**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\textsuperscript{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\textsuperscript{L206R} in 23.1%, CTNNB1\textsuperscript{S45P, S45F} in 23.1%, GNAS\textsuperscript{R201C} in 5.8%, and CTNNB1 + GNAS\textsuperscript{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\textsuperscript{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\textsuperscript{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.

**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 adenyl 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 vasodilation (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 ± 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 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
PRKACA mutations in adrenal Cushing’s syndrome

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 ARMC5 or PRKARIA 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 µg/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)
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 showed 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.
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
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 as 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 KCNJ5T584A, 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 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
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 germ-line 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 PRKACA12008 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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