

Aldosterone-Producing Cell Clusters Frequently Harbor Somatic Mutations and Accumulate With Age in Normal Adrenals

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Context: Aldosterone synthase (CYP11B2) immunohistochemistry and next-generation sequencing (NGS) have revealed the frequent presence of aldosterone-producing cell clusters (APCCs) harboring somatic mutations in aldosterone-regulating genes in adrenals from Americans without defined hypertension status.

Objective: Determine the frequency and somatic mutation status of APCCs in a Japanese non-hypertensive cohort.

Design, Setting, Patients, and Interventions: Adrenals from 837 consecutive autopsies at a Japanese institution, Tohoku University Hospital, were screened to select 107 unilateral adrenal glands from nonhypertensive patients. APCC score (APCC number/adrenal cortex area per case) was assessed by CYP11B2 immunohistochemistry. DNA from all APCCs and adjacent adrenal cortex was subjected to NGS using two panels targeting aldosterone-regulating genes.

Primary Outcome Measure: APCC frequency and somatic mutation spectrum.

Results: In 107 adrenals, 61 APCCs were detected (average of 0.6 APCCs per gland). APCC score was positively correlated with age ($r = 0.50$, $P < 0.0001$). NGS demonstrated high confidence somatic mutations in 21 of 61 APCCs (34%). Notably, 16 of 21 APCCs (76%) harbored somatic mutations in *CACNA1D*, the most frequently mutated gene in our previous studies of APCCs in Americans and CYP11B2-positive micronodules in cross-sectional imaging (computed tomography) negative primary aldosteronism (PA), whereas no APCCs harbored mutations in *KCNJ5*, the most frequently mutated gene in aldosterone-producing adenoma. APCC score was significantly lower than our previous cohort of unilateral computed tomography-negative PA.

Conclusions: APCCs are frequent in nonhypertensive Japanese adrenals, accumulate with age, and frequently harbor somatic mutations (most commonly in *CACNA1D*). The role of APCCs in PA pathobiology and non-PA hypertension warrants further investigation.

Abbreviations: APA, aldosterone-producing adenoma; APCC, aldosterone-producing cell cluster; CT, computed tomography; FFPE, formalin-fixed paraffin-embedded; H&E, hematoxylin and eosin; IHC, immunohistochemistry; IQR, interquartile range; NGS, next-generation sequencing; PA, primary aldosteronism; VF, variant allele frequency; ZG, zona glomerulosa.

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Freeform/Key Words: aldosterone-producing cell clusters, adrenal glands, CYP11B2, next generation sequencing, somatic mutations

Primary aldosteronism (PA) accounts for ~4% to 10% of all hypertensive patients [1, 2] and is one of the few curable forms of hypertension. Increased aldosterone levels, in addition to hypertension, have been reported to significantly increase indicators of cardiovascular disease when compared with blood pressure–matched essential hypertensives [3, 4]. The two major causes of PA are unilateral aldosterone-producing adenomas (APAs) and bilateral hyperaldosteronism based on the laterality of adrenal aldosterone oversecretion. Exome-based next-generation sequencing (NGS) of APAs has identified recurrent somatic mutations in four genes that cause autonomous aldosterone production through increasing intracellular calcium: *KCNJ5*, *CACNA1D*, *ATP1A1*, and *ATP2B3* [5–8]. To date, >50 somatic mutations in these genes have been reported, some of which have been functionally characterized [9]. *KCNJ5* is the most frequently affected gene in APAs, accounting for ~70% and 40% of Japanese and European populations, respectively, with mutations in *CACNA1D*, *ATP1A1*, and *ATP2B3* accounting for <10% each [10, 11]. Despite the identification of somatic mutations underlying aldosterone deregulation in APA, little is known about the cellular origin and development of PA.

Immunohistochemistry (IHC) for aldosterone synthase (CYP11B2), the rate-limiting enzyme for aldosterone synthesis, has been useful to identify aldosterone-producing adrenocortical cells. CYP11B2-expressing cells are negative for 11 β -hydroxylase (CYP11B1) and 17 α -hydroxylase (CYP17A), enzymes needed for cortisol production [12, 13]. Recently, the combination of CYP11B2 IHC and NGS has enabled new insights in adrenal zonation and aldosterone production. Nishimoto *et al.* [14] previously reported small CYP11B2-expressing adrenocortical cell clusters, termed aldosterone-producing cell clusters (APCCs), beneath the adrenal capsule in patients under both normal and renin-suppressed conditions (supporting renin–angiotensin autonomous CYP11B2 expression). Subsequently, we collected 42 adrenal glands from American kidney transplant donors and performed targeted NGS on DNA isolated from APCCs to assess the somatic mutation status of the aldosterone-regulating genes somatically mutated in APAs (*KCNJ5*, *CACNA1D*, *ATP1A1*, and *ATP2B3*). Unexpectedly, 8 of 23 (35%) APCCs in this cohort harbored previously reported aldosterone-deregulating somatic mutations [15], supporting autonomous aldosterone production in APCCs through somatic mutations. Interestingly, mutations in *CACNA1D* (6 of 23 APCCs, 26%) and *ATP1A1* (2 of 23 APCCs, 9%) were identified in APCCs, in contrast to *KCNJ5* being the most frequent somatic mutated gene in APAs. To explore the potential of APCCs in other forms of PA, we examined 25 Japanese adrenal glands that were diagnosed with unilateral PA without any detectable masses by cross-sectional imaging [computed tomography (CT)] [16]. In this cohort, adrenal glands frequently harbored multiple CYP11B2-positive cell clusters (identical to APCCs), and NGS identified recurrent somatic mutations in *CACNA1D* in 17 of 26 (65%) clusters.

Taken together, our previous results support APCCs as potential precursors of APAs (particularly *CACNA1D* mutated) and contributors to CT-negative PA. Likewise, whether APCCs may contribute to hypertension in patients without clinically increased aldosterone is unclear. Limitations of our previous study identifying somatic mutations in APCCs included the unknown hypertensive status of patients and an exclusively US cohort. Likewise, baseline APCC number and somatic mutation spectrum in a nonhypertensive cohort would be useful for comparison with our previous CT-negative PA cohort as well as future hypertensive (PA or essential) cohorts. Hence, in this study, we investigated APCC frequency and somatic mutation spectrum in a well-defined nonhypertensive, non-American population by performing targeted NGS (using a stringent two-panel approach) on a large consecutive cohort of adrenal

glands from a single institution Japanese autopsy cohort and compared results to our previous American (with unknown hypertensive status) and PA cohorts.

1. Materials and Methods

A. Human Normal Adrenal Glands

We retrospectively reviewed consecutive Japanese autopsy cases with available adrenal paraffin blocks (collected between 2001 and 2010) stored at Tohoku University Hospital (Sendai, Miyagi, Japan). Unilateral adrenal glands from all the cases that lacked any of the following exclusion criteria were retrieved: (1) patient age ≥ 65 years, (2) hypertension or its complications, (3) use of at least one antihypertensive drug, (4) adrenal tumors (benign or malignant), or (5) use of inhaled and/or internal steroids for >3 weeks. Hypertension or its complications were defined as blood pressure $>140/90$ mm Hg, acute coronary syndrome, atrial fibrillation, severe left ventricular hypertrophy, stroke, aneurysm of cerebral artery, aneurysm of thoracic and abdominal aorta, and severe atherosclerosis of aorta or chronic kidney disease (presence of any excluded a patient). The research protocol was approved by the ethics committee at Tohoku University Graduate School of Medicine (Sendai, Miyagi, Japan) and the Institutional Review Board of the University of Michigan (Ann Arbor, MI).

B. Adrenal Sectioning and IHC

Adrenal specimens were fixed in 10% formalin and embedded in paraffin after the autopsy. Formalin-fixed paraffin-embedded (FFPE) blocks from one adrenal were selected for evaluation based on visual inspection of the block (largest amount of cortical tissue) and availability. Selected blocks were uniformly serially sectioned into 14 slides, with the first and last slides used for hematoxylin and eosin (H&E) staining. The second slide was for CYP11B1 IHC and the second to the last was for CYP17A IHC. The third and third to the last slides were used for CYP11B2 IHC, and others in-between were left unstained for tissue capture and DNA isolation. H&E and IHC slides were sectioned at $4 \mu\text{m}$, with unstained slides for DNA isolation cut at $10 \mu\text{m}$. IHC for CYP11B2, CYP11B1, and CYP17A was performed as previously described using mouse monoclonal antibody for CYP11B2 (RRID: AB_2650562), rat monoclonal antibody for CYP11B1 (RRID: AB_2650563), and mouse monoclonal antibody for CYP17A (RRID: AB_2650564) [12, 13, 17].

C. APCC Detection and Image Analysis

Using the above-described IHC staining approach, APCCs were defined as previously described [15], with increased stringency, when all of the following criteria were met: (1) CYP11B2 IHC-positive cell clusters, (2) cluster present on both CYP11B2 IHC slides, (3) cluster twofold or more deep than adjacent zona glomerulosa (ZG), and (4) IHC-negative for CYP17A or CYP11B1. All of the slides were scanned on an Aperio AT2 (Leica Biosystems). The area of each APCC was calculated by determining its average area on the two CYP11B2 IHC slides using HALO (Indica Laboratories). The total adrenocortical area for each case was calculated as the average area on the two H&E slides using HALO. Then, to normalize intersection differences, an APCC score was calculated per case by dividing the number of APCCs by the total adrenocortical area.

D. NGS of APCCs and Adjacent Normal

Using a scalpel under a dissecting microscope (AmScope), APCCs were macrodissected from the sandwiched unstained slides, as well as CYP11B2-negative adjacent adrenal cortex (as a control). Genomic DNA was isolated from APCCs and the control area using the Qiagen AllPrep DNA/RNA FFPE kit and assessed for somatic mutations in aldosterone-regulating

genes using an updated, optimized version of our previous approach [15]. First, we sequenced separate DNA aliquots (0.3 to 20 ng) from each APCC using two different multiplex PCR panels (APAv1 and APAv2), targeting essentially the same genes but with different amplicons, enabling us to distinguish true mutations (present in both panels) from sequencing artifacts (present in only one). The panels contained genes previously reported as recurrently somatically mutated in APA (full coding sequence of *KCNJ5*, *CACNA1D*, *ATP1A1*, and *ATP2B3* on both panels) and genes associated with adrenal diseases (*GNAS* [18] and *CTNNB1* [19, 20] hotspots and the full *CACNA1H* [21] and *ARMC5* [22] genes only on the APAv2 panel). APCC genomic DNA was sequenced using APAv1 and APAv2 and the Ion AmpliSeq library kit 2.0 with barcode incorporation. CYP11B2-negative regions from each sample (controls) were processed as above except that they were only sequenced on the APAv2 panel. Templates were prepared using an Ion PGM template OT2 200 kit or an Ion PI Hi-Q OT2 200 kit (Life Technologies) according to the manufacturer's instruction. NGS was performed using an Ion PGM sequencing 200 kit v2 with Ion 318 chip on the Ion Torrent PGM sequencer, or Ion PI Hi-Q sequencing 200 kit with Ion PI chip v3 on the Ion Torrent Proton sequencer (Life Technologies). A subset of libraries was sequenced on both the PGM and Proton generating identical mutations (data not shown).

Data analysis was essentially as described previously [15], but with increased stringency and incorporation of results from both panels. For each APCC, variants called by default PGM/Proton low-stringency somatic variant filtering were further filtered to identify high-confidence somatic mutations by removing synonymous or noncoding variants, and applying an extensive set of filtering criteria, including removal of variants with flow-corrected read depth <300 in both panels, flow-corrected variant allele containing read <30 in both panels, variant allele frequency (VF; flow-corrected variant allele-containing read/flow-corrected read depth) <0.05 in both panels, flow variant allele calling forward to reverse read ratio <0.2 or >5 in both panels, or indels within homopolymer runs ≥ 4 . Variants occurring exclusively in reads containing other variants (single-nucleotide variants or indels) not present in nonvariant calling reads, or those occurring in the last mapped base of a read, were excluded. Variants passing default PGM/Proton low-stringency somatic variant filtering in any CYP11B2-negative regions were considered germline mutations or panel-specific artifacts, and excluded from all samples. For the genes only on APAv2 (*GNAS/CTNNB1/CACNA1H/ARMC5*), the variants were filtered by using an APCC library on APAv2 and matched to normal tissue as described previously [15]. Lastly, all variants that passed these filtering criteria in APCCs were visually confirmed in and Integrative Genomics Viewer (Broad Institute, <https://www.broadinstitute.org/igv>).

E. Statistical Analysis

Gaussian variables were reported as mean \pm standard deviation and non-Gaussian variables were described as median [interquartile range (IQR)]. The D'Agostino–Pearson method was used for normality test in each variable. A Spearman rank test for correlation was used for comparisons between at least one non-Gaussian parameter. An unpaired *t* test or Mann–Whitney test was used to compare two groups for Gaussian and non-Gaussian variables, respectively. A *P* value of <0.05 was considered a significant difference. Prism (version 7, GraphPad Software) software was used for all statistical calculations.

2. Results

A. Case Characteristics

We reviewed 837 consecutive autopsy cases from a single Japanese institution and collected 107 unilateral adrenal glands (Fig. 1) from Japanese patients who had no evidence of hypertension. Of the 107 cases, 60 were men and 47 women. The age at autopsy was a median of 35 (IQR, 6 to 52; *n* = 107), and systolic and diastolic blood pressures were a median of 110

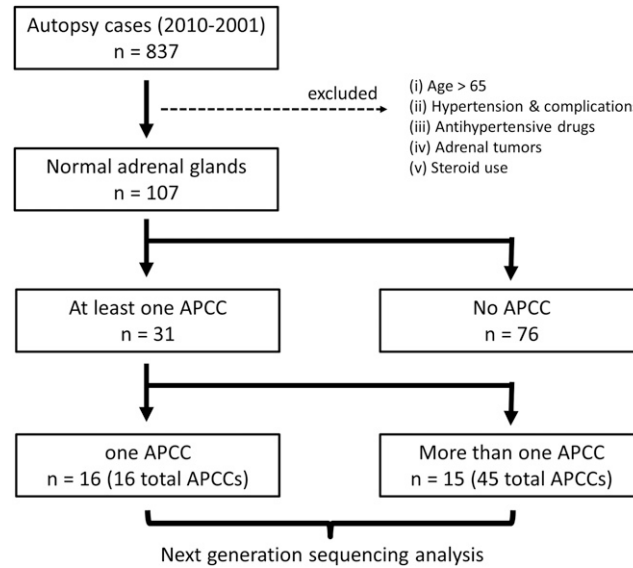


Figure 1. Adrenal gland collection of a nonhypertensive cohort. One hundred seven adrenal glands from patients without evidence of hypertension (exclusion criteria shown in figure) were collected from a consecutive single institution Japanese autopsy cohort. Sixty-one APCCs were detected in 31 cases, all of which underwent NGS analysis.

(IQR, 100 to 113; $n = 22$) and 63 (IQR, 59 to 78; $n = 21$), respectively. No significant difference between men and women was observed in age ($P = 0.29$) and systolic/diastolic blood pressure ($P = 0.70$ and $P = 0.75$, respectively). The causes of death were malignant neoplasms (27 of 107, 25%), congenital diseases (17 of 107, 16%), infectious diseases (11 of 107, 10%), other reasons such as gastrointestinal bleeding and perforation, pulmonary hypertension and diastolic cardiomyopathy (27 of 107, 25%), and unknown causes (25 of 107, 23%).

B. APCC Detection

In this large cohort of nonhypertensive adrenal glands, we detected 61 APCCs in 107 cases (0.6 APCC per adrenal on average) (Fig. 1; Supplemental Fig. 1). Thirty-two APCCs were detected in 16 males and 29 APCCs in 15 females. More than one APCC (multiple APCC) was found in 15 adrenals (eight males, seven females). The maximum number of APCCs was five in two cases (a 52-year-old man and a 53-year-old man). The youngest case with APCC was a 6-year-old boy. The median size per APCC was 0.15 mm^2 (IQR, 0.07 to 0.25), with no significant difference by sex ($P = 0.48$). There was no significant correlation between patient age and size of each APCC ($P = 0.12$). Although systolic and diastolic blood pressures were available for only a subset of subjects ($n = 22$ and 21 , respectively), there were no significant differences between those two groups in both systolic and diastolic blood pressure ($P = 0.09$ and $P = 0.41$, respectively).

C. APCC Score and Age Are Positively Correlated

Next, we determined an APCC score per case to normalize section area differences. The score ranged from 0 to 11.1 per cm^2 of adrenal cortex in men and from 0 to 9.8 in women. The APCC score was positively correlated with age ($r = 0.50$, $P < 0.0001$, $n = 107$) (Fig. 2). No significant difference in APCC score between male and female was observed ($P = 0.54$).

D. APCC Score Is Significantly Lower Than in Unilateral CT-Negative PA

Of interest, we compared the APCC score in this present cohort to the seven patients with unilateral CT-negative PA from our previously described cohort [16]. With APCC score

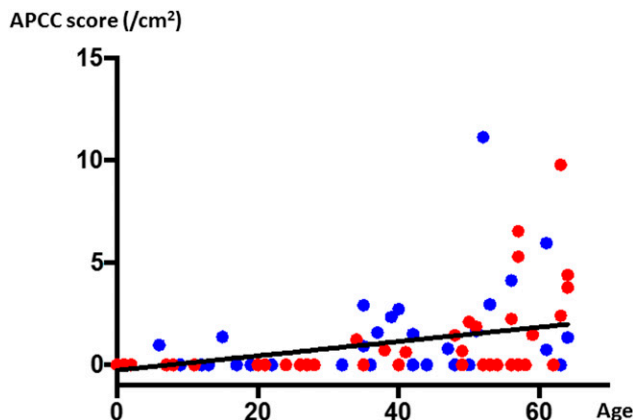


Figure 2. Age-associated increase in APCC score. APCC score (APCC number per adrenal/adrenal cortex area) for each of our 107 nonhypertensive cases were plotted vs age. A significant positive correlation was observed (Spearman rank correlation, $r = 0.50$, $P < 0.0001$). Men and women are shown in blue and red, respectively.

calculated exactly the same (using digital imaging), we observed a significantly lower APCC score in our present normotensive autopsy cohort compared with patients with unilateral CT-negative PA [median APCC score 0 (IQR, 0 to 0.8) vs 4.9 (IQR, 3.7 to 6.2), two-sided Mann-Whitney test, $P < 0.0001$].

E. Targeted NGS on APCCs Confirm Frequent Somatic CACNA1D Mutations

Targeted NGS was performed on all 61 APCCs and paired adjacent normal tissues to identify somatic mutations using a stringent two-panel approach (Fig. 3). We generated an average of 1,379,518 mapped reads yielding an average of 2223 covering reads over targeted bases, per sample. Average uniformity and on-target reads were 87.9% and 93.9%, consistent with high-quality results. Of 31 cases with at least one APCC, we identified a total of 30 high-confidence somatic mutations in 21 APCCs from 17 cases (10 males, 7 females) (Table 1; Supplemental Table 1; Supplemental Fig. 2). Of note, four adrenal glands (cases 26, 42, 56, and 59) were shown to harbor different somatic mutations in individual APCCs, supporting the different clonal development of multiple APCCs. There were significant differences in the size of APCCs between those with somatic mutations (both known and unreported) vs wild-type APCCs ($P = 0.047$ and $P = 0.047$, respectively).

Somatic mutations in *CACNA1D*, *ATP2B3*, and *ATP1A1* were observed in 16 of 61 (26%) [5 of 61 (8%) and 2 of 61 (3%) of the 61 total APCCs in the cohort, respectively; Fig. 4(a); Table 1]. Two APCCs (26A1 and 39A1) harbored both *CACNA1D* and *ATP2B3* somatic mutations. Five APCCs (17A2, 26A1, 36A1, 39A1, and 47A1) harbored more than one somatic mutation, all of which were previously unreported. The sequencing statistics of these five APCCs were similar to the remaining 56 APCCs (uniformity/coverage 84%/1510 in the former vs 81%/3230 in the latter), supporting that the identification of multiple somatic mutations is not due to sample degradation. No somatic mutation in *KCNJ5* was found in any APCC. Importantly, of 30 somatic variants, nine of 20 (45%) *CACNA1D*, none of eight (0%) *ATP2B3*, and one of two (50%) *ATP1A1* had been observed previously as somatic mutations in APAs [Fig. 4(b)]. We did not see age accumulation of somatic mutation status. Lastly, by comparing variants in APCC libraries on APav2 and matched normal tissue, no high-confidence somatic (or germline) mutations in *GNAS/CTNNB1/CACNA1H/ARMC5* were identified.

3. Discussion

The recent development of a monoclonal antibody for CYP11B2 [12] enabled several groups to identify aldosterone-producing cells in the outer adrenal ZG by IHC, even in the presence of

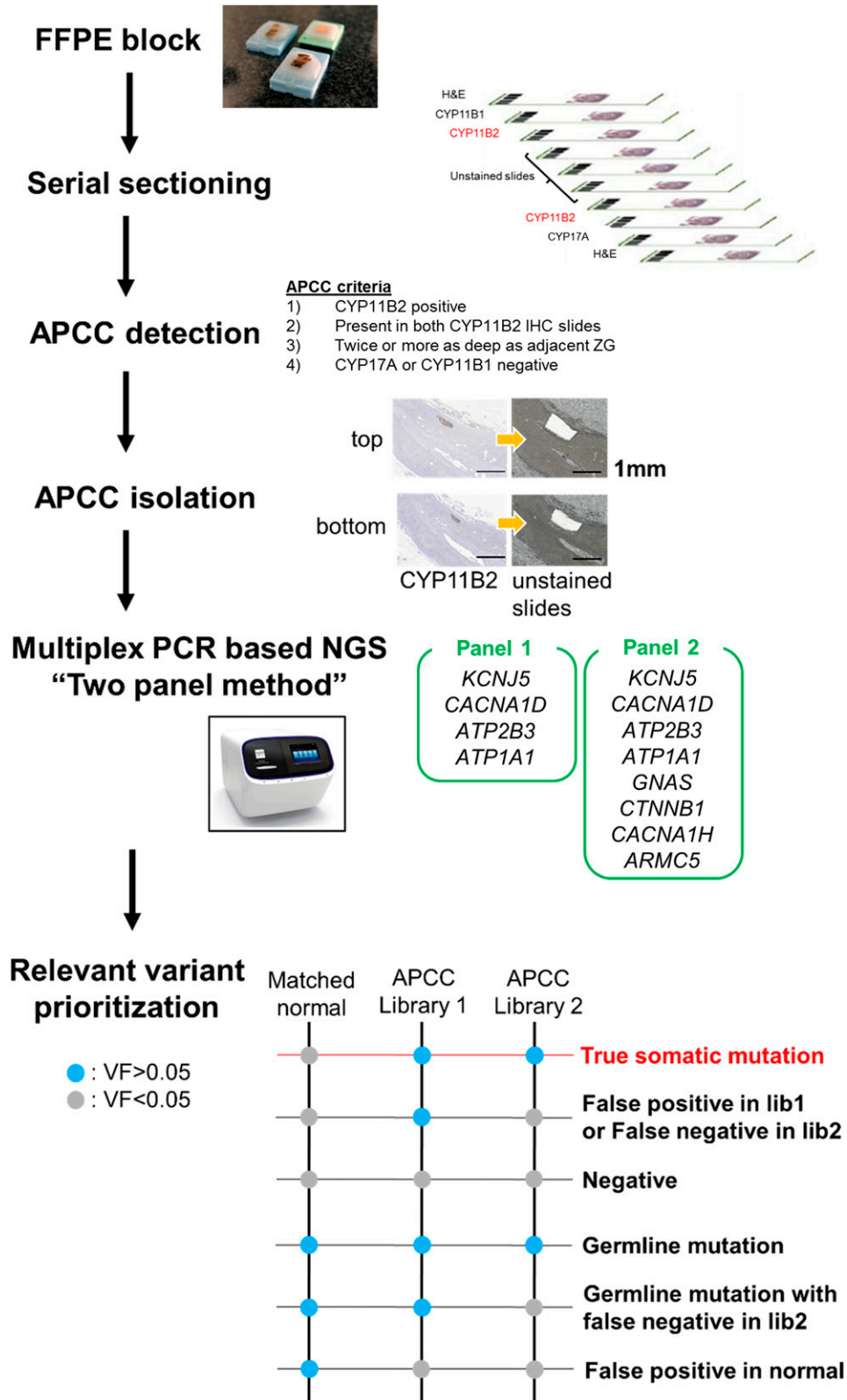


Figure 3. NGS approach to robustly detect somatic mutations in aldosterone-regulating genes from APCCs. FFPE blocks from our cohort were serially sectioned, and careful macrodissection of APCCs under a dissecting microscope was performed using H&E and CYP11B2 IHC to localize APCCs on unstained slides. After DNA extraction, separate

libraries were prepared from each APCC using two distinct NGS panels, which target the same genes but differ in amplicon composition. After stringent filtering, variants present in both APCC libraries but absent in normal adrenal cortex were considered true somatic mutations. Variants that were present only in one library were considered panel-specific errors. Variants that were present in any normal adrenal sample were considered nonpathogenetic changes, panel-specific errors, or germline variants. For the genes only on APAv2 (*GNAS/CTNNB1/CACNA1H/ARMC5*), the variants were filtered by using an APCC library on APAv2 and matched normal tissue.

repressed systemic renin–angiotensin activity [13–15]. Previously, using NGS, we found that APCCs in a United States cohort of renal transplant donors frequently harbored somatic gene mutations in aldosterone-regulating genes, analogous to those observed in APAs [15]. Of interest, in that cohort, *CACNA1D* was the most frequently mutated gene and no mutations were observed in *KCNJ5*, in contrast to APA cohorts, where *KCNJ5* somatic mutations were much more frequent than *CACNA1D* mutations [11, 23]. These results support APCCs as neoplastic alterations harboring somatic mutations known to drive autonomous aldosterone synthesis, suggesting a potential role for APCCs in PA and other hypertensive conditions.

In this study, we confirm the high frequency of somatic mutations in aldosterone-regulating genes in APCCs, as well as the unique somatic mutational spectrum in APCCs vs APAs (*CACNA1D* >>> *KCNJ5*). Importantly, our results suggest that although APCCs may serve as precursors for rare APAs (particularly those harboring *CACNA1D* mutations), they are unlikely to serve as the precursor to most APAs (those harboring *KCNJ5* mutations), unless *KCNJ5*-mutated APCCs progress so rapidly to clinically detectable APAs that they are rarely observed as APCCs. Of likely more relevance, we recently assessed (using similar methodology to our present study) a cohort of 25 adrenal glands from Japanese patients diagnosed with PA and no adrenal masses by CT [16]. In this CT-negative PA cohort, we identified aldosterone-expressing micronodules (analogous to APCCs) in most adrenal glands, as well as an increased APCC score (APCC number normalized to adrenal cortex area) compared with our present normotensive cohort (in the unilateral cases assessed exactly in this study). Of note, *CACNA1D* and *KCNJ5* somatic mutations were observed in 65% and 8%, respectively, of the aldosterone-expressing micronodules in the CT-negative PA cohort, in line with the spectrum seen in non-PA cohorts. Taken together, results from our present and previous studies imply that although APCCs, which frequently harbor *CACNA1D* somatic mutations, are unlikely precursors to most APAs, they may play a driving role in other forms of PA, particularly CT-negative disease.

Our present study was designed to address several limitations of our previous assessment of APCCs in both unselected and PA cohorts. For example, in our initial identification of somatic mutations in aldosterone-regulating genes in APCCs, the cohort lacked information on hypertension status and had American individuals only [15]. Likewise, in our study of CT-negative PA, no control, normotensive group was available for comparison of APCC number (or score) and somatic mutation status to evaluate the potential contribution of APCCs to clinical manifestations of PA [16]. Hence, in this study we sought to assess baseline APCC frequency and somatic mutation spectrum in a well-defined Japanese normotensive autopsy cohort.

In this study, we sought to carefully exclude all patients with any evidence of hypertension or its complications to remove this confounder for a baseline assessment of APCCs. We excluded patients ≥ 65 years of age, as they have been reported to have increased numbers of adrenal cortical nodules and adenomas, some of which are reportedly related to hypertension, compared with those < 65 years of age [24, 25], potentially resulting in an inappropriate inclusion of hypertensive cases and an incorrect calculation of cortex area and subsequent APCC score. As $\sim 90\%$ of PA patients are diagnosed by the age of 65 [26], we think that our current cohort is a useful “baseline” for normotensive controls. We also excluded cases with inhaled and/or internal steroid use for at least 3 weeks, as steroids can cause adrenal cortex atrophy (increasing APCC score through decreased cortical area) [27]. Importantly, although

Table 1. Somatic Mutations Identified in APCCs by Gene

Case	Sample	Age	Sex	Gene	Reference Allele	Variant Allele	Amino Acid Change	FDP	VF	FDP	VF
56	56A3	57	F	<i>CACNA1D</i>	G	C	G403R ^a	1992	0.15	2000	0.28
42	42A1	64	F	<i>CACNA1D</i>	C	T	S410L	1999	0.06	1992	0.06
39	39A1	64	M	<i>CACNA1D</i>	G	A	G457R	1549	0.08	1993	0.09
26	26A1	56	F	<i>CACNA1D</i>	C	T	R510X	699	0.12	1519	0.07
47	47A1	59	F	<i>CACNA1D</i>	C	T	P548L	1474	0.06	1621	0.05
52	52A2	53	M	<i>CACNA1D</i>	T	G	F747V ^b	964	0.07	1999	0.07
59	59A1	39	M	<i>CACNA1D</i>	T	G	F747V ^b	1996	0.07	1695	0.07
59	59A4	39	M	<i>CACNA1D</i>	T	G	F747C	2000	0.18	1986	0.15
76	76A1	35	M	<i>CACNA1D</i>	C	A	F747L ^a	1999	0.12	1986	0.13
51	51A1	50	F	<i>CACNA1D</i>	G	A	R990H ^a	352	0.14	217	0.18
87	87A1	42	M	<i>CACNA1D</i>	G	A	R990H ^a	1922	0.11	1997	0.11
42	42A3	64	F	<i>CACNA1D</i>	C	T	A998V ^b	321	0.17	435	0.28
26	26A1	56	F	<i>CACNA1D</i>	T	G	F1147C	1429	0.26	1181	0.32
94	94A1	38	F	<i>CACNA1D</i>	T	A	F1147L	1995	0.17	460	0.15
39	39A1	64	M	<i>CACNA1D</i>	C	A	F1248L	1486	0.22	2000	0.08
35	35A1	61	M	<i>CACNA1D</i>	C	G	P1336R ^a	464	0.14	2000	0.06
56	56A2	57	F	<i>CACNA1D</i>	G	A	V1338M ^b	1997	0.11	1906	0.1
31	31A1	37	M	<i>CACNA1D</i>	T	C	I1352T	2000	0.1	2000	0.1
47	47A1	59	F	<i>CACNA1D</i>	C	T	P1499L	445	0.08	1593	0.06
47	47A1	59	F	<i>CACNA1D</i>	G	A	W1836X	192	0.08	1481	0.1
26	26A1	56	F	<i>ATP2B3</i>	G	A	D77N	1998	0.05	1999	0.06
26	26A1	56	F	<i>ATP2B3</i>	G	A	E135K	1988	0.07	1993	0.07
26	26A3	56	F	<i>ATP2B3</i>	G	A	G270D	2000	0.26	179	0.99
17	17A2	57	F	<i>ATP2B3</i>	G	A	G325D	269	0.22	853	0.08
36	36A1	35	M	<i>ATP2B3</i>	C	T	A790V	348	0.1	595	0.06
36	36A1	35	M	<i>ATP2B3</i>	C	T	S1137F	544	0.05	518	0.08
17	17A2	57	F	<i>ATP2A3</i>	C	T	P1150L	1078	0.08	818	0.11
39	39A1	64	M	<i>ATP2B3</i>	C	T	A1157V	1999	0.06	1981	0.06
18	18A1	56	M	<i>ATP1A1</i>	T	G	L104R ^a	1999	0.13	611	0.12
16	16A2	61	M	<i>ATP1A1</i>	G	A	E687K	1998	0.14	235	0.07

All high-confidence somatic nonsynonymous variants that passed stringent filtering in APCCs are shown and sorted by gene and location. Variants with detectable read support in any normal tissue were excluded. Reference transcript sequences used for determining amino acid changes were NM_001128839.2 (*CACNA1D*), NM_021949.3 (*ATP2B3*), and NM_000701.7 (*ATP1A1*).

Abbreviation: FDP, flow-corrected read depth.

^aMutations that have been previously reported in sporadic APAs. See Supplemental Table 1 for somatic mutations sorted by case.

^bMutations that have been previously reported in sporadic APAs and functionally characterized.

some cases may have had undiagnosed or undescribed hypertensive conditions due to exclusion of that information from autopsy reports, we excluded >87% (730 of 837) of cases due to hypertension or hypertensive complications in an effort to generate a true normotensive cohort. Importantly, as it is difficult to obtain a large cohort of normal adrenal glands with full medical records and hormone data, our cohort may serve as a useful reference for other studies.

Our assessment and subsequent NGS of APCCs was dependent on their accurate detection by CYP11B2 IHC. We and others have previously demonstrated that APCCs consist of both ZG and zona fasciculata cells [15, 28]. In our cohort, however, lipid depletion [29] (Supplemental Fig. 1), which is frequently seen in autopsy specimens, precluded the distinction of zona fasciculata and ZG cells in APCCs. Therefore, we defined APCCs as CYP11B2-positive cell clusters where the depth was twofold or greater than the adjacent ZG cells. Nevertheless, this limitation is unlikely to impact the major findings from our study, as the high rate of somatic mutations in APCCs as defined herein (and in line with our previous studies) strongly supports their neoplastic nature.

If APCCs contribute to PA development, we postulated that they may increase during aging until diagnosis, which could not be assessed in our previous cohorts. Accurate

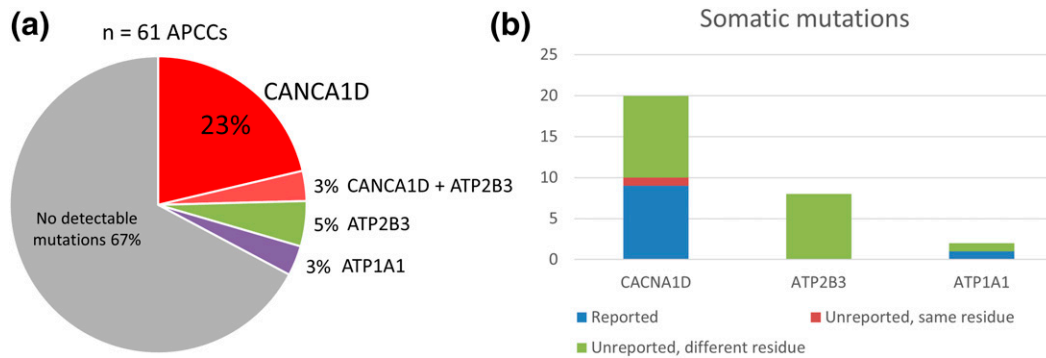


Figure 4. Somatic mutations identified in APCCs by NGS. (a) Somatic mutations in *CACNA1D*, *ATP2B3*, *ATP1A1*, and *KCNJ5* were observed in 16 of 61 (26%), 5 of 61 (8%), 2 of 61 (3%), and none of 61 (0%) of the 61 total APCCs in our Japanese normotensive cohort, respectively. Two APCCs (26A1 and 39A1) harbored both *CACNA1D* and *ATP2B3* somatic mutations. (b) Of 30 somatic variants, 9 of 20 (45%), none of eight (0%), and one of two (50%) in *CACNA1D*, *ATP2B3*, *ATP1A1*, respectively, were previously reported in aldosterone-producing adenomas.

determination of APCC number requires normalization based on the amount of adrenal cortex examined. Hence, in this study we determined an APCC score for each case, where the number of APCCs was normalized to adrenal cortex area using image analysis. Importantly, in our present cohort, we found a statistically significant age accumulation in APCC score (Fig. 2), which suggests that APCC presence or development may be an age-dependent phenomenon in a nonhypertensive cohort. Given that there was no significant correlation between the patient age and size of each APCC and that the detection of APCC was done with the same method and slide thickness regardless of age, we do not think that APCCs in a particular age group had been missed more than the others. Likewise, our APCC score approach can be used to enable comparison of APCCs across cohorts, as described above for our CT-negative unilateral PA cohort.

We previously reported frequent somatic mutation of aldosterone-regulating genes—most frequently *CACNA1D*—in our United States cohort of renal transplant donors without defined hypertension status [15]. In this study, to investigate baseline somatic mutation frequency and spectrum in this normotensive Japanese cohort, we optimized our targeted NGS approach through improved macrodissection and a two-panel approach (Fig. 4). Use of a dissecting microscope is more precise than traditional macrodissection and easier, faster, and less expensive than laser capture microdissection. To increase the reliability of NGS from these minute FFPE specimens, we developed a two-panel strategy, where separate aliquots of DNA isolated from each APCC were sequenced using two panels targeting the same genes, but with different amplicons, to remove panel-specific and technical artifacts (Fig. 4; Supplemental Fig. 2). This updated approach is more specific than our previous single-panel approach [15], as well as considerably more sensitive and requires less DNA for mutation detection than standard Sanger sequencing.

Using our extremely stringent NGS approach, we observed high-confidence somatic mutations in 21 of 61 (34%) APCCs. Similar to our previous study in a United States cohort, where we detected *CACNA1D* mutations in 35% of APCCs, *CACNA1D* was again the most frequently mutated gene in this normotensive cohort [16 of 61 APCCs harboring somatic *CACNA1D* mutations; Fig. 4(a); Table 1]. Also, similar to our previous study, we did not find any mutations in *KCNJ5* in APCCs in this Japanese cohort, where *KCNJ5* mutations in APA are far more frequent than in European subjects [10, 11]. Importantly, we have also confirmed that our approach was able to detect known somatic mutations in *KCNJ5* down to 7% VF by serial dilution of DNA from APAs harboring *KCNJ5* G151R or L168R somatic mutation (data not shown). Of note, one third of the somatic mutations in our present cohort (especially those in *CACNA1D*) were at residues previously reported as somatically mutated in APAs, supporting both our approach and the role of APCCs in autonomous aldosterone production [Fig. 4(b)].

We observed significant differences in the size of APCCs comparing APCCs with somatic mutations (both known and unreported) vs wildtype APCCs. We attribute this difference largely to the technical challenges of dissecting APCCs, with larger APCCs being easier to isolate with relatively high purity, increasing the ability to detect a somatic mutation above our variant allele frequency cutoff. This limitation also contributes to the relatively low variant allele frequency of observed somatic mutations, with the observed VF of prioritized somatic variants (average of 13%; Table 1) supporting the clonal nature of these alterations in APCCs given the limitations of CYP11B2 IHC-guided macrodissection of APCCs and consistent with our previous results using laser capture microdissection isolated APCCs [15]. Importantly, however, we cannot entirely exclude the possibility that some mutations identified in this study represent technical artifacts (false positives), and the challenges of dissecting minute lesions may contribute to some APCCs lacking somatic mutations (false negative due to insufficient tumor content).

Likewise, somatic mutations detected in larger APCCs may represent a mixture of those promoting growth advantage and passenger mutations, as well as subclonal mutations. For example, two nonsense somatic *CACNA1D* mutations and the nonclustered hotspot *ATP2B3* mutations identified herein are unlikely to be functionally active based on present knowledge of aldosterone-activating mutations and may well represent passenger alterations or technical artifacts. Alternatively, the present study identified several novel mutations that are likely to be functionally relevant, particularly the two *CACNA1D* mutations at the F1147 residue (F1147C and F1147L), which occurred in different APCCs at relatively high variant allele frequencies. Importantly, these variants occur in the S6 segment that lines the channel pore, analogous to other highly recurrent and functional *CACNA1D* mutations (e.g., *CACNA1D* F747L) [7, 8]. Likewise, the *CACNA1D* I1352T mutation occurs in the same voltage-sensing domain as the functionally confirmed *CACNA1D* P1336R mutation [8]. Importantly, however, functional studies will be needed to determine the impact of these novel mutations.

In summary, our results in a large cohort of normal adrenals from a Japanese population support an age-dependent accumulation of APCCs. Using an improved, highly robust protocol of macrodissection and two panel-targeted NGS, we identified aldosterone-activating somatic gene mutations in approximately a third of APCCs. Although *KCNJ5* is the most common somatically mutated gene in APAs, especially in Japanese, our results in this study (and in a previous United States population with undefined hypertension status as well as a CT-negative PA cohort) demonstrate that APCCs do not harbor recurrent somatic *KCNJ5* mutations, but instead frequently harbor somatic mutations in *CACNA1D*. Of particular interest, we observed a significantly lower APCC score in this normotensive cohort compared with our previous CT-negative PA cohort (where we hypothesized that APCCs are driving the observed hyperaldosteronism and hypertension). Hence, our results herein suggest the potential that APCC accumulation may also contribute to non-PA hypertension (with subclinical hyperaldosteronism) and provide the rationale for a prospective evaluation of whether APCC score (or frequency) differs substantially between non-PA hypertensive and nonhypertensive subjects.

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