Double adrenocortical adenomas harboring independent *KCNJ5* and *PRKACA* somatic mutations

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Abstract

Objective: Co-secretion of cortisol and aldosterone can be observed in adrenal adenomas. The aim of this study was to investigate the molecular characteristics of a co-existing aldosterone- and a cortisol-producing adenoma (CPA) in the same patient.

Design and methods: Two different adenomas within the same adrenal gland from a 49-year-old female patient with primary aldosteronism (PA) and Cushing's syndrome (CS) were studied. Multiple formalin-fixed paraffin-embedded tumor blocks were used for the analysis. Immunohistochemistry (IHC) was performed using a specific antibody against aldosterone synthase (CYP11B2). DNA and RNA were isolated separately from CYP11B2-positive and -negative tumor regions based on CYP11B2 IHC results.

Results: CYP11B2 IHC clearly demonstrated that three pieces from one adenoma were positive for CYP11B2 and the remaining three from the other adenoma were negative for CYP11B2. In quantitative real-time RT-PCR, *CYP11B2* mRNA was upregulated in CYP11B2-positive tumor specimens (219-fold vs CYP11B2-negative tumor specimens). Targeted next-generation sequencing (NGS) detected novel *KCNJ5* gene mutations (p.T148I/T149S, present in the same reads) and a *PRKACA* gene hotspot mutation (p.L206R) in the CYP11B2-positive and -negative tumors, respectively. Sanger sequencing of DNA from each tumor specimen (CYP11B2-positive tumor, $n=3$; CYP11B2-negative tumor, $n=3$) showed concordant results with targeted NGS.

Conclusion: Our findings illustrate the co-existence of two different adrenocortical adenomas causing the concurrent diagnosis of PA and CS in the same patient. Molecular analysis was able to demonstrate that the two diseases resulted from independent somatic mutations seen in double adrenocortical adenomas.

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Introduction

Primary aldosteronism (PA) is a common form of secondary hypertension caused by autonomous adrenal aldosterone production. PA is clinically characterized by hypertension and hypokalemia. Aldosterone-producing adenoma (APA) and idiopathic hyperaldosteronism are the major subtypes of PA. Adrenal Cushing syndrome (CS) is caused by glucocorticoid excess due to a cortisol-

producing adenoma (CPA), resulting in hypertension, obesity, type 2 diabetes, and vertebral osteoporosis. As a result of cortisol excess, CS patients present with unique clinical features such as easy bruising, skin atrophy, facial plethora, striae, and dorsocervical fat pad. Over the last decades, an increasing number of patients with subclinical Cushing syndrome (SCS) have been identified. SCS is often

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characterized by mild hypercortisolism without specific signs or symptoms for CS. In patients with PA, concurrent autonomous cortisol production has been increasingly recognized (1). The majority of the reported cases have had SCS, whereas overt CS has been rarely found.

Next-generation sequencing (NGS) has identified several somatic and germline mutations underlying sporadic APA and/or familial PA, such as mutations in an inwardly rectifying potassium channel (*KCNJ5*) (2), the P-type ATPase gene family (*ATP1A1* and *ATP2B*) (3), an L-type voltage-gated calcium channel (*CACNA1D*) (4, 5), and T-type voltage-gated calcium channel (*CACNA1H*) (6). These mutations alter the pathway involved in regulation of aldosterone production, such as potassium sensing, membrane potential regulation, intracellular Ca^{2+} levels, and steroidogenesis. More recently, recurrent activating mutations in *PRKACA*, encoding the catalytic subunit α of protein kinase A (PKA) have been identified in CPA (7). *PRKACA* gene mutations are found in 35–65% of the patients with CS (8). Herein, we report a case of PA and CS due to the co-existence of two adenomas harboring novel *KCNJ5* somatic mutations and a *PRKACA* somatic mutation.

Patient and methods

Case report

The patient was a 49-year-old African-American woman referred for further evaluation of endocrine hypertension. Her past medical history was positive for a pregnancy-associated deep venous thrombosis and her family history was negative for any adrenal or endocrine diseases. She had a 5-year history of high blood pressure and 3 years of hypokalemia necessitating replacement doses of 30–90 mmol/day of potassium. Over the course of the recent year, she had a weight gain of 27 kg (weight at presentation 154 kg). She noted some facial fullness and rounding and had recently been diagnosed with prediabetes. She complained of depression, easy bruising, and muscle weakness, particularly while climbing stairs. On examination, she was found to be obese with facial rounding and increased prominence of the supraclavicular fat pads. Blood pressure was 166/97 mmHg. There were some pale striae on the abdomen, no skin atrophy, but some acanthosis of the neck. Laboratory evaluation revealed an aldosterone of 45 ng/dL (1248 pmol/L) (4–31 ng/dL), a renin of 0.4 ng/mL/h (0.11 ng/L·s) (1–7 ng/mL/h), and a 24 h urine cortisol of 47.3 $\mu\text{g}/24\text{ h}$ (130.5 nmol/day) (20–90 $\mu\text{g}/24\text{ h}$), with a low morning adrenocorticotrophic hormone (ACTH) of <9 pg/mL (1.98 pmol/L)

(9–52 pg/mL). CT showed two adrenocortical adenomas in the right adrenal (2.7 and 3.0 cm). The posterior mass measured 4 Hounsfield Unit (HU) on unenhanced CT scan (lipid-rich adenoma), and the anterior mass measured 20 HU, with a percentage enhancement washout value of 63% (lipid poor adenoma) (9) (Fig. 1A, B and C). NP59 scan showed suppressed uptake in the contralateral gland. Both lesions were identified in the pathological specimen and the diagnosis was that of an adrenal adenoma in both masses. Following surgery, the patient felt significant mood improvement and blood pressure and potassium levels normalized. She lost 6 kg over the course of 3 months following surgery. She was diagnosed with adrenal insufficiency following surgery with stimulation to maximum of 1.4 $\mu\text{g}/\text{dL}$ (38.6 nmol/L) (>18 $\mu\text{g}/\text{dL}$) cortisol and did require glucocorticoid replacement therapy.

Materials

Multiple formalin-fixed paraffin-embedded (FFPE) tumor blocks were used for the analysis. This study was approved by Institutional Review Board of the University of Michigan (HUM00042749).

Immunohistochemistry

Immunohistochemistry (IHC) was performed using antibodies against aldosterone synthase (CYP11B2) (mouse monoclonal; 1:1500, Millipore, #MABS1251) (10) and cytochrome P450 17A1 (CYP17) (mouse monoclonal; 1:500, kindly provided by Dr Richard Parker, University of Alabama at Birmingham) as described previously (11).

DNA/RNA isolation

For each sample, six unstained FFPE slides were used for genomic DNA (gDNA) and RNA isolation. gDNA and RNA of CYP11B2-positive and -negative regions were isolated separately based on CYP11B2 IHC using the AllPrep DNA/RNA FFPE kit (Qiagen) as described previously (11).

Quantitative real-time RT-PCR

Total RNA was reverse transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems). Quantitative real-time RT-PCR (RT-qPCR) was performed in the ABI StepOnePlus Real-Time PCR systems (Applied Biosystems). *CYP11B2* primer/probe mixtures were prepared as described previously (12). Primer/probe mixtures for the

Figure 1

Imaging and histopathological findings of adrenal tumors. (A, B and C) Abdominal CT showing adrenocortical adenomas in the right adrenal. (A) Unenhanced CT scan. The Hounsfield Unit (HU) of the anterior tumor (arrowhead) and the posterior tumor (arrow) were 20 and 4 HU, respectively. (B) Enhanced CT at 100s. The HU of the anterior tumor (arrowhead) and posterior tumor (arrow) were 47 and 23 HU, respectively. (C) Delayed CT at 15min. The HU of the anterior tumor (arrowhead) and the posterior tumor (arrow) were 30 and 14 HU, respectively. (D, E, F, G, H and I) Histopathological findings of the anterior tumor (D, E and F) and the posterior tumor (G, H and I). The anterior tumor showed positive staining for CYP11B2 and the posterior tumor was negative for CYP11B2 (E and H). The posterior tumor had higher expression of CYP17 compared with the anterior tumor (F and I). Scale bars = 100 μ m. D and G, H&E; E and H, CYP11B2 IHC; F and I, CYP17 IHC. H&E, hematoxylin and eosin staining.

amplification of β -actin (*ACTB*, Hs01060665_g1) were purchased from Applied Biosystems. *ACTB* transcript was used for normalization of sample loading. Relative quantification was determined using comparative threshold cycle method (13).

Targeted NGS

For mutation analysis of CYP11B2-positive tumor specimen 1 (B2T1) and CYP11B2-negative tumor specimen 1 (T1) samples, 20ng of isolated gDNA was used for barcoded library generation by multiplexed PCR using a custom Ion AmpliSeq Panel and the Ion AmpliSeq Library kit 2.0 (Life Technologies) according to the manufacturer's instructions. The custom Ion AmpliSeq

Panel was designed to target genes previously shown to be mutated in APA or other adrenal hyperplasias/neoplasms. The panel contains 499 independent primer pairs targeting the entire coding regions of genes with reported germline or somatic mutations in PA (*KCNJ5*, *ATP1A1*, *ATP2B3*, *CACNA1D*, and *CACNA1H*), genes shown to harbor germline or somatic variants associated with adrenal hyperplasia (phosphodiesterase 11A (*PDE11A*), phosphodiesterase 8B (*PDE8B*), protein kinase, cAMP-dependent, regulatory, type 1 α (*PRKARIA*), *PRAKACA*, and armadillo repeat containing 5 (*ARMC5*)), and oncogene hotspots in guanine nucleotide-binding protein subunit α (*GNAS*) and catenin β 1, 88 kDa (*CTNNB1*). Template preparation and NGS of multiplexed templates were performed as described previously (11) using Ion 318 Chip v2 on the Ion Torrent Personal Genome Machine (PGM) Sequencer (Life Technologies). Data analysis methods for somatic variant identification are described in [Supplementary methods](#), see section on [supplementary data](#) given at the end of this article.

Direct sequencing of PCR products and subsequent analysis

Bidirectional Sanger sequencing analyses of *KCNJ5* and *PRKACA* gene for DNA samples isolated from each tumor piece were performed using primers as follows: *KCNJ5* forward 5'-GGACCATGTTGGCGACCAAGAGTG-3', reverse 5'-GACAAACATGCACCCCACCATGAAG-3'; *PRKACA* forward 5'-GGTGACAGACTTCGGTTTCGC-3', reverse 5'-CCTTGTGTAGCCCTGGAGCA-3' (14). For each PCR reaction, 20ng of template gDNA was used. Sanger sequencing of the purified products was undertaken on the Applied Biosystems 3730 XL sequencer. The PCR product from CYP11B2-positive specimen 3 (B2T3) DNA sample was inserted into pCR2.1-TOPO vector using a TOPO TA cloning kit (Invitrogen) for subsequent analysis. After chemical transformation using TOP10 competent cells, several carbenicillin-resistant clones were picked and sequenced.

Results

CYP11B2 IHC clearly demonstrated that the anterior adenoma showed positive staining for CYP11B2 (B2T1, B2T2 and B2T3) and the posterior adenoma was negative for CYP11B2 (T1, T2 and T3). CYP11B2-positive tumor specimens exhibited positive but lower expression of CYP17 compared with the

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Table 1 Somatic mutations identified by targeted NGS. All high-confidence somatic nonsynonymous variants identified in CYP11B2-positive tumor specimen 1 (B2T1) and CYP11B2-negative tumor specimen 1 (T1) are shown. The variant allele frequency in the matched tumor specimen, that is, T1 for B2T1 and B2T1 for T1, respectively, is shown for comparison.

Sample	Gene	Reference allele	Variant allele	Amino acid change	FAO	FDP	Variant allele frequency (FAO/FDP; %)	Variant allele frequency in matched tumor specimen (%)	Reference sequence
B2T1	<i>KCNJ5</i>	C	T	T148I	691	1999	35	0	NM_000890
B2T1	<i>KCNJ5</i>	A	T	T149S	702	1997	35	0	NM_000890
T1	<i>PRKACA</i>	A	C	L206R	553	1996	28	0	NM_002730

FAO, flow-corrected variant allele-containing read; FDP, flow-corrected read depth.

CYP11B2-negative tumor specimens (Fig. 1D, E, F, G, H and I). In RT-qPCR, mean of *CYP11B2* mRNA expression in three specimens from the CYP11B2-positive adenoma was 219-fold higher than that in CYP11B2-negative adenoma, confirming accurate sample collection. Targeted NGS identified novel *KCNJ5* mutations (c.443C>T and c.445A>T, p.T148I/T149S) in a specimen from the anterior adenoma (B2T1) and a *PRKACA* hotspot mutation (c.617T>G, p.L206R) in a specimen from the posterior adenoma (T1) (Table 1). Read level analysis in Integrative Genomics Viewer demonstrated that the *KCNJ5* mutations were present on the same reads (in cis) as shown in Supplementary Fig. 1. We further investigated mutational heterogeneity through assessing multiple tumor specimens. As shown in Fig. 2A and B *KCNJ5* mutations were identified in all CYP11B2-positive anterior adenoma specimens by Sanger sequencing, but not in CYP11B2-negative tumor specimens from the posterior adenoma. However, *PRKACA* mutations were identified only in CYP11B2-negative tumor specimens from the posterior adenoma (Fig. 2A and C). Subsequent analysis of PCR product from B2T3 DNA confirmed the NGS identified *KCNJ5* mutations (Fig. 2B).

Discussion

Adrenal tumors are common neoplasms and the prevalence increases with age. Most are benign cortical adenomas and some are associated with endocrine disorders such as PA and CS. Although adrenal tumors with concomitant autonomous aldosterone and cortisol secretion have been increasingly recognized, their molecular characteristics remain to be elucidated.

This study demonstrates that the adrenal tumors in our patient have different characteristics in terms of steroidogenic enzyme expression and mutation

status. *KCNJ5* encodes the G-protein-activated inwardly rectifying potassium channel 4 (GIRK4). Although the *KCNJ5* gene mutations observed in this study have not been reported previously, two somatic mutations involving T148 and T149 located near the selectivity filter of the GIRK4 channel pore have been identified in APA (p.T148_T149insT, p.T148_T149insR) and *in vitro* analyses demonstrated that both mutations caused membrane depolarization, raised intracellular Ca²⁺, and increased aldosterone production (15, 16), supporting a pathological potential of the *KCNJ5* mutations identified in this case (p.T148I/T149S). Additionally, considering higher conservation of T149 residue among orthologs and paralogs compared with T148 (2), the mutation altering the amino acid in position 149 likely has a more important pathophysiological role than the change in position 148. *PRKACA* encodes the catalytic subunit of cyclic AMP-dependent PKA. The p.L206R mutation is located in a highly conserved core of the domain responsible for the interaction between the regulatory and catalytic subunits of PKA (7). Functional experiments revealed that the mutation caused constitutive PKA activation, providing a molecular explanation for the development of CPA (7, 8). The mutation analysis suggests that the CYP11B2-positive tumor was the main source of autonomous aldosterone production and the CYP11B2-negative and CYP17-positive tumor was the main source of autonomous cortisol production in this case. It remains speculative whether the co-occurrence of two adenomas in a single patient might be caused by a common underlying genetic predisposition or simply a chance event. In most cases, the pathomechanism of hypercortisolism accompanying PA is believed to be co-secretion of cortisol and aldosterone by the same adenoma. Considering the rare co-appearance of APA and CPA in a single adrenal gland, the adenomas likely have occurred

Figure 2

CYP11B2 IHC and Sanger sequencing results of each tumor piece. (A) Low magnification scan of tumor pieces with CYP11B2 IHC (CYP11B2; brown). Scale bars=5 mm. (B) Results of Sanger sequencing of *KCNJ5* gene (upper panel) and subsequent analysis of PCR product (lower panel). *KCNJ5* gene mutations (p.T148I/T149S) were detected in B2T1, B2T2, and B2T3, but not in T1, T2, and T3. Subsequent analysis of PCR product (B2T3) showed both mutations were present in the same allele (clone A) and the other allele was WT (clone B). (C) Results of Sanger sequencing of *PRKACA* gene. *PRKACA* gene mutations (p.L206R) were identified in T1, T2, and T3, but not in B2T1, B2T2, and B2T3. IHC, immunohistochemistry; B2T1, CYP11B2-positive tumor specimen 1; B2T2, CYP11B2-positive tumor specimen 2; B2T3, CYP11B2-positive tumor specimen 3; T1, CYP11B2-negative tumor specimen 1; T2, CYP11B2-negative tumor specimen 2; T3, CYP11B2-negative tumor specimen 3. WT, Wild type.

independently through two independent genetic events rather than having a common progenitor of the two adenomas. There remains a possible involvement of a susceptibility gene to tumor development. However, more patients and families would be necessary to attempt to define this susceptibility locus.

In this study, we demonstrate a case of PA and CS due to two different adrenocortical adenomas. This is certainly a quite rare case. However, it is important to notice any hypercortisolism in patients undergoing adrenal surgery in order to consider the necessity of perioperative hydrocortisone supplementation. This study also has

implications for the pathologic distinction of nodular hyperplasia and double adenoma in endocrine neoplasia. Our finding of distinct mutations in discrete masses provides compelling evidence for a double adenoma model that also applies to other endocrine glands.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/EJE-16-0262>.

Declaration of interest

S A T has received honoraria from Thermo Fisher Scientific and had a separate sponsored research agreement with Thermo Fisher Scientific. None of the study described herein was supported by Thermo Fisher Scientific and they had no role in the data collection, interpretation, or analysis, and did not participate in the study design or the decision to submit for publication. The remaining authors have nothing to disclose.

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