Aldosterone-Producing Adenomas

Clinical Characteristics of Somatic Mutations in Chinese Patients With Aldosterone-Producing Adenoma


See Editorial Commentary, pp 507–509

Abstract—Recent studies have shown that somatic mutations in the KCNJ5, ATP1A1, ATP2B3, and CACNA1D genes are associated with the pathogenesis of aldosterone-producing adenoma. Clinical profile and biochemical characteristics of the mutations in Chinese patients with aldosterone-producing adenoma remain unclear. In this study, we performed DNA sequencing in 168 Chinese patients with aldosterone-producing adenoma and found 129 somatic mutations in KCNJ5, 4 in ATP1A1, 1 in ATP2B3, and 1 in CACNA1D. KCNJ5 mutations were more prevalent in female patients and were associated with larger adenomas, higher aldosterone excretion, and lower minimal serum K+ concentration. More interestingly, we identified a novel somatic KCNJ5 mutation (c.445-446insGAA, p.T148-T149insR) that could enhance CYP11B2 mRNA upregulation and aldosterone release. This mutation could also cause membrane depolarization and intercellular Ca2+ increase. In conclusion, somatic KCNJ5 mutations are conspicuously more popular than mutations of other genes in aldosterone-producing adenomas of Chinese patients. The T148-T149insR mutation in KCNJ5 may influence K+ channel selectivity and autonomous aldosterone production. (Hypertension. 2015;65:622-628. DOI: 10.1161/HYPERTENSIONAHA.114.03346.) • Online Data Supplement

Key Words: aldosterone ■ hypertension ■ KCNJ5 ■ potassium channel ■ somatic mutation

Primary aldosteronism (PA), known as the most common cause of endocrine hypertension, accounts for ≈10% of newly diagnosed hypertensive patients. It is characterized by the dysregulation of aldosterone production. Compared with essential hypertension patients, PA patients are at higher risk of cardio-cerebrovascular complications that deserves more attention.6,7 The Endocrine Society recommends screening for PA in patients with drug-resistant hypertension, hypokalemia, and so on. PA comprises both sporadic and familial forms.6,7 Aldosterone-producing adenoma (APA) and bilateral adrenal hyperplasia are the major causes of sporadic PA.6 It is of value to detect the molecular mechanisms of APA, a possibly curable disease, because adrenalectomy could partially reverse hypertension and cardiovascular damage.8

In 2011, Choi et al identified 2 somatic mutations (G151R, L168R) in the KCNJ5 gene (encoding a potassium inwardly rectifying channel) in 8 out of 22 sporadic APAs.9 The authors explained that KCNJ5 mutations resulted in a loss of K+ channel selectivity and constitutive aldosterone production. This discovery has provided new insight into the molecular mechanisms of APA pathogenesis. More recently, somatic mutations in 3 other genes, namely, ATP1A1 (encoding the α1 subunit of the Na+/K+-ATPase), ATP2B3 (encoding the plasma membrane Ca2+-ATPase 3), and CACNA1D (encoding the Ca2+1.3 channel), were revealed to increase aldosterone biosynthesis in APAs.10–12 Mutations of these genes could affect adrenal zona glomerulosa cell membrane potential and intracellular ionic homeostasis.

As these acquired mutations were only detected in resected APAs, the clinical implications of somatic mutations remain to be determined. Intriguingly, recent observations showed that KCNJ5 mutation carriers might exhibit a more florid phenotype with younger age,13,14 higher aldosterone level,13,14 and prominent cardiac damage.15 Currently, there has not been agreement on the correlation between KCNJ5 mutations and response to adrenalectomy.15,16 Therefore, a greater number of
cases need to be studied to evaluate the clinical characteristics of patients with and without somatic mutations in APAs. There has been no study investigating somatic mutations in APAs in China thus far.

In this study, we explored KCNJ5, ATP1A1, ATP2B3, and CACNA1D for mutations in 168 consecutive Chinese patients with sporadic APA and investigated the clinical and biochemical characteristics associated with somatic mutations.

Methods

An expanded Methods section is available in the online-only Data Supplement.

Subjects and Diagnostic Criteria

One hundred sixty-eight cases with APA (83 males and 85 females) were consecutively enrolled in the study from 2008 to 2014. All cases were hospitalized in the Department of Hypertension for diagnosis and then underwent adrenectomy in the Department of Urology (Ruijin Hospital, Shanghai Jiao Tong University School of Medicine). The diagnostic process of APA was formulated accordingly based on the recommendations of current guidelines (Figure S1 in the online-only Data Supplement).6,17 All subjects provided written informed consent, and the procedure got approval of the local ethics committee. The detailed procedure is shown in the online-only Data Supplement.

Sequencing of the KCNJ5, ATP1A1, ATP2B3, and CACNA1D Genes

Detailed data for DNA extraction and Sanger Sequencing are available in the online-only Data Supplement.

Western Blotting

Western blotting was performed to detect KCNJ5 protein expression in 50 APAs. Relative protein quantification was evaluated by Image J 1.44 (National Institutes of Health, Bethesda, MD).

Biophysical Properties of the KCNJ5T148-T149insR Mutation in Vitro

Adrenocortical carcinoma NCI-H295R cells or human embryonic kidney 293T cells were transiently transfected with KCNJ5T148-T149insR (or KCNJ5WT as control) and KCNJ3. The mRNA expression of CYP11B2 (aldosterone synthase gene) was assessed, and the supernatant was collected for aldosterone measurement. In addition, whole-cell voltage clamp recordings were performed.

Statistical Analysis

Statistical analyses were performed using the SPSS Statistics 19.0. Categorical variables were compared with chi-square test. Normally distributed continuous variables were presented as mean±SD and analyzed using Student’s t test or 1-way ANOVA. Skewed variables were analyzed using Mann–Whitney U test. If not appropriate, skewed variables were analyzed using logarithmic or square transformation before Student’s t test or 1-way ANOVA. Skewed variables were presented as mean±SD and analyzed using Student’s t test. If not appropriate, skewed variables were analyzed using Mann–Whitney U test. P<0.05 was considered as statistically significant.

Results

KCNJ5 Sequencing and Identification of a New KCNJ5 Mutation

Somatic KCNJ5 mutations were screened in 129 (76.8%) of the 168 APAs (Table 1). In these 129 cases, no KCNJ5 mutation was found in the normal adrenal tissue (n=116) or peripheral blood (n=87). The KCNJ5 mutations included G151R, L168R, and T158A mutations in 67, 60, and 1 case(s), respectively, and a novel insertion mutation (c.445-446insGAA, p.T148-T149insR) in a patient (Figure 1A). Of the 67 cases

<table>
<thead>
<tr>
<th>Country</th>
<th>Number</th>
<th>KCNJ5 Mutated,</th>
<th>G151R,</th>
<th>L168R,</th>
<th>Other Mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>CN</td>
<td>168</td>
<td>76.8</td>
<td>39.9</td>
<td>35.7</td>
<td>T158A (n=1)</td>
</tr>
</tbody>
</table>

Other Mutations Reference

T148-T149insR (c.445-446insGAA, n=1)

Somatic Mutations in Chinese APAs

Table 1. Prevalence of Somatic KCNJ5 Mutations in Aldosterone-Producing Adenomas in Various Study Populations

<table>
<thead>
<tr>
<th>Country</th>
<th>Number</th>
<th>KCNJ5 Mutated,</th>
<th>G151R,</th>
<th>L168R,</th>
<th>Other Mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>AU, UK</td>
<td>73</td>
<td>41.1</td>
<td>26.0</td>
<td>13.7</td>
<td>I157del (n=1)</td>
</tr>
<tr>
<td>F, G, I</td>
<td>380</td>
<td>33.9</td>
<td>20.0</td>
<td>13.9</td>
<td></td>
</tr>
<tr>
<td>J</td>
<td>23</td>
<td>65.2</td>
<td>52.2</td>
<td>13.0</td>
<td></td>
</tr>
<tr>
<td>I, J, US</td>
<td>47</td>
<td>38.3</td>
<td>17.0</td>
<td>21.3</td>
<td></td>
</tr>
<tr>
<td>AU, F, G, S</td>
<td>348</td>
<td>45.1</td>
<td>24.1</td>
<td>20.4</td>
<td>E145G (n=2)</td>
</tr>
<tr>
<td>F, G, I</td>
<td>308</td>
<td>38.3</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>US</td>
<td>64</td>
<td>32.8</td>
<td>18.8</td>
<td>14.1</td>
<td></td>
</tr>
<tr>
<td>CZ, D, UK</td>
<td>152</td>
<td>34.5*</td>
<td>20.7*</td>
<td>12.1*</td>
<td>L168R/E145K (n=1)*</td>
</tr>
<tr>
<td>I</td>
<td>127</td>
<td>24.4</td>
<td>15.0</td>
<td>7.9</td>
<td>T158A (n=1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ins T149 (c.446insAAC, n=1)</td>
</tr>
<tr>
<td>F, G, I</td>
<td>474</td>
<td>38.0</td>
<td>23.8</td>
<td>13.7</td>
<td>T158A (n=1)</td>
</tr>
</tbody>
</table>

AU indicate Australia; CN, China; CZ, Czech; D, Dutch; F, France; G, Germany; I, Italy; J, Japan; NA, not available; S, Sweden; UK, United Kingdom; and US, United States.

*The mutations were detected in a subset of DNA samples.

with recurrent G151R, 41 carried c.451G>A, and the remaining 26 carried c.451G>C. In addition, homology alignments of the protein sequences showed that all 4 identified mutations are highly conserved among most species (Figure S2A).

No somatic mutation was found in the KCNJ5 gene in 11 cortisol-producing adenomas, 10 adrenal pheochromocytomas, or 26 nonfunctional adrenal adenomas.

Sequencing of the ATP1A1, ATP2B3, and CACNA1D Genes

In the 39 cases without KCNJ5 mutations, 6 cases were found with somatic mutations in ATP1A1 (n=4, male), ATP2B3 (n=1, male), or CACNA1D (n=1, female; Table 2). These included a known ATP1A1 mutation (L104R, 3 cases) and a known CACNA1D mutation (G403R, 1 case), as well as a new ATP1A1 variant (c.304-309delATGTTA, p.M102-L103del; Figure 1B) and a new ATP2B3 variant (c.1264-1278delGTCACTGTGT-GCTGTCinsAGCACACTC, p.delV422-V426insSTL; Figure 1C). Of these, the ATP1A1 variants and the ATP2B3 variant were highly conserved in most species (Figures S2B and S2C). None of these mutations were found in the normal adjacent adrenal tissue (n=6) and peripheral blood (n=3).
Phenotypic Characteristics of Patients With and Without Somatic KCNJ5 Mutations

Carriers and noncarriers of the KCNJ5 mutations were similar in age at operation, duration of hypertension, baseline systolic blood pressure, diastolic blood pressure, plasma aldosterone concentration, urinary protein excretion, glomerular filtration rate, and left ventricle mass index ($P_{≥0.075}$; Table 3). Nonetheless, carriers of the somatic KCNJ5 mutations, compared with noncarriers, had a higher proportion of women (56.6% versus 33.3%; $P=0.02$), larger tumor size ($P=0.02$), higher urinary aldosterone excretion ($P=0.038$), and aldosterone–renin ratio ($P=0.007$), greater lateralization index ($P=0.052$), and lower plasma renin activity ($P=0.018$), and minimal serum K+ level ($P=0.022$). After adjustment for sex and age, all differences remained significant ($P_{≤0.053}$; Table 3).

The patient harboring the novel KCNJ5 T148-T149insR mutation was a 62-year-old female and had hypertension for >20 years. Systolic/diastolic blood pressures were 160/90 mm Hg under 3 antihypertensive drugs. The minimal serum K+ concentration was 2.1 mmol/L and aldosterone–renin ratio was 95 (ng/dL)/(ng/mL/h). A right adrenal mass (20 mm in diameter) showed aldosterone hypersecretion (lateralization index=5.6).

KCNJ5 Protein Expression in APAs

We performed western blotting in 31 APAs with KCNJ5 mutations and 19 APAs without the KCNJ5 mutations to investigate whether mutant KCNJ5 was associated with altered expression of KCNJ5 protein. We did not find significant change of the KCNJ5 protein expression in APAs with mutant KCNJ5 ($P=0.207$; Figure 2).

Biophysical Properties of the KCNJ5T148-T149insR Mutation in Vitro

We investigated the effect of the insertion mutation T148-T149insR in KCNJ5 on aldosterone expression by transiently cotransfecting KCNJ3 with mutant or wild-type KCNJ5 in H295R cells. At 48 hours after electroporation, T148-T149insR, compared with wild type and empty vector, could stimulate CYP11B2 mRNA upregulation by 9.8-fold ($P<0.001$) and 7.2-fold ($P<0.001$; Figure 3A), respectively. After adjustment for total protein, H295R cells expressing T148-T149insR released more aldosterone to the supernatant, 5.4-fold ($P<0.001$) and 5.0-fold ($P<0.001$) higher than cells expressing wild-type and empty vector (Figure 3B), respectively.

We further explored the underlying mechanism for the T148-T149insR mutation causing increased aldosterone production. We coexpressed KCNJ3 with mutant or wild-type KCNJ5 in 293T cells and recorded currents at voltages ranging from −100 mV to +60 mV by whole-cell recordings with physiological solutions and extracellular Na+ free solutions (Figure 4). KCNJ3/KCNJ5WT induced an inwardly rectifying current with a reversal potential of −23±3 mV. This current was remarkably decreased in the presence of BaCl2 by 90.4% at −100 mV, which was used to test the K+ channel sensitivity to barium. In contrast, KCNJ3/KCNJ5 T148-T149insR produced a current which was only partially blocked in the presence of BaCl2 by 28.3% at −100 mV. In addition, this mutant channel exhibited membrane depolarization with a less negative reversal potential (−3±1 mV). The substitution of choline for

Table 2. Prevalence of Somatic ATP1A1, ATP2B3, and CACNA1D Mutations in Aldosterone-Producing Adenomas in Various Study Populations

<table>
<thead>
<tr>
<th>Country</th>
<th>Number</th>
<th>ATP1A1 Mutated, %</th>
<th>ATP2B3 Mutated, %</th>
<th>CACNA1D Mutated, %</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CN</td>
<td>18</td>
<td>2.4</td>
<td>0.6</td>
<td>0.6</td>
<td>Our study</td>
</tr>
<tr>
<td>F, G, I</td>
<td>308</td>
<td>3.2</td>
<td>1.6</td>
<td>NA</td>
<td>10</td>
</tr>
<tr>
<td>US</td>
<td>64</td>
<td>1.6</td>
<td>3.1</td>
<td>7.8</td>
<td>11</td>
</tr>
<tr>
<td>CZ, D, UK</td>
<td>152</td>
<td>19.0*</td>
<td>NA</td>
<td>15.5*</td>
<td>12</td>
</tr>
<tr>
<td>F, G, I</td>
<td>474</td>
<td>5.3</td>
<td>1.7</td>
<td>9.3</td>
<td>21</td>
</tr>
</tbody>
</table>

CN indicates China; CZ, Czech; D, Dutch; F, France; G, Germany; I, Italy; NA, not available; UK, United Kingdom; and US, United States.

*The mutations were detected in a subset of DNA samples.
extracellular Na$^+$ markedly inhibited the currents of the mutant channel with a hyperpolarized potential (−23±2 mV).

We also studied the effect of KCNJ5 T148-T149insR on intracellular Ca$^{2+}$ homeostasis in cotransfected H295R cells with KCNJ3 and mutant KCNJ5 or wild-type KCNJ5. The intracellular Ca$^{2+}$ concentration as assessed by the Fura-2/AM ratio was higher in KCNJ3/KCNJ5 T148-T149insR cells than in KCNJ3/KCNJ5WT and empty vector cells both by 1.2-fold ($P<0.01$; Figure S3).

Discussion

Our study is the first to report somatic mutations in the KCNJ5 gene and several other genes in Chinese patients with APA. Indeed, 76.8% of the Chinese APAs had somatic KCNJ5 mutations, and in the absence of the KCNJ5 mutations, some, though not many, had somatic mutations in the ATP1A1, ATP2B3, and CACNA1D genes. Our observation on the remarkably high proportion of KCNJ5 mutations is in line with the result of a Japanese study where the prevalence was 65.2% in 23 Japanese APAs.\(^{14}\) In a review study across populations, the total prevalence of somatic KCNJ5 mutations in APAs was $\approx 40\%$.\(^{7}\)

Our observations on the characteristics of patients with somatic KCNJ5 mutations are also in accordance with the results of several other studies in different populations in several aspects.\(^{10,13,14,18–22}\) First, somatic KCNJ5 mutations were more common in female than male patients. Second, in the presence of KCNJ5 mutations, larger tumor size, higher urinary aldosterone, higher aldosterone–renin ratio, and later-alization index were observed, which suggested that excess aldosterone was related to the mutated APA. The patients with KCNJ5 mutations were more likely to be diagnosed

---

**Table 3. Clinical and Biochemical Characteristics of Aldosterone-Producing Adenomas With and Without KCNJ5 Mutations**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Wild type (n=33)</th>
<th>KCNJ5 mutated (n=129)</th>
<th>$P$</th>
<th>$P^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females, %</td>
<td>33.3</td>
<td>56.6</td>
<td>0.020</td>
<td></td>
</tr>
<tr>
<td>Age at operation, y</td>
<td>51±11</td>
<td>47±11</td>
<td>0.130</td>
<td></td>
</tr>
<tr>
<td>Duration of hypertension, y</td>
<td>10 (5–17)</td>
<td>7 (2–15)</td>
<td>0.101</td>
<td></td>
</tr>
<tr>
<td>Preoperative systolic BP, mmHg</td>
<td>151±20</td>
<td>146±16</td>
<td>0.194</td>
<td></td>
</tr>
<tr>
<td>Preoperative diastolic BP, mmHg</td>
<td>87±11</td>
<td>90±12</td>
<td>0.275</td>
<td></td>
</tr>
<tr>
<td>Preoperative PRA, ng/mL/h</td>
<td>0.6 (0.2–2.4)</td>
<td>0.3 (0.1–0.7)</td>
<td>0.018</td>
<td>0.022</td>
</tr>
<tr>
<td>Preoperative PAC, ng/dL</td>
<td>31.5 (21.2–44.5)</td>
<td>36.5 (22.3–47.7)</td>
<td>0.958</td>
<td></td>
</tr>
<tr>
<td>ARR, [ng/dL]/[ng/mL/h]</td>
<td>42 (29–136)</td>
<td>102 (50–277)</td>
<td>0.007</td>
<td>0.014</td>
</tr>
<tr>
<td>Minimal serum K$^+$ concentration, mmol/L</td>
<td>2.9 (2.5–3.0)</td>
<td>2.6 (2.2–2.8)</td>
<td>0.022</td>
<td>0.017</td>
</tr>
<tr>
<td>Lateralization index</td>
<td>6.4 (3.0–10.6)</td>
<td>10.9 (4.5–22.7)</td>
<td>0.052</td>
<td>0.053</td>
</tr>
<tr>
<td>Tumor size, mm</td>
<td>11 (8–16)</td>
<td>15 (12–20)</td>
<td>0.021</td>
<td>0.044</td>
</tr>
<tr>
<td>Urinary aldosterone excretion, (\mu g/24)h</td>
<td>16.6 (9.4–22.1)</td>
<td>23.3 (16.2–32.2)</td>
<td>0.038</td>
<td>0.047</td>
</tr>
<tr>
<td>Urinary protein excretion, (mg/24)h</td>
<td>135 (82–169)</td>
<td>109 (88–142)</td>
<td>0.221</td>
<td></td>
</tr>
<tr>
<td>Glomerular filtration rate, mL/min</td>
<td>77±18</td>
<td>85±18</td>
<td>0.075</td>
<td></td>
</tr>
<tr>
<td>Left ventricular mass index, g/m$^2$</td>
<td>120±27</td>
<td>119±30</td>
<td>0.850</td>
<td></td>
</tr>
</tbody>
</table>

ARR indicates aldosterone–renin ratio; BP, blood pressure; PAC, plasma aldosterone concentration; and PRA, plasma renin activity.

*$P$ value was adjusted for age and sex by analysis of variance.

---

**Figure 2. Effect of KCNJ5 mutations on KCNJ5 protein expression in aldosterone-producing adenomas (APAs).** KCNJ5 protein expression in APAs carrying wild-type KCNJ5 (n=19) and mutant KCNJ5 (n=31) was evaluated by western blotting. Data were presented as median (25th–75th percentile) and analyzed using Mann–Whitney $U$ test.

**Figure 3. Aldosterone production from NCI-H295R cells transiently electroporated with KCNJ3 and wild-type or mutant KCNJ5 (T148-T149insR).** At 48 h after transfection, the samples were collected and analyzed. A, CYP11B2 mRNA levels. B, Aldosterone concentration being adjusted for total protein. Data were presented as means±SD and assessed by 1-way analysis of variance followed by post hoc analysis. $\ast P<0.001$, n=5 for each group.
and receive adrenalectomy. Third, in the presence of KCNJ5 mutations, the minimal K⁺ concentration was lower. Because severe hypokalemia was a major indicator of PA, the patients with KCNJ5 mutations were therefore more likely to be screened for PA. These characteristics of patients with KCNJ5 mutations suggest that identification of the mutations may have prognostic significance.

In our study, the KCNJ5 mutations did not affect the expression of the protein. This finding is in agreement with the result of a previous report. We further investigated the function of the novel somatic KCNJ5 mutation (T148-T149insR). Our findings indicate that this mutation is capable of increasing aldosterone production, as several previously identified mutations (W126R, G151R, T158A, and Ins T149) in KCNJ5 also had the similar electrophysiological feature. Membrane depolarization in the adrenal zona glomerulosa cells could increase intracellular Ca²⁺ concentration through activation of voltage-gated Ca²⁺ channels and Na⁺/Ca²⁺ exchangers, which led to aldosterone biosynthesis.

The underlying mechanisms for the relationship between T148-T149insR in KCNJ5 and aldosterone biosynthesis remain incompletely understood. This novel mutation is positioned closely to the K⁺ channel selectivity filter and is highly conserved among disparate species. It is clear that the T148-T149insR mutation caused a loss of K⁺ ion selectivity and a less negative reversal potential, which might be accompanied with a pathological Na⁺ permeability similarly as the mutations at G151, L168R, del I157, and Ins T149 in KCNJ5 also had the similar electrophysiological feature. Membrane depolarization in the adrenal zona glomerulosa cells could increase intracellular Ca²⁺ concentration through activation of voltage-gated Ca²⁺ channels and Na⁺/Ca²⁺ exchangers, which led to aldosterone biosynthesis eventually.

Our finding on the mutations of ATP1A1 and ATP2B3 is also consistent with the results of a recent study in 2 aspects. First, KCNJ5 and ATP1A1 or ATP2B3 mutations were not concomitantly observed. Second, ATPase alterations showed male dominance.

Limitations
This work was conducted in a single center and not representative of the whole APAs. The prevalence of somatic KCNJ5 mutations is not so high in another Asian population. International multicenter studies may help reduce selection bias. In addition, long-term follow-up has not been accomplished, and we are unable to predict prognosis based on KCNJ5 mutations at present.

In summary, we investigated the prevalence of KCNJ5, ATP1A1, ATP2B3, and CACNA1D mutations in the Chinese patients with sporadic APA. Patients with the KCNJ5 mutations had clearly distinguishable phenotypes. The T148-T149insR mutation in KCNJ5 might influence K⁺ channel selectivity and autonomous aldosterone production.
Perspectives

The trigger for the occurrence of somatic mutations in APAs would be an interesting research theme. As there is currently no definite correlation between somatic KCNJ5 mutations and nodule formation, further investigation on this aspect is needed. In addition, easily accessible biomarkers of APAs should be explored in other possible biological samples.

Acknowledgments

We thank Chenlong Chu and Chenhui Zhao (Department of Urology, Luwan Branch of Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China) for helpful support on the storage of tissues. We also thank Kaida Ji and Ying Zhang (Ruijin Hospital, Shanghai Institute of Hypertension, Shanghai Jiao Tong University School of Medicine, Shanghai, China) for technical assistance on DNA isolation.

Sources of Funding

This study was supported by grants from National High Technology Research and Development Program 863 of China (2012AA02A516), grants from the State Key Program of the National Natural Science foundation of China (81230071), and grants from Shanghai Municipal Science and Technology Commission public service platform (12DZ2295200).

Disclosures

None.

References


**Novelty and Significance**

**What Is New?**

- The prevalence of KCNJ5 mutations in our Chinese subjects with aldosterone-producing adenoma was 76.8%. Mutations in ATP1A1, ATP2B3, and CACNA1D were rare.
- We found a novel insertion mutation (c.445-446insGAA, p.T148-T149insR) in KCNJ5 in a female patient and a novel mutation in ATP1A1 and ATP2B3, respectively.

**What Is Relevant?**

- KCNJ5 mutations were more prevalent in females, whereas ATPase mutations were males.
- KCNJ5 mutation carriers had larger adenomas, higher aldosterone production, and lower minimal serum K+ level.

- T148-T149insR in KCNJ5 might influence membrane voltage and Ca2+ homeostasis and increase CYP11B2 mRNA expression and aldosterone production.

**Summary**

Somatic KCNJ5 mutations have a distinctively high prevalence in sporadic aldosterone-producing adenomas in Chinese compared with ATP1A1, ATP2B3, and CACNA1D mutations. Patients with KCNJ5 mutations had distinguishable phenotypes. T148-T149insR in KCNJ5 might participate in the loss of K+ channel selectivity and increase aldosterone synthesis.
Aldosterone-Producing Adenoma

Clinical Characteristics of Somatic Mutations in Chinese Patients With Aldosterone-Producing Adenoma

Fang-Fang Zheng, Li-Min Zhu, Ai-Fang Nie, Xiao-Ying Li, Jing-Rong Lin, Ke Zhang, Jing Chen, Wen-Long Zhou, Zhou-Jun Shen, Yi-Chun Zhu, Ji-Guang Wang, Ding-Liang Zhu and Ping-Jin Gao
Online Supplement

Clinical Characteristics of Somatic Mutations in Chinese Patients with Aldosterone-Producing Adenoma

Fang-Fang Zheng\textsuperscript{1,2,3}, Li-Min Zhu\textsuperscript{1,3}, Ai-Fang Nie\textsuperscript{4}, Xiao-Ying Li\textsuperscript{4}, Jing-Rong Lin\textsuperscript{2}, Ke Zhang\textsuperscript{2}, Jing Chen\textsuperscript{1,3}, Wen-Long Zhou\textsuperscript{5}, Zhou-Jun Shen\textsuperscript{5}, Yi-Chun Zhu\textsuperscript{6}, Ji-Guang Wang\textsuperscript{1,3}, Ding-Liang Zhu\textsuperscript{1,3}, Ping-Jin Gao\textsuperscript{1,2,3}

\textsuperscript{1}State Key Laboratory of Medical Genomics, Shanghai Key Laboratory of Hypertension and Department of Hypertension, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China.
\textsuperscript{2}Laboratory of Vascular Biology and Key Laboratory of Stem Cell Biology, Institute of Health Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China.
\textsuperscript{3}Shanghai Institute of Hypertension, Shanghai, China.
\textsuperscript{4}Shanghai Institute of Endocrinology and Metabolism, Shanghai, China.
\textsuperscript{5}Department of Urology, Ruijin Hospital, Shanghai, China.
\textsuperscript{6}Department of Physiology and Pathophysiology, Shanghai Medical College, Fudan University, Shanghai, China.

Corresponding Author:
Ping-Jin Gao, Ruijin Hospital, Shanghai Institute of Hypertension, Shanghai Jiao Tong University School of Medicine, 197 Ruijin 2nd Rd, Shanghai 200025, China
E-mail: gaopingjin@sibs.ac.cn; tel: +86 21 64370045/610903; fax: +86 21 64458970
Supplemental Methods

Subjects
168 patients with primary aldosteronism (PA) recruited between 2008 and 2014 in the Hypertension Department (Ruijin hospital, Shanghai Jiao Tong University School of Medicine) were analyzed retrospectively. The PA workup was carried out according to the guidelines of Endocrine Society (Figure S1). In brief, patients from many provinces with the following profile or characteristic were attracted to our out-patient clinic for PA screening: hypertension and hypokalemia; hypertension and adrenal incidentaloma; resistant hypertension; etc. Before measuring plasma aldosterone concentration (PAC) and plasma rennin activity (PRA), mineralocorticoid receptor (MR) antagonists were withdrawn for at least 6 weeks, non-potassium sparing diuretics for 4 weeks, and β-blockers, angiotensin converting enzyme inhibitors (ACEIs) and angiotensin II type 1 receptor blockers (ARBs) for 2 weeks. In case of blood pressure control, non-dihydropyridine Ca\(^{2+}\) blockers and/or α1-blockers were prescribed. Patients with aldosterone-renin-ratio (ARR) >24 (ng/dL)/(ng/mL/h) at least two times at out-patient clinic were PA candidates. Then they were hospitalized for confirmation test. Intravenous saline loading test was applied. Patients with post-test PAC>6 ng/dL were diagnosed to have PA in our unit. The patients underwent the thin-sliced adrenal computed tomography (CT) scanning. Adrenal venous sampling (AVS) was carried out in patients who were candidates and were also willing to receive the adrenalectomy. A selectivity index (SI, adrenal cortisol to peripheral cortisol) ≥3.0 and a lateralization index (LI, aldosterone/cortisol ratio from high to low side) ≥2.0 without cosyntropin stimulation were used in our clinic, which was in line with an expert consensus. The aldosterone-producing adenomas (APAs) were verified by histopathology.

DNA Isolation
DNA was extracted from 168 APAs, matched peritumoral adrenal tissue from 122 patients and matched peripheral venous blood from 90 patients. DNA was also extracted from 11 cortisol-producing adenomas, 10 adrenal pheochromocytomas and 26 nonfunctional adrenal adenomas in accordance with the protocol (TIANamp Genomic DNA Kit, TIANGEN, China).

Sanger sequencing of KCNJ5 and other genes
The partial sequences of KCNJ5, ATP1A1, ATP2B3 and CACNA1D were amplified using primers described previously. The PCR conditions were similarly as follows: 95°C for 5 min; 35 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min; 72°C for 10 min using a GeneAmp PCR system 9700 (Applied Biosystems). Sanger sequencing of the purified PCR products was performed on an Applied Biosystems 3730xl DNA Analyzer.

Evaluation of KCNJ5 protein expression in APAs by western blotting
The adrenal tissue from 50 APAs was lysed in tissue protein extraction buffer (Promoton). The lysate of adrenal tissue was quantified by the BCA method. Equal amounts of total protein were subjected to SDS-PAGE in a 10% gel for KCNJ5 immunoblotting and transferred to a polyvinylidene difluoride membrane (Millipore) by electroblotting. The
membrane was blocked and incubated with anti-KCNJ5 (1:2000; Sigma) followed by horseradish peroxidase-conjugated secondary antibody (Santa Cruz). The immunoreactive bands were detected by enhanced chemiluminescence (GE healthcare) and quantified by Image J 1.44 (National Institutes of Health, Bethesda, MD).

The effect of the T148-T149insR mutation in KCNJ5 on aldosterone expression in NCI-H295R cells

The full-length cDNA of human KCNJ3 was subcloned into pDsRed-N1 vector. The cDNAs of human KCNJ5 WT and KCNJ5 T148-T149insR were subcloned into pIRES2-eGFP vector. The plasmid including KCNJ5 T148-T149insR sequence was constructed by site-directed mutagenesis (Invitrogen). All of the constructs were verified by DNA sequencing. Adrenocortical carcinoma NCI-H295R cells were cultured in DMEM/F12 (Gibco) supplemented with 2.5% nu-serum replacement (BD), 1% ITS+Premix (BD), and 1% L-glutamine (Gibco). 2×10^6 cells were electroporated with KCNJ3 (2 μg) and either KCNJ5 WT (2 μg) or KCNJ5 T148-T149insR (2 μg) by Amaxa nucleofection (program T16, Lonza, Germany). The empty vector was used as control. At 48 h after electroporation, total RNA was abstracted from NCI-H295R cells using TRIzol reagent (Invitrogen). 2 μg of total RNA was reverse transcribed into single-stranded cDNA by incubation with moloney murine leukemia virus reverse transcriptase (Promega). For quantification of CYP11B2 mRNA expression, real-time PCR was performed using the SYBR Premix Ex Taq kits (Takara) based on manufacturer’s protocol in ABI PRISM 7900 system (Applied Biosystems). β-actin was used as standard reference. Reactions were done at 95°C for 30 sec followed by 40 cycles of 95°C for 5 sec, 60°C for 30 sec in a total volume of 10 μL.

The aldosterone concentration in the supernatant of H295R cells was measured by radioimmunoassay (Beckman Coulter) and normalized to total cell protein. The cells were lysed in cell protein extraction buffer (Promoton). The lysate was quantified by the BCA method.

Electrophysiological experiment of the T148-T149insR mutation in KCNJ5 in Human embryonic kidney 293T cells

Human embryonic kidney (HEK) 293T cells were cultured in DMEM (Biowest) supplemented with 10% FBS (Gibco). KCNJ3 (1 μg) and either KCNJ5 WT (0.5 μg) or KCNJ5 T148-T149insR (0.5 μg) were transiently cotransfected in 293T cells in 35 mm dishes by X-tremeGENE HP DNA Transfection Reagent (Roche). Besides, empty vector was used as control. At 24 h after transfection, whole-cell voltage clamp recordings were performed on cotransfected cells (red and green fluorescence) using an EPC-10 amplifier (HEKA, Germany). The 293T cell was held at 0 mV and clamped from -100 mV to 60 mV in 20 mV increments which was repeated at least 3 times. The solutions were followed as: external solution (140 mM NaCl, 5 mM KCl, 1.8 mM MgCl2, 1.8 mM CaCl2 and 10 mM HEPES, pH=7.4) and pipette solution (140 mM KCl, 4 mM MgCl2, 1 mM CaCl2, 1 mM EGTA and 5 mM HEPES, pH 7.4). Moreover, 1 mM BaCl2 was added into the external solution to test the K+ channel sensitivity to barium.

Intracellular Ca^{2+} measurement in NCI-H295R cells
H295R cells (2×10^6) were electroporated with KCNJ3 (2 μg) and either KCNJ5\textsuperscript{WT} (2 μg) or KCNJ5\textsuperscript{T148-T149insR} (2 μg) by Amaxa nucleofection (program T16, Lonza, Germany). The empty vector was used as control. The electroporated cells were seeded on coverslips and the medium was changed after 24 h. After another 24 h, the cells were loaded with 5 μM Fura-2/AM for 45 min at 37°C in a KRBB solution containing: 119 mM NaCl, 4.75 mM KCl, 5 mM NaHCO\textsubscript{3}, 1.2 mM MgSO\textsubscript{4}, 1.18 mM KH\textsubscript{2}PO\textsubscript{4}, 2.54 mM CaCl\textsubscript{2}, 3 mM glucose and 20 mM HEPES (pH 7.4). The coverslips were placed in a superfusion chamber under IX71 inverted microscope (Olympus, Tokyo, Japan). Intracellular Ca\textsuperscript{2+} concentration was measured using the Video-Imaging-System (Till Photonics, Munich, Germany). Cells were illuminated by alternative excitation light of 340 nm and 380 nm wavelength, which was produced by a monochromator (Till Photonics, Munich, Germany). The images were captured with emission light of 510 nm wavelength using an image-intensifying CCD camera (SensiCam, PCO, Kelheim, Germany) and processed using an image processing system (TillVision, Till Photonics, Munich, Germany). Calcium concentrations were calculated as ratio of F340/F380.

**Orthologs**

Homology alignments of KCNJ5, ATP1A1 and ATP2B3 protein sequences among different sources were obtained from Genbank.

Multiple sequences of Homo sapiens (NP_000881.3), Pan troglodytes (XP_508857.3), Macaca mulatta (XP_001113260.1), Canis lupus familiaris (XP_546402.2), Bos taurus (XP_002699270.1), Mus musculus (NP_034735.3), Rattus norvegicus (NP_058993.1), and Gallus gallus (XP_417864.2) for KCNJ5 gene were analyzed.

Multiple sequences of Homo sapiens (NP_000692.2), Pan troglodytes (XP_513679.3), Macaca mulatta (NP_001253602.1), Canis lupus familiaris (NP_001003306.1), Bos taurus (NP_001070266.1), Mus musculus (NP_659149.1), Rattus norvegicus (NP_036636.1), and Gallus gallus (NP_990852.1) for ATP1A1 gene were analyzed.

Multiple sequences of Homo sapiens (NP_001001344.1), Macaca mulatta (XP_001083434.2), Canis lupus familiaris (XP_003435597.1), Bos taurus (NP_001178093.1), Cavia porcellus (XP_005000116.1), Mus musculus (NP_796210.2), Rattus norvegicus (NP_579822.1), and Danio rerio (NP_001002472.2) for ATP2B3 gene were analyzed.

**References**


Figure S1. The management of primary aldosteronism (PA) in our unit. ARR: aldosterone-renin-ratio; PAC: plasma aldosterone concentration; CT: computed tomography; LI: lateralization index; MR: mineralocorticoid receptor.
**Figure S2.** Homology alignments of KCNJ5, ATP1A1 and ATP2B3 protein sequences among different species. The red color represents that these related amino acids are conserved in most species.
**Figure S3.** The effect of T148-T149insR in KCNJ5 on intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]). NCI-H295R cells were transiently electroporated with KCNJ3 and either KCNJ5$^{WT}$ or KCNJ5$^{T148-T149insR}$. Empty vector was used as control. KCNJ3/KCNJ5$^{T148-T149insR}$ (n=7) induced higher [Ca$^{2+}$] than KCNJ3/KCNJ5$^{WT}$ (n=11) and empty vector (n=6). The data are presented as mean±SD and assessed by one-way ANOVA followed by post-hoc analysis. *P<0.01.
先兆子痛（摘要）

早发先兆子病、迟发先兆子病和妊娠期高血压者产后心血管疾病危险因素比较
Cardiovascular Disease Risk Factors After Early-Onset Preeclampsia, Late-Onset Preeclampsia, and Pregnancy-Induced Hypertension
Jan H.W. Veerbeek, Wietske Hermes, Anath Y. Breimer, Bas B. van Rijn, Steven V. Koenen, Ben W. Mol, Arie Franx, Christianne J.M. de Groot, Maria P.H. Koster

田野 译

观察性研究表明，怀孕期间伴随高血压会增加妇女终生患心血管疾病的危险。该风险与妊娠相关高血压病的严重程度以及发病时的胎龄有关。然而，在目前的产后心脑血管疾病危险因素筛查中，并没有调查这些因素的差异。我们通过3组有妊娠期高血压史的患者来评估产后心脑血管疾病危险因素的差异。我们对比了448例曾患有早发先兆子病患，76例曾患有迟发先兆子病和224例曾患有妊娠期高血压病的妇女。与曾患有迟发先兆子病以及曾患有妊娠期高血压的妇女对比，曾患有早发性先兆子病的妇女有明显较高的空腹糖(5.29 vs 4.80和4.83 mmol/L)，较高的胰岛素水平(9.12 vs 6.31和6.7 uIU/L)，较高的甘油三酯(1.32 vs 1.02和0.97 mmol/L)，较高的总胆固醇(5.14 vs 4.73和4.73 mmol/L)。曾有早发性先兆子病的妇女几乎一半会患上高血压，而作为对比的曾经患有妊娠期高血压的妇女和曾有迟发先兆子病的妇女，其患病率分别为39%和25%。我们的数据显示了妊娠高血压患者常见产后心脑血管疾病危险因素患病率的差异，并且建议其防治策略应根据病情的严重程度以及发病时的胎龄来分层进行处理。

（Hypertension. 2015;65:600-606.）

醛固酮增多症腺瘤（摘要）

中国原发性醛固酮增多症腺瘤患者的体细胞突变及临床特征研究
Clinical Characteristics of Somatic Mutations in Chinese Patients With Aldosterone-Producing Adenoma
Fang-Fang Zheng, Li-Min Zhu, Ai-Fang Nie, Xiao-Ying Li, Jing-Rong Lin, Ke Zhang,

高平进 译

近期研究发现，原发性醛固酮增多症腺瘤患者的发病与KCNJ5、ATP1A1、ATP2B3和CACNA1D基因体细胞突变相关。然而，中国人群的原发性醛固酮增多症腺瘤患者的基因体细胞突变及其临床特征仍未研究。本研究纳入168例经病理确诊的原发性醛固酮增多症腺瘤患者的肾上腺组织DNA进行测序，发现129例KCNJ5基因体细胞突变，突变发生率76.8%，高于欧美国家发生率。另有4例ATP1A1基因体细胞突变，1例ATP2B3基因体细胞突变和1例CACNA1D基因体细胞突变。临床特征显示女性患者的KCNJ5基因突变率高于男性患者。携带KCNJ5基因突变患者的肿瘤直径更大、醛固酮水平更高，血钾水平更低。有意思的是，我们还发现了1例KCNJ5突变新位点（c.445-446insGAA, p.T148-T149insR），该位点可使CYP11B2基因mRNA表达上调，醛固酮产生增加。同时该突变可引起细胞膜去极化和细胞内Ca²⁺浓度增加。以上研究提示，本中心纳入的原发性醛固酮增多症腺瘤主要表现为以KCNJ5基因为主的体细胞突变，其突变率远高于其它国家。新发现的KCNJ5基因T148-T149insR位点可能影响K⁺通道的选择性，并促进醛固酮的自主分泌。

（Hypertension. 2015 Mar;65(3):622-628.）