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Somatic mutations in adrenocortical carcinoma with primary aldosteronism or hyperreninemic hyperaldosteronism

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Abstract

Several somatic mutations specific to aldosterone-producing adenomas (APAs) have been described. A small proportion of adrenocortical carcinomas (ACCs) are associated with hyperaldosteronism, either primary aldosteronism or hyperreninemic hyperaldosteronism. However, it is unknown whether they harbor mutations of the same spectrum as APAs. The objective of this study is to describe the clinical phenotype and molecular genotype of ACCs with hyperaldosteronism, particularly the analysis for common APA-associated genetic changes. Patients were identified by retrospective chart review at a specialized referral center and by positive staining for CYP11B2 of tissue microarrays. Twenty-five patients with ACC and hyperaldosteronism were initially identified by retrospective chart review, and tissue for further analysis was available on 13 tumors. Seven patients were identified by positive staining for CYP11B2 in a tissue microarray, of which two were already identified in the initial chart review. Therefore, a total number of 18 patients with a diagnosis of ACC and features of either primary aldosteronism or hyperreninemic hyperaldosteronism were therefore included in the final study. Mutational status for a select list of oncogenes, tumor suppressor genes and genes known to carry mutations in APAs were analyzed by next-generation sequencing. Review of clinical data suggested autonomous aldosterone production in the majority of cases, while for some cases, hyperreninemic hyperaldosteronism was the more likely mechanism. The mutational landscape of ACCs associated with hyperaldosteronism was not different from ACCs with a different hormonal phenotype. None of the ACCs harbored mutations of known APA-associated genes, suggesting an alternative mechanism conferring aldosterone production.

Key Words

- ▶ primary aldosteronism
- ▶ adrenocortical carcinoma
- ▶ next-generation sequencing
- ▶ hyperreninemic hyperaldosteronism

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Introduction

Primary aldosteronism (PA) is the most common identifiable endocrine cause for hypertension and increased aldosterone levels are thought to pose health risk due to direct effects on the vasculature, heart and kidney. Most patients with PA have a benign adrenal adenoma or bilateral hyperplasia. Adrenocortical carcinoma (ACC), however, is a very rare cause of increased aldosterone levels, and a recent literature review identified only 58 reported patients (Seccia *et al.* 2005). ACC is a malignant tumor with an often unfavorable prognosis (Else *et al.* 2014a). Roughly 50–60% of ACCs produce active steroid hormones, most often glucocorticoids and/or adrenal androgens leading to Cushing syndrome and/or virilization and hirsutism. Only 3–5% of all ACC patients are reported to have suspected high aldosterone levels (Seccia *et al.* 2005, Else *et al.* 2014a,b). Currently it is unknown whether there are any genetic differences between aldosterone-producing and non-aldosterone-producing ACC with regards to their mutational landscape.

Most benign aldosterone-producing adenomas have mutations in ion channels affecting membrane conductance and polarization and subsequently intracellular calcium levels (Monticone *et al.* 2015). In particular, somatic mutations in *KCNJ5*, *ATP1A1*, *ATP2B3* and *CACNA1D* and a germline mutation in *CACNA1H* have been described (Choi *et al.* 2011, Beuschlein *et al.* 2013, Scholl *et al.* 2013, 2015).

Recently, the somatic mutational landscape of ACC has been described in three large studies (Assie *et al.* 2014, Juhlin *et al.* 2015, Zheng *et al.* 2016). The most common alterations are found in pathways involved in cell cycle regulation, telomere maintenance and WNT signaling. However, no mutations in any of the genes mutated in aldosterone-producing adenomas had been described in ACC.

In this study, our goal was to identify mutations in a set of genes known to confer aldosterone production in APA in ACCs associated with hyperaldosteronism.

Methods

Tissue microarray (TMA) building

Formalin-fixed, paraffin-embedded tissue blocks (FFPE) of 141 cases of adrenal cortical cancer, representing 128 patients, were obtained from the files of the Department of Pathology, University of Michigan Medical Center, Ann Arbor, MI, USA and contributing institutions. After pathological review, four TMAs were constructed

from the most representative non-necrotic area using the methodology of Nocito *et al.* (2001). Each case was represented in the array by three 1 mm diameter cores. Control tissue (ACA, tonsil, testes, kidney, liver and normal adrenal) and colored orientation cores were also arrayed. Cases were randomized regarding placement in the TMAs.

Immunohistochemistry

Formalin-fixed, paraffin sections from the four TMAs were cut at 5 microns and rehydrated to water. Heat-induced epitope retrieval was performed with FLEX TRS High pH Retrieval buffer (9.01) for 20 min. After peroxidase blocking, the antibody CYP11B2 mouse monoclonal (41–17B, kindly provided by Dr. Celso Gomez, clone RRID: AB_2650562) (Gomez-Sanchez *et al.* 2014) was applied at a dilution of 1:1500 at room temperature for 60 min. The FLEX HRP EnVision System was used for detection. DAB chromagen was then applied for 10 min. Slides were counterstained with Harris Hematoxylin for 5 s and then dehydrated and coverslipped.

TMA slides stained with CYP11B2 were scored based on staining distribution on a 1–3 grading system (<10%: 1+, 10–50%: 2+, >50%: 3+). Cases which showed possible staining were identified and corresponding full slides were cut and stained for CYP11B2 by the same method as described above. This cohort of full slides was then scored using the same methods as above. The cases identified to show true staining (2+ and 3+) were characterized by strong staining and contained a mixture of focal and diffuse positivity. Clinical data were then captured for the cases identified.

Retrospective chart review

Charts were reviewed for laboratory characteristics (pretreatment levels of renin, aldosterone (urine and serum) and potassium), mentioning of diagnosis of PA in physician notes, potassium replacement therapy. Any patient with a physician note stating aldosterone production was included in the initial study as well as patients with laboratory constellation suggestive of PA. The Endocrine Society Guideline suggestions for screening for PA were considered.

DNA extraction

Genomic DNA (gDNA) was extracted from unstained FFPE slides for AC100–112 and YY for AC113–119, using

the AllPrep DNA/RNA FFPE kit (QIAGEN) as described previously (Warrick *et al.* 2015).

Next-generation sequencing

For each sample, 20 ng of isolated gDNA was used for barcoded library generation by multiplexed PCR using two custom Ion AmpliSeq Panels covering aldosterone-associated genes (APAv1 for AC100-112 and APAv2 for AC113-119) and cancer-associated genes (OCPv2 for AC100-119) and the Ion AmpliSeq Library kit 2.0 (Life Technologies) according to the manufacturer's instructions. APAv1 was designed to target genes previously shown to be mutated in APA or other adrenal hyperplasias/neoplasms, contains 310 independent primer pairs targeting the entire coding regions of genes with reported somatic mutations in APA (*KCNJ5*, *ATP1A1*, *ATP2B3*, *CACNA1D*), genes shown to harbor germline or somatic variants associated with adrenal hyperplasia (phosphodiesterase 11A (*PDE11A*), phosphodiesterase 8B (*PDE8B*), protein kinase, cAMP-dependent, regulatory, type 1, (*PRKAR1A*)). APAv2 contains 499 independent primer pairs targeting the entire coding regions of genes in APAv1 and genes shown to harbor germline or somatic variants associated with other adrenal diseases (protein kinase, cAMP-dependent, catalytic, (*PRAKACA*) and armadillo repeat-containing 5 (*ARMC5*)), α -1H subunit of T-type voltage-dependent Ca(2+) channels (*CACNA1H*) and oncogene hot spots in guanine nucleotide-binding protein subunit (*GNAS*) and β -catenin (*CTNNB1*). Template preparation and NGS of multiplexed templates were performed as described (Warrick *et al.* 2015) using Ion 318 Chip v2 on the Ion Torrent Personal Genome Machine (PGM) sequencer (Life Technologies) for samples with APAv1 and Ion PI Chip v3 on the Ion Proton sequencer (Life Technologies) for samples with APAv2. Data analysis was performed essentially as described previously (Warrick *et al.* 2015).

Briefly, for the samples with APAv1, APAv2 and OCPv2 variants called by default PGM or Proton low stringency variant filtering were further filtered to identify potential driving somatic and germline mutations by removing synonymous or noncoding variants, those with flow corrected read depth (FDP) < AAA, flow corrected variant allele containing reads (FAO) < BBB, variant allele frequencies (FAO/FDP) < CCC, flow variant allele calling forward to reverse read ratio < 0.2 or > 5 or indels within homopolymer runs ≥ 4 . Variants occurring exclusively in reads containing other variants (single nucleotide variants or indels) or those occurring in the last mapped base of a

read were excluded. Variants with (1) allele frequencies > 0.5% in ESP6500 or 1000 genomes or (2) those reported in ESP6500 or 1000 genomes with observed variant allele frequencies between 0.40 and 0.60 or > 0.9 were considered germline variants unless occurring variants were at known hotspot locations. All variants were visually confirmed in Integrative Genomics Viewer (IGV, Broad Institute, <https://www.broadinstitute.org/igv/>) (Hovelson *et al.* 2015).

The study was approved by the Institutional Review Board at the University of Michigan and patients were either consented before the use of their biosamples for the analysis conducted in this study (HUM00024461) or secondary analysis of archived samples was granted with a waiver of consent (HUM00083056).

Results

We employed two different strategies to identify as many patients as possible with a diagnosis of ACC and concurrent aldosterone production. First, we reviewed charts of 422 patients with a pathologically confirmed diagnosis of ACC for features of hyperaldosteronism and second, we stained an ACC TMA for CYP11B2.

Identification of patients with ACC and aldosterone production

Patient records were reviewed for a mentioning of aldosterone production documentation of increased aldosterone levels (>15 ng/dL), a plasma aldosterone (ng/dL)-to-renin (ng/mL/h) ratio (ARR) of >20 or increased 24-h urine aldosterone. A total of 25 patients were identified and tissues of 13 patients were available for further evaluation by immunohistochemistry and sequencing (ACC 1–12, 15) (Table 1). Interestingly, despite the documentation of hyperaldosteronism in the setting of ACC, not all 13 patients were likely to have a diagnosis of PA. PA was the definitive diagnosis in at least three patients (ACC 1, 3, 6) as they had suppressed renin and aldosterone >20 ng/dL in the setting of hypertension and spontaneous hypokalemia, which are the criteria for the diagnosis of primary aldosteronism by the recent Endocrine Society guidelines, in which confirmatory testing can be omitted. Based on clinical features, aldosterone level and ARR, another five patients (ACC 2, 5, 7, 10, 12) had a likely diagnosis of primary aldosteronism. However, none of these patients underwent confirmatory testing at the time of initial diagnosis, likely due to the assumption of a malignant

Table 1 Patient and tumor characteristics.

ACC #	Sex	Age (years)	Side	Alive	OS (months)	Recurrence	RFS (months)	Identification	Stage	Grade	Size (cm)
1	M	66	R	No	91	Yes	62	CR	2	1	8
2	F	39	R	No	85	Yes	27	CR	2	1	6.5
3	F	69	L	Yes	48	Yes	19	TMA, CR	2	1	9
4	F	48	L	Yes	25	No	25	CR	3	1	16
5	F	52	L	Yes	59	No	59	CR	3	1	10
6	F	22	R	Yes	17	No	17	CR	3	1	13.5
7	F	43	L	No	34	Yes	23	CR	1	2	4.5
8	M	49	R	No	119	Yes	43	CR	2	2	8
9	F	29	L	Yes	37	No	37	CR	2	2	10.5
10	F	39	R	Yes	3	No	3	CR	2	2	12
11	F	51	R	No	33	Yes	31	CR	3	2	11
12	M	24	R	No	49	Yes	11	CR	3	2	9
13	F	61	R	No	6	n/a	n/a	TMA	4	2	25
14	F	60	R	No	8	n/a	n/a	TMA	4	2	25
15	F	49	L	No	21	Yes	7	TMA, CR	2	2	8.7
16	F	36	L	Yes	10	n/a	n/a	TMA	4	1	16
17	F	55	x	No	n/a	n/a	n/a	TMA	n/a	1	n/a
18	F	25	L	No	n/a	n/a	n/a	TMA	n/a	2	na
Total	3M/15F	45 (±14)	8L/9R	7 Yes/11 No	40 (±32)	8 Yes/5 No	28 (±16)	13 CR/7 TMA	1 (1) 2 (7) 3 (5) 4 (3)	1 (8) 2 (10)	12 (±6)

ACC, adrenocortical carcinoma; CR, retrospective chart review; F, female; L, left; M, male; OS, overall survival; R, right; RFS, recurrence free survival; TMA, tissue microarray.

mass and little impact on care. Only one patient with a low suspicion for PA underwent 24-h urine collection with a final value of 330 µg/L/24 h (ACC 12). Two patients had hyperreninemic hyperaldosteronism (HH) (ACC 11, 15) and for another three patients, clinical data were insufficient for subclassification of hyperaldosteronism into PA or hyperreninemic hyperaldosteronism (ACC 4, 8, 9). However, all three patients had spontaneous hypokalemia and required potassium supplementation in the absence of significant hypercortisolism.

In order to evaluate the 13 clinically identified subjects for molecular correlates of aldosterone production, tissue sections were stained for CYP11B2 protein. Nine of the 13 patients showed positive staining for CYP11B2. However, intensity and distribution varied greatly from only small islands or single cells with positive staining to nearly entire staining of the whole specimen (Fig. 1). Of the four patients whose specimen stained negative, two clearly had a clinical diagnosis of PA (ACC 1, 12), one only had a mentioning of PA in the clinical notes without any further data available (ACC 4), and another patient likely carried the diagnosis of hyperreninemic hyperaldosteronism as judged by available biochemical data (ACC 11). Therefore, it is likely that the source of aldosterone in patients with samples with negative staining was either the contralateral adrenal gland or a different subclone of the often large adrenal tumors, which were not available for analysis.

Identification of patients with ACC and aldosterone production by tissue microarrays (TMA) staining for CYP11B2

In the second separate approach, ACC TMAs, containing 141 different specimens, including specimens of 118 patients that were also part of the initial 422 patients available for chart review, were stained for CYP11B2 and full slides were used to confirm staining. From the CYP11B2-stained TMA slides, we identified 57 cases of possible positive CYP11B2 staining. Full slide CYP11B2 staining, confirmed a total of seven ACC samples with true positive staining (Fig. 1 and Table 1). Five of these samples were not included in the initial retrospective chart review.

Of the initial 13 patients identified by retrospective chart review, a total of 7 patients were included in the TMA. Two of these seven samples had high and moderate CYP11B2 staining and were correctly identified in this second approach. The remaining five patients showed only minimal focal staining or were entirely negative on CYP11B2 staining and therefore escaped identification by TMA staining.

Clinical data were subsequently collected from all patients identified by CYP11B2 staining. However, there was only a renin value for a single patient available, which was clearly elevated and suggestive of hyperreninemic hyperaldosteronism (ACC 14).

Figure 1

Representative IHC for CYP11B2. Left two panels – CYP11B2 (10× and 20×), right two panels H&E (10× and 20×).

The total number of patients identified as potential PA by the two methods was 18.

Demographics of identified patients

As described above, a total of 18 patients were identified as possible cases of PA. Of these patients, the sex distribution was 83% female (15/18) and 17% male (3/18) (Table 2). The age range was 22–69 with an average age of 45 years. One patient was diagnosed at stage 1, seven patients at stage 2 and five patients at stage 3. Three patients had metastasized ACC at the time of diagnosis. The stage at diagnosis was unavailable for two patients. Tumor grading was available for all tumors with eight tumors being low grade (≤ 20 mitosis per 50 hpf) and ten tumors being high grade (> 20 mitosis per hpf). Eight tumors were on the left side, nine on the right and one tumor specimen was obtained from a metastasis.

Somatic genetic alterations

DNA was isolated from FFPE samples and subjected to two different NGS custom panels, a panel sequencing mutations in genes known to be associated with PA in adrenal adenomas (Supplementary Table 1, see section on

supplementary data given at the end of this article) and the DNA component of the Oncomine Comprehensive Assay (OCA), a pan-cancer panel targeting ~135 genes recurrently altered through somatic mutation or high level copy number amplification/loss that is being used in the NCI MATCH trial (Supplementary Table 2). The average coverage for the samples on APAV1 was 401.5X and on APAV2 was 2046X. None of the ACCs, including the ACCs, which showed autonomous aldosterone production (true primary aldosteronism) (Table 2), harbored well-supported mutations in the aldosterone production-associated genes. One patient (ACC 14) had a likely germline variant of uncertain significance in *ATP2B3* (c.3518T, p.A1173V, NM_001001344).

Using the OCA, six ACCs had activating *CTNNB1* mutations and four ACCs displayed *TP53* mutations (Fig. 2A). In accordance with recently published molecular landscape analysis, deleterious mutations in several other known tumor suppressors were found (e.g. *CDKN2A*, *PRKAR1A*, *ATM*, *RB1*) (Assie *et al.* 2014, Juhlin *et al.* 2015, Zheng *et al.* 2016). Somatic genomic losses and gains also mirrored recent publications with amplifications of *TERT* and *PIK3R1* and losses of *CDKN2A* (Fig. 2B).

Interestingly, one tumor harbored a mutation in *APEX1*, which had been implied in regulation of the

Table 2 Endocrine characteristics of patients and tumor samples.

ACC #	HC	HA	Aldo (ng/dL)	Renin (ng/mL/h)	ARR	K	K suppl	HTN	Documentation	CYP11B2 score	Clinical diagnosis
1	No	No	30.9	0.2	154.5	3.4	Yes	Yes	PA	–	PA
2	No	No	51.3	1.4	36.6	2.5	Yes	Yes	PA	+	PA
3	Yes	No	22.4	0.1	224	2.3	Yes	Yes	PA	++	PA
4	No	No	n/a	n/a	n/a	2.3	Yes	Yes	PA	–	PA/HH
5	Yes	Yes	16.2	0.6	27.9	3.2	No	Yes	x	+	PA
6	No	No	22	0.5	44	3	n/a	Yes	PA	++	PA
7	No	No	51	2.1	24.3	2.8	Yes	Yes	PA	+	PA
8	No	No	n/a	n/a	n/a	bnr	Yes	Yes	PA	++	PA/HH
9	No	No	28	n/a	n/a	2.8	Yes	Yes	x	++	PA/HH
10	No	No	11.4	0.4	28.5	nl	No	Yes	PA	+++	PA
11	Yes	No	20.4	6.9	3	2.8	Yes	Yes	PA	–	HH
12	Yes	No	5.8*	0.5	11.6	2.3	Yes	Yes	PA	–	PA
13	Yes	Yes	n/a	n/a	n/a	nl	Yes	Yes	x	++	IND
14	Yes	No	n/a	47.4	n/a	nl	Yes	Yes	x	++	HH
15	No	Yes	221.5	6.3	35.2	2.8	Yes	Yes	PA vs HH	+++	HH
16	Yes	No	n/a	n/a	n/a	nl	n/a	Yes	x	++	IND
17	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	x	++	IND
18	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	x	++	IND

*Urine aldosterone 303 µg/24 h.

Aldo, aldosterone; ARR, aldosterone–renin ratio; HA, hyperandrogenemia; HC, hypercortisolism; HH, hyperreninemic hyperaldosteronism; IND, indeterminate; K, potassium; K suppl, potassium supplementation; PA, primary aldosteronism.

CYP11B2 locus (McManus *et al.* 2012). Two patients were found to have likely germline predisposing mutations causing Lynch syndrome (*MSH2*), which is in accordance with prior studies (Raymond *et al.* 2013). One of these patients in addition had the commonly observed *CHEK2* founder mutation (c.1100delC). Other variants in genes associated with hereditary diseases were likely somatic mutations (e.g. *APC*, *CDKN2A*, *BRCA2*).

Discussion

In summary, a total of 18 patients with a diagnosis of ACC and features of hyperaldosteronism were identified using the combined approach of retrospective chart review (13 patients) and TMA CYP11B2 staining (seven patients, two identified in both). It is important to note that not all ACC patients in this series had autonomous aldosterone production and true primary aldosteronism. We aimed to capture a maximum number of patients with ACC and concurrent hyperaldosteronism. In further interpretation of clinical data, the patients were subtyped into primary aldosteronism (PA), hyperreninemic hyperaldosteronism (HH), equivocal or indeterminate groups (Table 2). The diagnosis of primary aldosteronism rarely impacts therapy for ACC, which is most commonly treated surgically. Therefore, confirmatory testing, which is standard for primary aldosteronism due to benign causes, is seldom pursued. In our case series three patients fulfilled these

strict criteria and can be categorized as true PA. For another five patients the diagnosis of PA was likely based on clinical data and three patients had a possible diagnosis of PA. Interestingly, three patients could be characterized as hyperreninemic hyperaldosteronism.

In summary, the available clinical data suggest that aldosterone production is autonomous and independent from the renin-angiotensin system in the majority of ACCs associated with hyperaldosteronism, like in aldosterone-producing adenomas. There are several explanations that can be considered for the pathophysiology of hyperreninemic hyperaldosteronism, observed in a subset of patients: (1) ACCs could preserve responsiveness to angiotensin II (AngII), (2) in some cases elevated renin production might originate from the ACC as a paraneoplastic phenomenon or (3) more likely, be of renoparenchymal origin due to compression of the kidney or renal vasculature by the often large tumors (Iimura *et al.* 1986, Maas *et al.* 2007). Although *in vivo* evidence is lacking to support the first possibility, there is ample evidence from published studies using NCI-H295 derived subclones (e.g. HAC15) as a model for AngII responsiveness (Parmar *et al.* 2008). HAC15 cells express CYP11B2 upon AngII stimulation and produce aldosterone. In the latter two instances aldosterone production might have originated from the remaining ipsilateral adrenal tissue or the contralateral adrenal gland. These data are well in accordance with another large series that analyzed ACC

Figure 2

Mutational landscape of aldosterone producing ACC. (A) Upper panel: clinical and tumor characteristics. Lower panel: genes carrying variants, mutations and indels (B) left panel: amplifications and deletions derived from next-generation sequencing sets on respective chromosomes, right panel: clinical and tumor characteristics.

with hyperaldosteronism, in which at least 17% of all patients did not have suppressed renin levels (Seccia *et al.* 2005).

When relying on retrospective clinical data, it is a limitation that aldosterone levels are most commonly measured by immunoassay and therefore cross-reactivity with for example, 11-deoxycorticosterone cannot be excluded. The use of a TMA bears the risk of identifying ACCs with CYP11B2 staining, which do not have other prerequisites for aldosterone production (e.g. missing expression of other steroidogenic enzymes). However, the identification of the two patients with generalized staining with both methods out of a large cohort of patients is reassuring. Therefore, the inclusion of all of these cases in the molecular analysis is justified as they potentially represent ACCs with autonomous aldosterone production.

With regards to the somatic mutational profile we analyzed known oncogenes and tumor suppressor genes. The observed mutations are well in accordance with prior publications, suggesting involvement of WNT signaling and cell cycle regulation in the pathogenesis of ACC. In summary, no unique mechanisms of molecular tumorigenesis were observed in ACCs associated with hyperaldosteronism compared to other ACC.

The majority of APAs (70–90%) carry specific mutations in genes altering cell membrane polarization

and intracellular calcium levels. In ACC, however, we did not find any mutation in these genes. We only found a monoallelic variant of undetermined significance in *ATP2B3* (likely germline) and *APEX1*, which have been implicated in APA pathogenesis and *CYP11B2* gene regulation, respectively. Although our two-pronged approach of identification also included ACCs with hyperaldosteronism driven by hyperreninemic hyperaldosteronism, the molecular analysis and conclusion is still valid as the group included several ACCs with a greater likelihood for true autonomous aldosterone production. Although we cannot exclude that some subclones of aldosterone-producing ACC potentially carry mutations in the same genes as described in APA, our data are suggestive for a different mechanism of aldosterone production in aldosterone-producing ACCs. This is well in accordance with the general clinical observation that APAs rarely progress to ACC. Although case reports have shown the occurrence of ACC in patients with prior adrenal nodules or adenomas, it is still a matter of debate, whether ACCs arise from adenomas as shown for other organs (Nogueira *et al.* 2015, Belmioub *et al.* 2017). In addition, APAs might be diagnosed early and subjected to surgery and therefore progression is rarely observed.

In summary, aldosterone production in patients with ACC and hyperaldosteronism can be caused by different mechanisms and a full biochemical evaluation is needed

to define the underlying pathophysiology, primary aldosteronism or hyperreninemic hyperaldosteronism. ACCs associated with hyperaldosteronism harbor similar mutations and a similar profile of genomic gains and losses as ACCs without aldosterone production. As no classical APA-associated mutations were found, we conclude that molecular events conferring aldosterone production in ACC are likely different from those in aldosterone-producing adenomas.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/ERC-18-0385>.

Declaration of interest

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