

PRKACA mutations in cortisol-producing adenomas and adrenal hyperplasia: a single-center study of 60 cases

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Abstract

Objective: Cortisol excess due to adrenal adenomas or hyperplasia causes Cushing's syndrome. Recent genetic studies have identified a somatic *PRKACA*^{L206R} mutation as a cause of cortisol-producing adenomas. We aimed to compare the clinical features of *PRKACA*-mutant lesions with those of *CTNNB1* mutations, and to search for similar mutations in unilateral hyperplasia or tumors co-secreting aldosterone.

Design, patients, and methods: In this study, 60 patients with cortisol excess who had adrenalectomies at our institution between 1992 and 2013 were assessed, and somatic mutations were determined by Sanger sequencing. A total of 36 patients had overt Cushing's syndrome, the remainder were subclinical: 59 cases were adenomas (three bilateral) and one was classified as hyperplasia. Four tumors had proven co-secretion of aldosterone.

Results: Among cortisol-secreting unilateral lesions without evidence of co-secretion ($n=52$), we identified somatic mutations in *PRKACA* (L206R) in 23.1%, *CTNNB1* (S45P, S45F) in 23.1%, *GNAS* (R201C) in 5.8%, and *CTNNB1* + *GNAS* (S45P, R201H) in 1.9%. *PRKACA* and *GNAS* mutations were mutually exclusive. Of the co-secreting tumors, two (50%) had mutations in *KCNJ5* (G151R and L168R). The hyperplastic gland showed a *PRKACA*^{L206R} mutation, while patients with bilateral adenomas did not have known somatic mutations. *PRKACA*-mutant lesions were associated with younger age, overt Cushing's syndrome, and higher cortisol levels vs non-*PRKACA*-mutant or *CTNNB1*-mutant lesions. *CTNNB1* mutations were more significantly associated with right than left lesions.

Conclusions: *PRKACA*^{L206R} is present not only in adenomas, but also in unilateral hyperplasia and is associated with more severe autonomous cortisol secretion. Bilateral adenomas may be caused by yet-unknown germline mutations.

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Introduction

The steroid hormone cortisol is produced by the adrenal zona fasciculata and acts to increase gluconeogenesis and regulate inflammatory activity. Physiologically, the release of hypothalamic corticotropin-releasing hormone (CRH) induces secretion of corticotropin (ACTH) from the pituitary gland. The binding of ACTH to its G-protein-coupled melanocortin 2 receptor

activates adenylyl cyclase, leading to cAMP production. cAMP binds to the regulatory subunit of protein kinase A (PKA), causing the release of the catalytic subunit, which phosphorylates downstream targets that act to increase cortisol production and cell proliferation (1). A regulatory feedback mechanism prevents excess production of cortisol.

Autonomous production of CRH, ACTH, or cortisol causes endogenous Cushing's syndrome (2). Recently, recurrent somatic mutations in cortisol-producing adenomas have been identified by next-generation sequencing and confirmed in follow-up studies (3, 4, 5, 6, 7, 8). The most frequently mutated gene (35.4%, range 14.3–65.5% across published cohorts, [Supplementary Table 1](#), see section on [supplementary data](#) given at the end of this article) is *PRKACA*, and almost all mutations are accounted for by a single p.Leu206Arg (L206R) substitution in the encoded catalytic subunit of PKA. The mutation abolishes binding to the regulatory subunit and causes constitutive PKA activity, excess cortisol biosynthesis, and cell proliferation. Somatic hotspot mutations in the *CTNNB1* gene, encoding β -catenin, were found in 16.4% of tumors in one study (6), while the remaining authors did not systematically screen for such mutations ([Supplementary Table 1](#)). Activating *CTNNB1* mutations occur not only in adrenal adenomas, including hormonally inactive tumors, but also in malignancies of the adrenal gland and other organs. These activate Wnt signaling by preventing β -catenin degradation, thereby causing proliferation, but any role in cortisol production remains to be determined (9, 10). In mice, constitutive activation of β -catenin causes adrenal hyperplasia, the development of malignant characteristics, and, interestingly, primary aldosteronism in the absence of hypercortisolism (11). Mutations in *GNAS*, the stimulatory G-protein α -subunit associated with the melanocortin receptor 2, were initially discovered to be the cause of macronodular adrenal hyperplasias (12) and are less frequent causes of cortisol-producing adenomas (6). Mutations in *GNAS* and *PRKACA* are mutually exclusive and appear to be associated with small tumors, young age at presentation, and overt Cushing's syndrome (6).

The prevalence of hypertension in patients with Cushing's syndrome is ~80%. The factors that are thought to contribute to the hypertensive effects of cortisol excess include the mineralocorticoid activity of cortisol, activation of the renin–angiotensin system, enhanced vasoconstriction and blunted vasodilatation (13, 14). In rare cases, secretion of aldosterone is the main cause of hypertension and optional hypokalemia, and co-secretion of cortisol may play a minor role. Some of the corresponding tumors have been shown to carry activating mutations in the *KCNJ5* potassium channel gene that are specific to aldosterone-producing adenomas (15, 16, 17). Only one study screened for *PRKACA* mutations in these tumors, but did not report any (7). Similarly, no *PRKACA* mutations were found in 20 'pure' aldosterone-secreting adenomas (3).

To compare the features of subjects with *PRKACA* mutations vs *CTNNB1* mutations, in this study, we report the clinical and genetic characteristics of 60 patients with cortisol excess, who underwent adrenalectomy at our institution. Using Sanger sequencing, we confirm a high frequency of *PRKACA* mutations in cortisol-producing adenomas, but not in those co-secreting aldosterone. We show that *PRKACA* mutations are associated with a more severe clinical phenotype. Unexpectedly, we demonstrate the presence of a *PRKACA* mutation in unilateral adrenal hyperplasia.

Subjects and methods

Subjects and clinical data

Formalin-fixed paraffin-embedded (FFPE) tissue samples of 60 patients who underwent unilateral or bilateral adrenalectomy for Cushing's syndrome (11 with previously published genetic data (6) and two previously published as case reports (15)) at University Hospital Düsseldorf between 1992 and 2013 were analyzed. Matched normal tissue samples were available in 53 cases.

Hypercortisolism was diagnosed based on a combination of biochemical test results (elevated urinary free cortisol, increased late-night salivary or serum cortisol, suppressed plasma ACTH levels, nonsuppressible serum cortisol levels) and typical signs of hypercortisolism (e.g. muscle weakness, skin fragility and osteoporosis). Overt Cushing's syndrome was defined as either the presence of at least three abnormal biochemical test results or as typical signs plus at least two abnormal biochemical test results. Subclinical Cushing's syndrome was defined as at least one abnormal biochemical test result in the absence of clinical signs (3, 8).

Dexamethasone suppression tests typically consisted of a 1–2 mg overnight dose, with cortisol measured the following morning, and were used as a screening tool for nonsuppressible Cushing's syndrome (overt or subclinical). In rare cases, 8 mg dexamethasone tests were performed, which were considered indicative of adrenal Cushing's syndrome in case of nonsuppression of cortisol in the presence of suppressed ACTH.

Co-secretion of aldosterone was assumed based on elevated aldosterone levels in the presence of suppressed renin and confirmed by salt infusion testing, fludrocortisone suppression testing, and/or normalization of values after surgery. The patient characteristics are summarized in [Supplementary Table 2](#), see section on [supplementary data](#) given at the end of this article.

All specimens were independently reviewed by two pathologists and categorized by size and histology (absence of signs of malignancy, adenoma vs hyperplasia). Written informed consent was obtained before adrenalectomy, and the study was approved by the Institutional Review Board at the School of Medicine, Heinrich Heine University Düsseldorf.

DNA extraction, PCR, and sequencing

H&E staining was performed according to standard procedures in a clinical pathology laboratory. Areas of tumor and normal tissue were marked by a pathologist, and two 2 mm cores were obtained from each area. DNA was isolated using the BiOstic FFPE Tissue DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA), repaired with the PreCR Repair Mix (New England Biolabs, Beverly, MA, USA), and purified with the Agencourt Ampure XP PCR Purification System (Agencourt Bioscience, Beverly, MA, USA) according to the manufacturers' instructions.

Routine PCR amplification of known target genes from matched tumor and normal samples was performed using specific primers. As larger template regions failed to amplify, shorter template regions were amplified. The primer sequences are listed in [Supplementary Table 3](#), see section on [supplementary data](#) given at the end of this article. PCR purification and direct bidirectional Sanger sequencing were performed (Beckman Coulter Genomics, Takely, Essex, UK). All identified mutations were confirmed in independent PCRs.

Targeted capture and whole-exome sequencing

Targeted capture was performed at the Yale Center for Genome Analysis using the NimbleGen 2.1 Exome reagent, followed by sequencing on the Illumina HiSeq platform as previously described (6), and sequences were aligned to the human genome (hg19).

Statistical analysis

Data are shown as mean \pm s.d., unless otherwise indicated. Comparisons between two groups were performed using two-tailed Fisher's exact test or Mann-Whitney *U* tests, and *P* values <0.05 were considered to be significant.

Results

Characterization of the cohort

Among the 60 adrenalectomies for adrenal Cushing's syndrome (Fig. 1 and [Supplementary Table 2](#)), 57 were

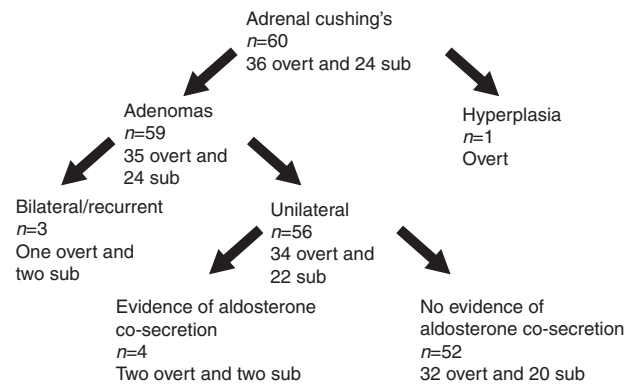


Figure 1

Overview of the cohort. Overt, overt Cushing's syndrome; sub, subclinical Cushing's syndrome.

unilateral (40 left and 17 right) and three were bilateral (one case with simultaneous bilateral adrenalectomy due to bilateral adenomas, two subsequent right and left adrenalectomies due to recurrence on the contralateral side). Between 1992 and 1997, typically, open adrenalectomies were performed, with a switch to laparoscopic surgery in 1997 and retroperitoneoscopic surgery in 2007. Pathology revealed single or multiple adrenal adenomas in 59 cases (two with associated nodular hyperplasia) and a single case of unilateral diffuse adrenal hyperplasia (Fig. 2 and [Supplementary Table 2](#)).

Among the 60 subjects, 36 presented with overt Cushing's syndrome, while 24 individuals were subclinical. In four cases (CS057–CS060), co-secretion of aldosterone was noted. Previous studies suggest a distinct, aldosterone-producing adenoma-like biology of tumors co-secreting cortisol and aldosterone (16). Similarly, we reasoned that bilateral or recurrent adenomas may be due to yet unknown germline mutations. Thus, these two groups were analyzed separately.

PRKACA and CTNNB1 mutations are frequent in cortisol-secreting tumors; PRKACA mutations are associated with a more severe phenotype

To determine the frequencies of known somatic mutations in patients with unilateral cortisol-secreting adenomas without evidence of aldosterone co-secretion ($n=52$), we performed PCR and direct Sanger sequencing of *PRKACA* (exon 7, encoding the L206R mutation (3, 4, 5, 6)), *CTNNB1* (exon 3 with hotspot mutations (6, 9)), and *GNAS* (exons 8 and 9 with known disease-causing mutations (6)) ([Supplementary Table 2](#) and [Table 1](#)). As available

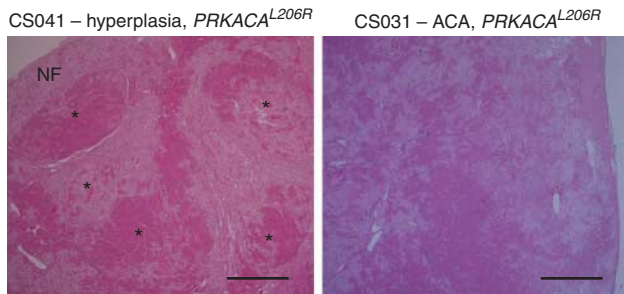


Figure 2

Histology of samples with *PRKACA*^{L206R} mutation (H&E). Left, sample CS041 with elements of normal fasciculata (NF), in addition rearrangement of zona fasciculata and reticularis and several hyperplastic micronodules (*). Right, sample CS031 with adrenocortical adenoma (ACA) shows typical areas of lipid-rich clear cells and oncocytic cells (eosinophilic). Scale bars, 1000 μm.

(46 samples), normal tissue samples were screened for the same mutations. With the exception of an extremely low peak in sample CS048 that was assumed to be tumor contamination, all mutations were confirmed to be somatic, i.e. absent from normal tissue. The age of patients at time of surgery was 49.8 ± 13.4 (s.d., range of 28–74) years. The mean tumor size was 3.6 ± 1.1 cm (range of 1.4–6), and the male:female ratio was 3:49. Thirty-two patients (61.5%) presented with overt Cushing's syndrome. Serum cortisol was 20.0 ± 8.0 μg/dl, urinary cortisol was 315.2 ± 318.5 μg/24-h, and ACTH was 4.2 ± 4.2 pg/ml. After dexamethasone suppression, serum cortisol was 12.5 ± 10.4 μg/dl.

Twelve patients (23.1%) carried the previously described c.617T>G (p.Leu206Arg) missense mutation in *PRKACA*. Another 12 subjects (23.1%) had a *CTNNB1* mutation of codon 45 (eight cases with c.133T>C (p.Ser45Pro); four instances of c.134C>T (p.Ser45Phe)). In three patients (5.8%), a c.601C>T (p.Arg201Cys) mutation in *GNAS* was found, and one patient (1.9%) had concomitant *CTNNB1* and *GNAS* mutations (c.133T>C (p.Ser45Pro) and c.602G>A (p.Arg201His) respectively) (Fig. 3 and Table 1).

Within this group, *PRKACA* mutations were associated with a significantly more severe phenotype (Table 1 and Fig. 4A, B and C). Patients presented at a significantly younger age than those without *PRKACA* mutations (37.7 ± 7.7 years vs 53.5 ± 12.6 years ($P < 0.0001$)) or with *CTNNB1* mutations (59.6 ± 10.9 years, $P < 0.0001$ vs *PRKACA*). Serum cortisol levels after dexamethasone suppression in those with *PRKACA* mutations were

significantly higher (24.6 ± 3.8 μg/dl vs 9.0 ± 8.9 μg/dl in those without *PRKACA* mutations ($P < 0.0001$) and 9.6 ± 11.8 μg/dl in those with *CTNNB1* mutations ($P = 0.0119$ vs *PRKACA*)).

All three male subjects in the cohort had *CTNNB1* mutations (one combined with *GNAS* mutation).

Subjects with *PRKACA* mutation had overt Cushing's syndrome in 11/12 cases, vs 21/40 of the remainder ($P = 0.0181$), and 5/12 with *CTNNB1* mutation only ($P = 0.0272$ vs *PRKACA*). Whereas *PRKACA* mutations were enriched in the subcohort with overt Cushing's syndrome (34.4% vs 23.1% overall), the opposite was the case for *CTNNB1* mutations (15.6% vs 25.0% overall).

PRKACA is mutated in a sample with diffuse hyperplasia

Remarkably, one of the somatic *PRKACA* mutations occurred in the single sample with apparently unilateral adrenal hyperplasia (CS041), a rare subgroup of adrenal Cushing's syndrome that has not been previously described to carry such mutations (Fig. 2 and Supplementary Table 2). Upon independent reassessment of several areas of the affected glands, this mutation was confirmed to be present in four hyperplastic areas, but absent from non-hyperplastic tissue (Supplementary Figure 1, see section on supplementary data given at the end of this article). No germline mutations in genes previously described to cause macronodular adrenal hyperplasia, and specifically, no *ARMCS5* or *PRKAR1A* mutations were identified by targeted capture and sequencing.

The subject with unilateral adrenal hyperplasia was evaluated for secondary hypertension at age 49 years. Markedly increased urinary cortisol (626 μg/24 h) was noted, ACTH was suppressed (<1.0 pg/ml), and urinary tetrahydroaldosterone (<10 μg/24 h), serum aldosterone (25 ng/l), metanephrin, and normetanephrin were normal. Plasma renin activity was not suppressed (5.47 ng/ml per h). After 2 mg dexamethasone, serum cortisol remained elevated (27.5 μg/dl). High-dose dexamethasone (8 mg) also failed to suppress cortisol (26.0 μg/dl). Imaging demonstrated a 26 mm right adrenal lesion, and retroperitoneoscopic right adrenalectomy was performed. No adenoma was identified macroscopically or microscopically. Pathology demonstrated regular differentiation into cortex and medulla. There was focal rearrangement of the fascicular and reticular zones with hyperplasia of the zona reticularis and scanty lymphocytic infiltration (Fig. 2). Baseline serum cortisol (13.9 μg/dl)

Table 1 Summary of patients with unilateral pure cortisol-producing lesions.

	All (n=52)	Subclinical (n=20)	Overt (n=32)	PRKACA (n=12)	Non-PRKACA (n=40)	CTNNB1 (n=13) ^a	WT (n=24)
Age at surgery (years)	49.8±13.4	56.6±11.7	45.6±12.8	37.7±7.7	53.5±12.6	59.6±10.9	50.2±13.2
Female (%)	49/52 (94.2)	17/20 (85.0)	32/32 (100.0)	12/12 (100.0)	37/40 (92.5)	10/13 (76.9)	24/24 (100.0)
Adenoma size (cm)	3.6±1.1 (n=49)	3.8±1.1 (n=18)	3.5±1.1 (n=31)	3.5±0.8 (n=12)	3.6±1.2 (n=37)	3.9±0.7 (n=12)	3.6±1.3 (n=23)
Left (%)	37/52 (71.2)	17/20 (85.0)	24/32 (75.0)	9/12 (75.0)	28/40 (70.0)	4/13 (30.8)	21/24 (87.5)
sCortisol (µg/dl)	20±8.0 (n=45)	19.3±7.2 (n=17)	20.4±8.5 (n=28)	24.7±4.0 (n=11)	18.5±8.4 (n=34)	20.3±5.8 (n=12)	17.4±8.4 (n=20)
uCortisol (µg/24 h)	315.2±318.5 (n=33)	134.0±147.1 (n=6)	355.5±333.8 (n=27)	372.8±151.4 (n=10)	290.2±368.8 (n=23)	438.4±339.1 (n=5)	197.8±257.4 (n=15)
sCortisol after DST (µg/dl)	12.5±10.4 (n=41)	7.0±4.6 (n=15)	15.6±11.5 (n=26)	24.6±3.8 (n=9)	9.0±8.9 (n=29)	9.6±11.8 (n=11)	8.5±7.2 (n=18)
ACTH (pg/ml)	4.2±4.2 (n=46)	5.1±3.5 (n=17)	3.6±4.5 (n=29)	1.6±1.4 (n=12)	5.1±4.4 (n=32)	4.8±4.3 (n=12)	5.6±4.8 (n=19)
PRKACA (%)	23.1 (n=12)	5.0 (n=1)	34.4 (n=11)				
CTNNB1, all ^a (%)	25.0 (n=13)	40.0 (n=8)	15.6 (n=5)				
GNAS, all ^a (%)	7.7 (n=4)	5.0 (n=1)	9.4 (n=3)				
CTNNB1 + GNAS (%)	1.9 (n=1)	5.0 (n=1)	0.0 (n=0)				
WT (%)	46.2 (n=24)	55.5 (n=11)	40.6 (n=13)				

Subclinical, subclinical Cushing's syndrome; overt, overt Cushing's syndrome; PRKACA, cases with PRKACA mutation; CTNNB1, cases with CTNNB1 mutation; sCortisol, serum cortisol; uCortisol, 24-h urinary cortisol; DST, dexamethasone suppression test; CTNNB1 + GNAS, subject with concurrent CTNNB1 and GNAS mutations; WT, subjects without mutations in genes screened.

^aOne case with CTNNB1 + GNAS mutation.

and ACTH (44.9 pg/ml) became normal 16 months after surgery, and a rise in ACTH and cortisol was observed after 100 µg CRH i.v. (ACTH 122.0 pg/ml after 30 min, 95.2 pg/ml after 60 min; cortisol 20.9 µg/dl after 30 min, 21.4 µg/dl after 60 min). Computed tomography 3 years after surgery showed some remaining right adrenal tissue and a normal left adrenal gland. Urinary cortisol (19.5 µg/24 h) was normal, supporting unilateral hyperplasia as the cause of prior hypercortisolism and arguing against asynchronous macronodular adrenal hyperplasia as the cause of disease.

CTNNB1 is more frequently mutated in the right adrenal gland

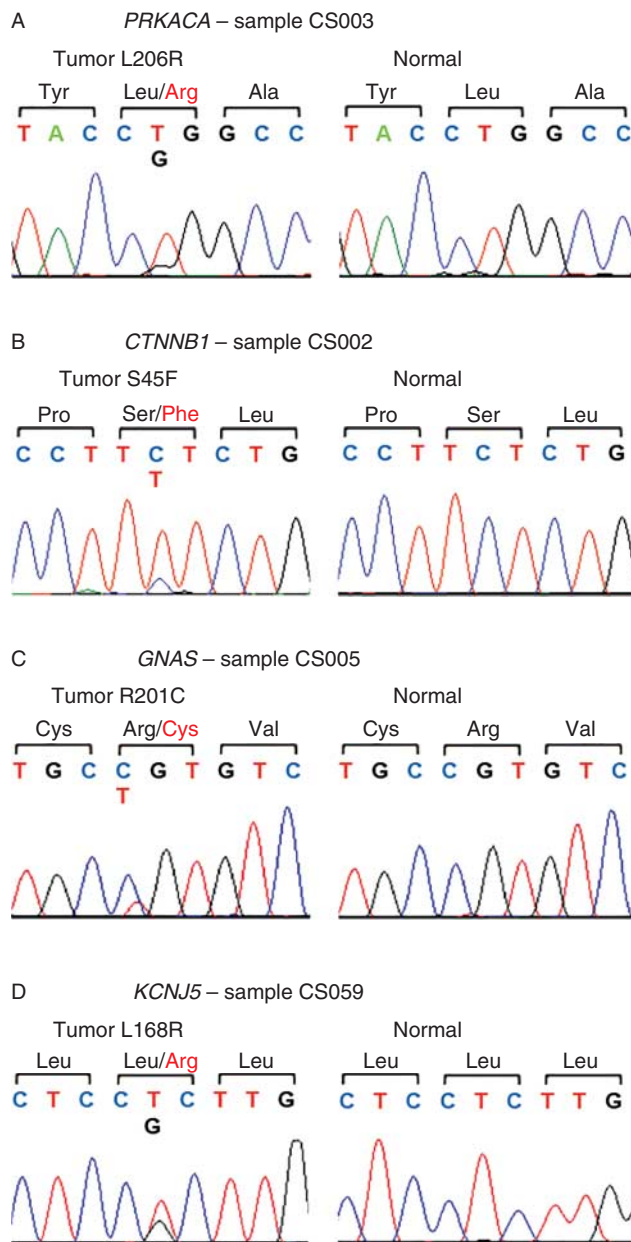
Both cortisol- and aldosterone-producing adenomas occur more frequently on the left side (18), but the underlying reasons remain to be determined. This effect was also seen in our cohort, with 37/52 (71.2%) of the pure cortisol secreting unilateral lesions occurring on the left side and 9/12 (75.0%) in the subset with PRKACA mutations. However, unexpectedly, right adrenal adenomas were overrepresented in our samples with CTNNB1 mutations

(9/13 samples with CTNNB1 mutations vs 6/39 without CTNNB1 mutations, $P=0.0006$, Fig. 4D).

Aldosterone and cortisol co-secreting adenomas show mutations in KCNJ5

In samples co-secreting aldosterone (Supplementary Figure 2, see section on supplementary data given at the end of this article), in addition to the previously mentioned genes, we also sequenced genes known to carry somatic mutations in aldosterone-producing adenomas: KCNJ5 (exon 2 encoding previously described p.Gly151Arg (G151R) and p.Leu168Arg (L168R) mutations (19)), ATP1A1 (exons 4 and 8 (20, 21)), ATP2B3 (exon 8 (20)), and CACNA1D (exons 6, 8, 8b, 17 and 34 (21, 22)).

Tumors co-secreting aldosterone and cortisol carried KCNJ5 mutations (G151R and L168R) in 50% (2/4) cases (Supplementary Table 2). In contrast, none of these patients showed PRKACA mutations, in line with previously published findings (7, 16). Mutations in genes other than KCNJ5 are considerably less frequent in aldosterone-producing adenomas. Accordingly, we did not detect mutations in ATP1A1, ATP2B3, or CACNA1D in this rather small cohort.

**Figure 3**

Somatic mutations in adrenal adenomas associated with Cushing's syndrome. Sanger DNA sequences (representative chromatograms) of tumor and normal DNAs for each gene with mutations in the study cohort. Mutations are not present in normal tissue. (A) *PRKACA* with heterozygous somatic mutation encoding L206R, (B) *CTNNB1* with heterozygous somatic mutations encoding S45F, (C) *GNAS* with heterozygous somatic mutation encoding R201C, and (D) *KCNJ5* with heterozygous somatic mutation encoding L168R. Additional mutations in the same genes and chromosomal positions are listed in Supplementary Table 4.

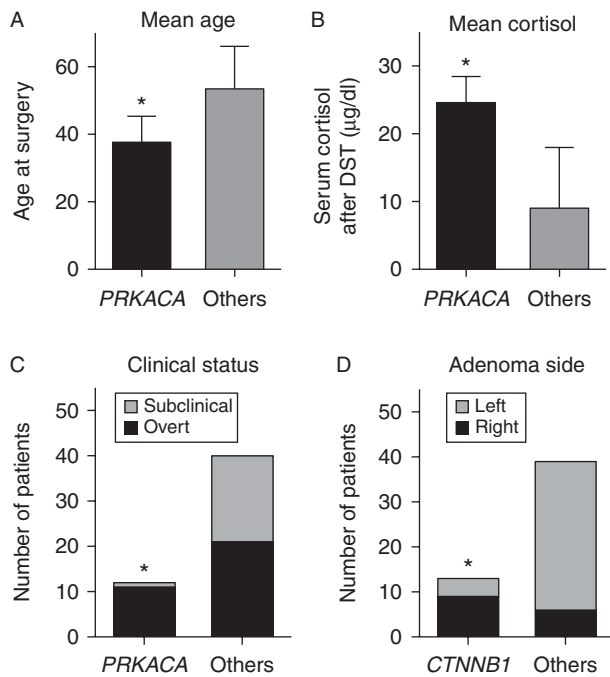
Bilateral or recurrent adenomas do not carry somatic or germline mutations at typical residues

None of the bilateral or recurrent adenomas carried mutations in any of the genes examined, pointing to germline mutations as the potential causes of these tumors (Supplementary Table 2). In the case of CS001 with bilateral adenomas, tumors from both sides were sequenced and tested negative for known mutations. To assess whether bilateral or recurrent adenomas may represent a variant of bilateral macronodular hyperplasia, we performed targeted capture and sequencing of the corresponding germline DNA in all cases. No mutations were detected in genes implicated in adrenal hyperplasia. In particular, all samples tested negative for mutations in *ARMC5*, *PRKAR1A*, *MEN1*, or *APC*.

Discussion

In this study, we ascertained an extensive cohort of patients with cortisol-producing adrenal lesions, covering more than 20 years, from a single institution. In this homogenous cohort, we confirmed the high frequency of mutations in *PRKACA* and *CTNNB1* in patients with cortisol-producing adenomas (3, 4, 5, 6). Interestingly, the frequency of *PRKACA* mutations has shown some variation across different studies. Our finding of *PRKACA* mutations in 23.1% of patients with unilateral adenomas producing cortisol only (34.4% of patients with overt Cushing's syndrome) is similar to the values reported by Goh *et al.* (6) (24 and 35% respectively), but considerably higher frequencies were reported in two Asian studies (4, 5) (Supplementary Table 1). In line with previous reports, we show that *PRKACA* mutations are associated with a more severe phenotype, i.e. higher cortisol levels after dexamethasone suppression as a sign of autonomous cortisol secretion, younger age at presentation, and overt Cushing's syndrome. Only one subject with such mutations was classified as having subclinical Cushing's syndrome. The pathophysiology of the *PRKACA*^{L206R} mutation, which abolishes binding of the regulatory PKA subunit and provides an autonomous signal for cortisol production, provides a plausible explanation for the severe phenotype observed.

Any role of *CTNNB1* mutations in hormone production is less obvious. In our cohort, *CTNNB1* mutations are associated with older age at surgery, lower post-dexamethasone cortisol levels and lower likelihood of developing overt Cushing's syndrome than *PRKACA* mutations. Similar or identical mutations have been

**Figure 4**

Clinical correlations of *PRKACA* and *CTNNB1* mutations in unilateral adenomas with cortisol production only. Age at surgery (A) and mean cortisol levels after dexamethasone suppression test (DST) (B) are compared between subjects with *PRKACA*-mutant tumors (*PRKACA*) and subjects with mutations in other genes or no identified mutations (others). Shown are mean \pm s.d. *PRKACA* mutations are associated with younger age at surgery and higher cortisol levels after DST. (C) Number of patients with subclinical vs overt Cushing's syndrome in both groups. *PRKACA* mutations are associated with overt Cushing's syndrome. (D) Side of adenoma (left vs right) in tumors with *CTNNB1* mutations vs tumors with other mutations or no identified mutations (others). *CTNNB1* mutations are associated with right adenomas. * $P < 0.05$ (Mann-Whitney *U* tests (A), (B) or Fisher's exact test (C), (D)). See text for details.

described in pure aldosterone-secreting adenomas or hormonally inactive adrenal tumors (9, 22). Interestingly, both in this study and in a prior study (4), simultaneous mutations in *GNAS* and *CTNNB1* were found in one tumor each, suggesting that cortisol production in these cases may be mainly caused by mutant *GNAS*. Of note, both these patients were male, and the mutation spectrum appears to be different in male vs female subjects (see below). *PRKACA* and *GNAS* mutations have so far been mutually exclusive in all studies (Supplementary Table 1), suggesting that mutation of either of the two genes is

sufficient to cause autonomous cortisol secretion, in line with known physiology. Whether additional somatic mutations, germline alterations, or epigenetic factors determine the hormonal status of the remaining tumors with *CTNNB1* mutations remains to be determined.

In line with a recent study from Japan (7), we do not observe *PRKACA* mutations in tumors co-secreting aldosterone and cortisol, suggesting that elevated PKA activity specifically upregulates cortisol secretion but not aldosterone secretion. However, tumors in the 'pure' cortisol-secreting adenoma group were not systematically screened for aldosterone co-secretion, therefore aldosterone secretion cannot be formally excluded in all.

We found *KCNJ5* mutations in tumors predominantly secreting aldosterone, with concomitant cortisol secretion, again in line with prior work (7, 16). *KCNJ5* mutations cause abnormal sodium permeability of the mutant potassium channel, leading to depolarization, activation of voltage-gated calcium channel, and calcium influx (19). It is conceivable that one or more of the signals downstream of calcium influx that have been implicated in aldosterone secretion are involved in cortisol production as well. Potential candidates include ATF2, CREB, and CREM that are activated by calmodulin kinases, but also act downstream of PKA (23). Consistent with this notion, an *in vitro* study using the HAC-15 human adrenocortical cancer cell line (24) showed slight upregulation of cortisol production after viral transduction with *KCNJ5*^{T158A}, a mutation implicated in primary aldosteronism.

Both cortisol- and aldosterone-producing adenomas are more frequent in the left adrenal gland and are more prevalent in females than in males (18), yet the underlying pathophysiology remains unclear. In the case of aldosterone-producing adenomas, *KCNJ5* mutations appear to fully account for the female preponderance (25), and this effect is not seen in patients with *CACNA1D* or ATPase mutations (20, 22). In our cohort, there were only three male individuals among the subjects with pure cortisol-producing adenomas, all with *CTNNB1* mutations (in one case combined with a *GNAS* mutation), so it seems possible that a similar gender effect as for *KCNJ5* mutations applies to *PRKACA* and tumors without known mutations. In our cohort, *CTNNB1* mutations were more frequent on the right side, while overall, tumors were more frequent on the left side. Previous genetic studies have not reported tumor side; therefore, it would be interesting to see whether this effect is present in other cohorts as well.

Another interesting observation is the finding of a *PRKACA* mutation in unilateral adrenal hyperplasia with

cortisol production, a rare phenotype that has not been studied in previous publications on *PRKACA* mutations. While a few cases of unilateral hyperplasia have been reported (reviewed by Takamura *et al.* (26)), typically, nodular hyperplasia was described. In contrast, macroscopic nodules were not detected in our case (Fig. 2). As demonstrated for *ARMC5* mutations in ACTH-independent macronodular hyperplasia, the nodular structure in bilateral disease is typically caused by independent somatic mutations in individuals with underlying germline mutations. We suspect that a similar mechanism accounts for the individuals in our cohort who presented with recurrent and/or bilateral cortisol-producing adenomas, another rare entity (27). Pathology in these cases was not typical of ACTH-independent macronodular hyperplasia or primary pigmented nodular adrenal disease, and no germline mutation characteristics of these diseases were found. Future genome- or exome-level sequencing of additional cases may reveal the underlying pathophysiology.

In conclusion, we confirm the high frequency of *PRKACA* and *CTNNB1* mutations in cortisol-producing adenomas and demonstrate that *PRKACA* mutations are associated with a more severe phenotype. For the first time, we identify the characteristic *PRKACA*^{L206R} mutation in a sample with diffuse unilateral hyperplasia. We suggest the presence of an unrecognized phenotype of adrenal Cushing's syndrome that features bilateral adenomas, possibly due to yet-unrecognized germline mutations. These samples warrant further studies.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/EJE-14-1113>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

U I Scholl and G Goh designed the study; M Haase, H S Willenberg, M Schott, and U I Scholl evaluated subjects and ascertained clinical

information; A-C Reis evaluated histological specimens and provided tissue cores; A Thiel performed Sanger sequencing and statistical analysis; and A Thiel and U I Scholl wrote the manuscript, with all authors contributing to a critical revision.

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