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## PRIMARY ALDOSTERONISM: KCNJ5 MUTATIONS AND ADRENOCORTICAL CELL GROWTH

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## Abstract

1 Aldosterone-producing adenomas (APA) with somatic mutations in the KCNJ5 potassium channel  
2 are a cause of primary aldosteronism. These mutations drive aldosterone excess but their role in  
3 cell growth is undefined. Our objective was to determine the role of KCNJ5 mutations in adrenal  
4 cell proliferation and apoptosis. The Ki67 proliferative index was positively correlated with  
5 adenoma diameter in APAs with a KCNJ5 mutation ( $r=0.435$ ,  $P=0.007$ ), a negative correlation was  
6 noted in adenomas with no mutation detected (NMD) ( $r=-0.548$ ,  $P=0.023$ ). Human adrenocortical  
7 cell lines were established with stable expression of cumate-inducible wild type or mutated KCNJ5.  
8 Increased cell proliferation was induced by low-level induction of KCNJ5-T158A expression  
9 compared with control cells ( $P=0.009$ ) but increased induction ablated this difference. KCNJ5-  
10 G151R displayed no apparent proliferative effect but KCNJ5-G151E and L168R mutations each  
11 resulted in decreased cell proliferation (difference  $P<0.0001$  from control cells, both comparisons).  
12 Under conditions tested, T158A had no effect on apoptosis but apoptosis increased with  
13 expression of G151R ( $P<0.0001$ ), G151E ( $P=0.008$ ) and L168R ( $P<0.0001$ ). We generated a specific  
14 KCNJ5 monoclonal antibody which was used in immunohistochemistry to demonstrate strong  
15 KCNJ5 expression in adenomas without a *KCNJ5* mutation and in the zona glomerulosa adjacent to  
16 adenomas irrespective of genotype as well as in aldosterone-producing cell clusters. KCNJ5-  
17 CYP11B2 double immunofluorescence showed markedly decreased KCNJ5 immunostaining in  
18 CYP11B2-positive cells compared with CYP11B2-negative cells in APAs with a KCNJ5 mutation.  
19 Together these findings support the concept that cell growth effects of KCNJ5 mutations are  
20 determined by the expression level of the mutated channel.

**Key words:** aldosterone-producing adenoma, adrenal gland, primary aldosteronism, KCNJ5, growth and apoptosis

## 21 **Introduction**

22 Unilateral primary aldosteronism (PA) is the most prevalent surgically-correctable form of  
23 hypertension. The constitutive production of aldosterone mainly originates from a unilateral  
24 aldosterone-producing adenoma (APA) and less often from unilateral hyperplasia (30% and 2% of  
25 cases of PA, respectively).<sup>1</sup> Major breakthroughs in understanding the pathophysiology of sporadic  
26 APAs have been made since the identification by Choi et al.<sup>2</sup> of somatic mutations in the *KCNJ5*  
27 gene (causing *KCNJ5*-G151R or *KCNJ5*-L168R missense mutations) in a high proportion of these  
28 tumors.<sup>2-4</sup> *KCNJ5* is an inwardly rectifying potassium channel (also called GIRK4, G protein coupled  
29 inwardly rectifying potassium channel) and the described mutations cause sodium ion  
30 conductance due to the loss of selectivity for potassium ions by the channel pore. In  
31 adrenocortical cells, the consequent membrane depolarization triggers opening of voltage-gated  
32 calcium channels and calcium ion influx ultimately activates aldosterone production.<sup>2, 5</sup>

33  
34 The identification of additional APA somatic mutations in the Cav1.3 calcium channel (*CACNA1D*)  
35 and in the  $\text{Na}^+/\text{K}^+$ -ATPase and  $\text{Ca}^{2+}$ -ATPase ion transporters (*ATP1A1* and *ATP2B3*, respectively)  
36 highlighted the importance of intracellular ion homeostasis and calcium signaling in aldosterone  
37 production<sup>6, 7</sup> and, together with somatic mutations in  $\beta$ -catenin (*CTNNB1*), these mutations can  
38 be detected in almost 90% of APAs.<sup>8</sup> In most populations, a predominance of *KCNJ5* mutations in  
39 APAs over other genotypes is reported<sup>3, 4, 9-11</sup> with a global prevalence of 43%.<sup>12</sup>

40  
41 A role for *KCNJ5* mutations in adrenal cell growth has not been defined. When initially described,  
42 *KCNJ5* mutations were proposed to result in both constitutive aldosterone production and cell  
43 proliferation<sup>2</sup> due to the established role of calcium signaling in both processes.<sup>13, 14</sup> A function in  
44 driving aldosterone excess has been demonstrated by expression of mutated forms of *KCNJ5* in

45 human adrenocortical cells *in vitro*<sup>5</sup> but a decrease in cell proliferation resulted from expression of  
46 KCNJ5-T158A.<sup>5</sup> This mutation (KCNJ5-T158A) has been identified in both sporadic APAs and a  
47 familial form of PA (called familial hyperaldosteronism type III)<sup>2, 15</sup> and the absence of an effect on  
48 cell proliferation *in vitro* is seemingly paradoxical to the massive cortical hyperplasia observed in a  
49 patient carrying the germline variant.<sup>2, 16</sup>

50

51 Aldosterone-producing cell clusters (APCC) are a histopathologic feature often found beneath the  
52 adrenal capsule under normal and pathologic conditions.<sup>17</sup> APCCs comprise tight nests of  
53 predominantly zona glomerulosa cells with intense immunohistochemistry staining for CYP11B2  
54 (aldosterone synthase). A notable proportion of APCCs carry mutations in CACNA1D, ATP1A1 and  
55 ATP2B3 but KCNJ5 mutations are curiously absent.<sup>17, 18</sup> Our objective was to establish the effects  
56 of KCNJ5 mutations on cell growth in human adrenocortical cells by specifically addressing their  
57 roles in cell proliferation and apoptosis.

58

## 59 **Methods**

60 The data that support the findings of this study are available from the corresponding author upon  
61 reasonable request.

62

## 63 ***Patient samples***

64 The study included 72 surgically resected adrenals from patients diagnosed with unilateral PA  
65 according to the Endocrine Society Guideline.<sup>19</sup> Patients were screened for PA using the plasma  
66 aldosterone-to-direct renin concentration ratio and diagnosis was confirmed by the intravenous  
67 saline load test according to local criteria.<sup>20</sup> Adenoma size was assessed from the diameter of the  
68 largest nodule at pathology and CYP11B2 immunohistochemistry was done on all adrenals and any

69 without a well circumscribed CYP11B2-positive adenoma were excluded. All participants gave  
70 written informed consent and the protocol was approved by the local ethics committee.

71

## 72 ***DNA sequencing***

73 Genomic DNA was extracted from dissected nodules from fresh frozen adrenal tissues, and DNA  
74 fragments were amplified using primers flanking mutation hot spot regions in *KCNJ5*, *ATP1A1*,  
75 *ATP2B3*, and *CACNA1D* before DNA sequencing as described elsewhere.<sup>21</sup>

76

## 77 ***Production of HAC15 stable cells lines with inducible KCNJ5 expression***

78 cDNAs encoding mutated and wild type forms of *KCNJ5* were prepared by Gateway cloning  
79 (ThermoFisher Scientific) in cumate inducible PiggyBac vectors (System Biosciences, Palo Alto, CA).  
80 Stable cell lines were established by co-transfection of human adrenocortical cells (HAC15 cells, a  
81 kind gift from Professor William E. Rainey, University of Michigan, Ann Arbor, USA) with the  
82 PiggyBac vector (carrying the human *KCNJ5* cDNA) and the Super PiggyBac transposase according  
83 to the manufacturer's instructions (System Biosciences, Palo Alto, CA). Transfected cells were  
84 selected with puromycin (4 µg/mL) in the presence of verapamil (10 µM) to inhibit the P  
85 glycoprotein.<sup>22</sup> The macrolide antibiotic roxithromycin (20 µM) was also included to inhibit any  
86 potential effects on cell growth of mutant *KCNJ5* channels<sup>23</sup> in the absence of the cumate inducer.  
87 Total RNA was extracted from stable cell lines after induction with cumate (10 µg/mL) for 72 h,  
88 reverse transcribed and the *KCNJ5* gene was sequenced to confirm the mutated or wild-type  
89 *KCNJ5* genotype of all cell lines.<sup>21</sup>

90

91

92

93 ***Cell proliferation and apoptosis assays***

94 HAC15 cells ( $2.5 \times 10^4$  cells/ well) stably transfected with wild type or mutated forms of *KCNJ5*  
95 (*T158A*, *G151R*, *G151E* or *L168R*) or empty vector were plated in 96-well plates and transcription  
96 was induced with 1  $\mu\text{g}/\text{mL}$  or 10  $\mu\text{g}/\text{mL}$  cumate in the absence of roxithromycin for 24 hours. Cell  
97 proliferation was determined with a WST-1 assay (Roche), and apoptosis was quantified by an  
98 Annexin V apoptosis assay (Promega).

99

100 ***Generation of monoclonal antibodies against human KCNJ5.***

101 A peptide corresponding to the N-terminal portion of human KCNJ5 (acetyl-36-ATDRTRLLAEGKKP-  
102 49-C) with the addition of a cysteine at the C-terminal end was synthesized by LifeTein LLC  
103 (Hillsborough, NJ) and conjugated to 5 mg of Imject<sup>TM</sup> Blue Carrier<sup>TM</sup> Protein (ThermoFisher  
104 Scientific) using Succinimidyl-6-(iodoacetyl)aminocaproate (Molecular Biosciences (Boulder, CO).  
105 Four Swiss Webster Female mice were immunized initially with 10  $\mu\text{g}$  of immunogen with  
106 Complete Freund's Adjuvant (Millipore-Sigma) followed by immunization using incomplete  
107 Freund's adjuvant every two weeks. After 2 months of biweekly immunizations, the mice received  
108 the immunogen in saline intraperitoneally and 3 days later were sacrificed using isofluorane  
109 anesthesia, blood was withdrawn and spleens removed under aseptic conditions. Spleen cells  
110 were then obtained and frozen in liquid nitrogen using DMEM media containing 20% newborn calf  
111 serum, 5% dimethylsulfoxide and 2.5% of polyethylene glycol 1,000.

112

113 After titers were performed on the serum, the spleen from the mouse with the higher titer was  
114 fused with PEG 1450 (ATCC.org) to the mouse myeloma SP2-mIL6-hIL21-hTERT cells and plated  
115 into 10 x 96 well plates. After 10 days the wells were screened by ELISA on plates coated with the  
116 acetyl-36-ATDRTRLLAEGKKP-49-C conjugated to chicken ovalbumin. Positive clones were then



117 screened by Western blotting of cell lysates from HEK 293T cells transduced with a tetracycline-  
118 inducible lentivirus containing the human *KCNJ5* sequence.<sup>5</sup> Clones which gave single bands of the  
119 appropriate molecular mass for *KCNJ5* on Western blots were subcloned using high density methyl  
120 cellulose<sup>24</sup> and were isotyped. The use of mice for the generation of monoclonal antibodies was  
121 approved by the University of Mississippi Medical Center IACUC.

122

### 123 ***Immunohistochemistry and immunofluorescence***

124 Formalin-fixed paraffin-embedded (FFPE) adrenal tissue sections (3  $\mu$ m) were used for CYP11B2  
125 immunohistochemistry to detect aldosterone synthase expression with a monoclonal antibody  
126 (clone 17B) diluted 1:200 as described<sup>25</sup> and *KCNJ5* immunohistochemistry was performed using  
127 the *KCNJ5* monoclonal antibody generated herein (clone # 36-33-5, dilution 1:2000). Double  
128 immunofluorescence CYP11B2 and *KCNJ5* staining used an anti-mouse IgG1 Alexa Fluor 488  
129 secondary antibody (to detect CYP11B2 primary antibody) and anti-mouse IgG2B Alexa Fluor 594  
130 (to detect *KCNJ5* antibodies) both diluted 1:200 (Invitrogen). A rabbit anti-PARP monoclonal  
131 antibody diluted 1:2000 (Cell Signaling) was used for immunofluorescence staining of cleaved  
132 PARP (poly-ADP ribose polymerase) with an anti-rabbit Alexa Fluor 594 secondary antibody diluted  
133 1:200 (Invitrogen).

134

### 135 ***Scoring adrenals for Ki67 proliferation index and *KCNJ5* immunostaining***

136 Ki67 immunohistochemistry was performed on FFPE adrenal sections (3  $\mu$ m) using a rabbit  
137 monoclonal antibody (clone # SP6 1:200 dilution, Sigma-Aldrich). The Ki67 proliferation index was  
138 assessed as the percentage of the manual count of intense Ki67 stained nuclei relative to the total  
139 hematoxylin stained nuclei which were quantified by color segmentation using ImageJ software.

140 Three separate fields of view were used for scoring and the final proliferation index was calculated

141 as the average of the 3 Ki67 scores.<sup>26</sup> To score KCNJ5 immunostaining intensity in adenomas and  
142 paired adjacent cortical tissue, a semi-quantitative score system was used in which intensity of  
143 immunohistochemistry staining was graded 0 to 4 for undetectable, low, moderate or high<sup>27</sup> from  
144 a field of view at x 20 magnification acquired from each adrenal sample. Both the Ki67  
145 proliferation index and H scores for CYP11B2 were evaluated by researchers blinded to mutational  
146 status and pathological reports of the assessed adrenals (HS and TAW). Adenoma sizes (to  
147 determine correlations with Ki67 index) were determined by the pathologist (TK) as the diameter  
148 of the largest nodule.

149

#### 150 ***Statistical analyses***

151 Statistical analyses were performed using SPSS, version 25.0 and Graphpad Prism version 7.0.  
152 Comparisons between two groups were determined using a *t* test or a Mann-Whitney test,  
153 multiple comparisons were analyzed by ANOVA with a Bonferroni test or Kruskal-Wallis tests with  
154 pairwise comparisons. Pearson's correlation coefficients were used to analyze univariate  
155 correlations.  $P < 0.05$  was considered significant.

156

#### 157 **Results**

##### 158 ***Clinical characteristics of patients with APA according to genotype***

159 Genotyping of 72 resected adrenals from patients with an APA, determined 39 APAs with a KCNJ5  
160 mutation (L168R, n=22; G151R, n=16, and T158A, n=1), 5 with a *CACNA1D* mutation, and 3 and 2  
161 APAs with *ATP1A1* or *ATP2B3* mutations, respectively. The remaining 23 APAs did not carry a  
162 mutation in known hotspots of target genes and were referred to as tumors with no mutation  
163 detected (NMD).

164

165 Patients with a KCNJ5-mutated APA were younger than patients with an NMD-APA (47.2 years  $\pm$   
166 10.4 *versus* 57.7 years  $\pm$  11.0,  $P= 0.001$ ) with a higher proportion of women than patients with an  
167 NMD-APA (82.1% of 39 patients *versus* 30.4% of 23,  $P<0.001$ ) or relative to the small group of  
168 patients with other somatic APA mutations (10.0 % of 10 patients,  $P<0.001$ ). The largest adenoma  
169 diameter at pathology was greater in KCNJ5-mutated APAs (17.0 mm [14.0-24.0]) compared with  
170 both NMD-APAs and APAs with other mutations combined (12.0 mm [8.0-25.0],  $P=0.019$  and 9.0  
171 mm [7.8-15.3],  $P=0.003$ , respectively). We noted a lower PAC in KCNJ5-mutated APAs compared  
172 with the group of APAs with a mutation in ATP1A1, ATP2B3 and CACNA1D combined (979 pmol/L  
173 [500-1470] compared with 1989 pmol/L [1624-3346],  $P=0.006$ ) (**Table S1**).

#### 174 175 ***Diverse proliferation in adenomas with or without a KCNJ5 mutation***

176 Ki67 proliferation index was assessed in a subset of adrenals (37 APAs with KCNJ5 mutations; 17  
177 designated NMD and 10 with either a CACNA1D, ATP1A1 or ATP2B3 mutation). Adenoma size was  
178 larger in APAs with a KCNJ5 mutation compared with NMD (17.0 mm [14.5-24.5] *versus* 12.0 mm  
179 [8.0-27.5],  $P=0.0327$ ). APAs with a KCNJ5 mutation had a lower proliferation index relative to APAs  
180 with NMD (0.9%  $\pm$  0.4 *versus* 1.2%  $\pm$  0.4,  $P=0.011$ ). The Ki67 proliferation index was positively  
181 correlated with adenoma diameter in KCNJ5-mutated APAs ( $r=0.4347$ ,  $P=0.0072$ ) in contrast to the  
182 negative linear correlation noted in NMD-APAs ( $r=-0.5484$ ,  $P=0.0226$ ) (**Figure 1**). There was no  
183 correlation of adenoma diameter with Ki67 index in the small group of APAs with a CACNA1D,  
184 ATP1A1 or ATP2B3 mutation combined. There was no significant difference in adenoma diameter  
185 between APAs with a L168R or a G151R mutation (L168R, 16.0 mm [15.0-27.3] *versus* G151R, 18.0  
186 mm [14.0-22.0],  $P=0.636$ ) or in Ki67 score (L168R, 1.0 %  $\pm$  0.4 *versus* G151R, 0.8 %  $\pm$  0.4,  $P=0.339$ ).

187

188

189 ***Effects of KCNJ5 mutations on cell growth in adrenocortical cells***

190 Stable HAC15 cell lines expressing KCNJ5 with different genotypes were established using the  
191 selection marker puromycin. Sensitivity to puromycin was increased in the presence of verapamil  
192 (10  $\mu$ M) (**Figure S1**) and the presence of KCNJ5 mutations was confirmed by Sanger sequencing.  
193 The cell viability of the KCNJ5-T158A HAC15 cell line was significantly higher compared with  
194 control cells (transfected with empty vector) after 24-hour induction with 1  $\mu$ g/mL cumate  
195 ( $P=0.0094$ ). This effect on cell proliferation was absent in cells with increased transcriptional  
196 induction of *KCNJ5-T158A* (10  $\mu$ g/mL cumate). KCNJ5-G151R had no apparent effect on  
197 adrenocortical proliferation *in vitro*, whereas decreased proliferation was observed in HAC15 cells  
198 with KCNJ5-G151E and L168R mutations ( $P<0.0001$  versus control cells, both comparisons) (**Figure**  
199 **2A**).

200

201 Higher levels of cell death by apoptosis were observed in cells with KCNJ5-G151R, G151E and  
202 L168R mutations ( $P<0.0001$ ,  $P=0.0078$ , and  $P<0.0001$  versus control cells, respectively) under the  
203 conditions tested (24-hour incubation with 1  $\mu$ g/mL cumate). Cells carrying the KCNJ5-T158A  
204 mutation did not induce apoptosis under the same conditions (**Figure 2B**). These observations  
205 were consistent with immunofluorescence detection of cleaved poly-ADP ribose polymerase  
206 (PARP), a hallmark of apoptosis, which showed increased numbers cells with positive cleaved  
207 PARP staining in the nuclei of *KCNJ5-G151E* and *L168R* transfected cells compared with control  
208 cells (**Figure S2**). HAC15 cells with KCNJ5-T158A and G151R mutations displayed a similar  
209 proportion of cleaved-PARP positive cells compared with control cells (**Figure S2**).

210

211

212

213 ***Generation of monoclonal antibodies against human KCNJ5***

214 There were 100 positive clones from the ELISA screen and of these, 2 clones (#33 and #68)  
215 displayed specific binding to KCNJ5 on Western blots of HEK 293T cell lysates transduced with a  
216 lentivirus carrying the human *KCNJ5* sequence. Clones #33 and #68 were subcloned to produce  
217 antibodies KCNJ5-33-5 and KCNJ5-68-15 and their specificity was validated by Western blotting  
218 (**Figure 3A**). The two clones were isotypized, clone KCNJ5-33-5 was IgG2b and the KCNJ5-68-15 was  
219 IgG2c. Both antibodies were used for immunohistochemistry of FFPE sections of resected adrenals  
220 from patients with an APA. Analysis of the cortical tissue adjacent to an adenoma demonstrated  
221 membrane and cytoplasmic staining with #68-15 quite diffuse throughout the cortex compared  
222 with predominant plasma membrane staining of zona glomerulosa cells with #33-5 (**Figure 3B, C**).  
223 Clone #33-5 was selected for further immunohistochemistry and immunofluorescence staining.

224

225 ***KCNJ5 expression in APAs varies according to genotype***

226 Immunohistochemistry using the KCNJ5 #33-5 monoclonal antibody was performed on 33 adrenal  
227 samples with various APA genotypes (*KCNJ5*, n=13; WT, n=10; *CACNA1D*, n=5; *ATP1A1*, n=3;  
228 *ATP2B3*, n=2). Adenomas of all adrenals showed positive-immunostaining for KCNJ5 and CYP11B2  
229 (**Figure 4, Figure S3**) with decreased intensity of KCNJ5 immunostaining in APAs with KCNJ5  
230 mutations compared with other adenomas (**Figure 4**). Semi-quantitative H score assessment of  
231 KCNJ5 immunostaining highlighted the decreased KCNJ5 expression in APAs with a KCNJ5  
232 mutation (**Figure 5A**, difference  $P < 0.0001$  for KCNJ5-mutated APAs *versus* NMD-APAs and APAs  
233 with *ATP1A1*, *ATP2B3*, *CACNA1D* mutations combined). There were no apparent differences in  
234 KCNJ5 immunostaining intensity between NMD-APAs *versus* APAs with *CACNA1D*, *ATP1A1*,  
235 *ATP2B3* mutations (**Figure 4, Figure 5A**). No differences in intensity of KCNJ5 immunostaining were

236 apparent between APAs with different KCNJ5 mutations (KCNJ5-G151R, L168R or T158A) (**Figure**  
237 **S3**).

238

239 KCNJ5 immunostaining was lower in all 13 tumors with *KCNJ5* mutations compared with the  
240 paired adjacent cortex (**Figure 4A and B, Figure 5B**). In contrast, the majority of APAs with other  
241 genotypes showed either increased or similar KCNJ5 immunostaining intensity in adenomas (75%  
242 of 20 adrenals) (**Figure 4C, Figure 5B**).

243

244 Double KCNJ5-CYP11B2 immunofluorescence was performed on APAs of different genotypes. Co-  
245 localization of KCNJ5 with CYP11B2 was demonstrated in all adrenals but a decrease of KCNJ5  
246 immunostaining was evident in CYP11B2-positive cells relative to CYP11B2-negative cells of the  
247 same adenoma carrying a KCNJ5 mutation (**Figure 4D, Figure S4**). This difference of KCNJ5  
248 immunostaining intensity was absent in APAs of other genotypes (**Figure S4**).

249

#### 250 ***Expression of KCNJ5 in aldosterone-producing cell clusters***

251 KCNJ5 and CYP11B2 immunohistochemistry and double KCNJ5-CYP11B2 immunofluorescence of  
252 APCCs showed moderate to high expression of KCNJ5 in APCCs (n=11) (**Figure 6A**) and the co-  
253 localization of the high-level KCNJ5 and CYP11B2 immunostaining (**Figure 6B**).

254

#### 255 **Discussion**

256 We demonstrate the diverse effects of KCNJ5 mutations on adrenocortical cell growth. We show  
257 an increase in adrenocortical cell proliferation with low-level transcriptional induction of KCNJ5-  
258 T158A and, under similar conditions, stimulation of apoptosis with KCNJ5-G151R, L168R and  
259 G151E. In adenomas with KCNJ5 mutations, CYP11B2-positive cells display strikingly reduced levels

260 of KCNJ5 expression compared with CYP11B2-negative cells of the same tumor and compared with  
261 CYP11B2-positive cells in APAs of other genotypes. We found decreased KCNJ5 immunostaining in  
262 KCNJ5-mutated APAs compared with paired adjacent cortical tissue in agreement with a previous  
263 study which also showed the absence of KCNJ5 mutations in the adjacent cortex.<sup>28</sup>

264

265 These observations indicate that only low-level expression of KCNJ5 mutations is compatible with  
266 adrenocortical cell survival. KCNJ5 mutations are absent (or at least rarely found) in APCCs which  
267 comprise tight nests of zona glomerulosa cells.<sup>17</sup> The cell toxicity of KCNJ5 mutations combined  
268 with the high KCNJ5 expression in the zona glomerulosa layer is consistent with the absence of  
269 KCNJ5 mutations in APCCs and the particular phenotype of KCNJ5-mutated APAs with a  
270 predominance of zona fasciculata cells over zona glomerulosa cells.<sup>29-31</sup>

271

272 It is unlikely that the differences in intensity of KCNJ5 immunostaining are due to diminished  
273 antibody binding to mutated KCNJ5 because the monoclonal antibody was raised against a peptide  
274 corresponding to an extracellular N-terminal sequence at positions 36-49 (ATDRTRLLAEGKKP), far  
275 removed from the KCNJ5 mutations which are located in or near the channel pore region. Further,  
276 KCNJ5 immunohistochemistry with a polyclonal antibody (binding to multiple epitopes) shows a  
277 similar reduction of KCNJ5 immunostaining compared with the adjacent cortex.<sup>28</sup>

278

279 As reported in other studies,<sup>4,12</sup> APAs with KCNJ5 mutations were larger than other APAs and we  
280 show a positive correlation between nodule diameter of tumors with a KCNJ5-G151R or L168R  
281 mutation with cell proliferation. The pro-apoptotic effects of G151R and L168R and the relatively  
282 larger adenoma diameter of tumors carrying these mutations suggests a selective pressure to  
283 override apoptosis in these tumors. KCNJ5-mutated APAs have distinct transcriptional profiles

284 compared with other APAs<sup>32-34</sup> which may result in the expression of specific pro-survival factors  
285 to counteract the pro-apoptotic effects of KCNJ5-G151R and L168R.<sup>35-37</sup> Conversely, in NMD-APAs,  
286 a decreased Ki67 index was noted with increasing tumor diameter such that NMD-APAs with large  
287 tumor diameters displayed relatively lower Ki67 indices. This is probably due to a decline in  
288 proliferation rate during the lifespan of the tumor, as described previously for sporadic  
289 parathyroid adenomas,<sup>38</sup> and potentially explained by the activation of anti-proliferation and pro-  
290 apoptotic mechanisms to self-regulate tumor growth.

291

292 KCNJ5 potassium channel mutations associated with PA display a loss of selectivity for potassium  
293 ions and aberrant sodium ion conductance.<sup>2,5</sup> This disturbance in channel conductance appears  
294 less severe in with KCNJ5-T158A because human embryonic kidney (HEK) cells expressing this  
295 mutant display an increased permeability ratio for potassium relative to sodium ions compared  
296 with cells expressing G151R or L168R.<sup>2</sup> Transduction of human adrenocortical cells with a  
297 lentivirus carrying the cDNA encoding the KCNJ5-T158A channel resulted in a decrease in cell  
298 proliferation compared with control cells.<sup>5</sup> Our data with the higher level of transcriptional  
299 induction of KCNJ5 concord with the observations of Oki et al.<sup>5</sup> but we did observe an increase in  
300 cell proliferation when the level of induction of KCNJ5-T158A gene expression was decreased.

301

302 Germline variants of KCNJ5 cause a familial form of PA called familial hyperaldosteronism type III  
303 (FH type III).<sup>39</sup> Patients with germline *KCNJ5-T158A* or *G151R* mutations present with a severe  
304 form of PA with extensive adrenocortical hyperplasia requiring bilateral adrenalectomy.<sup>2,16,40</sup>  
305 Patients with FH type III with a KCNJ5-G151E mutation display a relatively mild, medically-  
306 treatable clinical phenotype with apparently normal adrenals from computerized tomography  
307 scan results.<sup>41</sup> Patch clamp electrophysiology of HEK 293T cells transfected with *KCNJ5-G151E* and



308 *G151R*, demonstrated the increased sodium ion conductance of the G151E mutated channel and  
309 cell survival assays established the greater cell lethality induced by G151E relative to G151R.<sup>41</sup> Our  
310 study supports this suggestion because KCNJ5-G151E, but not G151R, caused a highly significant  
311 reduction in the viability of human adrenocortical cells. The increased cell toxicity associated with  
312 KCNJ5-G151E was inferred to limit adrenocortical cell mass and account for the milder phenotype  
313 of carriers of this germline variant<sup>41</sup> probably because only a subset of cells expressing low-levels  
314 of the mutated channel can survive and produce excess aldosterone.

315

### 316 **Strengths and limitations of the study**

317 The strength of our study is the production of stable human adrenocortical cell lines with inducible  
318 expression of KCNJ5 mutations to study the cell growth effects of sporadic and germline *KCNJ5*  
319 mutations. A further strength is the analysis of the proliferative status of a large cohort of APAs  
320 with genotype data that were homogeneously selected for surgery according to a stringent  
321 diagnostic flow chart that included adrenal venous sampling. Finally, we used highly specific  
322 monoclonal antibodies to demonstrate by immunohistochemistry and double  
323 immunofluorescence the variance in KCNJ5 and CYP11B2 expression in APAs according to  
324 genotype. A limitation of our study is that genotyping was performed on dissected pieces of  
325 adrenal nodule rather than targeted to CYP11B2 expressing regions. However, we minimized the  
326 potential genotyping of a non-functional nodule because we performed CYP11B2  
327 immunochemistry of all adrenals included in the study and those with non-functional nodules  
328 were excluded.

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331

332 **Perspectives**

333 KCNJ5 mutations cause cell lethality to a variable degree according to genotype and expression  
334 level. The proliferative function of KCNJ5 mutations *in vivo* is challenging to reproduce *in vitro*  
335 because any long-term chronic effects of potential survival factors is difficult to replicate in  
336 adrenal cell cultures. Transcriptome studies are planned to identify genes and signaling pathways  
337 which enable cell proliferation of adenomas with KCNJ5 mutations, despite the increased cell  
338 lethality caused by their expression, and which limit growth rates of tumors with no mutation  
339 detected.

340

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343

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356 **Conflicts of Interest/Disclosure**

357 None

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## Novelty and Significance

### What is New?

- Ki67 proliferation index is positively correlated with adenoma diameter in KCNJ5-mutated APAs, a negative correlation was noted in tumors with no mutation detected
- Adrenocortical cell expression of the sporadic and germline *KCNJ5-T158A* mutation caused cell proliferation at low induction of expression, other KCNJ5 mutations induced apoptosis
- The zona glomerulosa layer and aldosterone-producing cell clusters adjacent to adenomas show intense KCNJ5 immunostaining
- KCNJ5-mutated adenomas comprise CYP11B2-positive cells with a marked reduction of KCNJ5 immunostaining compared with CYP11B2-negative cells and APAs of other genotype

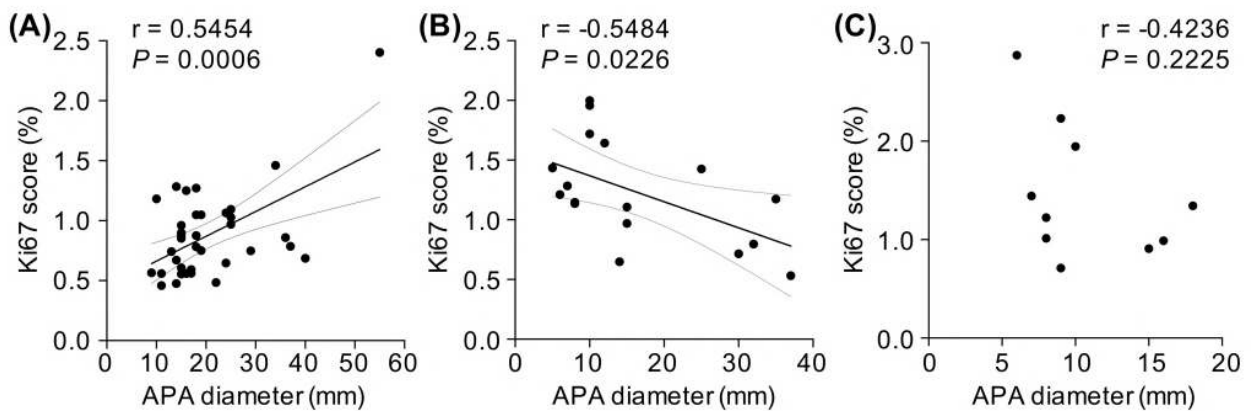
### What is relevant?

- KCNJ5 mutations in APAs are associated with increased adrenal cell proliferation
- KCNJ5 mutations may be absent from aldosterone-producing cell clusters due to the high level of KCNJ5 expression in the zona glomerulosa

### Summary

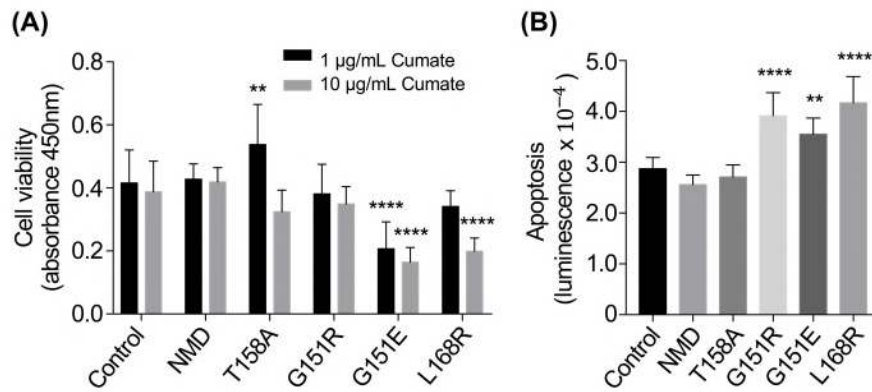
KCNJ5 mutations induce cell toxicity and their effects on adrenocortical cell growth are determined in part by the expression level of the mutated KCNJ5 potassium channel

## Figure Legends



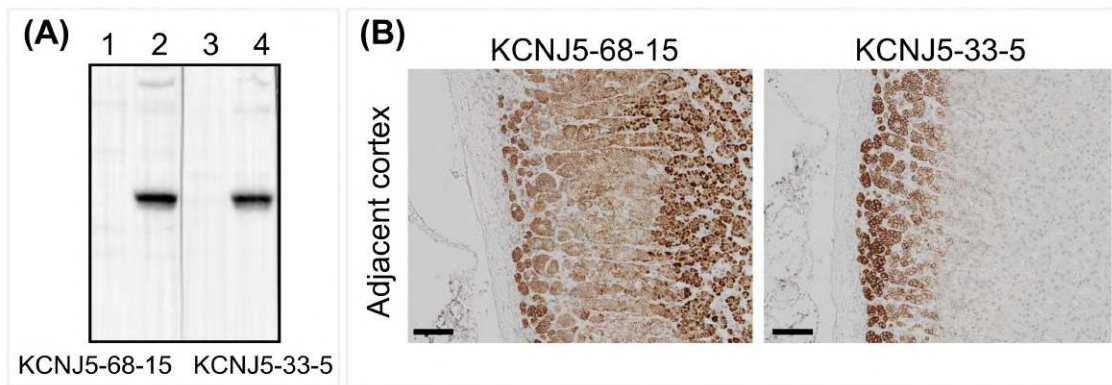
**Figure 1: Correlation of Ki67 score with nodule diameter according to genotype.**

Ki67 score was positively linearly correlated with APA diameter in KCNJ5-mutated APAs ( $r=0.4347$ ,  $P=0.0072$ ) (**Panel A**), whereas a linear negative correlation was observed in the group of tumors with no mutation detected (NMD) ( $r=-0.5484$ ,  $P=0.0226$ ) (**Panel B**). Ki67 index was not correlated with adenoma diameter in the small group of APAs with a CACNA1D, ATP1A1 or ATP2B3 mutation combined (**Panel C**). Ki67 score was derived using ImageJ software and calculated from the average intense Ki67 nuclei staining count divided by the total nuclei hematoxylin staining count from 3 fields of view. Lines represent the Pearson correlation (thick black line) and 95% CI (thin grey line). When the outlier in panel A is omitted, a positive linear correlation between Ki67 index and APA diameter is still observed ( $r=0.5454$ ,  $P=0.0006$ ).



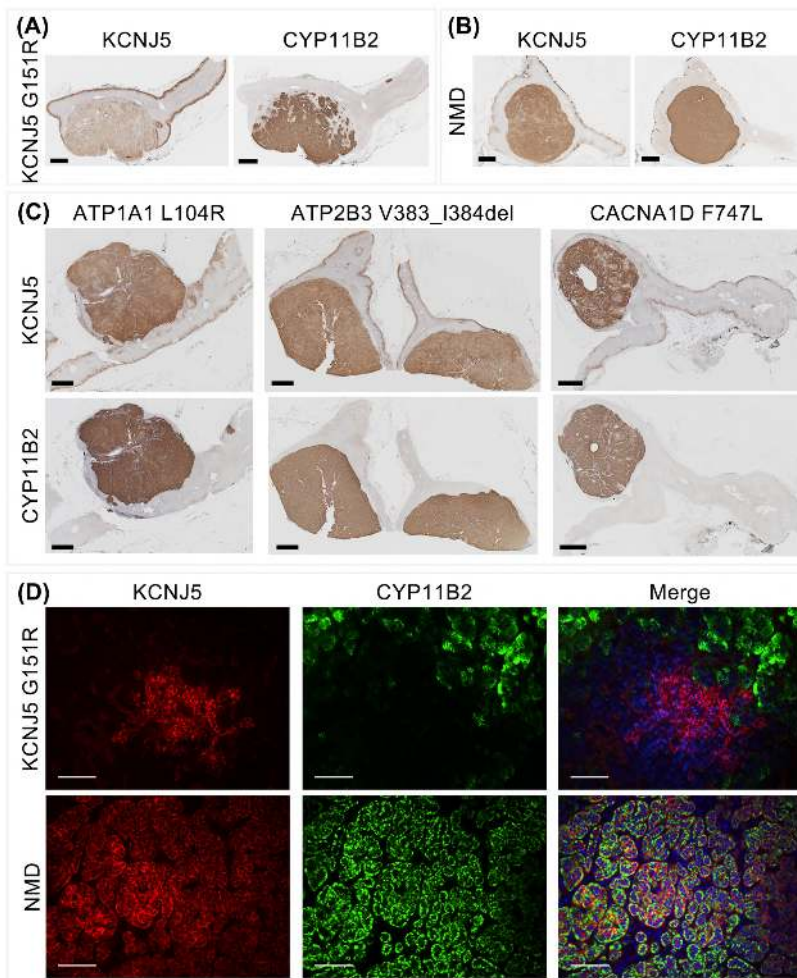
**Figure 2: Effects of KCNJ5 mutants on cell growth in adrenocortical cells.**

HAC15 cells stably transfected with wild type or mutated forms of *KCNJ5* (*T158A*, *G151R*, *G151E* or *L168R*) or empty vector (control) were used to measure cell viability (**Panel A**) or apoptosis (**Panel B**). Cell viability was measured using a WST-1 assay after 24-hour incubation with either 1 µg/mL or 10 µg/mL cumate (black and grey bars, respectively) to induce expression of KCNJ5 (**Panel A**). Apoptosis was measured using an Annexin V assay after 24-hour incubation with 1 µg/mL cumate (**Panel B**). Bars represent means of 6 separate experiments, error bars indicate SD. *P* values were calculated by ANOVA with a post hoc Bonferroni test, \*\*difference ( $P < 0.01$ ) from control, \*\*\*\* difference ( $P < 0.0001$ ) from control. NMD, no mutation detected



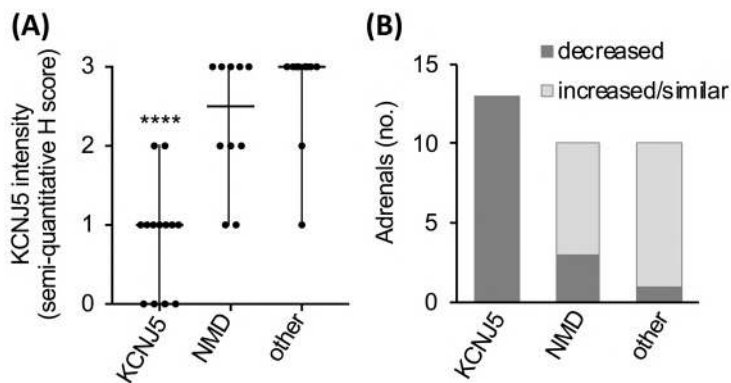
### Figure 3: Generation of KCNJ5 monoclonal antibodies

Monoclonal antibodies against KCNJ5 were produced by injection of mice with a synthetic peptide corresponding to the N-terminal portion of KCNJ5 (acetyl-36-ATDRTRLLAEGKKP-49-C). See methods for details. The specificity of antibodies KCNJ5-68-15 and KCNJ5-33-11 was validated by Western blotting of cell lysates of HEK 293T cells transduced with a tetracycline-inducible lentivirus containing the human KCNJ5 sequence (**Panel A**, uninduced [lanes 1 and 3] and tetracycline-induced [lanes 2 and 4]). KCNJ5 immunohistochemistry of adrenal cortex adjacent to an APA using KCNJ5-68-15 and KCNJ5-33-5 (**Panel B**). KCNJ5-68-15 resulted in staining of most of the cortical tissue with evident staining of nuclei (**Panel B, left**). KCNJ5-33-5 produced intense staining of the zone glomerulosa with clear localization to the plasma membrane (**Panel B, right**). Panel B scale bar = 100  $\mu$ m.



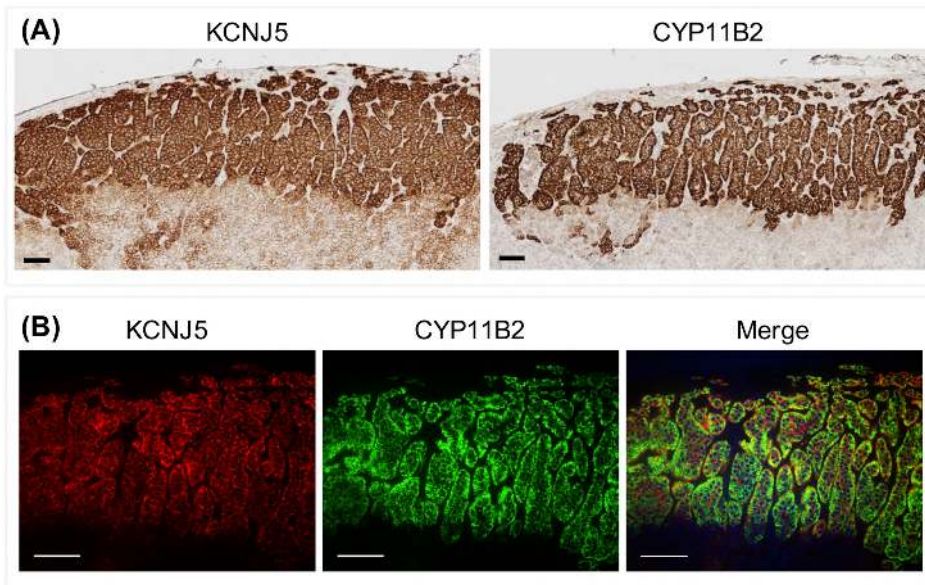
**Figure 4: Heterogeneous immunostaining of KCNJ5 in APA according to genotype.**

Immunohistochemical staining of KCNJ5 and CYP11B2 in an APA with a *KCNJ5* mutation or in an APA with no mutation detected (NMD) showing decreased KCNJ5 immunostaining in the adenoma with a *KCNJ5* mutation (**Panel A, Panel B**). APAs with *ATP1A1*, *ATP2B3* or *CACNA1D* mutations displayed intense KCNJ5 immunostaining (**Panel C**). Double immunofluorescence staining of KCNJ5 and CYP11B2 in an APA with a *KCNJ5* mutation compared with a NMD-APA (**Panel D**). KCNJ5 was intensely expressed in CYP11B2-negative cells in *KCNJ5*-mutated adenoma but markedly decreased KCNJ5 immunofluorescence was observed in CYP11B2-positive cells (**Panel D, upper panel**). In wild type APAs, KCNJ5 and CYP11B2 were co-localized to the same cells (**Panel D, lower panel**). DAPI staining (blue) was only included in the merged image. Panels A - C scale bar = 2  $\mu\text{m}$ ; panel D scale bar = 100  $\mu\text{m}$ .



**Figure 5: KCNJ5 immunostaining in APAs according to genotype.**

Semi-quantitative H score of KCNJ5 immunohistochemistry in adenomas according to genotype is shown **(Panel A)**. Horizontal Lines represent median, vertical lines represent range **(Panel A)**. *P* value was calculated by the Mann-Whitney test, \*\*\*\*difference ( $P < 0.0001$ ) from NMD or from other mutations combined. Relatively lower KCNJ5 immunostaining was noted in all adenomas with a KCNJ5 mutation compared with paired adjacent cortex, whereas 75% of 20 adenomas with other genotypes combined (NMD,  $n=10$ ; *ATP1A1*,  $n=3$ ; *ATP2B2*,  $n=2$ ; *CACNA1D*,  $n=5$ ) showed either increased or similar expression in APAs compared with paired adjacent cortex **(Panel B)**. NMD, no mutation detected; other, adenomas with *ATP1A1*, *ATP2B3* and *CACNA1D* mutation.



**Figure 6: KCNJ5 and CYP11B2 immunostaining of aldosterone-producing cell clusters.**

Immunohistochemistry (**Panel A**) and double immunofluorescence (**Panel B**) showed intense KCNJ5 staining in aldosterone-producing cell clusters and co-localization with CYP11B2. DAPI (blue) was only included in the merged image. Scale bar = 100  $\mu\text{m}$ .