozen tissues. DNA was amplified by PCR (details are provided in supplementary material). Bidirectional Sanger sequencing was performed using the ABI BigDye Terminator v.3.1 Cycle Sequencing Kit. The results of the sequencing analysis were evaluated using the Mutation Surveyor software (SoftGenetics). The results were confirmed after a second DNA extraction. Images of *in silico* analysis were prepared using the PyMOL software (www.pymol.org). The structure of the mouse full-length tetrameric RIIβ(2):Cα(2) holoenzyme (PDB entry 3TNP) (20) was used to display the PKA Cα and regulatory subunit (RIIβ) structures.

PKA activity assay

HEK293A cells were seeded at a density of 0.25 x 106 cells/well onto 6-well plates and allowed to grow for 24 h, before transfection with Effectene (Qiagen) according to the manufacturer´s instructions. Catalytic and regulatory subunits were co-transfected in a 1:8 ratio. All experiments were performed 48 h after transfection.

Cells were washed twice with phosphate-buffered saline at room temperature, 300 µl lysisbuffer (5 mM Tris-HCl, 2 mM EDTA, pH 7.4) were added and cells were scraped from the plate. Lysis was done using an Ultraturrax for 20 s on ice. Then samples were centrifuged at 50,000 x g for 30 min at 4°C to remove membranes. PKA subunit Cα RIIß expression in cell lysates were determined by Western blotting with specific antibodies (anti-PKA Cα (1:7000), #4782, Cell Signaling Technology; anti-PKA RIIß (1:1000), #610625, BD Transduction Laboratories), to use equal amounts of catalytic subunit for the PKA activity assay. PKA catalytic activity was measured with or without the addition of cyclic AMP (cAMP) using the PepTag non-radioactive cAMP-dependent protein kinase assay (Promega), following the manufacturer´s instructions. Images were acquired using a gel documentation system (Herolab) and analyzed with ImageJ software (http://rsbweb.nih.gov/ij). Activity of endogenously expressed PKA was subtracted and samples were normalized to expression levels of PKA Cα. Data are mean±s.e.m. of three independent experiments (two replicates per experiment).

Statistical analysis

Statistical analysis was done using Graphpad Prism 6. Results are shown as mean±SEM. Statistical analysis was performed using 2-way ANOVA followed by Bonferroni's post-hoc test to correct for multiple comparisons.

Results

The clinical history and the preoperative hormonal evaluation of the two patients are summarized in Table 1.

Patient 1

The clinical history of this patient has already been described (4). This 41-years old woman was referred to the Department of Internal Medicine of Radboud University Medical Center of Nijmegen (The Netherlands) in 2002 for clinical hypercortisolism, confirmed by elevated cortisol levels after 1-

mg dexamethasone suppression test (DST), high urinary free cortisol (UFC) and midnight cortisol values. The results of CRH testing and high-dose DST were atypical for ACTH-dependent hypercortisolism. However, basal ACTH was elevated, and the pituitary MRI showed a left pituitary microadenoma (7 mm). The inferior petrosal sinus sampling was indicative of a central source of ACTH. The patient underwent trans-sphenoidal surgery with removal of the pituitary microadenoma, confirmed by histological examination (positive ACTH staining). After surgery, the patient experienced clinical remission and normalization of UFC and morning cortisol levels. However, cortisol after 1-mg DST was still elevated. An abdominal CT-scan revealed a left adrenal macronodule. During the 12 months following surgery, the patient experienced recurrence of signs specific to hypercortisolism. Increase in UFC levels with undetectable ACTH were also recorded. The pituitary MRI at that time was unremarkable. The patient underwent left adrenalectomy and histological examination showed a 35-mm macronodule in the context of focal micronodular hyperplasia. Glucocorticoid replacement therapy was needed for one year after surgery. At the last follow-up, 15 years after surgery, the patient was free of recurrence.

Analysis of the left adrenal macronodule showed a c.617A>C (p.Leu206Arg) missense mutation. No adjacent adrenal cortex was available for genetic screening. The functional implications of this mutation have previously been described (13).

Patient 2

This patient, a 31-years old woman, was referred to the Endocrinology division of Ancona (Italy) in 2000 for clinical Cushing´s syndrome developed during the last 28 months. UFC and midnight cortisol levels were indicative of hypercortisolism, which was confirmed by the cortisol levels after 1-mg DST. Basal ACTH and stimulation tests with CRH and desmopressin were concordant with the diagnosis of CD. Cortisol level after high-dose DST was 166 nmol/L. A pituitary MRI-scan revealed a microadenoma. The patient was treated with trans-sphenoidal surgery and histological examination confirmed an ACTH-positive pituitary microadenoma. After surgery, the patient experienced persistence of clinical and biochemical hypercortisolism, accompanied by undetectable plasma ACTH

levels and no evidence of remnant adenoma at pituitary MRI. An abdominal CT-scan showed bilateral diffuse enlargement with a left–sided adrenal nodule. Two years after surgery, bilateral adrenalectomy was performed. Histological examination showed a left macronodule in the context of micronodular hyperplasia.

PRKACA analysis showed a missense mutation c.639G>T (p.Ser213Arg) in tissue extracted from the adrenal macronodule. No mutations were found in the adjacent hyperplastic tissue, supporting the concept that the mutation occurred at a somatic level. As depicted in Figure 1, *in silico* analysis of the mutation showed that Ser213 is located at the surface of the Cα subunit of PKA, in a region that adopts a "tip-like" structure, which is inserted into a complementary cavity of the regulatory subunit. Substitution with an arginine in position 213 could therefore impede the interaction between the regulatory and catalytic subunits, leading to unregulated activation of the latter. As shown in Figure 2, a PKA activity assay revealed that mutant cells expressing Cα subunit with Ser213Arg missense mutations had higher basal PKA activity than cells transfected with WT Cα subunit, in the absence of cAMP. The high basal PKA activity was not different from Leu206Arg mutation (Figure 2).

Discussion

In a subgroup of patients with CD, the progressive acquisition of functional autonomy by the adrenals has been claimed as a potential evolution of the natural history of the disease. The so-called "transition" from pituitary to adrenal hypercortisolism in CD is supposed to be a late feature of the disease that occurs mainly in the presence of nodular alterations of the adrenal gland. However, until now, this entity has been hypothesized only based on clinical and biochemical features, given that no studies have yet investigated the molecular mechanisms underlying this condition.

In this study, we screened *PRKACA* mutations in two patients carefully selected on the basis of ambiguous pre- and post-operative evaluations indicative of hypercortisolism of adrenal as well as pituitary origin. We aimed to test the hypothesis whether the acquisition of adrenal autonomy, presumed by the hormonal tests, can be driven by a gain of function of PKA. In fact, it is now well known that *PRKACA* somatic mutations, which lead to autonomous activation of PKA, are the

underlying cause of ACTH-independent hypercortisolism in more than 30% of patients with adrenal adenomas (12-19).

The adrenals of the two patients enrolled in this study showed somatic *PRKACA* mutations. Specifically, we found a p.Leu206Arg substitution in one patient (n. 1), which is the most frequent alteration in ACTH-independent Cushing´s syndrome, and a p.Ser213Arg substitution in the second one (n. 2). The p.Ser213Arg substitution has been already described in a recent study, in association with a 12-bp duplication, in a patient with adrenal Cushing´s syndrome (16). According to functional and *in silico* analysis, those mutations impede the interaction between Cα and the regulatory subunit of PKA by altering the structure of the former, causing a cAMP-independent increase in PKA activity (21, 22). The *in silico* analysis of the p.Ser213Arg substitution predicted a similar pathogenetic mechanism, even though no functional analysis was performed to confirm this hypothesis. Nonetheless, with respect to WT, the p.Ser213Arg missense mutation showed high basal PKA activity, similar to the known p.Leu206Arg mutation. Those data suggest a pathogenetic role also for this novel variant in inducing PKA activation.

The discovery of *PRKACA* somatic mutations in adrenals of a specific subset of patients with CD is novel and provides indirect evidence for the concept of an evolutionary transition from pituitary-dependent to adrenal-dependent hypercortisolism, sustained by the acquirement of PKA autonomous activity. The exact mechanism that leads to this entity is unknown. It is tempting to speculate that the continuous growth stimulation and the prolonged activation of steroidogenesis exerted by corticotropin could generate a favorable microenvironment that facilitates the onset of genetic aberrations resulting in adrenal nodularity. The higher sensitivity to ACTH of adrenal nodules compared to the hyperplastic adjacent cortex in CD, previously demonstrated in *in vitro* and *in vivo* studies (3, 7, 23, 24), could be a contributing factor. If true, it is feasible that those conditions may be associated with a more severe hypercortisolism than patients with Cushing's disease, even though this should be investigated in targeted studies. However, given the rarity of the occurrence of transition from pituitary- to adrenal-dependent Cushing's syndrome, the coexistence of autonomous pituitary

and adrenal masses sustained by independent genetic events cannot be ruled out and should be further investigated.

In summary, this study shows that *PRKACA* somatic mutations can be found also in adrenal nodules of a subset of patients with CD, in specific conditions such as long duration of hypercortisolism and nodular alterations of the adrenal gland. These findings provide important insights into the pathophysiology of adrenal gland hyperplasia in CD.

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Figure legends

Figure 1. *In silico* analysis of the Ser213Arg missense mutation.

The figures focus on the region of the catalytic subunit (green) of PKA that adopts a "tip-like" structure, which is inserted into a complementary cavity of the regulatory subunit (red). The upper part of the figure shows the wild-type situation in stick mode (A) and in space-filling representation (B), with serine in position 213 (arrow). In the lower part of the figure, the *in silico* replacement of arginine at position 213 in one possible conformation (arrow) is represented in stick (C) and space-filling (D) mode. Substitution of the serine with an arginine at position 213 is thus likely to cause steric hindrance, which is expected to interfere with the association between the two subunits of PKA.

Figure 2. PKA activity assay of the Ser213Arg missense mutation.

A. The Figure shows the PKA activity against a synthetic peptide substrate (kemptide). HEK293A cells were co-transfected with RIIβ and either wild-type (WT) or mutant (L206R, S213R) Cα subunits. PKA activity in cell lysates was then measured under basal condition or upon stimulation with cAMP (40 μM). The PKA activity measured in cells transfected with the empty expression vector (pcDNA) was subtracted. Data are mean±s.e.m. of three independent experiments. Data are statistically significant by two-way ANOVA. *P<0.05. **P<0.01 vs. WT basal by Bonferroni´s post hoc test. **B.** The upper part of the figure shows data (mean±s.e.m.) of all three experiments, as shown in A, without subtraction of the endogenous PKA activity. In the lower part of the figure, a representative Western blot of a single experiment, showing similar expression levels of RIIβ and the Cα subunits in the samples, is depicted.

351 **Table 1. Characteristics, medical history, and mutational status of the patients**

	Patient 1[†]	Patient 2
General Characteristics		
Age at diagnosis, year	41	31
Sex	Female	Female
Symptoms´ onset before diagnosis, months	24	28
Complications		
Hypertension	Yes	Yes
Diabetes	No	Yes
Osteoporosis	No	Yes (+ fractures)
Hormonal characteristics		
Midnight serum cortisol, nmol/L	440	773
24h-urinary free cortisol, nmol/day	323[*] (2X ULN)	1214 (4X ULN)
ACTH, pmol/L	18	18
1 mg DST, nmol/L	410	579
8 mg DST, nmol/L	No suppression[§]	166
CRH test		
ACTH	No increase	56% increase
Cortisol	No increase	35% increase
Additional hormonal tests	IPSS: ACTH central/periphery ratio 6.7 (baseline) and 6.6 (after CRH)	Desmopressin: ACTH 75% increase, cortisol 40% increase
Pituitary surgery		
Imaging	Microadenoma	Microadenoma
Pathological report	Microadenoma	Microadenoma
Persistence/remission	Clinical remission	Persistence
Adrenal surgery		
Imaging	Left adrenal mass	Pseudonodular hyperplasia
Adrenalectomy	Left	Bilateral
Pathological report	Macronodule in micronodular hyperplasia	Macronodule in micronodular hyperplasia
PRKACA mutations	Leu206Arg	Ser213Arg

ULN: upper limit of normal; DST: dexamethasone suppression test; CRH: corticotropin releasing hormone; IPSS: inferior petrosal sinus sampling.
[†] The medical history of patient 1 has been already previously published (Ref. 4).
[*] Mean of two values.
[§]The DST was performed with 7 mg of i.v. dexamethasone.

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