RESEARCH

Characterization of a mutated \textit{KCNJ5} gene, G387R, in unilateral primary aldosteronism

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Abstract

Somatic mutation in the \textit{KCNJ5} gene is a common driver of autonomous aldosterone overproduction in aldosterone-producing adenomas (APA). \textit{KCNJ5} mutations contribute to a loss of potassium selectivity, and an inward Na\textsuperscript{+} current could be detected in cells transfected with mutated \textit{KCNJ5}. Among 223 unilateral primary aldosteronism (uPA) individuals with a \textit{KCNJ5} mutation, we identified 6 adenomas with a \textit{KCNJ5} p.Gly387Arg (G387R) mutation, previously unreported in uPA patients. The six uPA patients harboring mutant \textit{KCNJ5}-G387R were older, had a longer hypertensive history, and had milder elevated preoperative plasma aldosterone levels than those APA patients with more frequently detected \textit{KCNJ5} mutations. CYP11B2 immunohistochemical staining was only positive in three adenomas, while the other three had co-existing multiple aldosterone-producing micronodules. The bioinformatics analysis predicted that function of the \textit{KCNJ5}-G387R mutant channel could be pathological. However, the electrophysiological experiment demonstrated that transfected G387R mutant cells did not have an aberrantly stimulated ion current, with lower CYP11B2 synthesis and aldosterone production, when compared to that of the more frequently detected mutant \textit{KCNJ5}-L168R transfected cells. In conclusion, mutant \textit{KCNJ5}-G387R is not a functional \textit{KCNJ5} mutation in unilateral PA. Compared with other \textit{KCNJ5} mutations, the observed mildly elevated aldosterone expression actually hindered the clinical identification of clinical unilateral PA. The \textit{KCNJ5}-G387R mutation needs to be distinguished from functional \textit{KCNJ5} mutations during genomic analysis in APA evaluation because of its functional silence.

Introduction

Primary aldosteronism (PA) is the leading cause of secondary hypertension (Douma \textit{et al.} 2008). Various genes that are implicated in excess aldosterone production in PA have been identified (Seidel \textit{et al.} 2019). Specifically, mutations in the \textit{KCNJ5}, \textit{CACNA1D}, \textit{CLCN2}, \textit{ATP1A1}, and \textit{ATP2B3} genes are associated with abnormal ion channels or pumps of the cell membrane (Seccia \textit{et al.} 2018).

In aldosterone-producing adenomas (APA), \textit{KCNJ5} is the most commonly identified mutated gene (Boulkroun \textit{et al.} 2012). \textit{KCNJ5} encodes the inward rectifying
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potassium channel Kir3.4 (which is a G protein-coupled, inward-rectifying K channel) participating in potassium selectivity and membrane potential. Mutant KCNJ5 could lead to more depolarization of aldosterone-producing cells, and, therefore, opening of membranous calcium channels increased intracellular calcium signaling and aldosterone production (Choi et al. 2011, Seidel et al. 2019). The majority of KCNJ5 mutations previously linked to APA are somatic mutations, such as p.Gly151Arg (G151R) (49.5–80.0%) and p.Leu168Arg (L168R) (20–45.1%) in Asians and Caucasians, although not in African Americans (Choi et al. 2011, Taguchi et al. 2012, Fernandes-Rosa et al. 2014, Wu et al. 2015, Hong et al. 2016, Nanba et al. 2020). While most KCNJ5 mutations in PA/APA are somatic (Seidel et al. 2019), germline KCN5 mutations are rare and found in inherited forms of PA, such as familial hyperaldosteronism type III (FH-III) (Geller et al. 2008).

Based on the tissue bank of our TAIPAI database for those surgically treated (adrenalectomy) patients with clinical APA (unilateral PA, uPA), we identified a specific mutation of KCNJ5-G387R, previously unreported in APA patients. The mutant KCNJ5-G387R confers an underlying arrhythmia-susceptibility that was known to cause a congenital long QT syndrome (Yang et al. 2010). Using bioinformatics analysis (Ng & Henikoff 2003, Adzhubei et al. 2010, Kircher et al. 2014), the potentially pathogenic phenotype of the KCNJ5-G387R mutation was predicted. Therefore, we investigated the accompanying clinical characteristics in the presence of the KCNJ5-G387R mutation and evaluated the functional properties of KCNJ5-G387R channels in APA.

Methods

Ethics approval of the study protocol

The study complied with the Declaration of Helsinki and was approved by the National Taiwan University Hospital Research Ethics Committee (No. 200611031R). All participants received comprehensive written information and signed a consent form before their inclusion in the study.

Study subjects

The present study enrolled APA patients who were registered in the TAIPAI database (Chan et al. 2019) after unilateral adrenalectomy between 2006 and 2018. The participating study centers included two tertiary medical centers and five regional hospitals (TAIPAI Study Group et al. 2009, Wu et al. 2009, 2011a,b,c, 2015, 2016, 2017a, Peng et al. 2018). General information about age, sex, BMI, systolic blood pressure (SBP) and diastolic blood pressure (DBP) were recorded, and biochemical data were collected. Post-operative clinical and biochemical parameters, including those at 12 months post-adrenalectomy, were also collected serially.

Diagnosis

All anti-hypertensive medications were discontinued for at least 21 days before PA confirmation tests. Doxazosin and/or diltiazem were administered to control markedly high blood pressure during the screening stage when necessary. The diagnosis of PA in hypertensive patients was based on the inappropriate hypersecretion of aldosterone and according to standard criteria (Chan et al. 2019) (detailed in the Supplementary Materials and methods, see section on supplementary materials given at the end of this article). Plasma aldosterone concentration (PAC; Biochem Immunosystems, Bologna, Italy) and plasma renin activity (PRA; Stillwater, MN, USA) were measured using commercial RIA kits.

Lateralization and subtype identification

uPA was identified among PA patients as follows (Chan et al. 2019): (1) lateralization of aldosterone secretion at adrenal venous sampling (AVS) or dexamethasone suppressing NP-59 SPECT/CT (Yen et al. 2009) or (2) pathologically proven adenoma or aldosterone-producing cell clusters (APCC; (multiple) aldosterone-producing micronodule(s); (M)APM) after adrenalectomy and positive immunohistochemical staining with aldosterone synthase CYP11B2 (Wu et al. 2017b) (Supplementary Materials and methods).

KCNJ5 mutation identification and structural biology

DNA was purified from surgically removed adrenal adenoma tissue and peripheral blood using a QIAamp DNA mini kit (Qiagen). PCR was performed using Platinum Taq high fidelity (Invitrogen) in a final volume of 50 µL. After DNase I treatment, 500 ng of total RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Promega) and random hexamers (Promega). The entire coding sequence (exons 2–3) and flanking regions of KCNJ5 gene were amplified and sequenced using gene-specific primers. Direct sequencing of PCR products was performed using the BigDye Terminator version 3.1

https://jme.bioscientifica.com
https://doi.org/10.1530/JME-20-0282
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cycle sequencing kit (Applied Biosystems) with a 3730 DNA Analyzer (Applied Biosystems).

Subsequently, we investigated the structural biology of the mutant KCNJ5 protein (Charmandari et al. 2012). Since the structure of the KCNJ5 protein is still not known, we used the substitution of the KCNJ2 (Kir2.1) protein, which had a known protein structure and is a member of the same family of inward-rectifying potassium channels (Bhave et al. 2010). The amino acid sequence of Kir2.1 (KCNJ2) was shown to be similar to the Kir3.4 (KCNJ5) (Tao et al. 2009, Bhave et al. 2010). The protein sequence features of Kir3.4 (KCNJ5) protein with the mutated sequence was submitted to the Phyre2 web service (http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index) (Kelley et al. 2015) and the structure was specified using chicken Kir2.1 as a template (Tao et al. 2009, Charmandari et al. 2012) (Fig. 1B). Bioinformatics analysis, combined annotation-dependent depletion (CADD) (Rentzsch et al. 2019), polyphen2 and sorting intolerant (Adzhubei et al. 2013) from tolerant (SIFT) (Ng & Henikoff 2003) (http:// pec630.rockefeller.edu:8080/MSC/) were used to predict the aberrant function of the mutant gene.

Tissue immunohistochemistry

Immunohistochemistry (IHC) was conducted using mouse MAB for CYP11B2, rat MAB for CYP11B1 (generous gifts from Professor Celso Gomez-Sanchez (Gomez-Sanchez et al. 2014)) and antibody for KCNJ5 protein (Atlas antibodies, Bromma, Sweden). Sections of paraffin-embedded adrenal tumors and the surrounding tissues were stained using the polymerized horseradish peroxidase (HRP)-anti-mouse conjugate method (Novolink; Novocastra Laboratories Ltd, Newcastle Upon Tyne, UK) according to the manufacturer’s protocol (Wu et al. 2017b). Images were acquired using Olympus BX51 fluorescence microscope with a built-in Olympus DP72 camera and processed using cell Sens Standard 1.14 software (Olympus).

Electrophysiology study

In preparation for the electrophysiology study, HEK293T cells (Chi et al. 2017) were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and incubated at 37°C in a humidified atmosphere with 10% CO2. A total of 2 μg of pIRESGFP-empty vector, pIRESGFP-WT hKir3.4, IRES-GFP-hKir3.4-Gly387Arg or IRES-GFP-hKir3.4-Leu168Arg plasmid mixed with Lipofectamine 2000 according to the manufacturer’s instructions were added to a 35 mm dish grown with HEK293T cells. Transfected cells were confirmed by visualization of GFP expression. Recordings were performed within 48 h after transfection. The detailed experimental procedure is described in the Supplementary Materials and methods (Peng et al. 2018).

Cell culture, transient transfection and detection of aldosterone production

HAC15 cells were cultured in DMEM/F12 medium (Gibco), as previously described (Peng et al. 2018). The plasmids expressing WT KCNJ5 or those with the L168R mutation were constructed by adding the indicated mutation to the WT KCNJ5 plasmid using PCR-assisted site-directed mutagenesis (Pfu DNA Polymerase, Thermo Scientific). The successful introduction of the L168R mutation was confirmed by a PCR-based direct sequencing method. HAC15 cells were transiently transfected with 2 μg pIRES-EGFP empty vector, pIRES-GFP-wild-type hKir3.4, or IRES-GFP-hKir3.4-Gly387Arg. Transfections were performed by Lipofectamine and Plus reagent (Invitrogen) according to the manufacturer’s instructions. Complementary DNAs encoded human Kir3.4 (GenBank accession number NM_000890). Successful transfection was confirmed by KCNJ5 protein expression. The methods of detecting cellular aldosterone production and CYP11B2 synthesis were detailed described in the Supplementary Materials and methods.

Statistical analysis

In the data of clinical parameters, continuous variables were expressed as the mean ± s.d. were expressed as the descriptive statistics. The Mann–Whitney U-test was applied to distinguish variables between different groups. The paired t-test and Wilcoxon signed-rank test were conducted to distinguish variables before and after adrenalectomy. The experimental differences between transfected gene groups and results of the electrophysiological recording were analyzed by one-way ANOVA with post hoc Tukey tests. A two-sided P-value < 0.05 was defined as statistically significant. All of the statistical analyses were performed using IBM SPSS statistics version 17 (IBM Corp) software.

Results

Identifying the KCNJ5 G387R mutation in unilateral primary aldosteronism

A total of 476 adenoma samples from laparoscopic adrenalectomy of the uPA patients were collected and...
sequenced for KCNJ5 mutations. In 223 APA patients harboring KCNJ5 mutations, the most common point mutation was G151R (n = 121, 54.2%), followed by L168R (n = 77, 34.5%). Unexpectedly, a rare KCNJ5 point mutation, G387R, was also found in both adrenal adenomas and peripheral blood cells (Monticone et al. 2013). The guanine-to-cytosine substitution at nucleotide 1159 (c.1159G>C) was identified in six uPA patients. As a consequence of the germline mutation (Monticone et al. 2013), the Kir3.4 amino acid residue 387 was substituted from glycine to arginine (p.Gly387Arg) (Fig. 1A). The structural biology analysis revealed that the mutation site of G387R is located on the C-terminal end of the inner cellular part of the KCNJ5 protein, which is far away from the channel core comprising the potassium selectivity filters (Fig. 1B).

The demographics of APA patients harboring mutant KCNJ5-G387R

The pre-op laboratory values (Table 1) and post-op outcomes (Table 2) of individual uPA patients with mutant KCNJ5-G387R, in comparison with other APA patients harboring common KCNJ5 mutations, are shown. All outlier data were included for statistical analysis. The six uPA patients harboring mutant KCNJ5-G387R were older (62.3 ± 6.8 y vs 47.7 ± 10.2 y, respectively; P < 0.05) and had a higher BMI (27.6 ± 3.4 kg/m² vs 24.9 ± 4.1 kg/m², respectively; P < 0.05) than those APA patients harboring other KCNJ5 mutations. The six uPA patients harboring mutant KCNJ5-G387R had a longer duration of hypertension before their PA diagnosis was confirmed than that of those patients harboring other KCNJ5 mutations (13.6 ± 7.2 years vs 6.3 ± 6.1 years, respectively; P < 0.05). The six uPA patients harboring mutant KCNJ5-G387R also had a more mildly elevated pre-operative PAC level than that of those APA patients with other KCNJ5 mutations (47.7 ± 28.7 ng/dL vs 63.9 ± 47.2 ng/dL, respectively; P < 0.05). Systolic blood pressure readings 12 months post-adrenalectomy were significantly improved in the uPA patients harboring KCNJ5-G387R mutation and the APA patients with previously identified KCNJ5 mutants linked to APA.

Criteria for pathogenicity of filtered variants

Disease-causing mutations were entered into HGMD (http://www.hgmd.org/), ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), and LOVD (http://www.lovd.nl/) if

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**Table 1** Demographics and pre-op lab values of APA patients with mutant KCNJ5-G387R.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Gender</th>
<th>Age (y)</th>
<th>Blood pressure (mmHg)</th>
<th>Aldosterone* (ng/dL)</th>
<th>PRA* (ng/mL/h)</th>
<th>ARR* (ng/dL/ng/mL/h)</th>
<th>K* (mEq/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Female</td>
<td>63.3</td>
<td>135/80</td>
<td>24.18</td>
<td>0.69</td>
<td>35.04</td>
<td>3.9</td>
</tr>
<tr>
<td>2</td>
<td>Female</td>
<td>63.9</td>
<td>148/70</td>
<td>38.86</td>
<td>0.17</td>
<td>228.59</td>
<td>4.4</td>
</tr>
<tr>
<td>3</td>
<td>Male</td>
<td>58.6</td>
<td>152/87</td>
<td>54.8</td>
<td>0.07</td>
<td>782.86</td>
<td>2.9</td>
</tr>
<tr>
<td>4</td>
<td>Male</td>
<td>50.8</td>
<td>173/105</td>
<td>13.66</td>
<td>0.78</td>
<td>17.51</td>
<td>2.3</td>
</tr>
<tr>
<td>5</td>
<td>Male</td>
<td>69.3</td>
<td>190/110</td>
<td>93.39</td>
<td>0.75</td>
<td>124.52</td>
<td>3.1</td>
</tr>
<tr>
<td>6</td>
<td>Female</td>
<td>67.9</td>
<td>143/90</td>
<td>61.3</td>
<td>0.14</td>
<td>437.86</td>
<td>4.0</td>
</tr>
</tbody>
</table>

*aObtained after withholding drugs that interfere with the renin-angiotensin system. APA, aldosterone-producing adenoma; ARR, aldosterone renin ratio; PRA, plasma renin activity.*
variants were present in the disease databases. Null variants (including frameshift mutations, nonsense mutations, obligatory splice sites mutations, and mutations affecting the initiation codon) identified in known APA genes, where loss of function is a known disease mechanism, were also considered to be probably disease-causing. These comprehensive searches through disease databases showed that the germline mutation G387R was related to a congenital disease associated with long QT syndrome. By way of bioinformatics analysis, all pathogenicity scores indicated a possible pathogenic mutation of KCNJ5-G387R (Supplementary Table 2).

**CYP11B2 immunohistochemical staining**

To identify the source of aldosterone production, CYP11B2 IHC staining was performed in adenomas exhibiting KCNJ5 mutations. The CYP11B2 IHC staining of KCNJ5 mutants previously linked to APA demonstrated CYP11B2 positivity within the adenomas (data not shown), which was a common finding for KCNJ5 mutation in APA (Yang et al. 2019). The IHC staining of adrenalectomy specimens from germline KCNJ5-G387R mutant patients is shown in Fig. 2. The results of IHC staining varied significantly among these six KCNJ5-G387R adenomas in regard to CYP11B2 expression. For three mutant KCNJ5-G387R patients, CYP11B2 expression was homogeneous or heterogeneous within the adenoma. In the other three patients, the CYP11B2 staining was negative in the adenoma, but positive in the subcapsular portion of the peri-tumoral region or adjacent para-tumoral tissues, indicating the presence of APCC/(M)APM (Nanba et al. 2013). The CYP11B1 IHC staining of mutant KCNJ5-G387R resected adrenal tissues demonstrated that the density of CYP11B1 stain was similar between tumors and the adjacent regions (Fig. 2). The KCNJ5 protein IHC staining showed various staining within adenomas in the patients with mutant KCNJ5-G387R (Monticone et al. 2012, Taguchi et al. 2012, Mohideen et al. 2019, Yang et al. 2019).

**Electrophysiology of the transfected mutated KCNJ5 channel in HEK293T cells**

To characterize the effects of mutations on the electrical activities of KCNJ5 channels, we expressed the KCNJ5 channel in HEK293T cells and examined ionic currents by the whole-cell patch-clamp study with a holding potential of 0 mV (Fig. 3). The current–voltage relationship was obtained by a series of 100 ms step-wise changes to various membrane potentials ranging from −120 mV to +60 mV with an increment of 20 mV and repeated at intervals of 10 s, which is long enough to return the conformation of the channels to the state at 0 mV. The control cell expressing GFP did not show any significant currents. The representative cell expressing WT showed an outward current of ~300 pA when the potential was held at 0 mV.

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Table 2  Demographics and lab values of APA patients with G387R mutant compared to APA patients with other KCNJ5 mutations.

<table>
<thead>
<tr>
<th>Variables</th>
<th>G387R mutation</th>
<th>Other KCNJ5 mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of participants</td>
<td>6</td>
<td>217</td>
</tr>
<tr>
<td>Age (years)</td>
<td>62.3 ± 6.8a</td>
<td>47.7 ± 10.2a</td>
</tr>
<tr>
<td>Sex, male (%)</td>
<td>50% (3/6)</td>
<td>43.8% (95/217)</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>68.2 ± 15.0</td>
<td>67.1 ± 14.3</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.6 ± 3.4a</td>
<td>24.9 ± 4.1a</td>
</tr>
<tr>
<td>Hypertension duration (years)</td>
<td>13.6 ± 7.2a</td>
<td>6.3 ± 6.1a</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>156.8 ± 20.6</td>
<td>154.9 ± 22.1</td>
</tr>
<tr>
<td>SBP (12 months)</td>
<td>130.2 ± 8.0</td>
<td>129.2 ± 15.1</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>90.3 ± 15.1</td>
<td>94.0 ± 15.5</td>
</tr>
<tr>
<td>DBP (12 months)</td>
<td>79.0 ± 10.1</td>
<td>81.6 ± 10.0</td>
</tr>
<tr>
<td>Aldosterone level (ng/dL)</td>
<td>47.7 ± 28.7a</td>
<td>63.9 ± 47.2a</td>
</tr>
<tr>
<td>Aldosterone level (12 months) (ng/dL)</td>
<td>17.0 ± 13.2</td>
<td>29.8 ± 19.4</td>
</tr>
<tr>
<td>PRA (ng/mL/h)</td>
<td>0.43 ± 0.34</td>
<td>0.42 ± 0.69</td>
</tr>
<tr>
<td>PRA (12 months) (ng/mL/h)</td>
<td>1.52 ± 1.66</td>
<td>3.33 ± 4.17</td>
</tr>
<tr>
<td>ARR (ng/dL per ng/mL/h)</td>
<td>271 ± 294</td>
<td>1224 ± 3042</td>
</tr>
<tr>
<td>ARR (12 months) (ng/dL per ng/mL/h)</td>
<td>241 ± 457</td>
<td>111 ± 531</td>
</tr>
<tr>
<td>K (mEq/L)</td>
<td>3.4 ± 0.8</td>
<td>3.2 ± 0.6</td>
</tr>
<tr>
<td>12 months after adrenalectomy K (mEq/L)</td>
<td>4.6 ± 0.6</td>
<td>4.3 ± 0.4</td>
</tr>
</tbody>
</table>

The analysis of continuous variables was conducted by Mann-Whitney U test. *P < 0.05 was regarded as significant. aObtained after withholding drugs that interfere with the renin-angiotensin system. ARR, aldosterone renin ratio; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; PA, primary aldosteronism; PRA, plasma renin activity; SBP, systolic blood pressure.
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and had inward currents when the potential was changed to −120 to −80 mV; above −80 mV, the evoked currents were decreasing and approaching the holding current. For cells expressing the L168R mutant channel, there was also an outward holding current and the evoked currents were similar to those of the WT. However, there was no outward current in cells expressing G387R mutation at the holding potential; changing the potential to −120 mV evoked a large inward current. Cells expressing WT and G387R had a similar current–voltage relationship, except that outward currents in those cells with the G387R mutant were about half of those in the WT cells but without significance; at +60 mV, 0.12 ± 0.07 pA, n = 6 vs 0.26 ± 0.23 pA, n = 25, P = 0.09. The cells expressing L168R showed no outward currents but significantly higher inward current (−1.06 ± 0.59 nA, n = 11, P < 0.01) at −120 mV than that of the WT (−0.60 ± 0.35 nA, n = 25) and G387R (−0.45 ± 0.25 nA, n = 6). Considering the similar current–voltage relationship between G387R and WT, the G387R mutation did not affect the channel as the classical KCNJ5-L168R mutant did. In regards to the results of the patch-clamp study, the KCNJ5-G387R mutation did not differ from the WT adrenal cells in influencing the potassium selectivity in aldosterone-producing cells.

The aldosterone production of transfected mutated G387R gene in adrenal HAC15 cells

We investigated whether mutant KCNJ5-G387R could affect aldosterone production in an in vitro study. The aldosterone level in the culture medium did not increase in transfected mutant KCNJ5-G387R cells, but it increased in mutated KCNJ5-L168R cells (Fig. 4A). Western blotting demonstrated that expression of the CYP11B2 protein, aldosterone synthase, increased in the cells transfected with the L168R mutant cells but not in those transfected with KCNJ5-G387R (Fig. 4B). Successful transfection of WT and mutant gene of KCNJ5 was confirmed by KCNJ5 protein expression of transfected cells (Fig. 4B).
Discussion

The genomic DNA of adrenal adenomas was sequenced and a germline G387R mutation in the gene of KCNJ5 (Kir 3.4) potassium channel was identified in 1.26% of our uPA patients who underwent adrenalectomy. Although the mutant KCNJ5-G387R was originally found in long QT syndrome (Yang et al. 2010) among congenital arrhythmic patients and was identified to be a disease-associated human genetic variant from current repositories, it has not been reported in uPA patients. Interestingly, the transfected G387R-mutant HEK cells did not have a distinct stimulated ion current as expected from another KCNJ5 mutant (L168R) and behaved similarly and with even weaker response when compared with the WT KCNJ5 cells in the patch-clamp experiment. The CYP11B2 expression and aldosterone levels in the cell cultural medium also confirmed that the transfected mutated G387R gene did not increase aldosterone production – implying that the mutant KCNJ5-G387R is not a contributing factor for aldosterone over-production in uPA. Clinical observation showed that the elevated PAC was milder in those uPA patients harboring KCNJ5-G387R mutation, in comparison to those uPA patients harboring other KCNJ5 mutations previously linked to uPA. This might actually pose challenges to early and straightforward diagnosis of uPA, as the aldosterone excess was not that obvious among these patients, leading to a longer duration of hypertension before the definitive APA diagnosis among these uPA patients with KCNJ5-G387R mutation.

The prevalence of KCNJ5-G387R mutation was 1.26% in our uPA patients compared to 0.01–0.07% in the general population (https://www.ncbi.nlm.nih.gov/snp/rs199830292). With a higher prevalence of the
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KCNJ5-G387R mutation uPA, this mutation could be linked to the pathogenesis of uPA. Meanwhile, bioinformatics analysis predicted a probable pathological phenotype of the KCNJ5-G387R mutation and gave us a reason to study the function of the KCNJ5-G387R mutation. We applied structural biology analysis, the results of which demonstrated that the G387R mutation site was located away from the channel core comprising the potassium selectivity filter (Prada et al. 2017). Although the mutation was at a distance from the channel core, there was still the possibility that the mutation could affect potassium selectivity (Murthy et al. 2014). Interestingly, however, we did not find that HAC15 cells expressing this G387R mutant gene showed increased production of aldosterone in our in vitro functional study, and clinically, those uPA patients with this G387R mutation actually had more mildly elevated plasma aldosterone levels than those of the other APA patients.

The PA patients with KCNJ5 mutations previously linked to APA are younger than WT KCNJ5 PA patients (Lenzini et al. 2015). Patients harboring KCNJ5 mutations also have larger tumor sizes and higher plasma aldosterone levels than WT KCNJ5 patients (Lenzini et al. 2015, Monticone et al. 2015). PA patients carrying germline KCNJ5 mutations (Charmandari et al. 2012, Scholl et al. 2012) had an early-onset disease presentation or dominant phenotype. However, our mutant KCNJ5-G387R patients had a late-onset disease and less pronounced aldosterone excess preoperatively compared to other KCNJ5 mutant patients.

Most PA harboring a somatic KCNJ5 mutation presents as APA (Nishimoto et al. 2015, Seccia et al. 2018); only a few studies reported their presence in APCC/mAPM (Omata et al. 2018, De Sousa et al. 2020). In our uPA patients, the expression of CYP11B2 was dominant within adenomas with KCNJ5 mutations previously linked to

Figure 4
Aldosterone production and expression of aldosterone synthase in transfected HAC15 cells. The aldosterone levels in the cultural medium and the CYP11B2 expression in transfected HAC 15 cells were measured at 72 h. (A) The aldosterone levels in the medium of cultured cells increased in mutant L168R cells but not in G387R mutant cells. The data were presented as the means ± s.d. of three independent experiments. *P < 0.01 when compared to the WT group. (B) The CYP11B2 expression increased in L168R mutant cells but did not increase in mutant G387R cells compared with WT KCNJ5 cells. The KCNJ5 protein expression in transfected cells represented the successful transfection. (C) The quantitative analysis of CYP11B2 and KCNJ5 protein expression was performed using Imagej software. The relative protein expression was normalized to GAPDH and then compared with the empty vector control. The data are presented as the means ± s.d. of three independent experiments (P < 0.05).

https://doi.org/10.1530/JME-20-0282
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Published by Bioscientifica Ltd.
Printed in Great Britain
uPA, while uPA harboring the KCNJ5-G387R mutation had varied CYP11B2 staining. Three (50%) patients had CYP11B2 expression within the adenoma, while another three patients had CYP11B2 expression in adjacent APCC/mAPM. The different histological expression patterns further supported the hypothesis that G387R mutant patients were quite different from other KCNJ5 mutations previously linked to uPA.

In addition to the KCNJ5-G387R mutation, no other mutations were found in these six adenomas. Based on the improvement of clinical and biochemical manifestations after unilateral adrenalectomy, it was preliminarily inferred that the KCNJ5-G387R mutation might be a functional mutation. However, the IHC staining of CYP11B2, aldosterone synthase, was inconsistent in these six adenomas. According to the result of the CYP11B2 IHC staining, not all of these six adenomas were functional adenoma. Therefore, KCNJ5-G387R mutation identified within these adenoma could not be considered to be the cause of excess aldosterone production. Our in vitro experimental results also support that the KCNJ5-G387R mutation is not a functional mutation. Compared with cells transfected with WT KCNJ5, electrophysiology studies did not show inward currents of transfected mutant KCNJ5-G387R cells. Simultaneously, CYP11B2 expression and aldosterone production in transfected mutant KCNJ5-G387R cells did not increase, while that in the transfected mutant KCNJ5-L168R increased. In brief, although the resected adrenal tissues of the six patients with uPA had aldosterone over-production, no functional mutation could be identified. Non-functional KCNJ5 mutation, such as G247R mutation, has also been reported in APA patients (Murthy et al. 2014). However, it rose a question that where the excess aldosterone came from. Although somatic KCNJ5 mutations are the main cause of APA in certain ethnic groups (Williams et al. 2015), there are still some APA patients who cannot be identified with specific genetic mutations (Williams et al. 2015). Therefore, the three positive CYP11B2 IHC staining adenoma were functional adenoma but no specific gene mutation could be found. In contrast, in the other three patients with negative CYP11B2 IHC staining adenomas, the over-production of aldosterone might be contributed by APCCs in adjacent adrenal tissues.

It is reasonable to speculate that there could also be APCC/mAPM located in the contralateral adrenal gland. Hence, it is conceivable that clinical uPA patients with multiple ipsilateral APCCs might have similar adrenocortical conditions (multiple APCCs) in the opposite adrenal gland (Kometani et al. 2018).

This assumption was supported by the findings of our group and others, showing that the lateralization index (LI) was significantly lower and contralateral suppression (CLS) was higher in the clinical uPA patients harboring APCCs than in those without APCCs. Contralateral APCCs could also contribute to a lesser biochemistry recovery after adrenalectomy in patients with mutant KCNJ5-G387R.

Yang et al. reported that heterozygous KCNJ5-G387R mutation is pathogenic for congenital long QT syndrome (Yang et al. 2010). They co-expressed Kir3.1 and Kir3.4 in HEK293 cell to mimic heterozygous expression of Kir3.4 of IKACH channels in patients with long QT syndrome. Cells transfected with Kir3.1/Kir3.4-G387R had a smaller current amplitude than those with WT or, in other words, there was a dominant negative effect of the Gly387Arg mutant (Yang et al. 2010). They concluded that Gly387Arg mutation contributed to loss of IKACH channel function and interfered with ventricular repolarization (Yang et al. 2010). In this study, we found that HEK293 and HAC15 cells expressing mutated KCNJ5-L168R, but not KCNJ5-G387R, had a larger inward ionic current and more aldosterone production, respectively, than those cells expressing WT KCNJ5. The current–voltage relationship of KCNJ5-L168R expressed in HEK cells shows a larger inward current than the WT at cell potentials lower than −70 mV and little outward current, indicating a facilitating depolarization effect of mutated KCNJ5-L168R resulting in enhanced secretion of aldosterone. In contrast, KCNJ5-G387R showed a similar current–voltage relationship to WT but had a smaller (~50%) outward current than WT.

We administered angiotensin II as an aldosterone secretagogue (Oki et al. 2012) but did not observe significantly different aldosterone production in cells expressing WT and KCNJ5-G387R (see supplementary materials). Cells at zona glomerulosa (ZG) have a negative resting membrane potential close to the equilibrium potential of K+ and show periodic oscillation in membrane potential (Hu et al. 2012). It is likely that patients carrying KCNJ5-G387R mutation, which has a reduced outward current, may not be capable to repolarize the membrane potential as fast as that done by the WT during the electric oscillation. The lengthened depolarization may enhance the Ca2+ influx and aldosterone release. However, HAC15 cells are poorly depolarized and do not have such electric fluctuation. The outward current of KCNJ5-G387R mutation is enough to maintain the membrane potential as that of cells expressing WT. Therefore, there is no difference in the stimulated secretion of aldosterone in HAC15 cells expressing KCNJ5-G387R or WT.
However, there is still the possibility for mutant G387R channel to affect aldosterone production. It seems to be different curves between mutant KCNJ5-G387R and WT channels in our electrophysiological studies although there is no significant difference between them. Dynamic spike and rapid electric oscillation in neuron cells may affect depolarization. In neurons, when the membrane potential is rapidly depolarized, the spike voltage threshold is lower to enhance synchronized excitatory inputs in response to the induced depolarization. (Azouz & Gray 2000, 2003). The ZG cells in fresh adrenal gland slices show spiking activity and electric fluctuation (Hu et al. 2012) like neurons. In this way, the natural ZG cells could be excitable at more negative membrane potential and the threshold of calcium entry is basically decreased. The mutant channel could increase aldosterone production by allowing spiking at more hyperpolarized membrane potentials. However, HAC15 cells lack the complete electrophysiology system to support the membrane potential spiking and have a resting potential at a poorly depolarized state. In the absence of spiking and electric fluctuation, the evoked action potentials as natural ZG cells for aldosterone production could not be observed (Bandulik et al. 2015). Therefore, there might be the fact that mutant G387R channel could actually increase aldosterone production in ZG cells but it could not be demonstrated in cellular experiments. Although the HAC15 cell line is a well-established human cell model of the ZG cell (Oki et al. 2012), there are issues with its biology that has not been entirely recognized.

Patients with the G387R mutation might not show a significant increase in aldosterone level at an early stage; this could lead to a delay in clinical diagnosis of uPA and could also dampen the severity of hyper-aldosteronism compared with other KCNJ5 mutants previously linked to uPA. While recording the ionic current, Yang et al. adopted a buffer system with the same K+ concentration (140 mM) inside and outside the cell to emphasize the inward current and maintain an equilibrium potential of 0 mV according to Nernst’s equation. In contrast, in our studies to mimic the normal physiological environment, the K+ concentration in the bath and pipette solution were 5.7 and 120 mM, respectively, and the calculated equilibrium potential of K+ was about −78 mV. Therefore, we could monitor not only the inward but the outward currents as well caused by the opening of K+ channels.

Overall, KCNJ5 mutation is common in our uPA patients. However, there could be incidental mutations without functional influence in KCNJ5, and incidental mutations might not be the cause of excess aldosterone production (functional silence). When clinicians are investigating the KCNJ5 mutations with altered function, such incidental mutations need to be distinguished from other KCNJ5 mutations, which are functionally pathognomonic.

There were limitations to the present study. First, the limited numbers of uPA patients in our study with mutant KCNJ5-G387R could limit the statistical power from which to draw a conclusion. Secondly, the mutant KCNJ5-G387R patients had less pronounced PAC than other commonly reported KCNJ5 mutant patients. Since the mutant KCNJ5-G387R was not a functionally active mutation that triggered aldosterone excess, we did not identify the specific cause of hyperaldosteronism in these patients. To exclude the possibility of other mutation(s) contributing to excess aldosterone production in these six adenomas, we sequenced their ATP1A1, ATP2B3 and CACNA1D genes, but no mutations were identified. However, excess aldosterone in three of the six affected patients might come from the adjacent APCC/mAPM. Because of our technical limitation in microdissection, we did not identify the possible mutations in these APCCs. But it did not affect the subject that the mutant KCNJ5-G387R was not the functional mutation in this study. Finally, although the HAC15 cell is a well-established cellular model for adrenal aldosterone production (Oki et al. 2012), they are not as hyperpolarized as ZG cells in adrenal glands (Bandulik et al. 2015). The HAC15 cells are not as excitable as ZG cells (Hu et al. 2012, Bandulik et al. 2015), and their electrical properties might not be sufficiently preserved. Thus, these cells might not be ideal for use to determine effects of KCNJ5 mutations on aldosterone production.

Conclusion

Our findings expanded on the clinical spectrum of phenotypes associated with germline KCNJ5-G387R mutant uPA and suggested that this mutation is less related to the pathogenesis of adrenal aldosterone overproduction. The G387R mutation did not affect the potassium selectivity of the KCNJ5 channel as other KCNJ5 mutants do and was not able to enhance CYP11B2 overexpression in HAC15 cells. We suggest that the germline KCNJ5-G387R mutant is not a functionally active mutation of KCNJ5 and has no role in uPA/APA as revealed by the results from HAC15 cells. Thus, some mutations might not change the function of the KCNJ5 channel and need to be distinguished from the traditional KCNJ5 mutations with altered function when evaluating uPA/APA patients.
Supplementary materials
This is linked to the online version of the paper at https://doi.org/10.1530/JME-20-0282.

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.

Funding
This study was supported by Taiwan National Science Council (104-2314-B-002-125-MY3, 106-2314-B-002-166-MY3, 107-2314-B-002-026-MY3), National Health Research Institutes [PH-102-SP-09], National Taiwan University Hospital (106-FTN20, 106-P02, UN106-014, 106-S3582, 107-S3805, 107-702, PC1246, VN109-09, 109-S4634, UN109-041) and Ministry of Science and Technology (MOST) of the Republic of China (Taiwan) [grant number, MOST 106-2321-B-182-002] and Mrs Hsiu-Chin Lee Kidney Research Fund.

Data availability statement
The data that support the findings of this study are available on request from Professor Vin-cent Wu. The data are not publicly available due to containing information that could compromise the privacy of research participants.

Author contribution statement
C-Y Pan and H-W Liao contributed equally to this work.

Acknowledgements
The authors greatly appreciate the Second Core Lab in the National Taiwan University Hospital for technical assistance and Mrs Hsiu-Chin Lee Kidney Research Fund. The authors also greatly appreciate English editing from Dr Yvonne Chueh, Akron General Hospital, Cleveland Clinic Foundation.

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The mutant KCNJ5-G387R in unilateral PA


Received in final form 18 August 2021
Accepted 31 August 2021
Accepted Manuscript published online 31 August 2021