

# Mosaicism for *KCNJ5* Causing Early-Onset Primary Aldosteronism due to Bilateral Adrenocortical Hyperplasia

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## BACKGROUND

Somatic variants in *KCNJ5* are the most common cause of primary aldosteronism (PA). There are few patients with PA in whom the disease is caused by germline variants in the *KCNJ5* potassium channel gene (familial hyperaldosteronism type III—FH-III).

## METHODS

A 5-year-old patient who developed hypertension due to bilateral adrenocortical hyperplasia (BAH) causing PA had negative peripheral DNA testing for any known genetic causes of PA. He was treated medically with adequate control of his PA but by the third decade of his life, due to worsening renal function, he underwent bilateral adrenalectomy.

## RESULTS

Focused exome sequencing in multiple nodules of his BAH uncovered a “hot-spot” pathogenic *KCNJ5* variant, while repeated Sanger sequencing showed no detectable DNA defects in peripheral blood

and other tissues. However, whole exome, “deep” sequencing revealed that 0.23% of copies of germline DNA did in fact carry the same *KCNJ5* variant that was present in the adrenocortical nodules, suggesting low level germline mosaicism for this PA-causing *KCNJ5* defect.

## CONCLUSIONS

Thus, this patient represents a unique case of BAH due to a mosaic *KCNJ5* defect. Undoubtedly, his milder PA compared with other known cases of FH-III, was due to his mosaicism. This case has a number of implications for the prognosis, treatment, and counseling of the many patients with PA due to BAH that are seen in hypertension clinics.

**Keywords:** blood pressure; familial hyperaldosteronism; genetics; hyperaldosteronism; hypertension; *KCNJ5*; mosaicism; primary aldosteronism.

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Primary aldosteronism (PA) is recognized as the most common hormonal cause of secondary hypertension.<sup>1</sup> Dr Jerome Conn first demonstrated that this condition was linked to adrenal adenomas in 1960s, after adrenalectomy of a unilateral adrenal adenoma resulted in the cure of resistant hypertension in a young female patient. In addition to aldosterone producing adenomas (APAs), PA is also commonly caused by bilateral adrenocortical hyperplasia (BAH), and rarely by unilateral hyperplasia, aldosterone-producing adrenocortical carcinomas, or familial hyperaldosteronism (FH). With the advent of genetic sequencing, more definitive characterization of the

underlying genetic defects causing APA was made possible in the last decade.<sup>2</sup>

Past work has established PA to be either caused by somatic disease-causing variants in adrenal tissue or more rarely, germline defects leading to FH.<sup>3,4</sup> FH type I (FH-I), otherwise known as glucocorticoid-remediable aldosteronism (GRA), is due to a cross-over of the promoter of the 11-beta-hydroxylase gene and the coding region of the aldosterone synthase gene causing aldosterone production stimulation by adrenocorticotrophic hormone (ACTH). FH type II (FH-II) historically does not have specific genetic defects to define it, though recent work has linked some

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cases to disease-causing variants in chloride voltage-gated channel 2 (*CLCN2*), which encode chloride channels in the adrenal glomerulosa.<sup>5</sup> The cause of the disease in a subset of families with FH-II has been mapped to chromosome 7p22.<sup>6</sup> FH type III (FH-III) is attributed to germline variants in potassium voltage-gated channel subfamily J member 5 (*KCNJ5*), which codes for an inwardly rectifying potassium channel.<sup>4,7</sup> FH type IV (FH-IV) is due to disease-causing variants in calcium voltage-gated channel subunit alpha1 H (*CACNA1H*), which encodes a T-type calcium channel.<sup>3</sup>

Choi *et al.*<sup>8</sup> first identified a relatively frequent genetic etiology for APA development: Twenty-two patients with PA due to an unilateral adrenal mass identified on computed tomography (CT) scan underwent genetic analysis. “High probability somatic mutations” were identified via genotyping of tumors on Illumina 1M-Duo chips and were confirmed with Sanger sequencing. After filtering for variants that altered protein function, the gene *KCNJ5* was noted to be mutated in multiple patients. Compared against blood DNA (considered the equivalent of germline tissue), these variants were found to be exclusive to the adrenal tumors. Additionally, functional characterization of the disease-causing variants in *KCNJ5* demonstrated that the loss of channel selectivity conferred susceptibility to membrane depolarization, leading to calcium influx and consequent stimulation of aldosterone production. Moreover, prolonged calcium stimulation also was found to foster zona glomerulosa growth, initially thought to explain the manifestation of adrenal adenomas.<sup>8,9</sup> In the same study, a germline disease-causing variant in *KCNJ5* was also shown to cause an inherited form of PA<sup>8</sup> associated with bilateral enlargement of the adrenal glands, which histologically was consistent with BAH: a father and his two daughters all developed severe hypertension and PA associated with BAH between ages 4 and 7. The hypertension was resistant to medical therapy and bilateral adrenalectomy was pursued in all three cases. Subsequent work established that somatic disease-causing variants in other genes, such as *ATP1A1* and *ATP2B3*, can lead to APA as well.<sup>10</sup> Disease-causing variants in *CACNA1D*, leading to increased calcium influx, were similarly linked to APA and when in the germline, to BAH. Indeed, these findings confirmed that dysregulated calcium influx is the common last step in APA formation among the different APA- and/or BAH-associated disease-causing variants.<sup>2,11</sup>

Mosaicism can occur when genetic defects arise postzygotically leading to genetically distinct cell lineages in an individual developing from a single zygote. The clinical presentation in mosaicism may vary according to the type of disease-causing variants, percent of mutated cells, and tissue distribution of the genetic defect. There are three main types of mosaicism: somatic mosaicism which occurs in nongerm cells and cannot be transmitted to the next generation, gonadal or germline mosaicism, which occurs only in germ cells or their precursors and may be inherited by the next generation, and mixed gonadal and somatic mosaicism.<sup>12–14</sup> Therefore, identification of mosaicism is of great importance to establish disease diagnosis, assess recurrence risk, and provide genetic counseling.<sup>15,16</sup>

Here, we show for the first time, a case of BAH due to a mosaic *KCNJ5* defect in a patient who was initially thought

to have FH-II. Our results showed that, indeed, there was very low-level mosaicism in the germline DNA, whereas all adrenocortical cells tested from 11 different nodules harbored the disease-causing variant *KCNJ5* p.G151R.

## MATERIALS AND METHODS

### Clinical studies

The patient was recruited to an ongoing large study on the genetics of primary aldosteronism (NIH protocol 00-CH-0160) first approved by the Institutional Review Board (IRB) of the Eunice Kennedy Shriver National Institute for Child Health & Human Development, Division of Intramural Research (DIR). The patient signed an informed consent for all clinical and genetic studies.

### Sanger sequencing

DNA was extracted from peripheral blood leukocytes, right and left adrenal fat and 11 adrenal nodules (5 left nodules, 6 right nodules) using DNA easy blood and tissue kit according to the manufacturer protocol (Qiagen, Hilden, Germany). Primers to amplify *KCNJ5* were designed to amplify its hotspot region (comprising the most frequent disease-causing variants in *KCNJ5*—c.451G>A and c.503T>G). Primers used for the PCR reaction were as follows: forward—5'-CAGCGCTACATGGAGAAGAG-3'; reverse 5'-CCACGTTGATGTCTGTCTGG-3'. Annealing temperature of 60 °C was used for the PCR amplification. Classical bidirectional Sanger sequencing was performed using a Genetic Sequencer ABI3500 (Applied Biosystems, Grand Island) to identify the disease-causing variants in *KCNJ5*.

### Mosaicism analysis by deep next generation sequencing

To determine the proportion of cells exhibiting the disease-causing variant in question, we amplified the specific region with targeted flanking primers. Each primer was composed of two parts: (i) the 3' end was complementary to the targeted genomic sequence; and (ii) all primers had appended to their 5' end either forward or reverse adaptor sequences to allow the addition of barcodes and Illumina sequencing adaptors. These appended sequences were: forward—5'-TCGTCGGCAGCGTCAGATGTGTAT AAGAGACAG [locus-specific sequence]; and reverse—5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG [locus-specific sequence]. The locus specific sequences used were: forward 5'-AAACCTCAGTGGCTTCGTGT-3' and reverse 5'-GAGGGTCTCCGCTCTCTTCT-3'. Amplicons generated from the sample DNA were then converted to sequenced libraries according to Illumina's 16S Metagenomic Sequencing Library Preparation kit. Libraries were sequenced on an Illumina MiSeq using version 2 chemistry and generated 2 × 150-bp paired-end reads. Read pairs were trimmed with cutadapt, joined via “PEAR,” and aligned to expected amplicons with BWA. Variant frequencies were evaluated by counting bases at the variant position in aligned reads. Normalization was done using the WT sample result as “zero.” The experiment was done in technical triplicates



and we tested one nodule and four germline DNA (left and right adrenal fat, peripheral blood and buccal swab) from the individual in the study. We also tested a germline DNA sample from the parents of the study individual and a germline DNA sample from an unaffected individual (wild type (WT) control).

### Immunostaining

Deparaffinized sections of adrenal tumors were immuno-stained using antibodies against CYP11B2 (rabbit antihuman NBP2-1389, Novus Biologicals, Colorado, EUA). Routine Hematoxylin & Eosin staining was performed at Histoserv, Inc. Slides were scanned in a  $\times 10$  objective and pictures were taken using Keyence BZ-X710 (Osaka, Japan) microscope.

## RESULTS

### Clinical case

An eleven-year-old Caucasian male was initially seen at the National Institutes of Health Clinical Center in 1998 for early-onset severe hypertension. He was diagnosed with hypertension at the age of 5 years and was initially evaluated for renal causes of hypertension. He was empirically treated with propranolol, with modest improvement in his hypertension, and subsequently switched to hydrochlorothiazide but developed hypokalemia and nifedipine monotherapy was trialed thereafter. He was subsequently referred to the National Institutes of Health and diagnosed with PA. Laboratory studies demonstrated an initial serum aldosterone of 24 ng/dl (reference range 3–21 ng/dl), with repeat value of 45.1 ng/dl, and undetectable plasma renin activity ( $<0.2$  ng/ml/hr).

He underwent confirmatory testing while on a high salt diet with intake of 109 mEq/day (equivalent to an oral salt load test). On the third day of the diet, his aldosterone level was 70.2 ng/dl and renin  $<0.2$  ng/ml/hr. Twenty-four-hour urinary aldosterone was 23.5 mcg/24 hr. CT of the adrenal glands showed bilateral enlargement, consistent with BAH (Figure 1). He underwent adrenal vein sampling, with results consistent with bilateral disease. GRA was ruled out as his aldosterone levels failed to suppress on dexamethasone and sequencing for a chimeric *CYP11B1/CYP11B2* gene was negative. He then underwent whole exome sequencing from peripheral DNA, which did not reveal a disease-causing variant (data not shown). His parents were phenotypically normal with negative genetic evaluations. He was started on spironolactone, but this caused gynecomastia. In 2004, he was transitioned from amiloride to eplerenone. Due to uncontrolled hypertension and worsening renal function, he underwent bilateral adrenalectomy in 2017. Histology showed that both adrenal glands had changes consistent with BAH with nodules measuring up to 3 cm in their greatest dimension (Supplementary Figure 1).

Long suspected to have a *de novo* case of a genetic form of PA, due to lack of family history and early age of presentation; classified initially as FH-II, he agreed to undergo additional genetic testing.



**Figure 1.** Bilateral adrenal hyperplasia (arrows) on computed tomography.

### Sanger sequencing analysis of tumor DNA samples revealed the presence of disease-causing variant *KCNJ5* p.G151R in 11 different adrenal nodules

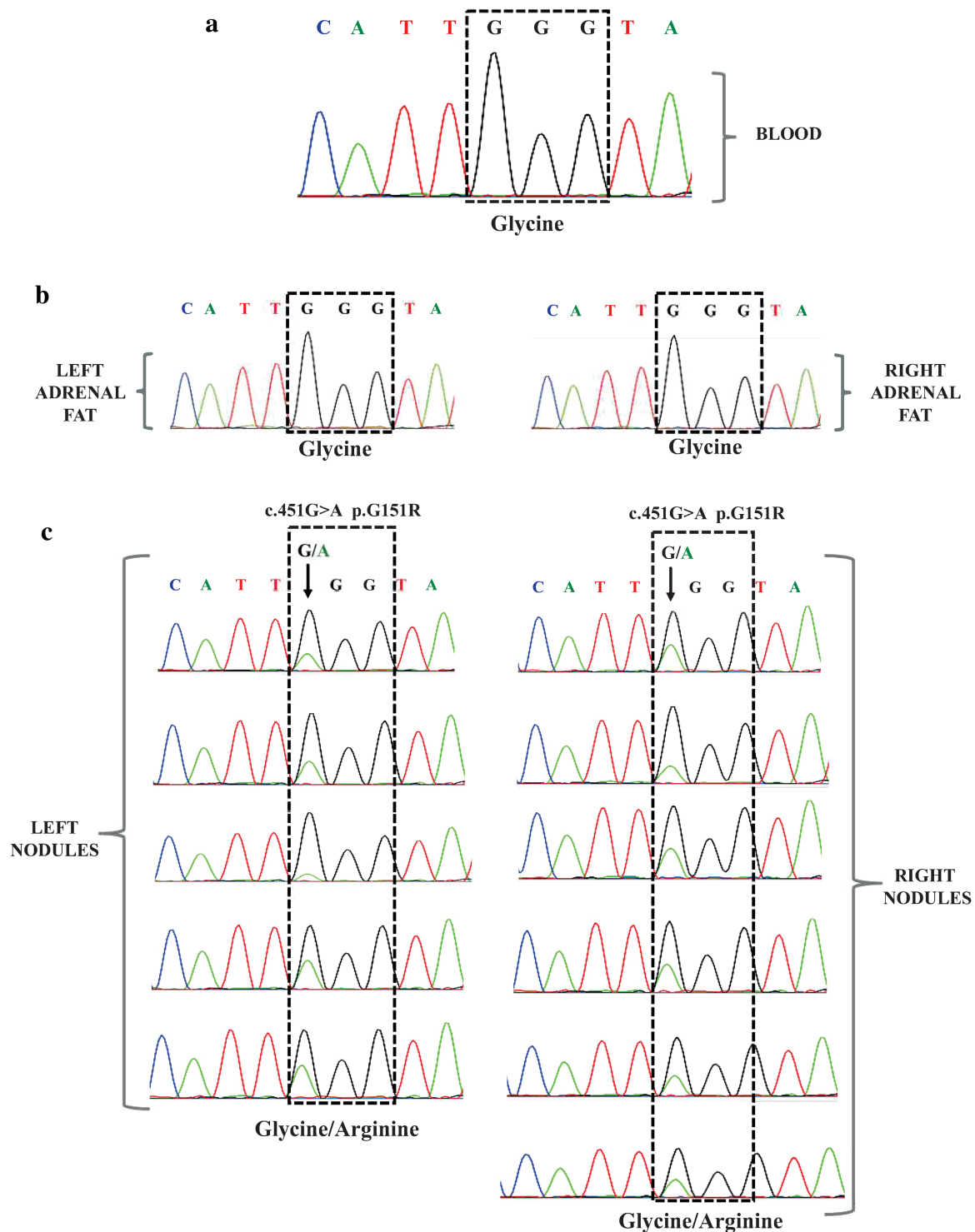
Screening for *KCNJ5* hotspot region p.G151R and p.L168R was performed in peripheral blood leukocytes, right and left adrenal fat DNA, and 11 different adrenal nodules by Sanger sequencing. The *c.451G>A* *KCNJ5* (p.G151R) defect was identified exclusively in the tumor samples; the mutant allele was present in all 11 nodules but not in the blood, nor in the adrenal fat samples (Figure 2). Genomic DNA from parents was analyzed and the *KCNJ5* *c.451G>A* defect, which was not present in their DNA (Supplementary Figure 2).

### CYP11B2 is highly expressed in the *KCNJ5*-mutated adrenal tumors

*CYP11B2* is highly expressed in APAs and therefore is a powerful histopathological tool to confirm excessive aldosterone production and localize aldosterone-producing cells in the adrenal gland.<sup>17</sup> As we found the disease-causing variant in *KCNJ5* in the nodules, we stained one section of a left and another of a right adrenal lesion for CYP11B2; our results showed high expression of CYP11B2 throughout the nodules (Figure 3).

### Deep sequencing analysis revealed mosaicism in *KCNJ5*

Considering the presence of the same *KCNJ5* sequence variant in all the adrenal tumors and no detectable changes in the genomic DNA, we sought to determine the proportion of cells exhibiting the pathogenic variant in question and therefore investigated whether this individual could harbor mosaicism for a *KCNJ5* defect. We performed

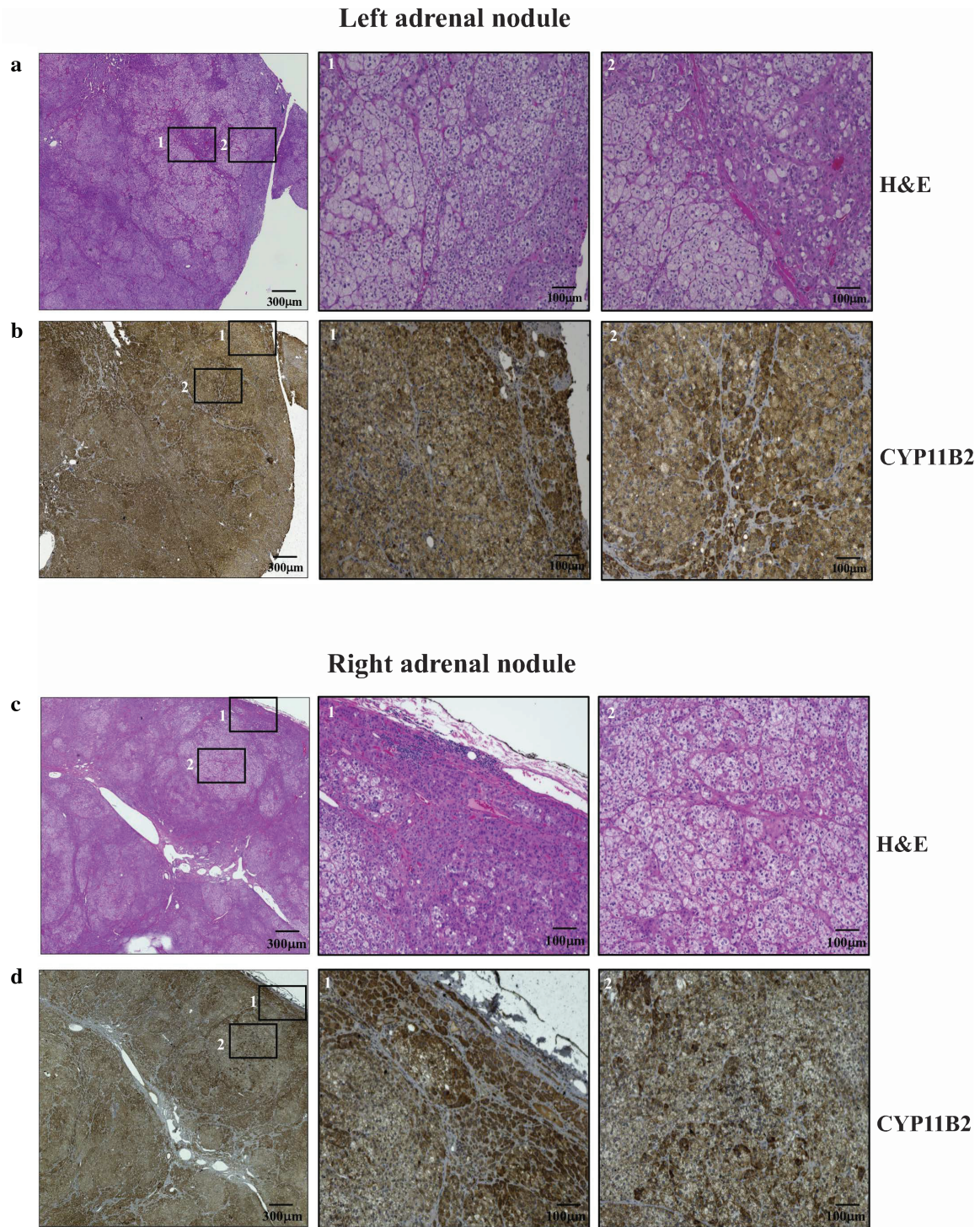


**Figure 2.** Electropherogram of part of *KCNJ5* sequence including the position c.451 in the DNA. No mutant alleles were detected in peripheral blood leucocytes DNA (a) or adrenal fat (b). The c.451G>A *KCNJ5* defect was detected in all the adrenal nodules analyzed, arrow denotes the location of the variant (c).

“deep” next generation sequencing (NGS) of right and left adrenal fat tissue, peripheral blood leukocytes, buccal swab, and tumor DNA (nodule). Approximately 39% of the mutant allele was identified in the nodule sample tested, while in peripheral blood leukocytes’ DNA, we found 0.23% of

the mutant and in other germline samples tested we found between 0.1% and 0.16% of the mutant allele (Table 1), suggesting the presence of very low levels of *KCNJ5* mosaicism at the germline level. The average number of reads per sample was 1,433,999.





**Figure 3.** Representative histopathological analysis of two adrenal nodules of a *KCNJ5* mosaic individual. Hematoxylin-eosin staining of a left adrenal nodule (a). Immunostaining for CYP11B2 of a left adrenal nodule (b). Hematoxylin-eosin staining of a right adrenal nodule (c). Immunostaining for CYP11B2 of a right adrenal nodule (d). Corresponding amplified images from the squares numbered with 1 and 2 in the lower magnification field can be seen in the adjacent images numbered as 1 and 2. Slides were scanned in a  $\times 10$  objective.

**DISCUSSION**

We describe the first patient with BAH caused by mosaicism for a *KCNJ5* pathogenic defect. Although germline defects are expected to cause no more than 10%

of all tumors,<sup>18</sup> NGS has uncovered many cases of causative defects that are present in a few cells. In endocrine tumor syndromes, mosaicism for a postzygotic mutational event is causative of McCune Albright syndrome (MAS) and *HIF2A*-related polycythemia-paraganglioma

**Table 1.** Frequency of variant KCNJ5 p.Gly151Arg detected by deep sequencing in different tissues of the individual in study and the parents

Patient code	Tissue sample	Gene	cDNA	Protein	Total number of reads	Mutation frequency (%)
ADT102.01 (patient)	Right adrenal fat	KCNJ5	c.451G>A	p.Gly151Arg	1,652,034	0.16
ADT102.01 (patient)	Left adrenal fat	KCNJ5	c.451G>A	p.Gly151Arg	1,393,092	0.11
ADT102.01 (patient)	Blood	KCNJ5	c.451G>A	p.Gly151Arg	1,114,362	0.23
ADT102.01 (patient)	Swab	KCNJ5	c.451G>A	p.Gly151Arg	1,397,124	0.10
ADT102.01 (patient)	Nodule	KCNJ5	c.451G>A	p.Gly151Arg	985,195	38.783
ADT102.02 (father)	Blood	KCNJ5	WT	WT	1,863,106	0.00
ADT102.04 (mother)	Blood	KCNJ5	WT	WT	1,885,344	0.00
Negative control (WT)	Blood	KCNJ5	WT	WT	1,181,736	0.00

Abbreviations: WT, wild type.

syndrome.<sup>19–21</sup> Mosaicism is also found in other tumor predisposition syndromes such as tuberous sclerosis. The implications of germline mosaicism for causative variants of tumor predisposition syndromes are immense in regard to genetic counseling for patients and their families, as well as for tumor surveillance which may affect the prognosis of the respective disorders. Germline mosaicism may explain why multiple affected offspring carrying an apparently *de novo* pathogenic variant may be born to unaffected parents.

Disease causing *KCNJ5* defects are present in approximately 24–82% of individuals diagnosed with APA, a percentage that varies between centers and ethnicity.<sup>22</sup> On the other hand, FH-III is extraordinarily rare.<sup>23</sup> It is possible that few cases of mosaicism exist within large cohorts of patients with APA and/or BAH but their identification would require NGS and a search for low-level mosaicism, as was apparent in our case.<sup>24</sup> Such patients may be those with multiple APAs and/or adrenocortical hyperplasia (BAH or other forms) upon histopathological evaluation of the excised tissue, which may be bilateral. Their identification is important not only to guide clinical care for the individual (since both adrenal glands may be affected) but also for the risk of transmission.

Is it possible that our patient's disease was due to multiple *de novo* disease-causing variants of the same type, in concurrent adenomas? Figure 3 demonstrates that all our patient's nodules were strongly positive for CYP11B2, as shown in other cases of PA<sup>9</sup>; however, this was also true for most of the surrounding tissue showing hyperplasia consistent with what has been described in FH-III.<sup>8</sup> Although multiple *de novo* disease-causing variants have been identified in heterogeneously staining nodules including *KCNJ5*, *CACNA1D*, and *ATP1A1*<sup>25</sup> in the same patient, given our patient's pattern of CYP11B2 staining, the identification of the *KCNJ5* p.G151R allele in all 11 nodules derived from both adrenal glands, and the discovery of low-level mosaicism for the same defect in blood, makes this possibility very unlikely.

In conclusion, a patient previously thought to have FH-II due to an unknown defect was found to have BAH due to mosaicism for a “hot-spot” disease-causing variant in *KCNJ5*. The finding has implications for the studies of patients with

aldosterone-producing lesions, the patients' management, prognosis and their genetic counseling.

#### SUPPLEMENTARY MATERIAL

Supplementary data are available at *American Journal of Hypertension* online.

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#### DISCLOSURE

The authors declared no conflict of interest.

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