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Genetics of aldosterone-producing adenomas with pathogenic *KCNJ5* variants

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Abstract

Somatic variants in genes that regulate intracellular ion homeostasis have been identified in aldosterone-producing adenomas (APAs). Although the mechanisms leading to increased aldosterone production in APA cells have been well studied, the molecular events that cause cell proliferation and tumor formation are poorly understood. In the present study, we have performed whole-exome sequencing (WES) to characterize the landscape of somatic alterations in a homogeneous series of APA with pathogenic *KCNJ5* variants. In the WES analysis on 11 APAs, 84 exonic somatic events were called by 3 different somatic callers. Besides the *KCNJ5* gene, only two genes (*MED13* and *ZNF669*) harbored somatic variants in more than one APA. Unlike adrenocortical carcinomas, no chromosomal instability was observed by the somatic copy-number alteration and loss of heterozygosity analyses. The estimated tumor purity ranged from 0.35 to 0.67, suggesting a significant proportion of normal cell infiltration. Based on the results of PureCN analysis, the *KCNJ5* variants appear to be clonal. In conclusion, in addition to *KCNJ5* somatic pathogenic variants, no significant somatic event that would obviously explain proliferation or tumor growth was observed in our homogeneous cohort of *KCNJ5*-mutated APA. The molecular mechanisms causing APA growth and tumorigenesis remain to be elucidated.

Key Words

- ▶ primary aldosteronism
- ▶ whole-exome sequencing
- ▶ somatic mutation
- ▶ *KCNJ5*
- ▶ CYP11B2

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Introduction

Primary aldosteronism (PA) has been recognized as a major cause of endocrine and drug-resistant hypertension that is associated with significant morbidity and mortality (Funder *et al.* 2016). Aldosterone-producing adenoma (APA) is one of the major subtypes of PA (Young 2007). Over the past decade, there has been a considerable

focus on defining the genetic bases of excess aldosterone production in PA. After the initial identification of somatic and germline *KCNJ5* variants in PA patients (Choi *et al.* 2011), different germline and somatic variants have been identified in familial and sporadic forms of PA (Zennaro *et al.* 2017, Monticone *et al.* 2018). The immediate

functional consequences of these alterations are disruption of intracellular ion homeostasis, which ultimately lead to increased aldosterone synthase (*CYP11B2*) transcription and inappropriate aldosterone production (Zennaro *et al.* 2017, Monticone *et al.* 2018). *KCNJ5* mutations are the most common somatic variants in APA with greater frequency in East Asian populations (Zennaro *et al.* 2017). However, the mechanism by which these variants provide growth advantage to adrenocortical cells and promote APA formation remains poorly understood.

Recent studies have demonstrated that in patients with PA and multiple adrenal nodules, somatic variants in different aldosterone-driver genes can be present in distinct *CYP11B2*-expressing, but not in *CYP11B2*-negative nodules within the same adrenal (Dekkers *et al.* 2014, Fernandes-Rosa *et al.* 2015, Nanba *et al.* 2016). Additionally, *in vitro* studies indicate that mutated *KCNJ5* channels do not confer growth advantage when expressed in the human adrenocortical HAC15 cell line (Oki *et al.* 2012). These observations suggest that the processes of nodule formation and autonomous aldosterone production may be independent events – the so-called ‘two-hit’ model (Zennaro *et al.* 2017). According to this model, an initial somatic event that confers growth advantage would induce tumor formation and a second mutational event (in any of the already characterized genes associated with APA) would be responsible for increased aldosterone production. However, no recurrent secondary mutations in growth-promoting genes have been reported in APA with mutations in aldosterone-driver genes so far. In this study, we used whole-exome sequencing (WES) to characterize the landscape of somatic alterations in a homogeneous series of APAs with *KCNJ5* variants. We were particularly interested in identifying novel recurrent mutations and somatic alterations that may contribute to APA tumorigenesis and in further characterizing *KCNJ5*-mutated tumors in terms of tumor purity, ploidy and clonal composition.

Patients and methods

Patients and samples

Fourteen APA from 14 patients with PA who underwent adrenalectomy at the University of Michigan or Yokohama Rosai Hospital were included in the present study. To study genetic alterations related to APA tumorigenesis, relatively large APAs (median: 30 mm, interquartile range: 23.8–30.0 mm) were studied. Clinical characteristics of studied patients are shown in Supplementary Table 1

(see section on [supplementary data](#) given at the end of this article). For somatic variant identification, control germline DNA either from peripheral blood ($n=5$) or matched adjacent normal adrenal ($n=6$) was used based on the availability. Peripheral blood or matched adjacent normal adrenal and APA tissue DNA was isolated using commercial kits (Nucleospin Blood (Macherey-Nagel), Nucleospin Tissue (Macherey-Nagel) or AllPrep DNA/RNA/Protein kit (Qiagen)). This study was approved by Institutional Review Board of the University of Michigan and Yokohama Rosai Hospital. Either a written informed consent was obtained from patients before the use of their biospecimens for the analysis (24-51) or a waiver of informed consent was granted for the use of specimens at the University of Michigan (HUM00083056).

Immunohistochemistry

Formalin-fixed paraffin-embedded tissue blocks were available from all but one patient (Case 10, APA-Y182). Immunohistochemistry (IHC) was performed using specific antibodies against *CYP11B2* (mouse monoclonal, Millipore, #MABS1251) (Gomez-Sanchez *et al.* 2014), 11 β -hydroxylase (*CYP11B1*, rat monoclonal) (Gomez-Sanchez *et al.* 2017), 17 α -hydroxylase (*CYP17A1*, mouse monoclonal) (Dharia *et al.* 2004), 3 β -hydroxysteroiddehydrogenase/ Δ 4-5 isomerase type II (*HSD3B2*, mouse monoclonal) (Gomez-Sanchez *et al.* 2017) and β -catenin (mouse monoclonal, BD Biosciences, #610153) essentially as described previously (Monticone *et al.* 2012, Nanba *et al.* 2016).

Whole-exome sequencing

DNA was fragmented to 300 bp using standard Covaris sonication (Covaris). Fragmented DNA was then prepared as a standard Illumina library using IntegenX (now Wafergen) reagents on the Apollo instrument, where the fragments were end repaired, A tailed and adapter ligated. Then, the samples were PCR amplified. Libraries were checked for quality and quantity by TapeStation (Agilent) and pooled for capture – six samples per pool. Each pool underwent exome capture using the Roche Nimblegen SeqCap EZ v3.0 using manufacturer's protocols (Roche NimbleGen). In brief, a total of 1 μ g prepared libraries was hybridized to the SeqCap EZ library for 72 h and then washed, and the captured DNA was recovered using Streptavidin Dynabeads (Life Technologies). The captured DNA was then amplified by PCR. The purified captured DNA was then clustered using the cBot (Illumina) and sequenced on a HiSeq 2500 (Illumina) high-output

paired-end 125bp run using version 4 reagents and manufacturer's protocols.

Data analysis

Paired-end reads were aligned to the hg19 assembly of the human genome using bwa-mem (Li 2013). Aligned reads were sorted and duplicates were marked with bamsort and bammarkduplicate tools, respectively, from biobambam2 (Tischler & Leonard 2014). Indel realignment and quality score recalibration were performed with GATK (McKenna *et al.* 2010). Coverage metrics and quality-control analysis of the alignments were performed with Qualimap2 (Okonechnikov *et al.* 2016). As an additional quality-control measure, we used NGSCheckMate (Lee *et al.* 2017) to rule out cross-contamination, sample mislabeling and unexpected similarities among samples from different patients. Somatic variants were called with three different callers: MuTect2, Lancet and LoFreq (Wilm *et al.* 2012, Cibulskis *et al.* 2013, Narzisi *et al.* 2017). Somatic calls were annotated with Annovar (Wang *et al.* 2010). We considered somatic variants all those called by at least two of the callers. We used the Integrative Genomics Viewer (IGV) to visually confirm the somatic alterations and the reported allele frequencies. We compared the final list of somatic variants to an internal database generated from other projects using our *in-house* pipeline to further reduce the possibility of calling recurrent sequencing artifacts. For samples which paired germline DNA were not available, we calculated the allele frequencies of pathogenic *KCNJ5* variants from the VCF files generated with samtools mpileup/bcftools (Li *et al.* 2009, Danecek *et al.* 2011). Finally, we used PureCN (Riester *et al.* 2016) to perform somatic copy-number alteration (SCNA) and loss of heterozygosity (LOH) analyses and to estimate tumor purity, ploidy and clonal composition. PureCN infers tumor purity and ploidy by simultaneously analyzing depth-of-coverage and variant allele frequencies from germline and tumor DNA.

Results

Histologic characteristics of *KCNJ5*-mutated APA

In accordance with previous studies (Azizan *et al.* 2012, Monticone *et al.* 2015), the *KCNJ5*-mutated tumor cells were mainly composed of lipid-rich clear cells. IHC was performed to assess the tumor expression of CYP11B2, which is essential for aldosterone biosynthesis, and to confirm the

tumor cell capacity to produce aldosterone (Fig. 1A and C). IHC for other steroidogenic enzymes, including CYP17A1, CYP11B1 and HSD3B2, was also performed ($n=13$). The CYP11B2 expression intensity was variable and most commonly exhibited a heterogeneous expression pattern (Supplementary Table 2). APA tumor cells also showed strong expression of HSD3B2 (Fig. 1D). The expression of CYP17A1 and CYP11B1, which are required for cortisol synthesis, was also observed in *KCNJ5*-mutated APA with some degree of variability (Fig. 1E and F, Supplementary Table 2). The results of 1 mg dexamethasone suppression test were available in ten cases (Supplementary Table 1). Of those, three cases showed non-suppressed cortisol after a dexamethasone suppression test (serum cortisol levels higher than 1.8 µg/dL). The APAs from those patients exhibited positive but variable expression of CYP17A1 and CYP11B1. We further performed IHC for β -catenin to determine a potential contribution to tumorigenesis or growth. The β -catenin IHC was not successful in one case (Case 1). All other cases ($n=12$) showed similar patterns of predominantly membranous staining, indicating the absence of Wnt/ β -catenin activation in the majority of the tumor cells (Supplementary Fig. 1). Only a few cases showed focal nuclear and/or cytoplasmic reactivity, which did not reach the staining intensity, usually observed in adrenocortical carcinomas with genetic changes known to activate the Wnt/ β -catenin pathway.

Somatic mutation analysis

We performed WES on 11 APA with paired germline DNA and 3 unpaired APA samples. The mean coverage and the percentage of targeted regions that were covered at least 20× were 90.45× (range 67.47–118.95×) and 91.12% (range 88.59–92.81%) for tumors and 89.45× (range 63.46–120.11×) and 91.45% (range 89.07–92.65%) for germline. Our NGSCheckMate analysis ruled out cross-contamination, sample mislabeling and unexpected similarities among samples from different patients (Supplementary Fig. 2). We identified 120 exonic somatic events that were called at least by 2 callers, including 109 single-nucleotide variants (SNVs) (22 synonymous, 81 nonsynonymous and 6 stopgain), 6 indels (4 frameshift deletions, 1 frameshift insertion and 1 nonframeshift insertion) and 5 splicing site variants, averaging 10.9 events per sample (range 5–16). Among these, 84 events were called by the three callers, 32 were called by Mutect2 and Lofreq, 3 by Mutect2 and Lancet and 1 by Lancet and LoFreq (Supplementary Fig. 3). Apart from *KCNJ5* variants, which were detected by the three

Figure 1

Representative histologic characteristics of *KCNJ5*-mutated aldosterone-producing adenoma. (A) Low magnification view of the adrenal gland with CYP11B2 IHC (Case 5, APA-Y80). The APA was identified by CYP11B2 IHC. Scale bar, 5 mm. (B, C, D, E and F) High magnification view of the APA, (B) H&E, (C) CYP11B2 IHC, (D) HSD3B2 IHC, (E) CYP17A1 IHC, (F) CYP11B1 IHC. Scale bars, 100 µm. APA, aldosterone-producing adenoma; IHC, immunohistochemistry; H&E, hematoxylin and eosin staining.

callers in all samples, only two other genes had variants in more than one APA: *MED13* (NM_005121:exon26:c.T5866G:p.Ser1956Ala, (APA-78 and APA-86)) and *ZNF669* (NM_001142572:exon4:c.A1052C:p.Glu351Ala, (APA-78 and APA-86)) (Supplementary Figs 4, 5, 6 and 7). Of note, the *MED13* and *ZNF669* variants were not confirmed by direct Sanger sequencing likely due to the low variant frequencies.

Among the identified mutated genes, six of them have been reported as cancer-associated (Futreal *et al.* 2004) (COSMIC v85): *JAK1*, *NAB2*, *FOXP1*, *ATM*, *MEN1* and *USP6*. Mutations in these genes have been shown to affect cellular processes that are frequently dysregulated in cancer and are thought to be tumor promoting. Additionally, we identified variants in *PDE11A* and *CYP17A1*, genes that are essential components of the adrenal steroidogenesis machinery and have been implicated in other adrenal diseases, such as forms of adrenal micronodular hyperplasia, cortisol-producing adenomas (CPAs) and congenital adrenal hyperplasia. Finally, we identified variants in other ionic channels such as *CACNA1E* and *KCNA1* (Supplementary Tables 3, 4, 5 and 6). Noteworthy, exome sequencing confirmed the presence of four previously identified *KCNJ5* variants in our cohort: c.T503G (p.L168R), c.G451A (p.G151R), c.G451C

(p.G151R) and c.477_478insATT (p.T149delinsTT) with allele frequencies ranging from 14 to 48% (Table 1).

SCNA and LOH analyses

In agreement with previous study (Choi *et al.* 2011), our SCNA and LOH analyses revealed that unlike a subset of ACA with *CTNNB1* mutations and adrenocortical carcinomas (ACCs) (Goh *et al.* 2014, Zheng *et al.* 2016), *KCNJ5*-mutated APA did not exhibit chromosomal instability (characterized by arm-level segmental loss and gains and/or alterations in ploidy). The diploid heterozygous state was maintained in all samples (Table 1 and Supplementary Fig. 8).

Tumor purity and clonal composition

KCNJ5-mutated APAs appear to bear a significant proportion of normal cell infiltration. Tumor purity in our cohort ranged from 0.35 to 0.67, which contrasts with ACC that exhibit high purity according to a recent molecular profiling study (Zheng *et al.* 2016). Furthermore, PureCN analysis also demonstrated that *KCNJ5* variants are heterozygous and likely clonal in all tumors, suggesting that these variants are early events in adenoma formation (Table 1 and Supplementary Fig. 8).

Table 1 Genetic characteristics of *KCNJ5*-mutated aldosterone-producing adenoma.

Case	Sample name	Tumor purity	Tumor ploidy	<i>KCNJ5</i> mutation	<i>KCNJ5</i> mutation allele frequency	# of mutated alleles per cell	Clonality
1	APA-59 ^a	0.57	2.00	p.G151R	0.44	1	Clonal
2	APA-62 ^a	0.64	2.00	p.L168R	0.29	1	Clonal
3	APA-78 ^a	0.67	2.00	p.L168R	0.37	1	Clonal
4	APA-86 ^a	0.64	2.00	p.T149delinsTI	0.34	1	Clonal
5	APA-Y80 ^a	0.36	2.00	p.L168R	0.14	1	Clonal
6	APA-Y108 ^b	0.59	1.98	p.G151R	0.26	1	Clonal
7	APA-Y110	NA	NA	p.G151R	0.24	NA	NA
8	APA-Y179 ^b	0.55	2.00	p.G151R	0.31	1	Clonal
9	APA-Y180	NA	NA	p.L168R	0.48	NA	NA
10	APA-Y182 ^a	0.35	1.99	p.L168R	0.26	1	Clonal
11	APA-Y183 ^b	0.55	2.02	p.L168R	0.18	1	Clonal
12	APA-Y184	NA	NA	p.G151R	0.22	NA	NA
13	APA-Y189 ^b	0.59	2.00	p.L168R	0.35	1	Clonal
14	APA-Y198 ^b	0.58	2.00	p.G151R	0.40	1	Clonal

^aDNA from matched adjacent normal was used as a control. ^bPeripheral blood DNA was used as a control.

NA, not available.

Discussion

In recent years, high-throughput molecular profiling studies have identified recurrent variants targeting different regulators of intracellular calcium homeostasis, including *KCNJ5*, *ATP1A1*, *ATP2B3* and *CACNA1D* in over half of APA. Despite the well-characterized effects of these variants in abnormal *CYP11B2* expression and aldosterone production, the contribution to cell growth remain poorly understood (Zennaro *et al.* 2017). So far, a growth-promoting effect of *KCNJ5* variants has not been demonstrated *in vitro* studies using HAC15 cells (Oki *et al.* 2012). Although it is difficult to assess a cell proliferation effect of these variants using highly proliferative H295R/HAC15 cells, which have a β -catenin mutation (Tissier *et al.* 2005), these *in vitro* studies suggest that alternative mechanisms might play a role in tumor formation. Interestingly, areas of cortical cell hyperplasia and other nodular lesions are frequently observed near APA. These areas are usually *CYP11B2* negative and do not bear aldosterone-driving gene variants (Dekkers *et al.* 2014). Furthermore, APAs often exhibit a heterogeneous immunostaining for *CYP11B2* (Nakamura *et al.* 2014), suggesting that mutated cells could be subclonal. Based on these observations, a 'two-hit' model in which tumor growth and increased aldosterone production are caused by independent molecular hits has been proposed (Zennaro *et al.* 2017). In fact, we observed at least two variants of uncertain significance in genes that have been previously associated with adrenocortical tumorigenesis: the *MEN1* NM_130804:exon3:c.T364C (p.Ser122Pro) and the *PDE11A* NM_001077197:exon5:c.C514T (p.Arg172Cys) variants,

both predicted to be pathogenic by SIFT, polyphen2 and CADD. However, both of these variants were somatic and, according to our PureCN analysis, none of these variants are in focal areas of LOH, suggesting that both are 'passenger' events. Although somatic variants in *MED13* and *ZNF669* were identified in more than one APA, the pathological role of these variants is unclear. *MED13* is a component of the Mediator Complex, a multiprotein complex critical for transcriptional activation. Mutations and altered expression of different Mediator components have been described in rare neurodevelopmental disorders and in a broad spectrum of benign and malignant tumors (Clark *et al.* 2015). The function and physiological relevance of *ZNF669* are currently unknown. Noteworthy, we did not identify variants in other genes previously described in APA, such as *CTNNB1* (encoding β -catenin), suggesting a dominant role for *KCNJ5* variants. These results are consistent with previous studies on APA genetics, in which *KCNJ5* and *CTNNB1* variants were mutually exclusive (Akerstrom *et al.* 2016, Wu *et al.* 2017). Previous reports have demonstrated that the activation of Wnt/ β -catenin pathway is frequent in APA regardless of the mutation status of *CTNNB1* (Boukroun *et al.* 2011, Berthon *et al.* 2014). However, the nonactivated state of β -catenin was predominantly observed in our cohort, suggesting that, unlike ACC (Mete *et al.* 2018), activation of Wnt/ β -catenin pathway may not be a common feature of *KCNJ5*-mutated APA. For better understanding of the role of Wnt/ β -catenin signaling pathway activation in APA development, further analysis including APA with variants in other aldosterone-driver genes, such as *CACNA1D*, *ATP1A1*, and *ATP2B3* will be needed.

Finally, our clonality analysis suggests that *KCNJ5*-mutated cells are a clonal population within the tumor rather than a subclone that acquired the ability to secrete aldosterone. Certain *KCNJ5* variants, including p.G151R and p.T158A, appear to have a potential impact on cell proliferation considering the fact that these variants have been identified both in sporadic APAs (as somatic variants) and in patients with massive hyperplasia (as germline variants), whereas a *KCNJ5* variant p.G151E has only been documented in individuals without massive adrenal hyperplasia as a germline variant (Choi *et al.* 2011, Mulatero *et al.* 2012, Scholl *et al.* 2012). Furthermore, in an early-onset PA patient, the identical somatic *KCNJ5* p.G151R variant has been identified in multiple CYP11B2-expressing hyperplastic lesions and APA in bilateral adrenals and the variant has not been observed in non-hyperplastic lesions (Tamura *et al.* 2017), supporting the potential role of the *KCNJ5* variant on cell proliferation.

Taken together, our results do not support the proposed 'two-hit' model. However, since our study included only a homogeneous population of APA with *KCNJ5* variants, the findings cannot be generalized. It is also important to note that the use of DNA from adrenal tissue adjacent to the tumors could have limited detection of somatic mutations considering a previous observation that small nodules are sometimes present in adrenal tissue adjacent to APA (Boukroun *et al.* 2010). The development of APA might be complex and heterogeneous. A recent case report describes the co-occurrence of a germline *APC* variant and a somatic *KCNJ5* variant in a young PA patient with multinodular adrenal hyperplasia (Vouillarmet *et al.* 2016). In the resected adrenal from the patient, the *KCNJ5* variant was identified only in a CYP11B2-expressing nodule, whereas biallelic *APC* inactivation due to LOH was observed in both CYP11B2-positive and -negative nodules (Vouillarmet *et al.* 2016), indicating 'two-hit' model as a possible mechanism of APA development at least for some cases. Epigenetic event may also be involved in adrenal nodule formation in some APAs. A recent study of integrated transcriptome and methylome analysis has demonstrated global hypomethylation in APA and upregulated genes with CpG hypomethylation in APA, including five potentially tumorigenesis-related genes (Murakami *et al.* 2015).

Interestingly, while our analysis is consistent with a relatively high proportion of normal cells in the tumors, we do not observe a significant infiltration of fibroblasts or inflammatory cells. In fact, although CYP11B2 expression is heterogeneous in some tumors, the majority of the cells, including CYP11B2-negative cells, exhibit

morphological features consistent with steroidogenic cells and express steroidogenic enzymes such as CYP17A1, HSD3B2 and CYP11B1 (Fig. 1). This observation suggests that the normal cell type that infiltrates *KCNJ5*-mutated APA are normal steroidogenic cells, consistent with a role for paracrine factors in mediating tumor growth.

In conclusion, our study did not identify recurrent somatic events that could explain proliferation or tumor formation in APA with *KCNJ5* variants. The mechanism by which *KCNJ5*-mutated tumors form and grow remains elusive.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/ERC-18-0364>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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