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Genetic, Cellular, and Molecular Heterogeneity in Adrenals With Aldosterone-Producing Adenoma

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See Editorial pp 927–929

Abstract—Aldosterone-producing adenoma (APA) cause primary aldosteronism—the most frequent form of secondary hypertension. Somatic mutations in genes coding for ion channels and ATPases are found in APA and in aldosterone-producing cell clusters. We investigated the genetic, cellular, and molecular heterogeneity of different aldosterone-producing structures in adrenals with APA, to get insight into the mechanisms driving their development and to investigate their clinical and biochemical correlates. Genetic analysis of APA, aldosterone-producing cell clusters, and secondary nodules was performed in adrenal tissues from 49 patients by next-generation sequencing following CYP11B2 immunohistochemistry. Results were correlated with clinical and biochemical characteristics of patients, steroid profiles, and histological features of the tumor and adjacent adrenal cortex. Somatic mutations were identified in 93.75% of APAs. Adenoma carrying *KCNJ5* mutations had more clear cells and cells expressing CYP11B1, and fewer cells expressing CYP11B2 or activated β-catenin, compared with other mutational groups. 18-hydroxycortisol and 18-oxocortisol were higher in patients carrying *KCNJ5* mutations and correlated with histological features of adenoma; however, mutational status could not be predicted using steroid profiling. Heterogeneous CYP11B2 expression in *KCNJ5*-mutated adenoma was not associated with genetic heterogeneity. Different mutations were identified in secondary nodules expressing aldosterone synthase and in independent aldosterone-producing cell clusters from adrenals with adenoma; known *KCNJ5* mutations were identified in 5 aldosterone-producing cell clusters. Genetic heterogeneity in different aldosterone-producing structures in the same adrenal suggests complex mechanisms underlying APA development. **(*Hypertension*. 2020;75:1034-1044. DOI: 10.1161/HYPERTENSIONAHA.119.14177.)** • **Online Data Supplement**

Key Words: adrenal cortex ■ aldosterone ■ arterial hypertension ■ mineralocorticoids ■ mutation ■ primary aldosteronism

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Primary aldosteronism (PA), caused by excessive aldosterone production from the adrenal cortex, is the most frequent form of secondary arterial hypertension with a prevalence of ≈5% of hypertensive patients in primary care and 10% in reference centers.[1,2] PA is characterized by hypertension associated with increased aldosterone levels, suppressed plasma renin, and often hypokalemia. Aldosterone-producing adenoma (APA) is a major subtype of PA.[3]

Somatic heterozygous mutations in *KCNJ5* (coding for the potassium channel GIRK4 [G protein-activated inward rectifier potassium channel 4]), *ATP1A1* (coding for the α1 subunit of the Na^+/K^+-ATPase), *ATP2B3* (coding for PMCA3, plasma membrane Ca^{2+}-ATPase type 3), and *CACNA1D* (encoding the Cav1.3 voltage-dependent calcium channel) were identified in APA.[4–7] They lead to increased intracellular calcium concentrations and activation of calcium signaling, which promotes aldosterone production by increasing the expression of *CYP11B2*, coding for aldosterone synthase. In addition, mutations in *CTNNB1*, coding for β-catenin, have been identified in 2% to 5% of cases with APA,[8–10] and a somatic mutation in *CLCN2*, coding for the chloride channel ClC-2 (chloride channel protein 2) mutated in familial hyperaldosteronism type II (FH-II) and

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early-onset PA,[11,12] has recently been identified in one APA.[13] The prevalence of somatic mutations in APA has been estimated at 54% by targeted sequencing of previously reported mutational hot spots.[14] However, recent studies applying CYP11B2 immunohistochemistry-guided next-generation sequencing (NGS) identified somatic mutations in ≤88% of APAs.[15,16]

It is unclear whether somatic mutations are entirely responsible for APA development, leading to both excessive aldosterone production and nodule formation. Recent studies have suggested that APA may derive from aldosterone-producing cell clusters (APCCs), adrenal structures found in normal adrenal glands, and carrying APA driver gene mutations.[17] This hypothesis is also supported by the description of possible APCC to APA translational lesions (pAATL), which also carry somatic mutations.[18] Other studies suggest a two-hit hypothesis, where somatic mutations are secondary events occurring in a previously remodeled adrenal cortex.[19–22] These 2 hypotheses may coexist, and both contribute to APA development.

The aim of this study was to investigate the genetic, cellular, and molecular characteristics of adrenals with APA to get insight into the mechanisms driving the development of aldosterone-producing structures. The specific questions we asked were as follows: (1) what is the genetic landscape of aldosterone-producing structures in adrenals with APA, assessed by CYP1B2 immunohistochemistry-guided NGS? (2) Is the well-known cellular and molecular heterogeneity in APA linked to genetic heterogeneity? (3) How does immunohistochemistry-guided NGS detection of mutations influence previously observed correlations with clinical, biological, and pathological parameters?

We performed genetic characterization of APA using a newly developed NGS kit, including all genes related to sporadic and familial PA (except *CLCN2*), on DNA extracted from formalin-fixed paraffin-embedded (FFPE) tissues following CYP11B2 immunohistochemistry. Results were correlated with clinical and biochemical characteristics of patients, their steroid profile, and with histological features of APA and adjacent adrenal cortex. APA heterogeneity was evaluated on the cellular, functional, and genetic level using immunohistochemistry, multiplex immunofluorescence multispectral image analysis, and CYP11B2 immunohistochemistry-guided NGS. Mutations were also assessed in aldosterone-producing lesions in the adrenal cortex adjacent to APA and in micronodular adrenals.

Subjects and Methods

The authors declare that all supporting data are available within the article (and in the online-only Data Supplement). An expanded Methods section is available in the online-only Data Supplement.

Patients

Patients included in this study were recruited within the COMETE (COrtico- et MEdullo-surrénale, les Tumeurs Endocrines) - HEGP (Hôpital Européen Georges Pompidou) protocol (authorization CPP 2012-A00508-35). Among the 67 patients undergoing adrenalectomy for lateralized PA at the Hypertension Unit of the HEGP between 2013 and June 2016, we included 49 patients for whom informed consent was available for research purposes. Screening and subtype identification of PA was performed according to institutional and Endocrine Society guidelines.[23–25] Thirty-nine of 40 patients with outcome data at 6 months after surgery had complete biochemical remission according to the Primary Aldosteronism Surgery Outcome criteria.[26] Further details are available in the online-only Data Supplement. All patients gave written informed consent for genetic and clinical investigation. Procedures were in accordance with the institutional guidelines. Two (1–5) (median [minimum to maximum]) FFPE blocs per patient were investigated for a total of 110 blocs. Two PA cases who underwent adrenalectomy at the University of Michigan were also included for the intratumoral genetic analysis. The use of the specimen for the analysis was approved by the Institutional Review Board at the University of Michigan (HUM00083056). The number of patient samples that underwent each procedure is described in Figure S1 in the online-only Data Supplement.

Pathological Analysis

Histological examination was performed on 4-µm sections stained with hematoxylin-eosin safran. Cellular composition was determined by examining for known features of zona fasciculata cells, in particular, a high cytoplasm-to-nucleus ratio. The main criterion used to determine zona glomerulosa (ZG) hyperplasia was the continuous character of the ZG; the second criterion, in case of discontinuity of the ZG, was its thickness (≥200 µm) measured with a micrometer integrated in the microscope.[27] All microscopic examinations were performed with a Leica microscope.

Immunohistochemistry

Detailed description of the immunohistochemistry procedure is available in the online-only Data Supplement. For the cases from the University of Michigan, CYP11B2 immunohistochemistry was performed as described previously.[28]

Multiplex Immunofluorescence

Multiplex immunofluorescence staining was performed with the Opal 7-Color Manual IHC Kit (PerkinElmer, Waltham). Details are available in the online-only Data Supplement.

Analysis and Quantification of Histological Features

Images were acquired using a Vectra automated imaging system (PerkinElmer). Quantifications were done either by blinded observation using Phenochart (CYP11B2, CYP11B1, and GIRK4) or by automated image analysis and feature selection (β-catenin, colocalization) using the InForm image analysis package (both programs from PerkinElmer). Details are available in the online-only Data Supplement.

DNA Isolation

Somatic DNA of APA was extracted from fresh frozen tissue using QIamp DNA midi kit (Qiagen, Courtaboeuf Cedex, France) for Sanger sequencing. Somatic DNA and RNA were extracted from FFPE sections using AllPrep DNA/RNA FFPE kit (Qiagen) for targeted NGS. Before DNA/RNA extraction of FFPE tissue, APA, aldosterone-producing nodules, non–aldosterone-producing nodules, and APCCs were identified by CYP11B2 immunohistochemistry. Based on the CYP11B2 immunohistochemistry, the areas of interest were delimited and isolated for DNA/RNA extraction by scraping unstained FFPE sections guided by the CYP11B2 immunohistochemistry slide using a scalpel under a Wild Heerbrugg or Olympus microscope. DNA from peripheral blood leukocytes was prepared using an automated platform at the Genetics Department of the HEGP.

Sanger Sequencing and Targeted NGS

Targeted Sanger sequencing of *KCNJ5*, *CACNA1D*, *ATP1A1*, *ATP2B3*, *CTNNB1*, and *CLCN2* is described in the online-only Data Supplement. The list of primers used for Sanger sequencing is indicated in Table S1.

NGS was performed using a newly developed NGS kit, covering all coding exons and intron-exon junction of the *KCNJ5* (NM_000890), *ATP1A1* (NM_000701), *ATP2B3* (NM_0010001344), *CTNNB1* (NM_001904), *CACNA1D* (NM_001128839.2 and NM_000720), *APC* (NM_000038.5), *CACNA1H* (NM_021098 and NM_001005407), *PRKACA* (NM_002730), and *ARMC5* (NM_002730) genes (MASTR_PA kit; Multiplicom/Agilent, Santa Clara, CA). The 9 target genes were amplified with a total of 3

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multiplex polymerase chain reactions generating 593 amplicons. Further details are available in the online-only Data Supplement.

For the 2 cases from the University of Michigan, targeted NGS using ion torrent technology and variant calls were performed as described previously.[28]

CYP11B2 quantitative PCR

RNA isolated from FFPE based on CYP11B2 immunohistochemistry findings was used for quantitative real-time PCR. cDNA was generated using High-Capacity cDNA archive kit (Applied Biosystems). To assess *CYP11B2* mRNA expression, quantitative PCR was performed in the ABI StepOnePlus Real-Time PCR systems (Applied Biosystems). Primer/probe mixtures for *CYP11B2* were prepared as described previously.[29] β-actin (ACTB; Hs01060665_g1) transcript was used as a reference gene. The delta-delta threshold cycle method was used for the calculation of fold changes over adjacent normal adrenal.

Liquid Chromatography coupled to tandem Mass Spectrometry Steroid Profiling

Thirteen steroids were measured simultaneously with steroid profiling: 18-oxocortisol, 18-hydroxycortisol, aldosterone, cortisone, cortisol, 11-deoxycortisol, 21-deoxycortisol, 18-hydroxy-11-deoxycorticosterone, 11-deoxycorticosterone, 18-hydroxycorticosterone, corticosterone, 17-hydroxyprogesterone, delta-4-androstenedione in a 13-minute run. The complete steroid profiling procedure is described in the online-only Data Supplement.

Statistical Analyses

Details on statistical analyses are available in the online-only Data Supplement. Unsupervised analyses were conducted using principal component analysis with variable normalization and uniform manifold approximation and projection.[30,31]

Results

Identification of Somatic Mutation in APA

Recurrent somatic mutations in genes coding for *KCNJ5*, *CACNA1D*, *ATP1A1*, *ATP2B3*, and *CTNNB1* were searched for by Sanger sequencing on 48 DNA samples extracted from fresh frozen APA tissue. We identified 21 (44%) *KCNJ5* mutations, 5 (11%) *CACNA1D* mutations, 4 (8%) *ATP2B3* mutations, and 4 (8%) *ATP1A1* mutations (Figure S2A; Table S2). Of the 48 samples, 14 negative samples were further investigated by CYP11B2 immunohistochemistry-guided NGS on DNA extracted from FFPE sections of APA, using a newly developed NGS kit, which includes all coding exons and intron-exon junctions of genes described in sporadic and familial PA (except *CLNC2*). This allowed identification of 11 additional somatic mutations (Table 1). The total frequency of somatic mutations after targeted NGS was 93.75% (45 of 48 samples): 44% (21 of 48) of *KCNJ5* mutations, 27% (13 of 48) of *CACNA1D* mutations, 13% (6 of 48) of *ATP1A1* mutations, and 10% (5 of 48) of *ATP2B3* mutations. Three APAs remained mutation negative (Figure S2B; Table S2). No additional mutations were identified in those 3 APAs by sequencing all coding exons and intron-exon junctions of *CLCN2*.

Patients with *KCNJ5* mutations were more frequently women and young patients, whereas patients with *CACNA1D*, *ATP1A1*, and *ATP2B3* mutations were more frequently men. There were no differences among mutational groups in adenoma size, potassium concentration, plasma aldosterone and plasma renin concentrations, systolic and diastolic blood pressure, treatment score, and follow-up parameters (Table 2).

Cellular and Molecular Heterogeneity of APA

To search for correlations between genotypes established by immunohistochemistry-guided NGS and morphological characteristics of APA, we investigated the cellular composition and performed immunohistochemistry for markers of cell identity and function. APAs are composed in variable proportion of clear and compact cells.[32] In this study, the majority of APAs (70.8%) were mainly composed of clear cells.

Table 1. Somatic Mutations Identified in Aldosterone-Producing Adenoma by CYP11B2 Immunohistochemistry-Guided NGS

Patient	Sanger-Mutated Gene (FFT)	NGS-Mutated Gene (FFPE)	c.DNA	Amino Acid Change	Read Depth	VAF, %
Patient 5	Neg	*CACNA1D*	c.1207G>C	p.Gly403Arg	821	31
Patient 8	Neg	*CACNA1D*	c.3044T>C	p.Ile1015Thr*	4655	35
Patient 10	Neg	*CACNA1D*	c.1856G>C	p.Arg619Pro	254	37
Patient 12	Neg	*ATP1A1*	c.311T>G	p.Leu104Arg	4808	37
Patient 22	Neg	*CACNA1D*	c.3044T>C	p.Ile1015Thr*	4189	32
Patient 25	Neg	Neg				
Patient 32	Neg	Neg				
Patient 34	Neg	*CACNA1D*	c.2239T>G	p.Phe747Val	8279	36
Patient 39	Neg	Neg				
Patient 42	Neg	*CACNA1D*	c.1229C>T	p.Ser410Leu	3356	38
Patient 44	Neg	*CACNA1D*	c.4057G>A	p.Val1353Met	4056	32
Patient 46	Neg	*CACNA1D*	c.4057G>A	p.Val1353Met	5462	35
Patient 49	Neg	*ATP1A1*	c.303_308delAATGTT	p.Ser101_Leu104del	4133	30
Patient 56	Neg	*ATP2B3*	c.1270_1275delGTGCTG	p.Val424_Leu426del	2305	47

Nomenclature of *CACNA1D* mutations is according to NM_001128839.2. FFPE indicates formalin fixed paraffin embedded; FFT, fresh frozen tissue; Neg, negative; NGS, next-generation sequencing; and VAF, variant allele frequency.

*Previously unreported variant.

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Table 2. Correlation of Clinical, Biological, and Immunohistochemistry Characteristics With Mutational Status in Patients With APA

Characteristics	*KCNJ5* (n=21)	*CACNA1D* (n=13)	*ATP2B3* (n=5)	*ATP1A1* (n=6)	Neg (n=3)	*P* Value
Female, n (%)	15/71.4	2/15.4	1/20	0/0	0/0	0.0008
Age at PA dg (y)	40 (33–47)	51 (38.5–55)	51 (40.5–64.5)	59.5* (45.25–71)	47 (47–55)	0.0209
Lowest plasma K, mmol/L	3.1[a] (2.6–3.2)	2.8 (2.6–3.2)	2.65[b] (1.95–2.9)	2.7 (2.375–3.55)	2.7 (2.1–3.1)	0.6665
Systolic BP, mm Hg	146[a] (131–154)	145 (136–152.5)	147[b] (137–152.5)	137.5 (123.3–149.3)	150.5[c] (140–161)	0.7406
Diastolic BP, mm Hg	92[a] (80–104)	90 (86.5–94)	90.5[b] (88.25–95)	82 (78.75–86.5)	85[c] (79–91)	0.2638
Treatment score, n	2[d] (1–3)	2 (2–3)	3[b] (2.25–3)	3.5[e] (1.75–4)	2 (2–2)	0.3842
Plasma aldosterone, pmol/L	1162[a] (926–1420)	639 (534.5–984.5)	815[b] (474.8–1311)	1963[e] (547.5–45928)	933 (532–1508)	0.1418
Plasma rennin, mU/L	1[a] (1–3.2)	1.15 (1.1–2.5)	1[b] (1–1.4)	2.1[e] (1–17)	1 (1–3.2)	0.774
ARR, pmol/mU	232.4 (185.2–284)	127.8 (106.9–159.7)	163 (94.95–262.1)	392.6 (109.5–5196)	186.6 (106.4–301.6)	0.0799
Adenoma size, mm	15 (12–20)	10 (9–15.5)	8 (4–17)	12 (9.75–23.75)	18 (10–47)	0.1069
ZG hyperplasia, n (%)	21/100	7 / 53.85	4/80	6/100	2/50	0.0067
Clear cells (tertile)	3 (3–3)	3 (1–3)	1† (1–2.5)	1.5* (0.75–3)	2 (2–2)	0.0013
18-oxocortisol, nmol/L	0.98[f] (0.57–2.61)	0.15†[g] (0.1–0.39)	0.15 (0.1–0.65)	0.14[b] (0.11–0.25)	0.27 (0.1–0.29)	0.0015
18-hydroxycortisol, nmol/L	4.10[f] (3.13–6.94)	1.30‡[g] (0.56–1.91)	1.95 (1.11–3.22)	2.09[b] (0.98–2.47)	2.03 (0.93–2.6)	0.0016
Aldosterone synthase expression (tertile)	1 (1–1.5)	3‡ (2–3)	3* (2–3)	2.5* (2–3)	2 (1–3)	<0.0001
11β-hydroxylase expression (tertile)	3 (1–3)	1† (0–1.5)	2 (0–2)	1* (0.75–1)	1 (1–2)	0.0038
GIRK4 expression APA<ADJ, n (%)	18/85.7	1/7.7	0/0	1/16.7	0/0	<0.0001
β-Catenin–activated cells (tertile)	1 (1–1)	2* (1–3)	3* (2–3)	2.5* (1.75–3)	3* (2–3)	0.0011

Quantitative variables are reported as medians and interquartile range and compared with Krustal-Wallis test. Categorical variables are reported as number and compared with χ^2 test. Percentages of aldosterone synthase and 11β-hydroxylase–expressing cell, β-catenin–activated cells, and clear cells are reported as 4 categories: 0, absent; 1, 1% to 33%; 2, 34% to 66%; 3, 67% to 100%. Relative expression of GIRK4 (G protein-activated inward rectifier potassium channel 4) represents the comparison of the GIRK4 intensity in APA vs the adjacent ZG. $P<0.05$ was considered significant. ADJ indicates adjacent; APA, aldosterone-producing adenoma; ARR, aldosterone-to-renin ratio; BP, blood pressure; K, potassium; Neg, negative; PA, primary aldosteronism; and ZG, zona glomerulosa.

Number variation based on the availability of clinical data and samples for steroid measurements: [a]n=19; [b]n=4; [c]n=2; [d]n=20; [e]n=5; [f]n=16; [g]n=12.

*$P\leq0.05$, †$P\leq0.01$, ‡$P\leq0.001$, vs *KCNJ5*.

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Correlation of the mutational status with cellular composition showed that APAs carrying *KCNJ5* mutations have higher percentage of clear cells (P=0.0008) in comparison with APAs carrying other or without identified mutations (Table 2).

Analysis of aldosterone synthase (CYP11B2), 11β-hydroxylase (CYP11B1), GIRK4, and β-catenin expression in APA showed important tumor heterogeneity (Figure 1A; Table 2). APAs carrying *KCNJ5* mutations had significantly lower percentage of cells expressing CYP11B2 in comparison with APAs carrying *CACNA1D*, *ATP1A1*, and *ATP2B3* mutations and higher percentage of cells expressing CYP11B1 in comparison with APAs carrying *CACNA1D* and *ATP1A1* mutations (Table 2). As previously reported,[33] GIRK4 expression in APAs carrying *KCNJ5* mutation was lower compared with the adjacent ZG (Table 2). The number of cells with activated β-catenin (nuclear or cytoplasmic expression) was lower in APA with *KCNJ5* mutations compared with APAs carrying *ATP2B3*, *CACNA1D*, and *ATP1A1* mutations or without mutations (Table 2).

Interestingly, in serial sections, we observed expression of GIRK4 and activated β-catenin in areas of APA expressing CYP11B2 but not CYP11B1 (Figure 1A). In contrast, in regions expressing CYP11B1, β-catenin was mainly localized to the cell membrane (inactivated; Figure 1A). Multiplex immunofluorescence on selected areas of 13 APAs revealed cellular coexpression of CYP11B2 with GIRK4 and activated β-catenin in the majority of cells, independent of the mutational status of APA (Figure 1B and 1C through 1F). A certain number of cells coexpressed 17α-hydroxylase (CYP17A1) with CYP11B2 (Figure 1B and 1C through 1F; Figure S3).

To investigate whether cellular and molecular heterogeneity of APA was related to different genetic defects within 1 tumor, we have sequenced areas with or without CYP11B2 expression within the same APA by CYP11B2 immunohistochemistry-guided NGS. A total of 10 areas positive and mostly negative for CYP11B2 expression were extracted from 2 APAs (Figure 2A, top). Despite heterogeneous expression of CYP11B2, all sequenced areas in both APAs were positive for the same *KCNJ5* mutation with similar variant allele frequencies (Figure 2B). These findings were replicated in the sequencing analysis of 5 areas from 2 APAs from the University of Michigan (Figure 2A, bottom; Figure 2B; Figure S4). Regions within the same APA with different levels of *CYP11B2* mRNA expression carried the identical somatic *KCNJ5* mutation, with similar variant allele frequencies (Figure 2A, bottom; Figure 2B; Figure S4).

Somatic Mutations in Different Lesions Expressing Aldosterone Synthase in Adrenals With Micronodular Hyperplasia or APA

Morphological and histological characterization of the adjacent adrenal cortex of 48 APAs revealed ZG hyperplasia,

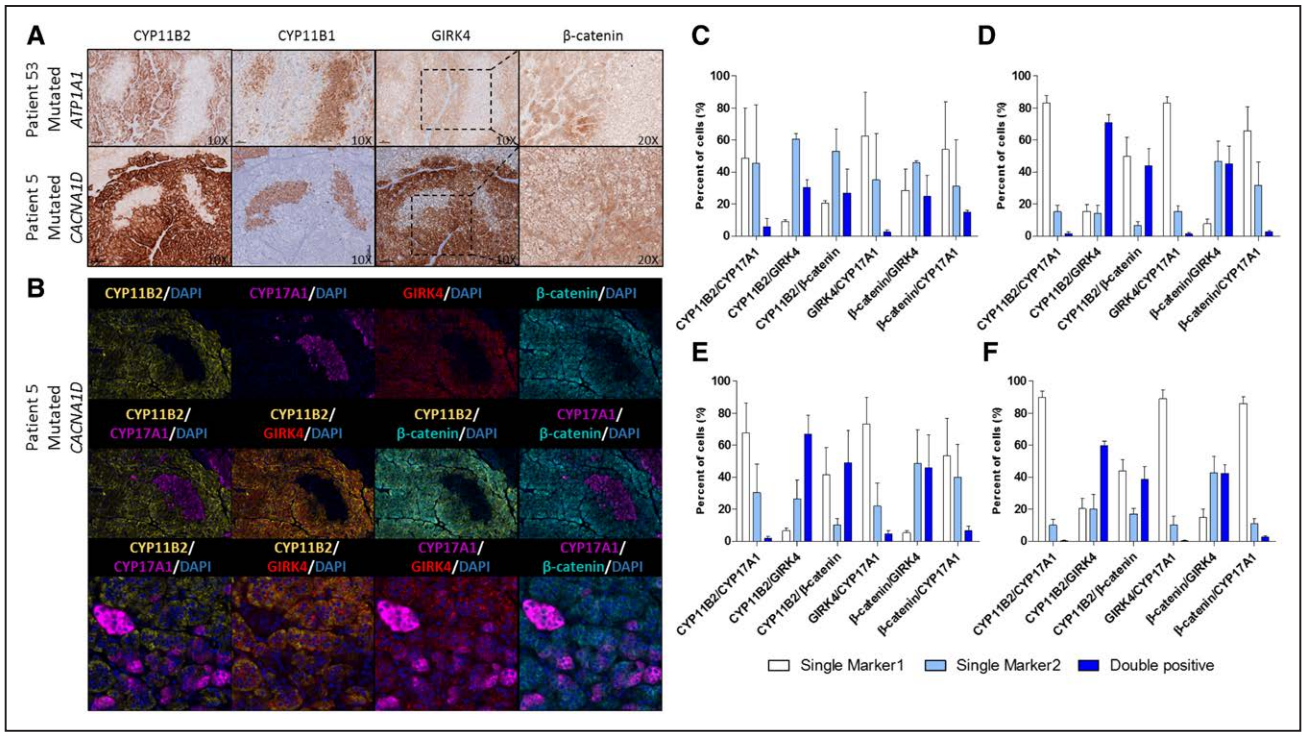


Figure 1. Molecular heterogeneity of aldosterone-producing adenoma (APA). **A**, Example of aldosterone synthase (CYP11B2), 11β-hydroxylase (CYP11B1), GIRK4, and β-catenin staining in APA. **B**, Colocalization of CYP11B2, CYP17A1 (17α-hydroxylase), GIRK4 (G protein-activated inward rectifier potassium channel 4), and β-catenin by multiplex immunofluorescence in APA. **C**–**F**, Automatic quantification of colocalization of different proteins in 13 APAs carrying mutations in *KCNJ5* (**C**; n=2), *CACNA1D* (**D**; n=5), *ATP2B3* (**E**; n=3), and *ATP1A1* (**F**; n=3). For each couple of colocalized proteins, marker 1 refers to the first marker indicated in the legend and marker 2 to the second one.

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mostly negative for CYP11B2, in a majority of adrenals carrying an APA (40 of 48), being more frequent in APA with *KCNJ5* mutations (Table 2). APCC and secondary nodules expressing or not expressing CYP11B2 were also observed (Figures S5 and S6), including multiple APCCs and 1 pAATL in 1 adrenal with micronodular hyperplasia (Figure S7).

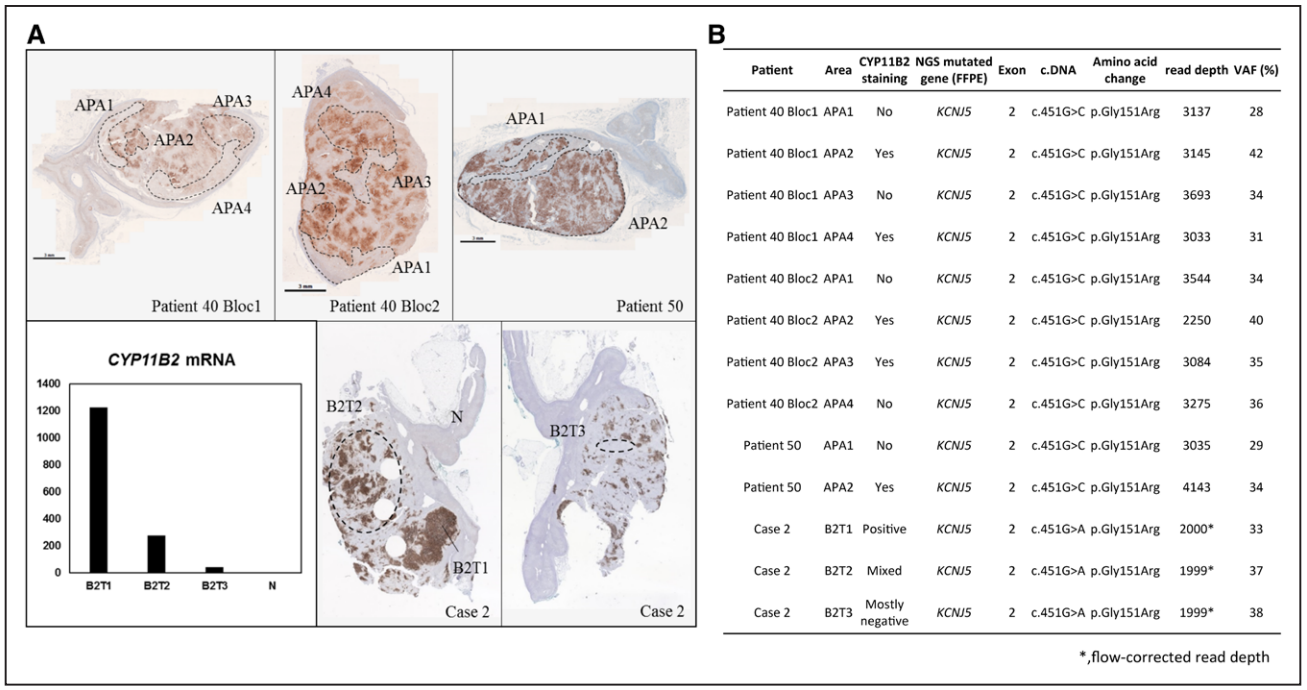
The mutational status of 6 APCCs and 1 pAATL from one micronodular adrenal and of 57 APCCs from 9 adrenals carrying an APA was assessed by CYP11B2 immunohistochemistry-guided NGS (Figures S5 and S7). In the adrenal with micronodular hyperplasia, *CACNA1D* mutations were identified in 4 of 6 APCCs and in the 2 portions (expressing and nonexpressing CYP11B2) of the pAATL (Figure S7). Remarkably, within the same adrenal, different lesions presented different *CACNA1D* mutations (APCC1, p.Ser652Leu; APCC3, p.Ala998Val; APCC4 and APCC6, p.Arg990His; pAATL, p.Gly403Arg; Figure S7).

From the 57 APCCs adjacent to an APA investigated, 15 carried mutations in APA driver genes (Figure S5; Table 3; Table S3). Interestingly, different APCCs within the same adrenal presented different mutations, with distinct mutational status compared with the APA. In 3 adrenals (patient 4, patient 34, and patient 41), a *CACNA1D* mutation was identified in the APA and different *CACNA1D* mutations were observed in APCC within the same adrenal. In 1 adrenal (patient 49), an *ATP1A1* mutation was identified in the APA and different *CACNA1D* or *ATP1A1* mutations were observed in APCC. Finally, in 1 adrenal (patient 52), a *KCNJ5* mutation was observed in the APA and 2 APCCs carried different *CACNA1D* mutations (Table 3; Figure S5). Remarkably, known *KCNJ5* mutations were also identified in 5 APCCs adjacent to APA. In 3 adrenals (patient 4, patient 5, and patient 41), the APA carried a *CACNA1D* mutation, while *KCNJ5* mutations were identified in APCC, with different mutations identified in 2 distinct APCCs from patient 4. In one adrenal (patient 52), a *KCNJ5* mutation was identified in the APA and a different *KCNJ5* mutation in APCC (Table 3; Figure S5).

CACNA1H variants previously described in patients with familial forms of PA[34] were also identified in APCC. In one adrenal with APA carrying a *CACNA1D* mutation (patient 41), the *CACNA1H* p.Pro2083Leu variant was identified in 1 APCC and the variant p.Ser196Leu was identified in a second APCC. These 2 APCCs also carried a *CACNA1D* mutation. The *CACNA1H* p.Ser196Leu was also identified in 1 APCC of a second adrenal with APA carrying a *CACNA1D* mutation (patient 44). A *CTNNB1* mutation (p.Ser37Phe) previously described in corticosteroid-producing adenoma[35] was identified in 1 APCC in 1 adrenal with APA harboring a *KCNJ5* mutation (patient 52). Previously unreported *PRKACA* mutations were identified in 2 APCCs, from which one carries 2 mutations (patient 25, p.Ser213Asn; patient 52, p.Cys200Tyr, p.Cys201Asp; Table 3; Figure S5).

A peculiar structure expressing CYP11B2 was observed in the adrenal of patient 5 (Figure 3). This structure extends deep into the adrenal cortex. NGS of the region near the capsule (structure 1) and the inner region (structure 2; Figure 3; Table 3) identified a *CACNA1H* variant (p.Pro2083Ser) in the inner area but no genetic abnormality in the subcapsular region. This variant affects the same codon as a *CACNA1H* mutation associated with familial PA (p.Pro2083Leu).[34]

Three secondary nodules expressing CYP11B2 and 2 nonexpressing CYP11B2 from adrenals with APA were also sequenced.



B

Patient	Area	CYP11B2 staining	NGS mutated gene (FFPE)	Exon	c.DNA	Amino acid change	read depth	VAF (%)
Patient 40 Bloc1	APA1	No	*KCNJ5*	2	c.451G>C	p.Gly151Arg	3137	28
Patient 40 Bloc1	APA2	Yes	*KCNJ5*	2	c.451G>C	p.Gly151Arg	3145	42
Patient 40 Bloc1	APA3	No	*KCNJ5*	2	c.451G>C	p.Gly151Arg	3693	34
Patient 40 Bloc1	APA4	Yes	*KCNJ5*	2	c.451G>C	p.Gly151Arg	3033	31
Patient 40 Bloc2	APA1	No	*KCNJ5*	2	c.451G>C	p.Gly151Arg	3544	34
Patient 40 Bloc2	APA2	Yes	*KCNJ5*	2	c.451G>C	p.Gly151Arg	2250	40
Patient 40 Bloc2	APA3	Yes	*KCNJ5*	2	c.451G>C	p.Gly151Arg	3084	35
Patient 40 Bloc2	APA4	No	*KCNJ5*	2	c.451G>C	p.Gly151Arg	3275	36
Patient 50	APA1	No	*KCNJ5*	2	c.451G>C	p.Gly151Arg	3035	29
Patient 50	APA2	Yes	*KCNJ5*	2	c.451G>C	p.Gly151Arg	4143	34
Case 2	B2T1	Positive	*KCNJ5*	2	c.451G>A	p.Gly151Arg	2000*	33
Case 2	B2T2	Mixed	*KCNJ5*	2	c.451G>A	p.Gly151Arg	1999*	37
Case 2	B2T3	Mostly negative	*KCNJ5*	2	c.451G>A	p.Gly151Arg	1999*	38

*,flow-corrected read depth

Figure 2. Genetic and molecular heterogeneity in aldosterone-producing adenoma (APA). **A**, Heterogeneous aldosterone synthase (CYP11B2) staining in APA from 3 patients. DNA was extracted from 4 CYP11B2-positive regions and 4 regions with mostly negative expression of CYP11B2 from patient 40, 1 CYP11B2-positive region and 1 region mostly negative for CYP11B2 from patient 50 (**top**), and 2 CYP11B2-positive regions with different degree of its expression and 1 tumor region mostly negative for CYP11B2 from case 2 (**bottom**, **right**). Determination of *CYP11B2* mRNA expression from different tumor regions from case 2 (**bottom**, **left**). **B**, Results of next-generation sequencing (NGS) performed in multiple tumor regions with different CYP11B2 expression levels in APA. B2T indicates CYP11B2-expressing tumor region; FFPE, formalin fixed paraffin embedded; N, adjacent normal adrenal; and VAF, variant allele frequency. *Flow-corrected read depth.

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In one adrenal (patient 4; Figure 3; Figure S6), 2 secondary nodules expressing CYP11B2 carry *CACNA1D* mutations different from the *CACNA1D* mutation observed in the APA (Figure S6). No mutations were identified in a secondary nodule expressing CYP11B2 of one adrenal (patient 34) whose APA carries a *CACNA1D* mutation. No mutations in APA driver genes were identified in 2 nodules not expressing CYP11B2 from 2 adrenals carrying APA with *CACNA1D* (patient 42) and *ATP2B3* (patient 56) mutations (Figure S6). In patient 56, the large nodule was considered to be the APA before immunohistochemistry.

18-Hydroxycortisol and 18-Oxocortisol Are Increased in APA With *KCNJ5* Mutations

Peripheral blood steroid profiles were measured in 40 patients (16 *KCNJ5*, 12 *CACNA1D*, 5 *ATP1A1*, 4 *ATP2B3*; 3 without identified mutations). No significant differences were observed between mutational groups in peripheral concentrations of 11-deoxycorticosterone, corticosterone, 18-hydroxycorticosterone, 18-hydroxy-11-deoxycorticosterone, aldosterone, 17-hydroxyprogesterone, 11-deoxycortisol, 21-deoxycortisol, cortisol, cortisone, delta-4-androstenedione (Table S4). Only 18-hydroxycortisol and 18-oxocortisol were significantly increased in *KCNJ5* mutation carriers (medians of 18-hydroxycortisol and 18-oxocortisol concentrations of 4.10 nmol/L [P<0.001] and 0.98 nmol/L [P<0.01], respectively) when compared with *CACNA1D* mutation carriers (18-hydroxycortisol and 18-oxocortisol median of 1.30 and 0.15 nmol/L, respectively; Table 2). When distinguishing mutations based on the underlying molecular mechanism, depolarizing mutations (*KCNJ5* and *ATP1A1*) showed higher 18-hydroxycortisol, 18-oxocortisol, and 11-deoxycorticosterone levels compared with calcium mutations (*CACNA1D* and *ATP2B3*). However, multivariate and supervised analyses did not allow to predict the APA mutation status based on steroid profiles (Figure S8). We were not able to identify any cluster of mutation or mutation types (depolarizing versus calcium mutations) in the unsupervised analyses (principal component analysis and uniform manifold approximation and projection). Although uniform manifold approximation and projection is optimized for clustered representation, those present were highly heterogeneous even when grouping by mutation type, with the exception of a small cluster (5 patients) of pure *KCNJ5* mutations. The cross-validated random forest models yielded accuracies of 0.52 and 0.57 for mutations and mutation types, respectively, using 2 predictors per tree. In the model predicting for the mutation, all classification errors are elevated except for predicting *KCNJ5* mutations (0.25 classification error), with 18-hydroxy- and 18-oxocortisol having the highest Gini importance measures. Interestingly, steroid output was associated with distinct histological functional features of APA. In particular, lower proportion of cells expressing CYP11B2 in APA was associated with higher levels of 18-oxocortisol, 18-hydroxycortisol, 18-hydroxy-11-deoxycorticosterone, corticosterone, and 11-deoxycorticosterone but not aldosterone levels. Lower proportion of cells expressing CYP11B1 and clear cell percentage in APA were associated with lower levels of 18-oxocortisol and 18-hydroxycortisol (Tables S5 through S7).

Discussion

In this study, CYP11B2 immunohistochemistry-guided NGS, using a newly developed NGS kit, allowed identifying

Table 3. Mutational Status of APCC and 1 Peculiar Structure Expressing Aldosterone Synthase in Adrenals With APA Identified by CYP11B2 Immunohistochemistry-Guided NGS

Patient	Structure	CYP11B2 Staining	Sanger-Mutated Gene (FFT)	NGS-Mutated Gene (FFPE)	Exon	c.DNA	Amino Acid Change	Read Depth	VAF, %
Patient 4 Bloc2	APA	Yes	*CACNA1D*	NS	23	c.2993C>T	p.Ala998Val		
Patient 4 Bloc2	APCC1	Yes	NS	*KCNJ5*	2	c.452G>A	p.Gly151Glu	168	15
Patient 4 Bloc2	APCC3	Yes	NS	*CACNA1D*	14	c.1963C>T	p.Leu655Phe*	289	58
Patient 4 Bloc2	APCC4	Yes	NS	*KCNJ5*	2	c.343C>T	p.Arg115Trp	670	10
Patient 5	APA	Yes	Neg	*CACNA1D*	8	c.1207G>C	p.Gly403Arg	821	31
Patient 5	APCC4	Yes	NS	*KCNJ5*	2	c.343C>T	p.Arg115Trp	799	8
Patient 5	Structure1	Yes	NS	Neg					
Patient 5	Structure2	Yes	NS	*CACNA1H*	35	c.6247C>T	p.Pro2083Ser	3079	7
Patient 25 Bloc2	APA	Yes	Neg	Neg					
Patient 25 Bloc2	APCC1	Yes	NS	*PRKACA*	7	c.638G>A	p.Ser213Asn*	713	12
Patient 34 Bloc2	APA	Yes	Neg	*CACNA1D*	16	c.2239T>G	p.Phe747Val	8279	36
Patient 34 Bloc4	APCC2	Yes	NS	*CACNA1D*	23	c.2969G>A	p.Arg990His	309	9
Patient 41 Bloc1	APA	Yes	*CACNA1D*	NS	23	c.2968C>G	p.Arg990Gly		
Patient 41 Bloc1	APCC2	Yes	NS	*CACNA1D*	16	c.2239T>G	p.Phe747Val	1414	9
Patient 41 Bloc1	APCC3	Yes	NS	*CACNA1D*	16	c.2239T>G	p.Phe747Val	1189	25
Patient 41 Bloc1	APCC4	Yes	NS	*CACNA1D*	33	c.4057G>A	p.Val1353Met	242	8
				CACNA1D	24	c.3044T>C	p.Ile1015Thr*	539	10
				CACNA1H	35	c.6248C>T	p.Pro2083Leu	244	13
Patient 41 Bloc1	APCC5	Yes	NS	*KCNJ5*	2	c.452G>A	p.Gly151Glu	38	31
Patient 41 Bloc3	APCC1	Yes	NS	*CACNA1D*	23	c.2968C>T	p.Arg990Cys*	1231	15
				CACNA1H	5	c.587C>T	p.Ser196Leu	204	21
Patient 44 Bloc7	APA	Yes	Neg	*CACNA1D*	33	c.4057G>A	p.Val1353Met	4056	32
Patient 44 Bloc15	APCC3	Yes	NS	*CACNA1H*	5	c.587C>T	p.Ser196Leu	166	8
Patient 49 Bloc6	APA	Yes	Neg	*ATP1A1*	4	c.303_308delAATGTT	p.Ser101_Leu104del	4133	30
Patient 49 Bloc4	APCC	Yes	NS	*CACNA1D*	16	c.2241C>A	p.Phe747Leu	5333	21
Patient 49 Bloc8	APCC	Yes	NS	*ATP1A1*	4	c.311T>G	p.Leu104Arg	3751	27
Patient 52 Bloc6	APA	Yes	*KCNJ5*	NS	2	c.451G>A	p.Gly151Arg		
Patient 52 Bloc3	APCC3	Yes	NS	*CACNA1D*	16	c.2250C>G	p.Ile750Met	6362	18
Patient 52 Bloc3	APCC6	Yes	NS	*PRKACA*	7	c.599G>A	p.Cys200Tyr*	409	21
				PRKACA	7	c.602G>A	p.Gly201Asp*	410	18
Patient 52 Bloc3	APCC7	Yes	NS	*CTNNB1*	3	c.110C>T	p.Ser37Phe	1755	10
Patient 52 Bloc6	APCC2	Yes	NS	*CACNA1D*	6	c.776T>A	p.Val259Asp	912	39
Patient 52 Bloc6	APCC4	Yes	NS	*KCNJ5*	2	c.472A>G	p.Thr158Ala	268	18

Only APCC-carrying mutations are shown. Nomenclature of *CACNA1D* mutations is according to NM_001128839.2. APA indicates aldosterone-producing adenoma; APCC, aldosterone-producing cell cluster; FFPE, formalin fixed paraffin embedded; FFT, fresh frozen tissue; Neg, negative; NGS, next-generation sequencing; NS, not sequenced; and VAF, variant allele frequency.

*Previously unreported variant.

somatic mutations in 93.75% of APAs. Those were associated with different clinical and histological characteristics and hybrid steroid output. APA showed important cellular and molecular heterogeneity, which was not associated with different somatic genetic events. In contrast, APCC adjacent to APA and from adrenals with micronodular hyperplasia carried different somatic mutations, including known *KCNJ5* mutations.

Somatic mutations in APA were previously reported by us and others with a frequency around 50%.[14,36] Recent studies, performing immunohistochemistry-guided NGS, have indicated that the frequency of somatic mutations may be much higher.[15,16] Using a newly developed NGS kit applied to DNA extracted from FFPE after CYP11B2 immunohistochemistry, we identified somatic mutations in APA previously described

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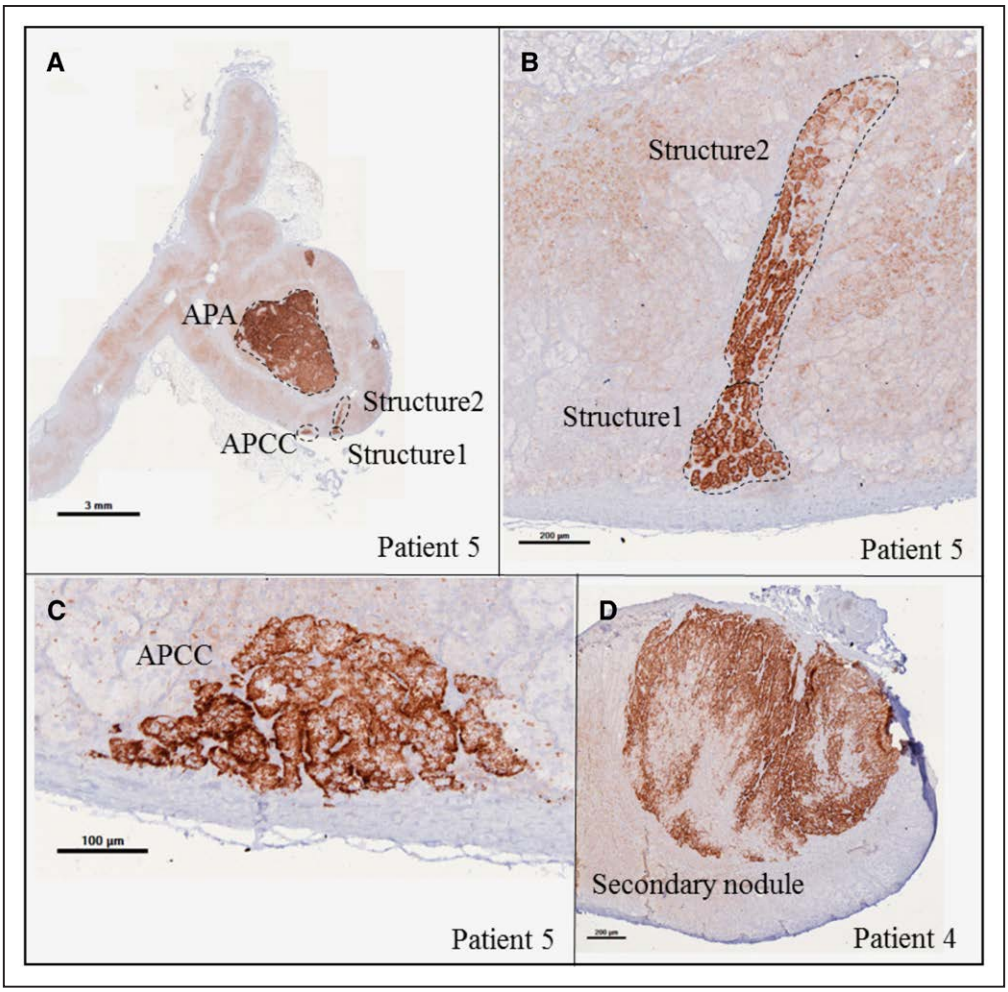


Figure 3. Different structures expressing CYP11B2 in adrenals with aldosterone-producing adenoma (APA). Immunohistochemistry showing CYP11B2 expression in APA and aldosterone-producing cell cluster (APCC) in patient 5 (**A**, low magnification; **C**, high magnification). One structure expressing CYP11B2 extends deep into the adrenal cortex (**A**, low magnification; **B**, high magnification). A secondary nodule expressing CYP11B2 in patient 4 (**D**).

as mutation negative by Sanger sequencing on fresh frozen tissue. The overall prevalence of somatic mutations in this study is 93.75%, even higher than in previous studies performing targeted NGS using a different technology.[15,16] *CACNA1D* mutations were the main genetic abnormality identified by CYP11B2 immunohistochemistry-guided NGS, with an overall prevalence of 27%. The higher prevalence of somatic mutations may be explained by better coverage of driver genes and by a better identification of aldosterone-producing structures.

In agreement with previous studies,[9,14,36–38] including those performing immunohistochemistry-guided NGS,[15,16] *KCNJ5* mutations were associated with female sex and younger patients. In contrast, we did not confirm the previously observed association between the mutational status and adenoma size.[9,14,39–41] Although this may be due to a smaller sample size of the present study, it is likely that this difference stems from a larger heterogeneity of the different mutational groups, due to improved detection rates of *CACNA1D* and ATPase mutations using NGS. Hybrid steroids 18-hydroxycortisol and 18-oxocortisol were significantly different across mutational groups, with increased concentrations in *KCNJ5*-mutated patients, confirming other studies.[42–45] Consistently, we also observe a correlation between the cellular and molecular composition of APA and the production of 18-hydroxycortisol and 18-oxocortisol. Indeed, higher levels of 18-hydroxycortisol and 18-oxocortisol in APA were associated with less CYP11B2 expression, more CYP11B1 expression, and with higher percentage of clear cells—a hallmark of *KCNJ5*-mutated tumors. However, neither individual mutations nor mutation types could be predicted by the steroid profile using multivariate analyses with cross-validated random forests, except marginally for *KCNJ5* mutations. This finding contrasts with previous studies, in which a 7-steroid fingerprint was able to correctly classify 92% of APAs according to genotype.[42] The reason may again be related to the increased detection rate of somatic mutations, with enrichment of APA carrying *CACNA1D*, *ATP1A1*, and *ATP2B3* mutations and near disappearance of mutation-negative APA. As mutation-negative APA made up ≈50% of previous series, they contributed in a major way to differences between groups. We cannot exclude, however, that the differences may be, in part, explained by the smaller sample size of the present study.

In this study, APA carrying *KCNJ5* had less cells expressing CYP11B2 and more cells expressing CYP11B1 than the other mutational groups. However, there was no difference in aldosterone output between groups, suggesting that overall aldosterone output depends not only on the number of cells but

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also on the expression level and size of tumor. A recent study applying quantitative digital image analysis on whole APA reported indeed no difference in aldosterone synthase expression between APA genotypes.[46]

As reported previously,[21,47,48] GIRK4 expression in *KCNJ5*-mutated APA was decreased in comparison with the adjacent ZG. Furthermore, *KCNJ5* mutations were associated with a mostly CYP11B2-negative ZG hyperplasia. Although ZG hyperplasia was shown to be frequent in adrenals with APA, no correlations with *KCNJ5* mutations were observed in previous studies performing mutation detection without immunohistochemistry-guided NGS.[46,47] These data need to be confirmed in larger series of patients.

Analysis of markers of cell function in APA revealed some specific cellular characteristics. Cells expressing CYP11B2 coexpress GIRK4 and exhibit nuclear/cytoplasmic localization (activation) of β-catenin. On the contrary, cells expressing CYP11B1 (or CYP17A1) do not express CYP11B2 or GIRK4 and exhibit a membranous (nonactivated) localization of β-catenin. This profile is mainly associated with clear cells. These results suggest inhibition of the Wnt/β-catenin pathway in clear cells of APA, possibly via the cAMP/PKA (protein kinase A)-signaling pathway, which is involved in the differentiation of zona fasciculata cells.[49,50] We also observed a certain number of cells coexpressing 17α-hydroxylase and CYP11B2, explaining the observed secretion of hybrid steroids 18-hydroxycortisol and 18-oxocortisol, as reported previously.[51]

Although we observed important cellular and molecular heterogeneity in APA, these features are not associated with different genetic defects. Tumor regions with different CYP11B2 expression levels shared the identical *KCNJ5* mutation with similar variant allele frequencies. This is in line with recent work demonstrating a clonal origin for *KCNJ5*-positive adenoma.[52] CYP11B2-negative regions in APA carrying *KCNJ5* mutations may indicate a repression of *CYP11B2* activity in some tumor cells by unknown mechanisms. Alternatively, there might exist dynamic functional states of APA cells (similar to ZG cells in conditions of high- or low-salt diet), with transition between periods of high and low CYP11B2 expression. Such a mechanism has been described for pancreatic β-cells.[53] There might, however, be exceptions to this, as described in previous studies, where different mutations were detected in separate parts of APA expressing or not expressing CYP11B2.[28]

Somatic *CACNA1D*, *ATP1A1*, and *ATP2B3* mutations were described in APCC from normal adrenals and image-negative unilateral hyperplasia; a *KCNJ5* mutation was reported only once in an APCC from a patient with idiopathic hyperaldosteronism.[17,54,55] Here, we identified somatic mutations in APCC from the adrenal cortex adjacent to APA, including *KCNJ5* mutations in 5 APCCs, suggesting that *KCNJ5* mutations in APCC could be more prevalent than observed previously. The 3 different *KCNJ5* mutations identified were previously described in APA and FH-III.[4,41,56] In all cases, variants identified in APCC were different from the mutation found in the APA from the same adrenal. Similar results were observed for CYP11B2-positive nodules from adrenals with APA, which carried different mutations. Furthermore, 6 APCCs and 1 pAATL carried different *CACNA1D* mutations within the same micronodular adrenal. This supports a different clonal origin of each aldosterone-producing structure, as previously observed in multiple nodules in micronodular adrenals with APA.[21]

In summary, CYP11B2 immunohistochemistry-guided NGS allowed identifying somatic mutations in 93.75% of APAs, which are associated with different clinical and histological characteristics and hybrid steroid output. The prevalence of somatic mutations is much higher than previously established mutation rates. CYP11B2 immunohistochemistry-guided NGS should, therefore, be the recommended technique for detecting mutations in APA, particularly when establishing clinical and biological correlations to the mutation status. Finally, an important genetic heterogeneity was identified in different aldosterone-producing structures in the same adrenal, suggesting multiple events underlying APA development.

Strengths and Limitations of the Study

This study explored for the first time a large number of morphological and functional features of APA and the adjacent adrenal gland and correlated them to the mutational status determined by immunohistochemistry-guided NGS. A limitation of this study is the limited quantity and quality of DNA extracted from APCC, which led to detection of a large number of variants and the impossibility of confirming those variants using a different technique. New somatic mutations described in this study, although not functionally characterized, affect the same amino acids as known mutations, which, together with bioinformatics analyses, suggests a high probability of pathogenicity according to current guidelines for attributing pathogenicity to variants of unknown significance.

Perspectives

Until now, all studies investigating correlations between clinical, biological, and histological characteristics and the mutational status of APA were performed using targeted Sanger sequencing on DNA extracted from fresh frozen tissue samples. Our results provide new insight on how the mutational groups evolve when applying immunohistochemistry-guided NGS and how this might affect the previously observed correlations. They suggest that identification of surrogate biomarkers for the mutation status requires applying immunohistochemistry-guided NGS, in particular, for identifying *CACNA1D*, *ATP1A1*, and *ATP2B3* mutations. We also show that somatic mutations, including *KCNJ5* mutations, are present in APCC from adrenals with APA and that those mutations are different from the ones carried by the APA itself. The description of different mutational events in APCC and secondary nodules from adrenals with APA opens new perspectives for the understanding of the pathogenic mechanisms leading to APA development.

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Disclosures

None.

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Novelty and Significance

What Is New?

- Somatic mutations are present in 93.75% of aldosterone-producing adenoma (APA) and are associated with different clinical and histological characteristics and hybrid steroid output.
- Aldosterone-producing cell clusters adjacent to APA carry different somatic mutations, including known *KCNJ5* mutations.
- Heterogeneous CYP11B2 expression in *KCNJ5*-mutated adenoma is not associated with genetic heterogeneity.

What Is Relevant?

- CYP11B2 immunohistochemistry-guided next-generation sequencing increases the detection rate of somatic mutations and affects their clinical and biochemical correlates. It should be the recommended technique for mutation detection when searching for surrogate biomarkers to predict the mutation status.

Summary

CYP11B2 immunohistochemistry-guided next-generation sequencing allowed identifying somatic mutations in 93.75% of APAs. Those were associated with different clinical and histological characteristics and hybrid steroid output. APA showed important cellular and molecular heterogeneity, which was not associated with different somatic genetic events. Aldosterone-producing cell clusters adjacent to APA and other aldosterone producing structures carried different somatic mutations, including known *KCNJ5* mutations.

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