Abstract—Primary aldosteronism is the most common form of secondary hypertension. Somatic mutations in KCNJ5, ATP1A1, ATP2B3, and CACNA1D are found in aldosterone-producing adenoma. In addition, adrenals with aldosterone-producing adrenocortical nodules show corticial remodeling and frequently multiple secondary nodules. Our aim was to investigate whether different aldosterone-producing nodules from the same adrenal share the same mutational status. Aldosterone synthase expression was assessed in multinodular adrenals from 27 patients. DNA of 37 aldosterone-producing secondary nodules was extracted from formalin-fixed paraffin-embedded tissues and genotyped for KCNJ5, ATP1A1, ATP2B3, and CACNA1D mutations. Among 17 adrenals with a somatic mutation in the principal nodule, 4 showed the same mutation in a secondary nodule, whereas 10 had no mutation in any of the known genes. In 1 adrenal harboring the KCNJ5 p.Gly151Arg mutation in the principal nodule, the same mutation was present in 2 secondary nodules, but no mutation was found in a third nodule. Finally, in 2 adrenals with a CACNA1D mutation in the principal nodule, a KCNJ5 mutation was identified in the secondary nodule. Among 10 adrenals without mutations in the principal nodule, 1 carried a KCNJ5 mutation in the secondary nodule. No mutations were detected in 7 aldosterone-producing cell clusters from 6 adrenals. No association was observed between the presence of mutations in secondary nodules and clinical parameters. In conclusion, different mutations are found in different aldosterone-producing nodules from the same adrenal, suggesting that somatic mutations are independent events triggered by mechanisms that remain to be identified. (Hypertension. 2015;66:1014-1022. DOI: 10.1161/HYPERTENSIONAHA.115.05993.) • Online Data Supplement

Key Words: adrenal cortex ■ aldosterone ■ hyperaldosteronism ■ mineralocorticoids ■ mutation ■ potassium channels
of similar mutations leading to familial hyperaldosteronism type 3 associated with massive bilateral hyperplasia, has indicated that this genetic abnormality was sufficient to drive both increased cell proliferation and autonomous aldosterone production. Recent work, however, has shown that cells transfected with mutant GIRK4 presented lower proliferation than cells transfected with wild-type GIRK4, or even apoptosis, which was because of the massive increase in intracellular Na⁺ concentration. This has led to the hypothesis that mutant K⁺ channels may cause sufficient Na⁺ permeability for tumor development but not as great as to cause cell death. We have shown that zona glomerulosa (ZG) hyperplasia, increased nodulation, and decreased vascularization are major features of adrenal cortex adjacent to APA, suggesting that KCNJ5 mutations could indeed occur within a proliferating cortex, leading to growth advantage, clonal expansion, and tumor formation. Alternatively, they may represent isolated events leading to APA formation, with adrenal cortex hyperplasia being secondary to reduced vascularization and tissue hypoxia. Indeed, no germline mutations are present in subjects carrying one of the different somatic mutations in APA, and mutations are absent in somatic DNA from peritumoral cortices from adrenals carrying somatic KCNJ5 mutations in the corresponding APA. Recently, Deckers et al have investigated the genotypes of multinodular adrenals and identified 2 aldosterone synthase positive nodules harboring 2 different recurrent KCNJ5 mutations in the same adrenal, supporting the hypothesis of independent events leading to nodulation and aldosterone overproduction.

The aim of this study is to get further insight into the sequence of events leading to nodulation and aldosterone overproduction in adrenals with APA. To this purpose, we have extensively characterized aldosterone production in secondary nodules from multinodular adrenals and investigated the presence of somatic KCNJ5, ATP1A1, ATP2B3, and CACNA1D mutations in these nodules. We have also investigated whether recurrent somatic mutations are present in aldosterone producing cell clusters (APCC), specific structures that have been suggested as the starting point for the development of APA.

Subjects and Methods
An expanded Methods section is available in the online-only Data Supplement.

Patients
Patients with PA from the COMETE-HEGP (Cortico-et Medullo-Surrénale, les Tumeurs Endocrines–Hôpital Européen Georges Pompidou) cohort were recruited between 2002 and 2012 within the COMETE network. Methods for screening and subtype identification of PA were performed according to institutional and Endocrine Society guidelines. In patients diagnosed with PA, a thin slice computed tomographic scan or magnetic resonance imaging of the adrenal and an adrenal venous sampling was performed to differentiate between unilateral and bilateral aldosterone hypersecretion. All patients gave written informed consent for genetic and clinical investigation. Procedures were in accordance with institutional guidelines. Further details are available in the online-only Data Supplement.

Pathological Analysis
Details are available in the online-only Data Supplement.

DNA Isolation and Sanger Sequencing
Details are available in the online-only Data Supplement.

Droplet Digital Polymerase Chain Reaction
Droplet digital polymerase chain reaction (ddPCR) was performed on a QX-100 system (Bio-Rad) using Bio-Rad assays comprising primers and probes designed for the detection of the KCNJ5 mutations c.451G>A and c.451G>C, leading to the amino acid substitution p.Gly151Arg. Further details are available in the online-only Data Supplement.

Statistical Analyses
Quantitative variables are reported as means±SD (Gaussian distribution) or medians and interquartile range (non-normal distribution), and compared with unpaired t test or Mann–Whitney test, respectively. Categorical variables are reported as percentages and compared with Fisher exact test. A P value <0.05 was considered significant for comparisons between 2 groups.

Results
Identification of Functional Secondary Nodules in Adrenals With APA
Based on the histopathologic description of adrenals resected for lateralized PA performed in the pathological department of the Hospital Européen Georges Pompidou, adrenals from 27 patients were selected on whom we performed haematoxylin–eosin–safran (HES) staining to identify secondary nodules in peripheral adrenal tissue (Figure 1A). All adrenals investigated in this study had functional APA as demonstrated by immunohistochemistry (IHC) of aldosterone synthase or in situ hybridization (ISH) of CYP11B2. Clinical and biological characteristics of this group of patients were comparable with the other PA patients with APA of the Paris cohort, with no difference in the age at diagnosis of PA (44 years [40–58] versus 40 years [34–47]; P=0.07), sex (35% of females versus 54% of females; P=0.06), clinical and biochemical signs of PA, APA diameter, number of preoperative medications, and follow-up characteristics (Table 1). No differences were observed when comparing patients with multinodular adrenals to those with a single adenoma (Table S2 in the online-only Data Supplement). Although median values of the lateralization index are lower and postoperative systolic blood pressure and number of medications are higher in patients with multinodular adrenals, this difference was not statistically significant. This might be indeed because of a lack of power of this study. After the identification of secondary nodules, CYP11B2 in situ hybridization or aldosterone synthase immunohistochemistry was performed to characterize the functionality of the secondary nodules (Figure 1B). Thirty-seven functional secondary nodules were identified in 27 adrenals (Table 1). In 18 adrenals, we identified 1 secondary nodule, in 8 adrenals 2 secondary nodules, and in 1 adrenal 3 secondary nodules. In addition, in 5 adrenals, we identified 1 APCC and in 1 adrenal 2 APCC. APCC are specific subcapsular cell clusters expressing high levels of CYP11B2 and are composed of morphological ZG cells in contact with the capsule and inner columnar zona fasciculate–like cells. Expression
of CYP11B2 throughout the entire APCC suggested that these structures possess an intermediate phenotype between cells from ZG and from zona fasciculata.22

Presence of Somatic Mutations in Secondary Functional Nodules
We genotyped hot spot regions of the KCNJ5, CACNA1D, ATP1A1, and ATP2B3 genes in 37 functional secondary nodules obtained from the 27 adrenals with an APA with known mutation status.13 Eight APA presented a KCNJ5 mutation (p.Gly151Arg), 7 presented a CACNA1D mutation (p.Phe747Leu, p.Val259Asp, p.Pro1336Arg, p.Ala998Val, p.Ile750Met, p.Gly403Arg and p.Val1151Phe), 2 had an ATP1A1 mutation (p.Val332Gly and p.Leu104Arg), and in 10 APA no mutation in any of the 4 known genes was identified (Table 2).

Genotyping results of secondary nodules are described in Table 2. Among 17 adrenals with a somatic mutation identified in the APA, 4 showed the same mutation in a secondary nodule (Figure 2A; Figure S1A–S1D). All these APA were carrying the KCNJ5 mutation p.Gly151Arg (adrenals 1, 2, 9, and 17). In 10 adrenals with a somatic mutation in the APA (adrenals 5, 6, 13, 14, 16, 18, 20, 21, 26, and 27), among which 5 carrying CACNA1D mutations, 3 with KCNJ5 mutations, and 2 with ATP1A1 mutations, no mutation in any of the known genes was detected in 13 functional secondary nodules analyzed (Figure 2B; Table 2). In 1 adrenal harboring the KCNJ5 p.Gly151Arg mutation in the APA (adrenal 3), we identified the same mutation in 2 functional secondary nodules, but no mutation in a third functional secondary nodule (Figure S1E). In 2 adrenals harboring a CACNA1D mutation in the APA (p.Ala998Val and p.Gly403Arg in adrenals 15 and 24 respectively), a KCNJ5 p.Gly151Arg was identified in the secondary nodules (Figure 2C and 2D; Figure S1F and S1G). We also analyzed functional secondary nodules from 10 adrenals without mutations in the APA. In 1 adrenal, we identified a KCNJ5 mutation (c.451G>A; p.Gly151Arg) in a secondary nodule (adrenal 4, Figure 2E; Figure S1H); no mutations were identified in the remaining 9 adrenals (Table 2). We did not observe differences in the cellular composition between APA and secondary nodules positive for KCNJ5 mutations (Table S3). Finally, no mutation was detected in 7 APCC regions from 6 adrenals (data not shown).

To verify the absence of small proportions of KCNJ5 mutations in secondary nodules negative for KCNJ5 mutations that could have been missed by Sanger sequencing, we performed ddPCR analysis of the KCNJ5 mutations c.451G>A and c.451G>C, leading to the amino acid substitution p.Gly151Arg in secondary nodules negative for

![Figure 1. Immunohistochemical features of multinodular adrenal glands.](http://hyper.ahajournals.org/)

A, Haematoxylin–eosin–safran (HES) staining and aldosterone synthase immunohistochemistry (IHC) of an adrenal gland resected for lateralized primary aldosteronism showing an aldosterone-producing adenoma (APA) and secondary micronodules. Left, HES staining with the identification of secondary nodules in an adrenal carrying an APA. Right, Aldosterone synthase IHC positive in 1 secondary adrenal nodule. B, HES staining (top) and CYP11B2 in situ hybridization (bottom) in an adrenal carrying 1 APA and 1 aldosterone-producing cell cluster region.
KCNJ5 mutations. In adrenals 26 and 21, with KCNJ5 mutation c.451G>C identified in APA, no KCNJ5 mutations were identified in secondary nodules by ddPCR (Figure 2B; Figure S2A). ddPCR analysis of adrenals 7 and 8, without KCNJ5 mutations identified in APA, did not reveal KCNJ5 mutations in the secondary nodules (Figure S2B and S2C), confirming the results of Sanger sequencing.

We also performed ddPCR analysis of the KCNJ5 mutation p.Gly151Arg in the 9 secondary nodules positive for a KCNJ5 mutation to confirm the genotype and determine the proportion of mutational events (Table S3). We identified 38% (adrenal 1), 12% (adrenal 2), 33% and 13% (adrenal 3, nodules A and B), 15% (adrenal 9), and 13% (adrenal 17) of the mutant allele in the secondary nodules (Figure 2A; Figure S2D and S2E; Table S3). In the corresponding principal nodules, 39% (adrenal 1), 40% (adrenal 2), 36% (adrenal 3), 38% (adrenal 9), and 33% (adrenal 17) of the mutant allele were detected (Figure S2F and S2G; Table S3). In adrenals 15 and 24, carrying a CACNA1D mutation in APA and the KCNJ5 mutation c.451G>A in the secondary nodules, the mutant A allele was present in a proportion of 18% (adrenal 15) and 15% (adrenal 24) in the secondary nodule (Figure 2C and 2D) and the absence of mutated KCNJ5 alleles was confirmed in the APA (Table S3). In adrenal 4, no mutated KCNJ5 alleles were identified in the APA (Table S3), and 29% of the mutated allele A was identified in the secondary nodule (Figure 2E). In addition, in the principal nodule of adrenal 13, positive for an ATP1A1 mutation by Sanger sequencing, no mutated KCNJ5 alleles were detected in the ddPCR analysis.

Clinical Correlates of the Presence of Mutations in Secondary Nodules

We compared clinical and biochemical findings between patients with and without identified mutations in secondary nodules (Table S4). Remarkably, all patients with a mutation in the secondary nodule harbored the KCNJ5 p.Gly151Arg mutation. There was no differences between the 2 groups in the age at PA diagnosis (44 years [39–53] versus 48 years [42–52]; P=0.3) and sex distribution (37.5% of females versus 31.5% of females; P=0.5). There was no association between the presence of mutations in the secondary nodules and preoperative plasma aldosterone or renin levels, the aldosterone/renin ratio, or the number of medications taken before surgery. There was also no association with postoperative blood pressure outcome as measured by blood pressure and treatment score at follow-up, cure or improvement of hypertension.

Discussion

Although the functional link between somatic mutations and aldosterone production is clearly established, it is still not clear whether and how mutations lead to increased proliferation and nodulation. Here, we report the results of the genotyping of somatic mutations in KCNJ5, ATP1A1, ATP2B3, and CACNA1D in 37 aldosterone-producing secondary nodules from 27 adrenals carrying an APA with known mutation status. In 13 adrenals, we identified the same mutation status between the APA and the secondary nodule. In 8 adrenals, we identified the somatic mutation


KCNJ5 p.Gly151Arg in at least 1 secondary nodule. In 14 adrenals, the mutation status was different between the APA and the secondary nodule. In 10 adrenals with an APA harboring a known mutation, no mutations were identified in the secondary nodule. Remarkably, in 1 adrenal without mutation in APA, we identified a KCNJ5 mutation in the secondary nodule, whereas in 2 adrenals harboring a CACNA1D mutation in the APA, a KCNJ5 mutation was identified in the secondary nodule.

We have observed in many adrenals carrying an APA the presence of macronodulations or micronodulations of the peripheral adjacent cortex. We have previously shown that adrenal cortex remodeling, decreased vascularization, and ZG hyperplasia are major features of adrenals.

\[
\begin{array}{|c|c|c|c|c|c|}
\hline
\text{Adrenal} & \text{Genotype APA} & \text{Secondary} & \text{Size of Secondary} & \text{Genotype Secondary} & \text{APPC, n} \\
\text{Nodule} & \text{Nodule} & \text{Nodule (Macro/Micro)} & \text{Nodule} & \\
\hline
 & & B & Micro & KCNJ5 p.Gly151Arg & \\
 & & C & Micro & Neg & \\
5 & CACNA1D p.Phe747Leu & A & Micro & Neg & \\
6 & CACNA1D p.Val259Asp & A & Micro & Neg & \\
7 & Neg & A & Micro & Neg & \\
8 & Neg & A & Micro & Neg & \\
10 & Neg & A & Micro & Neg & 1 \\
11 & Neg & A & Micro & Neg & \\
12 & Neg & A & Micro & Neg & \\
13 & ATP1A1 p.Val332Gly & A & Macro (6 mm) & Neg & \\
14 & CACNA1D & A & Micro & Neg & \\
16 & ATP1A1 p.Leu104Arg & A & Micro & Neg & 1 \\
19 & Neg & A & Micro & Neg & \\
20 & CACNA1D p.Ile750Met & A & Micro & Neg & \\
22 & Neg & A & Micro & Neg & \\
23 & Neg & A & Micro & Neg & \\
24 & CACNA1D p.Gly403Arg & A & Micro & Neg & \\
25 & Neg & A & Micro & Neg & \\
26 & KCNJ5 p.Gly151Arg & A & Macro (5 mm) & Neg & \\
27 & CACNA1D & A & Micro & Neg & \\
\end{array}
\]

APA indicates aldosterone-producing adenoma; and APCC, aldosterone-producing cell cluster.

*Secondary nodules were defined as macroscopic nodules if larger than 5 mm of diameter and microscopic nodules if smaller than 5 mm located in a hyperplastic adrenal cortex.
Although a certain degree of nodularity can be observed in normal adrenals associated with older age and severity of hypertension, we observed an increase of nodularity of the cortex adjacent to an APA when compared with control adrenals. This raises the possibility that remodeling of the adrenal cortex precedes the development of an APA, and thus, that somatic mutations could be a secondary event in APA development. Alternatively, they may represent isolated events leading to APA formation, with increased nodulation being secondary to reduced vascularization and tissue hypoxia or to the secretion of factors promoting nodularity by the APA itself. However, genetic...
background could also play a role in the nodulation of adrenals with APA. A recent study performed on 56 patients with PA showed the presence of germline heterozygous ARMC5 variants in subjects of African origin.\textsuperscript{25} ARMC5 mutations were described in primary macronodular adrenal hyperplasia with Cushing’s syndrome, with patients showing a germline mutation on one allele and a second somatic abnormality on the other allele.\textsuperscript{26} ARMC5 codes for armadillo repeat containing 5, which is likely to be a tumor suppressor gene. Tumors carrying ARMC5 mutations are supposed to be polyclonal, as different nodules carry different type of mutations. In vitro studies of the mutations observed in patients with PA did not identify a link between these mutations and increased aldosterone production,\textsuperscript{27} suggesting that they could represent a genetic predisposition for nodule formation preceding the occurrence of somatic mutations responsible for inappropriate aldosterone production.

A recent study has assessed the genotypic characteristics of nodules from adrenals with a solitary nodule or multinodular adrenals of 53 patients with PA.\textsuperscript{18} Adrenals were classified as harboring an adenoma characterized by one well demarcated or encapsulated nodule without nodulation in the adjacent adrenal cortex, or as a nodular hyperplasia if the presence of multiple nodules with an increase in cortex thickness or a distortion of the surrounding adrenal cortex was observed. Most adrenals contained only 1 nodule positive for aldosterone synthase harboring or not a mutation in 1 of the known genes. Only in 1 case, a KCNJ5 mutation was observed in a secondary nodule expressing aldosterone synthase.\textsuperscript{18} In this study, all adrenals carried a well-defined larger nodule defined as the APA and expressing aldosterone synthase and a different number of macronodulations or micronodulations in the peripheral adjacent adrenal cortex. Only nodules expressing CYP11B2 or aldosterone synthase were included in our analysis to discriminate nodules with the capacity of aldosterone production and, therefore, candidates to somatic mutations associated to inappropriate aldosterone secretion.

Among the 37 secondary nodules investigated in this study, we have identified somatic mutations in 9 secondary nodules from 8 adrenals with APA. In all cases, we identified a KCNJ5 p.Gly151Arg mutation (c.451G>C or c.451G>A), the most frequent somatic mutation associated with aldosterone production.\textsuperscript{13} In 28 secondary nodules, we did not observe a KCNJ5 mutation in the secondary nodules analyzed. In different studies, only 50% of APA carried a somatic mutation in the 4 investigated genes,\textsuperscript{13,17,27} thus somatic mutations in genes not yet described and associated with aldosterone production cannot be excluded. Interestingly, in 2 adrenals with APA positive for CACNA1D mutations, we observed a KCNJ5 mutation in a secondary nodule. In addition, in 1 adrenal without a mutation in APA, a KCNJ5 mutation was observed in the secondary nodule. In this adrenal, aldosterone synthase was highly expressed in the APA and in the secondary nodule (Figure S3). Different somatic mutations in nodules from the same adrenal were previously described in primary macronodular adrenal hyperplasia, where the same germline ARMC5 mutation was associated with different, nodule-specific, somatic ARMC5 alterations.\textsuperscript{26} More recently, Dekkers et al\textsuperscript{18} identified 2 different KCNJ5 mutations in 2 nodules from the same adrenal in a patient with APA. The discordance of mutation status between APA and secondary nodules in the same adrenal suggests that a specific genetic or epigenetic hit could be responsible for the remodeling in the adrenal cortex resulting in nodule formation. In this case, the somatic mutations described in KCNJ5, CACNA1D, ATP1A1, or ATP2B3 genes could represent second hits occurring in a previously altered adrenal cortex, being responsible only for the excessive aldosterone secretion. Alternatively, a somatic mutation may lead to the formation of an APA followed by secondary mutations and nodule formation triggered by changes in the tumor microenvironment. Against this hypothesis is however the fact that in multinodular adrenals, Dekkers et al\textsuperscript{18} identified somatic mutations only in aldosterone-expressing nodules.

Some authors suggested that APCC, isolated subcapsular cell clusters strongly expressing CYP11B2 without an apparent fibrous capsule, share morphological characteristics with cells composing an APA and could eventually be the starting point for the development of an APA.\textsuperscript{19} To investigate a possible continuum between APCC and APA development, we have genotyped 7 APCC structures from 6 adrenals with APA; no somatic mutations in the 4 target genes were identified. These data do not support the possible origin of APA from APCC and corroborate previous findings showing different molecular characteristics between APA and APCC.\textsuperscript{16} However, we cannot exclude that APCC might represent precocious structures in which an event inducing an abnormal proliferative state happened before the occurrence of one of the known somatic mutations.

We have compared clinical and biological characteristics of patients with PA enrolled in this study with patients of our entire cohort. We did not find differences in the clinical presentation of PA and the follow-up between patients from the 2 groups. This lack of difference may be because of the fact that we did not analyze the adrenals of all patients of the Paris cohort for the presence of peripheral cortical micronodules. As the patients were randomly selected for the presence of multinodular adrenals, the frequency of somatic mutations in the APA is slightly different compared with that previously reported.\textsuperscript{13} We did not observe differences in the disease presentation in patients carrying adrenals with secondary nodules positive for somatic mutations and patients carrying adrenals with secondary nodules negative for somatic mutations. However, given the small number of patients in each group, the comparison may lack statistical power to identify genotype–phenotype correlations.

Interestingly, in 2 adrenals with APA positive for CACNA1D mutations, we observed a KCNJ5 mutation in a secondary nodule. In addition, in 1 adrenal without a mutation in APA, a KCNJ5 mutation was observed in the secondary nodule. In this adrenal, aldosterone synthase was highly expressed in the APA and in the secondary nodule (Figure S3). Different somatic mutations in nodules from the same adrenal were previously described in primary macronodular adrenal hyperplasia, where the same germline ARMC5 mutation was associated with different, nodule-specific, somatic ARMC5 alterations. More recently, Dekkers et al identified 2 different KCNJ5 mutations in 2 nodules from the same adrenal in a patient with APA. The discordance of mutation status between APA and secondary nodules in the same adrenal suggests that a specific genetic or epigenetic hit could be responsible for the remodeling in the adrenal cortex resulting in nodule formation. In this case, the somatic mutations described in KCNJ5, CACNA1D, ATP1A1, or ATP2B3 genes could represent second hits occurring in a previously altered adrenal cortex, being responsible only for the excessive aldosterone secretion. Alternatively, a somatic mutation may lead to the formation of an APA followed by secondary mutations and nodule formation triggered by changes in the tumor microenvironment. Against this hypothesis is however the fact that in multinodular adrenals, Dekkers et al identified somatic mutations only in aldosterone-expressing nodules.
Perspectives
Although the functional link between somatic mutations in KCNJ5, ATP1A1, ATP2B3, and CACNA1D and aldosterone production has clearly been established, it is less clear whether and how these mutations also lead to adrenal cortex cell proliferation and nodule formation. Adrenals with APA show increased nodulation and cortical remodeling with ZG hyperplasia and often multiple secondary aldosterone-producing nodules. Our work shows the presence of different recurrent somatic mutations in different aldosterone-producing nodules from a same adrenal, suggesting that somatic mutations are independent events triggered by mechanisms that remain to be identified. Our results also show that in some cases a genetic defect is present in the secondary nodule but absent in the APA, challenging our current genetic classification of APA and the correlations with clinical measures that have been previously established. It will be particularly relevant in the future to identify additional genetic, epigenetic, or local environmental factors predisposing to somatic mutagenesis and to establish the sequence of events leading to APA formation.

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Disclosures
None.

References
What Is New?

• Mutations in different genes were observed among aldosterone-producing adenoma (APA) and functional secondary nodules in the same adrenal.
• One adrenal without somatic mutation identified in the APA carried a KCNJ5 mutation in a secondary aldosterone-producing nodule.

What Is Relevant?

• Recurrent somatic mutations in KCNJ5 were identified in secondary nodules of adrenals with APA.
• A different mutation status between APA and secondary nodules suggests independent genetic events leading to nodulation and aldosterone overproduction.

Summary

Different mutations in APA and functional secondary nodules were identified in the same adrenal cortex. Our data suggest that somatic mutations found in APA are independent events leading to increased aldosterone production. The sequence of events underlying formation of aldosterone-producing nodules remains to be elucidated.

Novelty and Significance

• All aldosterone-producing cell cluster are negative for somatic mutations in KCNJ5, CACNA1D, ATP1A1, and ATP2B3.


Different Somatic Mutations in Multinodular Adrenals With Aldosterone-Producing Adenoma

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Different somatic mutations in multinodular adrenals with aldosterone producing adenoma

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Running Title: Mutations in multinodular adrenals with APA
Expanded Subjects and Methods

Patients

AVS procedures were performed under standardized conditions. Mineralocorticoid receptor antagonists were systematically discontinued. Bilateral simultaneous AVS without cosyntropin stimulation was performed in the morning after a night sleep in the supine position, by the same experienced vascular radiologist. Blood samples were then collected simultaneously from each adrenal vein and the inferior vena cava for the measurement of cortisol and aldosterone concentrations. The criteria for primary aldosteronism are selectivity index (cortisol in adrenal vein / cortisol in inferior vena cava) >2 and lateralization index [(aldosterone/cortisol) in dominant side / (aldosterone/cortisol) in non-dominant side] ≥5. The diagnosis of adrenocortical adenoma was histologically confirmed after surgical resection. A final diagnosis of APA, diagnosed by CT scanning and AVS, was considered “proven” when the following conditions were satisfied: 1) histological demonstration of adenoma; 2) normalization of hypokalemia, if present; 3) cure or improvement of hypertension; 4) normalization of ARR and/or suppressibility of aldosterone under saline load. All patients gave written informed consent for genetic and clinical investigation within each individual institution. Procedures were in accordance with institutional guidelines.

Pathological analysis

Histological annotations were performed on APA and peritumoral adjacent tissue after Haematoxylin-Eosin-Safran (HES) staining. Aldosterone synthase immunohistochemistry was performed using the mouse monoclonal anti-human CYP11B2-41-17 antibody. After deparaffination, the slides were subjected to antigen retrieval using Trilogy (Cell Marque Corporation, Rocklin, CA) for 30 min at 98°C. Endogenous peroxidases were inactivated by incubation in 3% hydrogen peroxide (Sigma-Aldrich; St Louis, MO USA) in water for 10 min. The slides were blocked using filtered Tris 0.1 M pH 7.4, 10% horse serum and 0.5% SDS for 1 hr. Aldosterone synthase antibody (hCYP11B2-41-17B clone 1/1000 dilution) was incubated in Tris 0.1 M pH7.4, 10% horse serum and 0.2% Tween-20 overnight at 4°C. The slides were then washed 3 times in PBS with 0.2% Tween-20 and incubated with the ImmPress IgG mouse secondary antibody (ref) for 30 min at room temperature. The slides were developed using diaminobenzidin (Vector Laboratories) and counterstained with hematoxilin (Sigma-Aldrich; St Louis, MO USA). CYP11B2 in situ hybridization was performed as previously described. In the negative control reactions, the primary antibodies were omitted from the dilution buffer for immunohistochemistry and sense probe were used for in situ hybridization, which in all instances resulted in a complete absence of staining. All microscopic examinations were done on a Leica microscope.

DNA isolation and Sanger sequencing

DNA was extracted from formalin fixed paraffin embedded (FFPE) fixed tissues. To this purpose, adrenal glands were cut into 4-mm-thick slices after formalin fixation. Before extracting the DNA, nodules producing aldosterone were identified on immediately consecutive tissue slices by immunohistochemistry or in situ hybridization. On each corresponding slide, all aldosterone producing nodules were demarcated by felt pen. Of each demarcated nodule, tissue sections were manually microdissected and submitted to DNA extraction using the QIAamp DNA FFPE Tissue Kit (Qiagen). Paraffin was dissolved in xylene and then removed. The sample were lysed under denaturing conditions with proteinase K. Formalin crosslinking was reversed with incubation at 90°C. DNA was bound by the silica-based membrane of the QIAamp DNA FFPE Tissue Kit (Qiagen), while residual components
were washed away. Finally, the DNA was eluted from the membrane and used for subsequent genotyping of recurrent somatic mutations by Sanger sequencing and droplet digital (dd)PCR. *KCNJ5*, *CACNA1D*, *ATP1A1* and *ATP2B3* DNA were amplified using exonic primers for the amplification of amplicons ranging from 93 to 131 bp (Table S1). PCR was performed on 100 ng of DNA in a final volume of 25 µl containing 0.75 mM MgCl₂, 400 nM of each primer, 200 µM deoxynucleotide triphosphate and 1.25 U Platinum Taq DNA Polymerase (Invitrogen). An annealing temperature of 60°C was used for all amplicons. Direct sequencing of PCR products was performed using the ABI Prism Big Dye Terminator® v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on an ABI Prism 3700 DNA Analyzer (Applied Biosystems). All sequencing reactions were performed twice in forward and reverse sequencing.

**Droplet Digital PCR (ddPCR)**

Each ddPCR reaction mixture (20 µL) contained 50 ng of DNA template, 1 µL of 20X WT (HEX) and mutant (FAM) assays and 10 µL of 2X Bio-Rad ddPCR Supermix. The reaction mixture was mixed with 70 µL Bio-Rad droplet generator oil and partitioned into 15,000–20,000 droplets by using the Bio-Rad QX-100 droplet generator (Bio-Rad). The droplets of individual samples were separately applied to each well of a 96-well PCR reaction plate. PCR conditions were 10 min at 95°C, 40 cycles of denaturation for 30 s at 95°C and extension for 60 s at 60°C with ramp rate of 2.5°C.s⁻¹, followed by 10 min at 95°C and a hold at 4°C. After PCR amplification, the plate was transferred to the Bio-Rad QX-100 droplet reader (Bio-Rad). Each droplet in the well was checked for HEX or FAM fluorescence to count the number of droplets that yielded positive/negative results. Bio-Rad’s QuantaSoft software version 1.3.2.0 was used to quantify the copies of target DNA and the threshold for a positive signal was determined according to the software instructions. Droplets beyond the fluorescence threshold were counted as a positive event. We used positive HEX/positive FAM events to identify the presence and the proportion of target mutations.
References
<table>
<thead>
<tr>
<th>Gene</th>
<th>Exon</th>
<th>Forward Primers</th>
<th>Reverse Primers</th>
<th>Amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>KCNJ5</strong></td>
<td>2-A</td>
<td>TGTTGAAAAACCTCAGTGGCT</td>
<td>AATCCCCCTCTGGACACTT</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>2-B</td>
<td>GTATGGCTTCCGAGTCATCAC</td>
<td>CCCACCATGAAGGCATTGACG</td>
<td>94</td>
</tr>
<tr>
<td><strong>ATP1A1</strong></td>
<td>4</td>
<td>CAAGTTTTGTCGGCAGCTC</td>
<td>TCTGTAGCAGCTTGGATGC</td>
<td>96</td>
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<tr>
<td></td>
<td>8</td>
<td>AGTACACCTGGCTGAGGCTG</td>
<td>TGCCTCTTACCCTGACAGTG</td>
<td>93</td>
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<td><strong>ATP2B3</strong></td>
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<td>AGTGCACGCCCTGGGTATGTA</td>
<td>GCTGTCACCATCTCCTTAGCT</td>
<td>113</td>
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<tr>
<td><strong>CACNA1D</strong></td>
<td>6</td>
<td>CATTGGAGTGCCCTCCCTGG</td>
<td>GGTTCCCCTCCTCCTACATAGC</td>
<td>109</td>
</tr>
<tr>
<td></td>
<td>8A</td>
<td>TTGAATTGCCCTGGGTATGTA</td>
<td>AGAGGTGTCATTACCCCGT</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>8B</td>
<td>TGGCCATGGGTGTATTTTG</td>
<td>GATCCGACTTGCTTACCCCT</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>CTTCCCTGAGCAAAGTTAGTG</td>
<td>CCAAACAGCCTGCATCCCAAAG</td>
<td>118</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>AGTAGGTGTGGTGTGCCCCTAA</td>
<td>GCCTCCTTCTCTGAGCAGTG</td>
<td>120</td>
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<tr>
<td></td>
<td>23</td>
<td>CGTCTTTCTGCTCTCCTC</td>
<td>CGGGGAGGAGGAGAATCAAAAAAC</td>
<td>131</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>CATCATCTACATCATCATTG</td>
<td>TGTCCAGCTCAGTTTATAC</td>
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<td>32</td>
<td>GTGCTGAGCCAAGTGCCCTT</td>
<td>AGATGGCTGGAGCTTACCT</td>
<td>113</td>
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* NM_000890; † NM_000701.7; ‡NM_021949.3; § NM_001128839.2; || NM_000720.3
Table S2. Phenotype of PA patients with secondary adrenal nodules selected in this study and compared to the patients with uninodular adrenals from the Paris cohort*.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Multinodular adrenals</th>
<th>Uninodular adrenals</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>27</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>gender F (%)</td>
<td>35</td>
<td>47</td>
<td>0.43</td>
</tr>
<tr>
<td>Age of diagnosis (years)</td>
<td>44 (40;58)</td>
<td>43 (35;50)</td>
<td>0.09</td>
</tr>
<tr>
<td>Duration of hypertension (years)</td>
<td>3 (1.0;13.5)</td>
<td>4.5 (1.0;8.7)</td>
<td>0.8</td>
</tr>
<tr>
<td>Preoperative SBP (mmHG)</td>
<td>147 (138;160)</td>
<td>139 (130;157)</td>
<td>0.11</td>
</tr>
<tr>
<td>Preoperative DBP (mmHg)</td>
<td>91 (81;96)</td>
<td>88 (83;97)</td>
<td>0.9</td>
</tr>
<tr>
<td>Minimal plasma K (mmol/L)</td>
<td>3.1 (2.6;3.4)</td>
<td>3.2 (2.8;3.4)</td>
<td>0.9</td>
</tr>
<tr>
<td>Plasma Aldosterone (pmol/L)</td>
<td>823 (570;1230)</td>
<td>776 (547;1481)</td>
<td>0.8</td>
</tr>
<tr>
<td>ARR (pmol/mU)</td>
<td>160 (109;246)</td>
<td>155 (109;262)</td>
<td>0.7</td>
</tr>
<tr>
<td>Anti-hypertensive drugs (n)</td>
<td>3 (2;4)</td>
<td>2 (1;3)</td>
<td>0.06</td>
</tr>
<tr>
<td>Lateralization index</td>
<td>10 (6.5;28)</td>
<td>16 (10;34)</td>
<td>0.08</td>
</tr>
<tr>
<td>APA size (mm)</td>
<td>13 (10;18)</td>
<td>15 (11;20)</td>
<td>0.15</td>
</tr>
<tr>
<td>Time of follow up (months)</td>
<td>8.5 (6;13.5)</td>
<td>7 (5;12)</td>
<td>0.38</td>
</tr>
<tr>
<td>Post op SBP (mmHG)</td>
<td>131 (125;139)</td>
<td>124 (118;134)</td>
<td>0.06</td>
</tr>
<tr>
<td>Post op DBP (mmHG)</td>
<td>82 (77;90)</td>
<td>81 (77;84)</td>
<td>0.24</td>
</tr>
<tr>
<td>Post op plasma K (mmol/L)</td>
<td>4.1 (3.9;4.6)</td>
<td>4.1 (3.9;4.3)</td>
<td>0.5</td>
</tr>
<tr>
<td>Post op plasma aldosterone (pmol/L)</td>
<td>98 (46;246)</td>
<td>97 (60;183)</td>
<td>0.9</td>
</tr>
<tr>
<td>Post op anti-hypertensive drugs (n)</td>
<td>1 (0;2)</td>
<td>0 (0;1)</td>
<td>0.24</td>
</tr>
</tbody>
</table>

SBP: systolic blood pressure; DBP: diastolic blood pressure; ARR: aldosterone to renin ratio.

*, This comparison includes only the adrenals annotated by the same pathologist (TM), for which information on macro-or micronodulation was available on the routine pathological report.
Table S3. Cellular composition and proportion of *KCNJ5* mutant alleles in *KCNJ5* mutated secondary nodules.

<table>
<thead>
<tr>
<th>Adrenal Patient</th>
<th>Mutation Status</th>
<th>Size (mm)</th>
<th>Cellular* composition</th>
<th><em>KCNJ5</em> mutant allele (%)</th>
<th>APA</th>
<th>Secondary nodule Patient</th>
<th>Size (mm)</th>
<th>Cellular composition</th>
<th><em>KCNJ5</em> mutant allele (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>KCNJ5</em></td>
<td>13</td>
<td>ZFL</td>
<td>39%</td>
<td>A</td>
<td>ZFL</td>
<td>13</td>
<td>ZFL</td>
<td>38%</td>
</tr>
<tr>
<td>2</td>
<td><em>KCNJ5</em></td>
<td>15</td>
<td>ZFL</td>
<td>40%</td>
<td>A</td>
<td>ZFL</td>
<td>15</td>
<td>ZFL</td>
<td>12%</td>
</tr>
<tr>
<td>3</td>
<td><em>KCNJ5</em></td>
<td>20</td>
<td>ZFL</td>
<td>36%</td>
<td>A</td>
<td>ZFL</td>
<td>20</td>
<td>ZFL</td>
<td>33%</td>
</tr>
<tr>
<td>4</td>
<td>neg</td>
<td>11</td>
<td>ZGL</td>
<td>0%</td>
<td>A</td>
<td>ZGL</td>
<td>11</td>
<td>ZGL</td>
<td>29%</td>
</tr>
<tr>
<td>9</td>
<td><em>KCNJ5</em></td>
<td>13</td>
<td>ZFL</td>
<td>38%</td>
<td>A</td>
<td>ZFL</td>
<td>13</td>
<td>ZFL</td>
<td>15%</td>
</tr>
<tr>
<td>15</td>
<td><em>CACNA1D</em></td>
<td>6</td>
<td>ZFL</td>
<td>0%</td>
<td>A</td>
<td>ZFL</td>
<td>6</td>
<td>ZFL</td>
<td>18%</td>
</tr>
<tr>
<td>17</td>
<td><em>KCNJ5</em></td>
<td>26</td>
<td>ZFL</td>
<td>33%</td>
<td>A</td>
<td>ZFL</td>
<td>26</td>
<td>ZFL</td>
<td>13%</td>
</tr>
<tr>
<td>24</td>
<td><em>CACNA1D</em></td>
<td>8</td>
<td>ZFL</td>
<td>0%</td>
<td>B</td>
<td>ZFL</td>
<td>8</td>
<td>ZFL</td>
<td>15%</td>
</tr>
</tbody>
</table>

ZFL: Zona fasciculata-like; ZGL: Zona glomerulosa-like; *the predominant cellular composition is indicated.*
Table S4. Phenotype of patients with or without mutations in the secondary nodule.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>KCNJ5 Positive secondary nodule</th>
<th>Negative Secondary nodule</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>8</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>gender F (%)</td>
<td>37.5</td>
<td>31.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Age of diagnosis (years)</td>
<td>44 (39;53)</td>
<td>48 (42;52)</td>
<td>0.3</td>
</tr>
<tr>
<td>Systolic BP (mmHG)</td>
<td>142 (133;168)</td>
<td>152 (141;160)</td>
<td>0.3</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>91 (85;93)</td>
<td>90 (81;97)</td>
<td>0.97</td>
</tr>
<tr>
<td>Minimal plasma K (mmol/L)</td>
<td>3.4 (2.8;3.4)</td>
<td>3.0 (2.6;3.4)</td>
<td>0.3</td>
</tr>
<tr>
<td>Plasma Aldosterone (pmol/L)</td>
<td>795 (471;1026)</td>
<td>831 (584;1309)</td>
<td>0.55</td>
</tr>
<tr>
<td>ARR (pmol/mU)</td>
<td>154 (104;240)</td>
<td>166 (109;246)</td>
<td>0.95</td>
</tr>
<tr>
<td>Anti-hypertensive drugs (n)</td>
<td>3 (2;4)</td>
<td>2 (1;3)</td>
<td>0.06</td>
</tr>
<tr>
<td>APA size (mm)</td>
<td>13 (9;18)</td>
<td>15 (10;20)</td>
<td>0.42</td>
</tr>
<tr>
<td>Systolic BP at FU (mmHg)</td>
<td>128 (123;141)</td>
<td>135 (122;138)</td>
<td>0.95</td>
</tr>
<tr>
<td>Treatment score at FU (n)</td>
<td>1 (0;2)</td>
<td>2 (1;3)</td>
<td>0.36</td>
</tr>
<tr>
<td>Adjusted change in SBP (mmHg)*</td>
<td>30 ± 21</td>
<td>29 ± 18</td>
<td>0.89</td>
</tr>
<tr>
<td>Hypertension cure (%)</td>
<td>37.5</td>
<td>26.5</td>
<td>0.65</td>
</tr>
<tr>
<td>Significant BP improvement (%) †</td>
<td>62.5</td>
<td>53%</td>
<td>0.69</td>
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</table>

BP: blood pressure; ARR: aldosterone to renin ratio; FU: follow-up.
* change in systolic BP adjusted for change in treatment score by analysis of covariance
† hypertension cure or adjusted change in SBP > 20 mmHg
**Figure S1. Sanger sequencing chromatograms of DNA from APA and secondary nodules.**

In adrenals 1 (A), 2 (B), 9 (C) and 17 (D) we identified the same mutation, KCNJ5 p.Gly151Arg, in both APA and secondary nodule. E. Sequencing of APA and secondary nodules of Adrenal 3. The KCNJ5 mutation p.Gly151Arg is present in the APA and in the secondary nodules A and B, but absent in the secondary nodule C. F. Sequencing of APA and secondary nodule of Adrenal 15. A CACNA1D mutation p.Ala998Val is present in the APA, whereas a KCNJ5 mutation p.Gly151Arg is present in the secondary nodule. G. Sequencing of APA and secondary nodule of Adrenal 24. A mutation CACNA1D p.Gly403Arg is present in the APA whereas a mutation KCNJ5 p.Gly151Arg is present in the secondary nodule B. H. Sequencing of APA and secondary nodule of Adrenal 4. No KCNJ5 mutation was observed in the APA, while a KCNJ5 mutation p.Gly151Arg is present in the secondary nodule.
Figure S3. Aldosterone synthase expression in adrenal 4 with an APA without mutation (A) and a mutated secondary nodule (B). Upper panels: x40 Bottom panels: x200