Adrenal Nodularity and Somatic Mutations in Primary Aldosteronism: One Node Is the Culprit?

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Context: Somatic mutations in genes that influence cell entry of calcium have been identified in aldosterone-producing adenomas (APAs) of adrenal cortex in primary aldosteronism (PA). Many adrenal glands removed for suspicion of APA do not contain a single adenoma but nodular hyperplasia.

Objective: The objective of the study was to assess multinodularity and phenotypic and genotypic characteristics of adrenals removed because of the suspicion of APAs.

Design and Methods: We assessed the adrenals of 53 PA patients for histopathological characteristics and immunohistochemistry for aldosterone (P450C18) and cortisol (P450C11) synthesis and for *KCNJ5*, *ATP1A1*, *ATP2B3*, and *CACNA1D* mutations in microdissected nodi.

Results: Glands contained a solitary adenoma in 43% and nodular hyperplasia in 53% of cases. Most adrenal glands contained only one nodule positive for P450C18 expression, with all other nodules negative. *KCNJ5* mutations were present in 22 of 53 adrenals (13 adenoma and nine multinodular adrenals). An *ATP1A1* and a *CACNA1D* mutation were found in one multinodular gland each and an *ATP2B3* mutation in five APA-containing glands. Mutations were always located in the P450C18-positive nodule. In one gland two nodules containing two different *KCNJ5* mutations were present. Zona fasciculata-like cells were more typical for *KCNJ5* mutation-containing nodules and zona glomerulosa-like cells for the other three genes.

Conclusions: Somatic mutations in *KCNJ5*, *ATP1A1*, or *CACNA1D* genes are not limited to APAs but are also found in the more frequent multinodular adrenals. In multinodular glands, only one nodule harbors a mutation. This suggests that the occurrence of a mutation and nodule formation are independent processes. The implications for clinical management remain to be determined. **(*J Clin Endocrinol Metab* 99: E1341–E1351, 2014)**

Classically, endocrinologists consider the cause of primary aldosteronism to be either a unilateral aldosterone-producing (micro)adenoma (APA) or bilateral adrenal hyperplasia. The first is best treated by laparoscopic adrenalectomy, whereas the latter requires therapy with mineralocorticoid receptor antagonists (1). Correct pre-

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Abbreviations: APA, aldosterone-producing (micro)adenoma; APCC, aldosterone-producing cell cluster; ARR, aldosterone to renin ratio; AVS, adrenal venous sampling; BMI, body mass index; BP, blood pressure; CT, computed tomography; HE, hematoxylin and eosin; SIT, saline infusion test; ZF, zona fasciculata; ZG, zona glomerulosa.

operative diagnosis of an APA is confirmed by improvement or cure of hypertension and hypokalemia, the hallmarks of aldosteronism, after unilateral adrenalectomy. Numerous authors also regard the presence of an APA at pathological examination proof of a correct preoperative diagnosis and claim to find single adenomas in all excised glands (2–5). However, in our experience and that of others, in many cases the removed gland does not contain a single adenoma but demonstrates various patterns of macronodular or micronodular hyperplasia (6–16).

Adrenal glands removed because of the suspicion of APA have other remarkable features. Many nodules do not have the appearance of aldosterone-producing zona glomerulosa (ZG) cells, as would be expected, but of zona fasciculata (ZF) cells, which normally produce cortisol (6, 17). Immunohistochemically, adrenal nodules may express both p450C11, or cortisol synthase, encoded by *CYP11B1*, and p450C18, or aldosterone synthase, encoded by *CYP11B2*, suggesting that they are capable to produce both cortisol and aldosterone (13, 18–20). Furthermore, in the surrounding preexistent cortical tissue, small extranodular cell clusters are observed with strong p450C18 and no p450C11 expression, that leave normal cortex zonation intact (18). The function of these so called aldosterone-producing cell clusters (APCCs), which are present in both normal and pathological conditions, is unknown (13, 18). Another striking finding in the surrounding preexistent adrenal tissue in APAs is the almost ubiquitous presence of zona glomerulosa thickening where atrophy would be expected (21).

An explanation for some histopathological findings might be found in the recently discovered somatic mutations of *KCNJ5*, *ATP1A1*, *ATP2B3*, and *CACNA1D* in adrenal glands (22–24). *KCNJ5*, first described in APAs by Choi et al (22), encodes the inward rectifying potassium channel Kir3.4 that is present in the adrenal cortex and when mutated generates calcium influx of the adrenocortical cell, thus inducing activation of aldosterone synthesis. In addition, Choi et al hypothesize that these mutations in *KCNJ5* promote growth of aldosterone secreting cells into APAs because the mutation was present in 8 of 20 APAs studied. Other researchers confirmed the presence of the somatic *KCNJ5* mutations in about 20%–40% of resected APAs (8, 23–30). The recently discovered *ATP1A1, ATP2B3*, and *CACNA1D* mutations, accounting for about 7% of resected APAs, are also likely to increase intracellular calcium (23, 24).

Until now, most studies on these mutations have been performed on reportedly solitary APAs and lack data of the so-often-present hyperplasia (25–29). One study that assessed additional hyperplasia in the resected adrenals found *KCNJ5* mutations in 40% of the samples classified as adenoma with associated hyperplasia (8). However, this study lacks histopathological details of the glands that did or did not contain a *KCNJ5* mutation. A histopathological feature that has been reported is that adenoma tissue with the *KCNJ5* mutation resembles ZF cells and that adenoma tissue with *ATP1A1* and *CACNA1D* mutations resembles ZG cells (24, 26). This led us to systematically assess the multinodularity and phenotypic and genotypic characteristics of the adrenals removed because of the suspicion of APA.

Materials and Methods

Subjects

We reexamined all retrievable adrenals (n = 53) of patients with PA operated in the Radboud University Medical Center Nijmegen between 1997 and 2010 (n = 65, Table 1). All included patients had hypertension resistant to three or more drugs and/or hypertension accompanied by hypokalemia. The diagnosis of primary aldosteronism was confirmed by iv saline infusion test (SIT; n = 45), oral salt loading test (n = 3), or captopril suppression test (n = 1) performed after the cessation of medication and correction of hypokalemia in accordance to the current guidelines (1). In four patients (numbers 2, 8, 20, and 33, Table 2), no confirmation test was performed because of the potential risk of medication withdrawal that is necessary to create optimal conditions for a correct interpretation of the test results. In these patients the diagnosis was based on the triad of hypertension, hypokalemia, and an increased aldosterone to renin ratio (ARR). The diagnosis of unilateral APA was based on adrenal venous sampling (n = 36) or computed tomography (CT) scan (n = 17), which was used because adrenal venous sampling (AVS) was not yet available (before 2004, n = 9), considered too hazardous because of the need for medication withdrawal (n = 2) or was unsuccessful (n = 6). AVS was performed under continuous ACTH stimulation (5 μg/h) using a selectivity index of ≥2.0 or greater and a lateralization index of 4.0. For a lateralization index between 3.0 and 4.0, the decision to operate was reached

Table 1. Patient Characteristics (n = 53)

	Included (n = 53)	Not Included (n = 12)	*P* Value
Gender, male/female	30/23	5/7	NS
Age, y	50 ± 10	43 ± 13	NS
BMI, kg/m^2	27.4 ± 5.0	26.4 ± 4.6	NS
SBP, mm Hg	168 ± 26	182 ± 32	NS
DBP, mm Hg	99 ± 13	104 ± 18	NS
DDD[a]	4.0 (0.3–9.7)	2.3 (0.0–3.7)	<.01
Potassium, mmol/L	3.2 ± 0.6	3.4 ± 0.5	NS
Aldosterone, nmol/L[a]	0.78 (0.34–2.20)	0.85 (0.46–1.30)	NS

Abbreviations: DDD, defined daily dosages of antihypertensive medication (http://www.whocc.no/atc_ddd_index/); DBP, diastolic BP; NS, not significant; SBP, systolic BP.

[a] Median (range).

by consensus, based on clinical details and CT scan results (n = 3). Postoperative follow-up information was available for 3 months in seven and for at least 1 year in 46 patients. We defined outcome of surgery as cured, improved, or failed (Table 3) (27). The study was approved by the Medical Ethics Committee, who waived the requirement for informed consent, absent in earlier cases, because the use of anonymous or coded leftover material for scientific purposes is part of the standard treatment contract with patients in hospitals in The Netherlands. However, they set the condition that no genotyping of normal tissues (ie, germline genotyping) was to be performed.

Histopathological phenotype

All adrenal glands resected were cut into 4-mm-thick slices after formaline fixation. These slices were assessed macroscopically, including the description of nodularity. Representative material was sampled for microscopical evaluation. These hematoxylin and eosin (HE) slides of all adrenal glands were assessed twice by an experienced pathologist (B.K.), who was blinded to patient characteristics and genotype results. Histopathological phenotyping of the glands consisted of assessment of ZG thickening (continuous ZG and/or ZG thickness ≥200 μm as measured by a micrometer), nodule diameter, and the cellular composition of the nodule(s) (Figure 1). The cellular composition of the lesions was determined to be ZG-like (predominantly compact cells), ZF-like (predominantly foamy or lipid-rich cells), or a combination of both. Additionally, nodules were assessed for the presence of atypical cells, identified by nuclear enlargement, presence of nucleoli, and/or nuclear hyperchromasia. In case of multiple nodules within one specimen, we assessed all nodules separately. Finally, we classified all adrenal glands as containing either of the following: 1) adenoma (one well demarcated or encapsulated nodule, with the adjacent adrenal cortex resembling normal adrenal tissue without nodulation) or 2) nodular hyperplasia (the presence of multiple nodules; slight disturbances in the adrenal cortex were defined as a nodule in case they caused an increase in cortex thickness or caused distortion of the surrounding adrenal cortex).

Immunohistochemistry

We performed immunohistochemistry on p450C18 and p450C11 expression in all glands to assess the functional differentiation of the adrenal cells for aldosterone and cortisol secretion, respectively. Immunohistochemistry was performed using the antibodies and protocols previously described by Nishimoto et al (18) We defined the antigen expression areas as the percentage of the surface of the adrenal node expressing the antigen. Expression was qualified as weak or strong, in comparison with expression in preexistent tissue to correct for background staining (Table 2). Additionally, all slides were screened for the presence of APCCs. APCCs were defined as cell clusters within the adrenal cortex that exhibit conventional cortex zonation (ie, no nodulation with increase of cortex thickness or distortion of surrounding tissue) with marked p450C18, but no p450C11, expression (18). We did not classify elongated p450C180-positive cell clusters (<0.2 mm) spreading over and merging with the ZG as APCC but regarded them to be part of the conventional adrenocortical zonation with sporadic expression of p450C18 in the zona glomerulosa.

Genotyping

On each HE slide, all conspicuous nodules were demarcated by the pathologist (B.K.) by felt pen. Of each nodule demarcated, three 20-μm sections were manually microdissected. Genomic DNA was extracted from all separate nodules by overnight digestion with proteinase K and analyzed separately. For the mutation analysis, the crude extract was subsequently used to amplify the regions spanning the mutations. Primers used were as follows: *KCNJ5*, 5′-TTGGCGACCAAGAGTGGATTCCTT-3′ and 5′-CACCATGAAGGCATTGACGATGGA-3′; *ATP1A1* exon 4, 5′-CCACTACTCCTGAATGGATC-3′ and 5′-TCCTCTTCTGTAGCAGCTTG-3′; *ATP1A1* exon 8, 5′-CTCTCATCCTTGAGTACACC-3′ and 5′-TGCAAGCTGATCTGAGTCAG-3′; and *ATP2B3* exon8, 5′-GATTGAGACGT TTGTCGTGG-3′ and 5′-CCTTGACAGAGTAAGCTAAGG-3′. These were then analyzed by dideoxy sequencing. DNA samples of 50 of 53 patients were genotyped using custom TaqMan genotyping assays (Applied Biosystems) for the *CACNA1D* substitution mutations encoding c.T776A, c.G1207C, c.C2250G, and c.C4007G encoding p.Val259Asp, p.Gly403Arg, p.Ile1750Met, and p.Pro1336Arg, respectively (24).

Genotype-phenotype analysis

We compared *KCNJ5*, *ATP1A1*, *ATP2B3*, and *CACNA1D* genotype results of adrenals classified as either adenoma or nodular hyperplasia to histopathological characteristics, immunohistochemistry, patient characteristics, and treatment outcome.

Statistical analysis

All data are presented as mean and SD or, in the case of skewed distributions, as median and range. To assess the significance of difference between the histological classes, between glands with or without mutation, between demographic data, between histopathological characteristics, and between treatment outcome, we used the χ^2 test and Fisher's exact test for discrete data and one-way ANOVA (with Bonferroni correction) and Mann-Whitney *U* test (two samples) or Kruskal-Wallis test (multiple samples) for continuous data with and without a normal distribution, respectively. Forward stepwise binary logistic regression analysis (Likelihood Ratio method) was performed to determine the relation between patient characteristics and classification as a single adenoma or nodular hyperplasia and between patient characteristics and the presence of a mutation. Correlation between P450C18 and P450C11 expression was calculated using Spearman's rho. $P < .05$ was considered significant. We used IBM SPSS statistics 20 and GraphPad Prism 5 for Windows for statistical analysis.

Results

Subjects and adrenal glands

The clinical features of the 53 patients whose adrenal glands were reexamined are shown in Table 1. Except for medication intake, there were no significant differences in patient characteristics between those patients that could be included and those that could not (Table 1). The 53 glands studied contained 98 nodules (one to seven nodes per adrenal gland), which were all assessed separately (Ta-

Table 2. Clinical, Pathological, Genetic, and Histochemical Characteristics of 53 Adrenal Glands Removed Because of Suspected Unilateral Disease in Patients With Primary Aldosteronism

	Preoperative								Pathology			
Number	M/F	Age, y	SBP	DBP	K^+	Ald	ARR	CT/AVS	Classification	ZG	ZG Ø (μm)	Node
KCNJ5 mutations												
1	F	29	182	103	3.0	0.78	0.16	CT	Adenoma	Y	278	1
2	F	26	186	112	2.6	1.55	0.14	CT	Adenoma	Y	226	1
3	F	44	132	82	2.3	1.38	0.35	CT	Adenoma	Y	110	1
4	M	47	180	115	2.7	0.89	0.19	CT	Adenoma	Y	140	1
5	M	40	179	101	3.5	0.90	0.45	AVS	Adenoma	Y	230	1
6	F	30	142	97	3.5	0.40	0.20	AVS	Adenoma	Y	164	1
7	F	53	144	88	3.4	0.45	0.06	AVS	Adenoma	Y	255	1
8	F	51	162	92	3.8	1.74	0.24	CT	Adenoma	Y	174	1
9	F	44	166	106	2.8	0.87	0.09	AVS	Adenoma	Y	350	1
10	F	47	139	79	4.2	0.43	0.22	AVS	Adenoma	Y	182	1
11	F	51	220	120	3.3	1.10	0.12	CT	Adenoma	N	164	1
12	F	31	188	110	2.2	0.79	0.15	AVS	Adenoma	Y	252	1
13	F	40	147	92	3.2	0.55	0.18	AVS	Adenoma	Y	237	1
14	F	55	184	98	3.2	0.59	0.08	AVS	Multinodular	Y	200	1 2 3
15	F	53	149	79	2.8	0.85	0.43	AVS	Multinodular	Y	260	1 2 3
16	F	42	164	98	3.3	0.76	0.04	CT	Multinodular	Y	151	1 2 3
17	M	61	180	90	3.0	0.53	0.27	AVS	Multinodular	N	130	1 2 3
18	M	54	165	91	4.4	1.40	0.47	AVS	Multinodular	N	178	1 2 3
19	M	60	184	112	3.6	1.23	0.20	AVS	Multinodular	N	52	1 2
20	F	71	145	99	4.7	1.66	0.49	CT	Multinodular	Y	310	1 2 3
21	M	58	173	93	3.3	2.20	0.55	AVS	Multinodular	Y	134	1 2
22	M	54	197	114	3.9	0.68	0.08	AVS	Multinodular	Y		1 2 3 4 5 6 7
ATP2B3 mutations												
23	M	56	147	88	3.2	1.39	0.25	AVS	Adenoma	Y	180	1
24	F	62	144	85	2.6	0.89	0.22	CT	Adenoma	Y	365	1
25	M	43	154	96	3.8	0.78	0.39	AVS	Adenoma	Y	184	1
26	M	49	222	111	2.4	0.72	0.24	AVS	Adenoma	Y	144	1
27	M	42	198	139	3.1	0.98	0.49	CT	Adenoma	Y	183	1
ATP1A1 mutations												
28	M	48	186	87	3.0	1.16	0.17	AVS	Multinodular	N	179	1 2
CACNA1D Mutations												
29	M	46	150	98	3.0	0.65	0.14	AVS	Multinodular	Y	238	1 2 3
No mutations												
30	F	49	151	90	2.3	8.20	1.03	AVS	Adenoma	N	155	1
31	F	24	138	98	3.1	0.61	0.20	AVS	Adenoma	N	80	1
32	F	43	167	113	3.6	1.10	0.10	AVS	Adenoma	Y	176	1
33	F	69	181	94	2.9	0.54	0.18	CT	Multinodular	Y	268	1 2
34	M	48	158	108	2.6	0.55	0.02	CT	Multinodular	Y	233	1 2
35	M	49	168	87	2.9	0.48	0.10	AVS	Multinodular	Y	89	1 2
36	M	49	122	82	2.6	0.53	0.27	AVS	Multinodular	Y	98	1 2
37	M	63	214	106	3.8	0.77	0.39	AVS	Multinodular	Y	235	1 2
38	F	62	212	98	3.4	1.10	0.22	AVS	Multinodular	Y	138	1 2
39	M	36	147	100	3.8	0.81	0.20	AVS	Multinodular	Y	172	1 2

(*Continued*)

Table 2. Continued

Genetics			(Immuno) Histology						Postoperatively
Gene	Mutation	Size, mm	Cell Comp.	Atypic Cells, %	SB	p450C18 (Stain), %	p450C11 (Stain), %	APCC, n	Outcome
KCNJ5	c.503T>G (p.Leu168Arg)	23	ZF	5	n	25 (S)	33 (W)	0	Cured
KCNJ5	c.503T>G (p.Leu168Arg)	23	ZF	25	n	80 (W)	30 (W)	2	Impr
KCNJ5	c.503T>G (p.Leu168Arg)	15	ZG + ZF	10	N	70 (S)	60 (W)	0	Cured
KCNJ5	c.503T>G (p.Leu168Arg)	14	ZG + ZF	30	Y	33 (S)	100 (S/W)	0	Impr
KCNJ5	c.503T>G (p.Leu168Arg)	14	ZF	40	N	70 (S)	30 (S)	1	Impr
KCNJ5	c.503T>G (p.Leu168Arg)	13	ZF	25	N	40 (S)	0	1	Cured
KCNJ5	c.503T>G (p.Leu168Arg)	12	ZF	20	N	50 (S)	50 (W)	1	Impr
KCNJ5	c.451G>C (p.Gly151Arg)	17	ZG + ZF	5	N	53 (S/W)	66 (W)	3	Impr
KCNJ5	c.451G>C (p.Gly151Arg)	12	ZF	5	N	50 (S/W)	20 (S/W)	2	Impr
KCNJ5	c.451G>C (p.Gly151Arg)	11	ZG + ZF	5	N	40 (S)	40 (W)	2	Cured
KCNJ5	c.451G>A (p.Gly151Arg)	21	ZF	5	N	50 (S/W)	50 (W)	2	Failed
KCNJ5	c.451G>A (p.Gly151Arg)	12	ZF	5	N	40 (S)	25 (W)	1	Failed
KCNJ5	c.451G>A (p.Gly151Arg)	10	ZF	5	N	10 (S)	5 (S)	3	Cured
KCNJ5	c.503T>G (p.Leu168Arg)	20	ZF	0	N	20 (W)	50 (W)	2	Cured
wt		6	ZG + ZF	5	N	0	40 (S)		
wt		5	ZG + ZF	25	N	0	40 (S)		
wt		16	ZG + ZF	10	N	0	50 (S)	9	Failed
KCNJ5	c.503T>G (p.Leu168Arg)	15	ZG + ZF	30	N	45 (S/W)	0		
wt		4	ZF	5	N	0	60 (S)		
KCNJ5	c.503T>G (p.Leu168Arg)	13	ZF	20	N	25 (S)	40 (W)	1	Impr
wt		2	ZF	5	N	0	0		
wt		1.5	ZF	5	N	0	0		
KCNJ5	c.503T>G (p.Leu168Arg)	11	ZG + ZF	30	N	40 (S/W)	40 (S)	0	Failed
wt		4	ZF	5	N	20 (S/W)	40 (S)		
wt		6	ZG + ZF	10	N	0	40 (S)		
KCNJ5 KCNJ5	c.451G>A (p.Gly151Arg)	19	ZG + ZF	20	N	70 (S/W)	25 (W)	3	Impr
KCNJ5	c.503T>G (p.Leu168Arg)	7	ZF	25	N	70 (S)	0		
wt		4	ZG + ZF	5	N	0	40 (W)		
KCNJ5	c.451G>A (p.Gly151Arg)	19	ZF	25	N	15 (S)	50 (W)	0	Impr
wt		5	ZF	0	N	0	50 (W)		
KCNJ5	c.451G>A (p.Gly151Arg)	17	ZG + ZF	25	N	30 (S/W)	80 (W)	0	Failed
wt		5	ZG + ZF	0	N	0	60 (W)		
wt		5	ZG + ZF	0	N	0	70 (W)		
KCNJ5	c.451G>A (p.Gly151Arg)	15	ZF	25	N	25 (S)	10 (W)	0	Cured
wt		2.5	ZF	0	N	0	0		
KCNJ5	c.433G>C (p.Glu145Gln)	9	ZG + ZF	30	N	70 (W)	20 (S)	0	Impr
wt		9	ZF	5	N	0	40 (S)		
wt		7	ZF	10	N	0	60 (S)		
wt		7	ZF	10	N	50 (S)	50 (S)		
wt		6	ZF	5	N	0	40 (S)		
wt		5	ZG + ZF	5	N	0	40 (S)		
wt		3	ZF	0	N	0	40 (S)		
ATP2B3	c.1272_1277del (p. (Leu425_Val426del))	9	ZG	0	N	100 (S)	0	0	Failed
ATP2B3	c.1272_1277del (p. (Leu425_Val426del))	9	ZG + ZF	0	N	80 (S)	10 (S)	0	Cured
ATP2B3	c.1272_1277del (p. (Leu425_Val426del))	7	ZG	0	Y	90 (S)	0	0	Cured
ATP2B3	c.1272_1277del (p. (Leu425_Val426del))	8	ZG	0	N	60 (W)	20 (W)	0	Impr
ATP2B3	c.1269_1274del (p. (Leu425_Val426del))	8	ZG	0	Y	90 (S)	0	1	Impr
ATP1A1	c.311T>G (p.Leu104Arg)	13	ZG	15	Y	80 (S)	30 (W)	1	Impr
wt		10	ZF	10	N	0	30 (S)		
wt		6	ZG + ZF	5	N	0	40 (S)	3	Failed
CACNA1D	c.2250C>G (p.Ile1750Met) 1750Met)	5	ZG	0	Y	100 (S)	0		
Wt		3	ZG	0	Y	100 (S)	0		
wt		19	ZG + ZF	40	N	80 (S)	50 (W)	0	Impr
wt		10	ZG + ZF	0	N	80 (W)	20 (W)	0	Cured
wt		5	ZG	5	N	90 (S)	0	0	Cured
wt		23	ZG + ZF	5	N	0	66 (S/W)	4	Failed
wt.		4	ZG + ZF	0	N	0	0		
wt		18	ZG	0	Y	100 (S)	0	3	Impr
wt		5	ZG + ZF	0	N	0	40 (S)		
wt		10	ZF	0	N	100 (S)	10 (S)	3	Impr
wt		3	ZF	0	N	100 (S)	0		
wt		9	ZG + ZF	0	Y	100 (S)	0	2	Impr
wt		2	ZF	5	N	0	50 (W)		
wt		8	ZG + ZF	10	N	100 (S)	10 (W)	0	Failed
wt		4	ZG + ZF	0	N	0	70 (S)		
wt		8	ZG	0	Y	100 (S)	25 (W)	3	Impr
wt		4	ZF	0	N	0	70 (S)		
wt		7	ZF	0	N	80 (S)	0	0	Failed
wt		3	ZG + ZF	0	N	0	40 (S)		

(*Continued*)

Table 2. Continued

	Preoperative								Pathology			
Number	M/F	Age, y	SBP	DBP	K^+	Ald	ARR	CT/AVS	Classification	ZG	ZG Ø (μm)	Node
40	M	62	211	112	2.6	0.78	0.09	AVS	Multinodular	Y	250	1 2
41	M	62	226	120	3.2	0.95	0.48	AVS	Multinodular	Y	135	1 2
42	M	56	160	88	2.8	0.34	0.03	AVS	Multinodular	Y	80	1 2
43	M	61	140	90	2.9	2.08	1.04	AVS	Multinodular	Y	237	1 2
44	M	53	179	125	3.7	0.44	0.10	AVS	Multinodular	Y	300	1 2
45	M	59	148	98	2.9	0.50	0.16	AVS	Multinodular	Y	125	1 2 3
46	M	45	171	99	2.9	1.51	0.76	AVS	Multinodular	Y	463	1 2 3 4
47	M	55	132	76	4.4	0.40	0.02	AVS	Multinodular	Y	250	1 2 3 4
48	M	63	196	115	3.4	0.34	0.03	CT	Undefined§	Y	124	1
49	M	53	149	83	3.8	0.68	0.23	CT	Undefined§	n.a.	n.a.	1
Mutation not assessable												
50	F	44	200	120	3.5	0.81	0.06	CT	Adenoma	Y	265	1
51	F	48	126	82	4.0	1.10	0.52	CT	Adenoma	N	73	1
52	M	58	169	94	3.3	0.53	0.05	CT	Multinodular	N	80	1 2
53	M	48	151	105	3.1	0.45	0.09	AVS	Multinodular	Y	274	1 2

Abbreviations: ald, aldosterone (nanomoles); APCC, n, number of aldosterone-producing cell clusters, not assessable because of fragmented adrenal gland; impr, improved; cell comp., cell composition; DBP, diastolic blood pressure; diag, diagnostic strategy; F, female; K^+, serum potassium; M, male; n, no; n.a., not assessable; S, strong; SB, spironolactone bodies; SBP, systolic blood pressure; W, weak; wt, wild-type; y, yes; ZG Ø, ZG thickness (in micrometers).

ble 2). Two adrenal glands that were severely damaged during adrenalectomy and consisted of tissue fragments only (numbers 48 and 49) could not be classified as adenoma or nodular hyperplasia, and in one of these, ZG characteristics could not be assessed. These two glands were counted as containing one nodule each. One nodule could not be assessed for immunohistochemistry because the immunostainer had not covered the entire specimen (number 41). DNA analysis was unsuccessful in four nodules (numbers 50, 51, 52.1, and 53.2) because of DNA quality.

Histopathological phenotype

The resected adrenal glands were classified as containing either a solitary adenoma or nodular hyperplasia in 23 of 51 and 28 of 51 of the cases, respectively (Table 4). Using multivariate analysis, solitary adenomas were more often found in female patients and in younger patients (not shown). We found no relationship between body mass index (BMI), blood pressure (BP), potassium or aldosterone levels at presentation, and histopathological classification. Adrenals of patients in whom the diagnosis of unilateral APA was based on CT scan contained a solitary adenoma more frequently than the adrenals of patients in whom adrenalectomy was based on AVS. Most adrenals (43 of 52, 83%) demonstrated ZG hyperplasia, which did not differ significantly between glands with solitary adenoma and those with multiple nodules. Of the 98 individually assessed nodular structures, 15 (15%) were composed of ZG-like cells, 38 (39%) of ZF-like cells, and 45 (46%) of a combination of the two. The size of the largest nodule in each adrenal did not differ significantly between those classified as adenoma and those classified as nodular hyperplasia.

Immunohistochemistry

Most adrenal glands (42 of 52 assessable glands) contained one single nodule positive for p450C18 expression (ie, aldosterone production), with all other nodules in the same gland, when present, being negative. With the exception of two cases (numbers 15 and 29), this always concerned the largest nodule present. Five glands (numbers 17, 18, 22, 29, and 35) contained an additional nodule positive for p450C18 staining, whereas five other samples (numbers 33, 42, 43, 46, and 53) showed p450C18 expression in none of the nodules studied. Mutated nodules always expressed p450C18. P450C11 expression (ie, cortisol production) was present in most of the nodules

Table 2. Continued

Genetics			(Immuno) Histology						Postoperatively
Gene	Mutation	Size, mm	Cell Comp.	Atypic Cells, %	SB	p450C18 (Stain), %	p450C11 (Stain), %	APCC, n	Outcome
wt		10	ZG + ZF	5	N	80 (S)	50 (W)	1	Impr
wt		5	ZF	5	N	0	50 (S)		
wt		9	ZG	0	Y	80 (S)	40 (S/W)	1	Impr
wt		7	ZG	0	N	n.a.	n.a.		
wt		6	ZG + ZF	5	N	0	40 (S)	2	Impr
wt		4	ZG	5	N	0	40 (S)		
wt		6	ZF	5	N	0	66 (W)	2	Failed
wt		3	ZG + ZF	0	N	0	50 (S)		
wt		4	ZG + ZF	0	N	100 (S)	0	0	
wt		3	ZG + ZF	0	N	0	40 (S)		
wt		10	ZG	5	N	70 (S)	0	2	Impr
wt		5	ZG + ZF	10	N	0	40 (S)		
wt		4	ZG + ZF	5	N	0	40 (S)		
wt		15	ZF	20	N	0	50 (W)	0	Failed
wt		4	ZG + ZF	0	N	0	40 (S)		
wt		4	ZG + ZF	0	N	0	40 (S)		
wt		4	ZG + ZF	0	N	0	40 (S)		
wt		10	ZG + ZF	5	N	0	80 (S)	4	Cured
wt		4	ZG	0	N	0	80 (S)		
wt		3	ZG + ZF	25	Y	100 (S)	0		
wt		2.5	ZF	5	N	0	20 (W)		
wt		5	ZG + ZF	20	N	60(S)	40 (S)	1	Impr
wt		10	ZG + ZF	5	Y	80 (S)	30 (S)	0	Failed (rec.)
n.a.		19	ZF	5	N	30 (S/W)	66 (S/W)	0	Impr
n.a.		10	ZF	20	N	20 (W)	80 (S)	0	Cured
n.a.		16	ZF	10	N	10 (S)	60 (S)	—	Impr
wt		6	ZF	5	N	0	40 (S)		
wt		5	ZG + ZF	5	N	0	40 (S)	2	Impr
n.a.		4	ZG + ZF	0	N	0	50 (S)		

and was inversely related to the P450C18 expression ($r_s = -0.504$, 95% confidence interval -0.644 to -0.330, $P < .0001$). APCCs were found in 29 of the glands (55%), ranging from one to nine APCCs in total number in the assessed slides, with cell cluster diameters ranging from 0.2 mm to 1.2 mm. However, because the slides did not represent the whole gland, it is likely that this number is higher. We could not establish a relation between the presence of APCCs and patient characteristics, histopathology, or immunohistochemistry of the adrenal gland.

Genotyping: *KCNJ5*

KCNJ5 mutations were present in 13 (62%) and nine (32%) of the assessable adrenals classified as a solitary adenoma and nodular hyperplasia, respectively, adding up to a total of 22 affected glands (42%). The mutation was more frequently present in female patients compared with male patients (65% vs 23%, $P < .01$). No relationship between age, BMI, BP, potassium, or aldosterone levels and the presence of a *KCNJ5* mutation was found in univariate or multivariate analysis. Nodules containing

Table 3. Criteria for Cure or Improvement of PA at Follow-Up

Definition	Criteria
Cure	DBP less than 90 mm Hg and SBP less than 140 mm Hg, no antihypertensive medications, serum potassium 3.5 mmol/L or greater
	Normal SIT (posttest aldosterone <0.28 nmol/L) or ARR less than 0.09 nmol/mE
Improvement	DBP less than 90 mm Hg and/or SBP less than 140 mm Hg on the same or reduced number of medications (or reduced number of defined daily doses as described by the World Health Organization) or a reduction in DBP by at least 15 mm Hg on the same or reduced number of medications
	Serum potassium 3.5 mmol/L or greater
	Normal SIT (posttest aldosterone <0.28 nmol/L) or ARR less than 0.09 nmol/mE
Failure	No change or inability to meet above criteria for cure or improvement

Abbreviations: DBP, diastolic BP; NS, not significant; SBP, systolic BP.

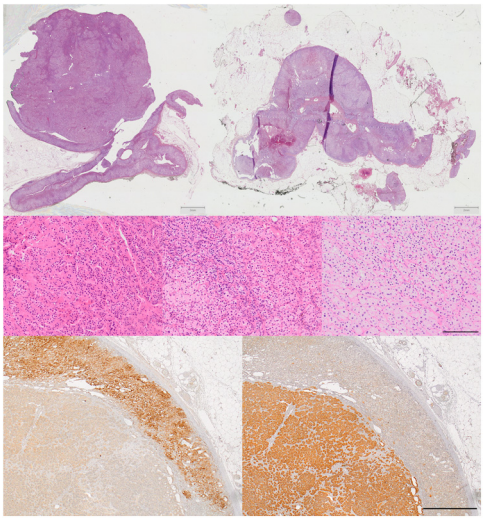

Figure 1. Examples of histopathological and immunohistochemical features of adrenal glands in primary aldosteronism. Upper row, left panel, Solitary adenoma; right panel, multinodular hyperplasia (HE staining); middle row, left panel, ZG-like cells; middle panel, ZG-like + ZF-like cells; right panel, ZF-like cells (HE staining) lower row, immunohistochemistry; left panel, p450C11 staining, 0% of adenoma cell surface positive; right panel, p450C18, 100% of adenoma cell surface positive.

the *KCNJ5* mutation consisted of ZF-like cells more often compared with those wild-type for *KCNJ5* (61% vs 28%, $P = .04$). *KCNJ5* mutations were never present in nodules consisting of only ZG-like cells. Nodules with the *KCNJ5* mutation showed more atypical cells than those without the mutation [median 20% (range 0%–40%) vs median: 5% (range 0%–40%) $P < .001$].

Genotyping: *ATP1A*, *ATP2B3*, and *CACNA1D*

ATP2B3 mutations were found in five nodules of adrenal glands (9%), all classified as solitary adenoma (numbers 23–27). Four of five patients were male and in these patients the mutated nodules consisted of ZG-like cells only. None of the nodules showed atypical cells. Nodules containing an *ATP2B3* mutation were significantly smaller than those containing a *KCNJ5* mutation (8.2 mm vs 14.9 mm, $P < .001$) and had a higher P450C18 expression (94% vs 44%, $P < .001$) and lower p450C11 expression (6% vs 36%, $P = .02$). One *ATP1A1* and one *CACNA1D* mutation were both found in two male patients (numbers 28 and nr 29, respectively) in a nodule of a multinodular gland, consisting of ZG-like cells only.

Multinodular adrenal glands

Regardless of the number of nodules in the 11 of 28 multinodular glands that contained a somatic mutation of one of the four genes we studied, the mutation was present in only one of the nodules in each individual gland. All other nodules within the same adrenal did not contain one of these somatic mutations, except for gland number 18 that contained two P450C18 positive nodules, each containing a different *KCNJ5* mutation (Leu168Arg and Gly151Arg) (Figure 2). The first nodule consisted of both ZG-like and ZF-like cells, whereas the second consisted of only ZF-like cells. Both nodules expressed P450C18 in a relatively high percentage of the cells and contained many atypical cells. A third nodule within the same adrenal gland was negative for both *KCNJ5* mutations and P450C18 expression.

Treatment outcome

At follow-up 13 patients (25%) were cured, 26 (49%) had improved, and 14 (26%) had no improvement. One patient had recurrence of disease because of incomplete adrenalectomy (number 40). Four patients did not undergo repeated biochemical testing because they were lost to follow-up (n = 3) or deceased (n = 1, melanoma). Patients whose adrenal contained a solitary adenoma were cured more often at follow-up compared with patients with an adrenal gland showing nodular hyperplasia (Table 4). In those with nodular hyperplasia, there was no correlation between the number of nodules and treatment outcome. Of the five cases that did not express p450C18 in any of the adrenal nodules, three had treatment failure. Two of these three had been diagnosed by AVS and one by CT scanning.

Of the 29 patients with a proven mutation, nine (31%) were cured, 13 (45%) improved, and seven (24%) had failure of treatment compared with 3 (15%), 10 (50%), and seven (35%) of the 20 patients without a proven mutation, respectively, which was not significantly different. Neither did we find a difference in the treatment outcome between the patients with different types of mutations.

Discussion

The present study shows that a majority of adrenal glands removed because of the suspicion of a unilateral APA demonstrate hyperplasia instead of adenoma, similar to the observation recently reported by Iacobone et al (14). *KCNJ5* mutations were present in 42% of the glands studied, which is in line with previous studies (8, 25, 26, 28–30). As in these studies, *KCNJ5* mutations in our cohort were more often present in nodules with a ZF-like cell type and were more frequently found in female patients (8, 25). The *ATP2B3*, *ATP1A1*, and *CACNA1D* mutations, present in seven patients, were predominantly present in ZG-like nodules of male patients. Mutations were found in adrenals with solitary adenomas and in adrenals with nodular hyperplasia. In a study that focused more on clinical details, Akerstrom et al (8) described the presence of

Table 4. Differences in Patient Characteristics, Histopathology, Genotyping and Treatment Outcome in Adrenal Glands Containing Either Adenoma or Nodular Hyperplasia

	Adenoma (n = 23, 23 Nodules)[a]	Nodular Hyperplasia (n = 28, 73 Nodules)[a]
Patient characteristics		
Gender, male	6/23 (20%)	22/28 (73%)[b]
Age, y	43.2 ± 9.7	54.9 ± 8.0[b]
Diagnostic strategy		
CT scan	11/23 (49%)	5/28 (18%)
AVS	12/23 (52%)	23/28 (82%)
Histopathological characteristics		
ZG hypertrophy	19/23 (83%)	23/28 (82%)
Size (largest) nodule, mm	12 (5–23)	10 (4–23)
Cell type		
ZF-like	11/23 (48%)	27/73 (37%)
ZG-like	5/23 (22%)	10/73 (14%)
ZG+ZF-like	7/23 (30%)	36/73 (49%)
Genotyping		
KCNJ5	13/23 (57%)	9/28 (32%)
ATP1A1	0/23 (0%)	1/28 (4%)
ATP2B3	5/23 (22%)	0/28 (0%)[c]
CACNA1D	0/23 (0%)	1/28 (4%)
Wild type	3/23 (13%)	15/28 (54%)[c]
Not assessable	2/23 (9%)	2/28 (7%)
Treatment outcome		
Cured	10/23 (43%)	3/28 (10%)[c]
Improved	10/23 (43%)	16/28 (53%)
Failed	3 /23 (14%)	11/28 (37%)

[a] Two glands could not be classified because they contained either adenoma or nodular hyperplasia due to severe tissue damage.

[b] Different from adenoma (significance level $P < .0001$).

[c] Different from adenoma (significance level $P < .05$).

KCNJ5 mutations in adrenals classified as adenoma with associated hyperplasia as well.

Our study adds to previous reports that most of the removed glands, regardless of whether they are classified as adenoma or nodular hyperplasia, contain one nodule, usually the largest in multinodular glands, expressing p450C18 and that mutations were found in only these p450C18-positive nodules. It can be surmised that the remaining p450C18-positive nodules (like 30, 31, 32, 34.1, etc) contain other, hitherto unidentified, mutations that cause aldosterone hypersecretion. This would lead to the hypothesis that in each (multinodular) gland the aldosterone hypersecretion can be attributed to one (or rarely two) mutated nodule(s). Whether the mutations found are causative in the development of the nodules remains to be proven because, if so, we would expect that within one gland each nodule contains a mutation, which was not the case in our study. A more plausible explanation is that the mutations are causative in aldosterone hypersecretion but not in nodulation itself, which is also suggested by the functional effects of the mutations (22, 24). The overall hypothesis that we propose therefore is that some individuals for some reason develop multinodular adrenal cortices with ZG thickening and that only if a mutation occurs, for instance in *KCNJ5*, *ATP1A1*, *ATP2B3*, or *CACNA1D*, but possibly also in other as-yet-unidentified genes, the clinical syndrome of PA develops. An intriguing question then is whether the contralateral adrenal gland is normal or that similar changes, perhaps to a lesser degree, are present. Because we clearly cannot obtain these contralateral glands, we cannot answer this question. Recent case reports describe the development of APAs on the contralateral or ipsilateral site in patients operated for APA (31, 32), which might be explained by newly arisen mutations in these glands. The questions, however, why a patient develops (unilateral or bilateral) multinodular cortices and why simultaneous ZG thickening occurs remain unanswered.

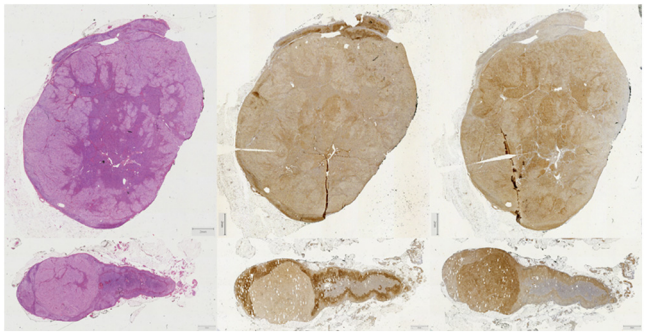

Figure 2. Two mutated nodules within one adrenal gland (number 26, Table 2). From left to right, HE staining, p450C11 staining, and p450C18 staining. Upper panels, Node 1 with KCNJ5 (c.451G>A) mutation. Bottom panels, Node 2 with KCNJ5 (c.503T>G) mutation.

Our study showed some interesting associations between the histopathological phenotype, immunohistochemistry, and the genotype. For instance, KCNJ5 mutations were present in adrenal glands classified as both solitary adenoma or nodular hyperplasia, whereas ATP2B3 mutations were found only in solitary adenomas. ATP1A1 and CACNA1D were both found in a multinodular gland. KCNJ5-mutated nodules were rather large, often consisted of ZF-like cells and showed a relatively high number of atypical cells. On the contrary, all five ATP2B3-mutated tumors were less than 1 cm, consisted mainly of ZG-like cells and had no atypical cells, which was also the case in the tumor with the CACNA1D mutation. Concerning immunohistochemistry, most KCNJ5 mutations had strong staining for both P450C18 and P450C11. All ATP2B3 mutations had a strong staining for P450C18, whereas staining for P450C11 was absent or weak, suggesting predominant expression of aldosterone synthase. The number of mutations is, however, not large enough to determine whether these patients have higher aldosterone levels or higher BP, nor can we derive yet from the histological features with 100% certainty which mutations should be looked for in the first place.

In five patients no nodules positive for p450C18 expression were found. This can be explained by several mechanisms. First, aldosterone production can be attributed to APCCs. Four of the five adrenals lacking a p450C18-positive nodule contained multiple APCCs. In their cohort Nanba et al (13) also found APCCs in adrenals containing a p450C18-negative nodule. However, because APCCs are also present in normal adrenal tissues and their ontogeny is unknown (18), it is unclear whether these cell clusters can be responsible for the aldosterone excess in PA. Second, the cross-section of the adrenal gland that was chosen by the pathologist may have missed the nodule responsible for aldosterone production in the multinodular adrenals. Especially for micronodular glands without evident nodules at macroscopy, this might have been a problem. Third, it is possible that, despite thorough patient screening, the initial diagnosis of primary aldosteronism was not accurate because specificity for the ARR and saline infusion test may not be 100% (33–35). Also, the diagnosis of unilateral APA established by AVS and/or CT scan could have been inaccurate with the consequence that the patient was falsely operated because CT is known for its possible misclassification, just like AVS is susceptible to interpretative error (24, 36, 37).

The clinical implication of our findings, in terms of prediction of treatment outcome, remains to be determined. We did not find a difference in treatment outcome between patients with different types of mutations. However, if it were possible to determine whether a mutation is present in an adrenal gland before adrenalectomy, this might be most helpful for the decision to proceed to adrenalectomy or not. As yet, there is no possibility to assess the presence of somatic mutations in adrenal glands in vivo, but perhaps new forms of specific imaging or composition of adrenal venous blood might provide this information.

Our study had some limitations. A diagnostic work-up was not performed uniformly because our retrospective study spanned a long period of time in which diagnostic strategies changed from CT scan to AVS. Although it has not been indisputably established, some clinicians and researchers regard CT scan to be potentially misleading in PA diagnostic work-up (24). Because solitary adenomas were more easily diagnosed by CT scan in our study, it is possible that the use of CT scan has led to an inclusion bias toward patients with a solitary adenoma. However, because this study was not primarily designed to evaluate the prevalence of unilateral adrenal hyperplasia, this is of minor importance. Another limitation associated with the retrospective approach of the study is that the follow-up data of some patients were incomplete. However, essential information on outcome could be retrieved for all patients included. We could not assess the presence of germline mutations in our patients, but it is unlikely that germline mutations were present, given that in all multinodular glands at least one of the nodules did not contain mutations in the four genes, although this does not exclude the possibility of mosaic mutations.

In conclusion, the concept that primary aldosteronism is caused by either an APA or bilateral adrenal hyperplasia needs to be reconsidered. Most adrenal glands with supposedly unilateral aldosterone production display multinodular pathology. In these cases the largest nodule is generally p450C18 positive, and in more than half of all cases, this nodule also contains a *KCNJ5*, *ATP1A1*, *ATP2B3*, or *CACNA1D* mutation. These mutations probably occur after nodule formation because in multinodular samples only one of the nodules contains the mutation and because in one of our cases there were even two nodules that each contained a different KCNJ5 mutation. These findings and the presence of ZG hypertrophy need further investigation to understand the pathogenesis of primary aldosteronism. The relevance of these findings for clinical management remains to be determined.

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References

1. **Funder JW, Carey RM, Fardella C, et al.** Case detection, diagnosis, and treatment of patients with primary aldosteronism: an endocrine society clinical practice guideline. *J Clin Endocrinol Metab*. 2008; 93:3266–3281.
2. **Mulatero P, Stowasser M, Loh KC, et al.** Increased diagnosis of primary aldosteronism, including surgically correctable forms, in centers from five continents. *J Clin Endocrinol Metab*. 2004;89: 1045–1050.
3. **Satoh F, Abe T, Tanemoto M, et al.** Localization of aldosterone-producing adrenocortical adenomas: significance of adrenal venous sampling. *Hypertens Res*. 2007;30:1083–1095.
4. **Lo CY, Tam PC, Kung AW, Lam KS, Wong J.** Primary aldosteronism. Results of surgical treatment. *Ann Surg*. 1996;224:125–130.
5. **Fogari R, Preti P, Zoppi A, Rinaldi A, Fogari E, Mugellini A.** Prevalence of primary aldosteronism among unselected hypertensive patients: a prospective study based on the use of an aldosterone/renin ratio above 25 as a screening test. *Hypertens Res*. 2007;30:111–117.
6. **Enberg U, Volpe C, Hoog A, et al.** Postoperative differentiation between unilateral adrenal adenoma and bilateral adrenal hyperplasia in primary aldosteronism by mRNA expression of the gene CYP11B2. *Eur J Endocrinol*. 2004;151:73–85.
7. **Scholl UI, Nelson-Williams C, Yue P, et al.** Hypertension with or without adrenal hyperplasia due to different inherited mutations in the potassium channel KCNJ5. *Proc Natl Acad Sci USA*. 2012;109: 2533–2538.
8. **Akerstrom T, Crona J, Delgado Verdugo A, et al.** Comprehensive re-sequencing of adrenal aldosterone producing lesions reveal three somatic mutations near the KCNJ5 potassium channel selectivity filter. *PloS One*. 2012;7:e41926.
9. **Murashima M, Trerotola SO, Fraker DL, Han D, Townsend RR, Cohen DL.** Adrenal venous sampling for primary aldosteronism and clinical outcomes after unilateral adrenalectomy: a single-center experience. *J Clin Hypertens (Greenwich)*. 2009;11:316–323.
10. **Proye CA, Mulliez EA, Carnaille BM, et al.** Essential hypertension: first reason for persistent hypertension after unilateral adrenalectomy for primary aldosteronism? *Surgery*. 1998;124:1128–1133.
11. **Tresallet C, Salepcioglu H, Godiris-Petit G, Hoang C, Girerd X, Menegaux F.** Clinical outcome after laparoscopic adrenalectomy for primary hyperaldosteronism: the role of pathology. *Surgery*. 2010; 148:129–134.
12. **Weisbrod AB, Webb RC, Mathur A, et al.** Adrenal histologic findings show no difference in clinical presentation and outcome in primary hyperaldosteronism. *Ann Surg Oncol*. 2013;20:753–758.
13. **Nanba K, Tsuiki M, Sawai K, et al.** Histopathological diagnosis of primary aldosteronism using CYP11B2 immunohistochemistry. *J Clin Endocrinol Metab*. 2013;98:1567–1574.
14. **Iacobone M, Citton M, Viel G, et al.** Unilateral adrenal hyperplasia: a novel cause of surgically correctable primary hyperaldosteronism. *Surgery*. 2012;152:1248–1255.
15. **Sigurjonsdottir HA, Gronowitz M, Andersson O, et al.** Unilateral adrenal hyperplasia is a usual cause of primary hyperaldosteronism. Results from a Swedish screening study. *BMC Endocrine Disorders*. 2012;12:17.
16. **Quillo AR, Grant CS, Thompson GB, Farley DR, Richards ML, Young WF.** Primary aldosteronism: results of adrenalectomy for nonsingle adenoma. *J Am Coll Surg*. 2011;213:106–112; discussion 112–113.
17. **Ganguly A.** Cellular origin of aldosteronomas. *Clin invest*. 1992; 70:392–395.
18. **Nishimoto K, Nakagawa K, Li D, et al.** Adrenocortical zonation in humans under normal and pathological conditions. *J Clin Endocrinol Metab*. 2010;95:2296–2305.
19. **Shigematsu K, Yamaguchi N, Nakagaki T, Sakai H.** A case of unilateral adrenal hyperplasia being difficult to distinguish from aldosterone-producing adenoma. *Exp Clin Endocrinol Diabetes*. 2009; 117:124–128.
20. **Fallo F, Pezzi V, Barzon L, et al.** Quantitative assessment of CYP11B1 and CYP11B2 expression in aldosterone-producing adenomas. *Eur J Endocrinol*. 2002;147:795–802.
21. **Boulkroun S, Samson-Couterie B, Dzib JF, et al.** Adrenal cortex remodeling and functional zona glomerulosa hyperplasia in primary aldosteronism. *Hypertension*. 2010;56:885–892.
22. **Choi M, Scholl UI, Bjorklund P, et al.** K+ channel mutations in adrenal aldosterone-producing adenomas and hereditary hypertension. *Science*. 2011;331:768–772.
23. **Beuschlein F, Boulkroun S, Osswald A, et al.** Somatic mutations in ATP1A1 and ATP2B3 lead to aldosterone-producing adenomas and secondary hypertension. *Nat Genet*. 2013;45:440–444.
24. **Azizan EA, Poulsen H, Tuluc P, et al.** Somatic mutations in ATP1A1 and CACNA1D underlie a common subtype of adrenal hypertension. *Nat Genet*. 2013;45:1055–1060.
25. **Azizan EA, Murthy M, Stowasser M, et al.** Somatic mutations affecting the selectivity filter of KCNJ5 are frequent in 2 large unselected collections of adrenal aldosteronomas. *Hypertension*. 2012; 59:587–591.
26. **Azizan EA, Lam BY, Newhouse SJ, et al.** Microarray, qPCR, and KCNJ5 sequencing of aldosterone-producing adenomas reveal differences in genotype and phenotype between zona glomerulosa- and zona fasciculata-like tumors. *J Clin Endocrinol Metab*. 2012;97(5):E819–E829.
27. **Rundback JH, Sacks D, Kent KC, et al.** Guidelines for the reporting of renal artery revascularization in clinical trials. American Heart Association. *Circulation*. 2002;106:1572–1585.
28. **Monticone S, Hattangady NG, Nishimoto K, et al.** Effect of KCNJ5 mutations on gene expression in aldosterone-producing adenomas and adrenocortical cells. *J Clin Endocrinol Metab*. 2012;97:E1567–E1572.
29. **Taguchi R, Yamada M, Nakajima Y, et al.** Expression and mutations of KCNJ5 mRNA in Japanese patients with aldosterone-producing adenomas. *J Clin Endocrinol Metab*. 2012;97:1311–1319.
30. **Xekouki P, Hatch MM, Lin L, et al.** KCNJ5 mutations in the National Institutes of Health cohort of patients with primary hyperaldosteronism: an infrequent genetic cause of Conn's syndrome. *Endocr Relat Cancer*. 2012;19:255–260.
31. **Rizek P, Gorecki P, Lindenmayer A, Moktan S.** Laparoscopic adrenalectomy for bilateral metachronous aldosteronomas. *JSLS J Soc Laparoendosc Surg*. 2011;15:100–104.
32. **Calvo-Romero JM, Ramos-Salado JL.** Recurrence of adrenal aldosterone-producing adenoma. *Postgrad Med J*. 2000;76:160–161.
33. **Schwartz GL, Chapman AB, Boerwinkle E, Kisabeth RM, Turner ST.** Screening for primary aldosteronism: implications of an increased plasma aldosterone/renin ratio. *Clin Chem*. 2002;48:1919–1923.
34. **Jansen PM, Boomsma F, van den Meiracker AH, Dutch ARRAT Investigators.** Aldosterone-to-renin ratio as a screening test for primary aldosteronism—the Dutch ARRAT Study. *The Neth J Med*. 2008;66:220–228.
35. **Salva M, Cicala MV, Mantero F.** Primary aldosteronism: the role of confirmatory tests. *Horm Metab Res*. 2012;44:177–180.
36. **Kempers MJ, Lenders JW, van Outheusden L, et al.** Systematic review: diagnostic procedures to differentiate unilateral from bilateral adrenal abnormality in primary aldosteronism. *Ann Intern Med*. 2009;151:329–337.
37. **Kline GA, Harvey A, Jones C, et al.** Adrenal vein sampling may not be a gold-standard diagnostic test in primary aldosteronism: final diagnosis depends upon which interpretation rule is used. Variable interpretation of adrenal vein sampling. *Int Urol Nephrol*. 2008; 40:1035–1043.