Characteristics of Japanese aldosterone-producing adenomas with KCNJ5 mutations

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Abstract. Somatic mutations in KCNJ5 gene have been identified in patients with adrenal aldosterone-producing adenomas (APAs). We previously reported that Japanese patients with APAs had distinct characteristics from patients in Western countries; i.e. they had a high frequency of KCNJ5 mutations and exhibited a frequent association with cortisol co-secretion. Therefore, APAs among Japanese patients may have different features from those in Western countries. We added recent cases, examined 47 cases (43% male) of APAs, including clinicopathological features, KCNJ5 mutations, and the mRNA levels of several steroidogenic enzymes, and compared the results obtained to those reported in other countries. While the prevalence of KCNJ5 mutations is approximately 40% in Western countries, 37 APA cases (78.7%) showed mutations: 26 with p.G151R and 11 with p.L168R. Although a significant gender difference has been reported in the frequency of KCNJ5 mutations in Europe, we did not find any gender difference. However, the phenotypes of Japanese patients with mutations were similar to those of patients in Western countries; patients were younger and had higher plasma aldosterone levels, lower potassium levels, and higher diastolic blood pressure. Reflecting these phenotypes, APAs with mutations had higher CYP11B2 mRNA levels. However, in contrast to APAs in Western countries, Japanese APAs with mutations showed lower CYP11B1, CYP17A1, and CYP11A1 mRNA levels. These findings demonstrated that Japanese APA patients may have distinct features including a higher prevalence of KCNJ5 mutations, no gender difference in the frequency of these mutations, and characteristics similar to the zona glomerulosa.

Key words: KCNJ5, APAs, Adrenocortical adenomas, Steroidogenic enzymes

PRIMARY ALDOSTERONISM (PA) has been reported to affect at least 6~10% of patients with essential hypertension [1-5] and is caused mostly by an adrenal adenoma (aldosterone-producing adenoma, APA) or by adrenal hyperplasia (idiopathic hyperaldosteronism, IHA). Although the tumorigenesis of APA remained unclear, somatic mutations of the potassium channel KCNJ5 were identified by Choi et al. in 2011 [6]. KCNJ5 belongs to a family of G protein-sensitive inwardly rectifying potassium channels and contains two transmembrane domains that surround the region harboring the potassium ion selectivity filter. Two recurrent mutations (G151R and L168R) have been reported to induce increased sodium conductance and membrane depolarization, activating voltage-gated Ca2+ channels. Increases in intracellular Ca2+ levels then promote aldosterone production, and the chronic Ca2+ stimulation may promote cellular proliferation, resulting in an adenoma [6].

We and others have recently confirmed above two mutations, G151R and L168R, in the KCNJ5 gene in
Japan, Australia, the United Kingdom, Europe, and Asia other than Japan [7-10]. Azizan et al. reported a prevalence of 38% (10 of 27) in Australia and 44% (20 of 46) in the United Kingdom. Boulkroun et al., who examined the large number of APA cases to date, 380, found that 129 patients (34%) had mutations in Europe [9]. We found a higher frequency, 24 out of 33 (approximately 73%) APAs, in Japan [7, 11-13]. Several differences of clinical features have been reported between Japanese and Western APA patients. In Western countries, approximately one third of patients with PA have APA, a small fraction have GRA, and the rest are idiopathic [1, 4, 5, 14, 15]. In contrast, most Japanese (~80%) with PA have APA [16-18]. A gender difference in the frequency of KCNJ5 mutations, approximately 65% in women and 25% in men, has been reported in Europe [10]. However, no significant gender difference was detected in our previous study [7]. Furthermore, we and others reported the co-secretion of cortisol in approximately 30% of Japanese patients with APAs, which was markedly higher than that in patients in Western countries [19-24]. In an attempt to identify biochemical differences between Japanese and Western APA patients, we examined the clinicopathological features of patients with APAs with and without KCNJ5 mutations and also analyzed several steroidogenic enzyme mRNAs including CYP11B2 (aldosterone synthetize), CYP11B1, CYP17A1, CYP12A2, HSD3B2, (3β-hydroxysteroid dehydrogenase), and CYP11A1 (cholesterol side chain cleavage cytochrome P-450), which may reflect the production of hormones and the adrenal zone of origin.

**Subjects and Methods**

**Subjects**

We reviewed the medical records of 47 patients with APAs, who were operated on at Gunma University Hospital between 2007 and 2016. Thirty-three patients had been included in our previous study [7, 11-13]. Each subject was given written informed consent, and the study was approved by the Ethics Committee on human research of Gunma University. The diagnosis of PA was performed as reported previously [1, 7, 19]. The mean age of patients was 51 ± 13 y (mean ± SD) (20 males and 27 females). All patients had a high plasma aldosterone/ plasma renin concentration ratio (ARR) and a unilateral adrenal cortical mass evident on CT, and the pathology of all removed tumors confirmed the diagnosis of adrenocortical adenoma. The mean tumor size (maximum diameter) on CT was 17 ± 7 mm. We measured plasma aldosterone levels in most cases with the RIA SPAC-S Aldosterone kit, TFB; plasma renin activity (the RIA Renin IRMA KIT “Daiichi” TFB); plasma cortisol (the RIA Cortisol kit “TFB” TFB); and ACTH (ECLIA Eclusys ACTH produced by Roche Diagnostics).

RNA extraction and detection of mutations in KCNJ5 cDNA by direct sequencing

All APA specimens and some normal parts of the adrenal cortex were put into liquid nitrogen immediately after removal during surgery. Total RNA was extracted and cDNA was reverse-transcribed and sequenced with specific primer sets as reported previously [7, 22].

Real-time RT-PCR for steroidogenic enzymes in APAs

In order to measure the mRNA levels of steroidogenic enzymes in each adenoma, 0.5 μL of cDNA was subjected to real-time PCR in triplicate using TaqMan probes and an Applied Biosystems 7500 sequence detection system as reported previously [25, 26]. The TaqMan probes for CYP11B2 (Hs01597732_m1), CYP11B1 (Hs01596404_m1), 17β-hydroxylase (CYP17A1) (Hs01124136_m1), cholesterol side-chain cleavage enzyme (CYP11A1) (Hs00167984_m1), CYP21A2 (Hs00365734_g1), 3β-hydroxysteroid dehydrogenase (HSD3B2) (Hs00605123_m1), and GAPDH (Hs99999905_g1) were from Applied Biosystems. The each mRNA level relative to that of GAPDH was evaluated at least twice using a standard curve as described in ABI User Bulletin #2. The mRNA level for each patient was calculated with the median value for the normal adrenal cortex being set as 1.0.

Immunohistochemistry

Immunohistochemistry was performed as described previously [7]. Briefly, a polyclonal anti-KCNJ5 antibody (#HPA017353) was purchased from Sigma. Samples were fixed in 10% (wt/vol) phosphate-buffered formalin at room temperature overnight and embedded in paraffin wax. Tumors were cut at a thickness of 4 μm. Sections were stained with the antibody, and only with non-specific IgG and a secondary antibody as a negative control.
Statistical analysis
All results are described as the median or mean ± SD for continuous variables. Fisher’s exact test was used to compare the gender ratio. Group comparisons were examined with an ANOVA and the Student’s t-test for normally distributed data, or the Wilcoxon rank-sum test or Mann-Whitney test for non-normally distributed data for continuous variables. All tests for significance and the P values were two-sided with a level of significance of 5%. Statistical analyses were performed using JMP 10.0.2 (SAS Institute Inc. Cary, NC, USA).

Results

Somatic mutations in the KCNJ5 gene and their gender-differences in Japanese APAs
Thirty-seven out of 47 patients with APAs had somatic mutations in the KCNJ5 gene: 16 with p.G151R, c.451G>A, 10 with p.G151R, c.451G>C, and 11 with p.L168R, c.503T>G. All mutations observed were heterozygous. Comparisons of these mutations between sexes revealed, as shown in Fig. 1A, 4 males with wild-type alleles and 15 males with p.G151R (10 with p.G151R, c.451G>A, and 5 with p.G151R, c.451G>C). Of note, the mutation p.L168R, c.503T>G was only detected in 1 male. In contrast to males, 6 females had wild-type alleles, 11 had p.G151R (6 with p.G151R, c.451G>A, 5 with p.G151R, c.451G>C), and 10 had p.L168R, c.503T>G. Overall, mutations were identified in 80.0% of males and 77.8% of females, a difference that was not significant.

Summary of studies on somatic mutations in the KCNJ5 gene in APAs in Asian countries
Table 1 shows a summary of the clinical features of APA patients with KCNJ5 mutations in Asian countries reported by April 2016. The overall prevalence of KCNJ5 mutations was reported to be between 37 and 47% in European studies [27]. The KCNJ5 mutation rate was higher in Asian countries: China (76.8%) [28], Taiwan (59.5%) [29], Korea (71.2%) [30] and Japan (Kitamoto et al. 69.4% [31] and the present study 76.3%), than in European countries. Mutated APA patients in Europe were more often females, whereas no gender difference was observed in Taiwan and Japan (China; 43.6% in males, Taiwan; 43.6% in males, Korea; 33% in males and Japan (Kitamoto et al., 50% in males and the present study 43.2% in males)).

Clinicopathological features of APA patients with and without KCNJ5 mutations
Since it currently remains unclear why these KCNJ5 mutations are more common in patients in Asia, we herein examined the clinicopathological features of patients with APAs with and without KCNJ5 mutations including age, gender, plasma aldosterone concentration (PAC), plasma renin activity (PRA), serum potassium level, and morning systolic and diastolic blood pressures at the time of admission after a 30-min bed rest.

As shown in Fig. 1B, mean age was significantly younger in patients with KCNJ5 mutations (48 ± 12 years) than in those without (64 ± 9). No significant difference was observed in the maximum size of tumors in CT scans (15 ± 7 without mutations vs. 17 ± 7 with, Fig. 1C). Plasma aldosterone levels were significantly higher in patients with KCNJ5 mutations than in those without (median, 56.1 vs. 25.7 ng/dL, p<0.01, Fig. 1D), whereas no change was observed in PRA (the median, 0.1 vs. 0.2 ng/mL/hr). In addition, serum potassium levels (the median, 2.8 vs. 3.2 mEq/L, p<0.05, Fig. 1E), and serum chloride levels (the median, 102 vs. 105 mEq/L, p<0.05, Fig. 1F) were significantly lower in patients with KCNJ5 mutations, whereas diastolic blood pressure was significantly higher (the median, 93 vs. 80 mmHg, p<0.01, Fig. 1G). There was no significant difference in systolic blood pressure in patients with or without KCNJ5 mutations (the median, 147 vs. 138 mmHg).

Expression of steroidogenic enzymes in APAs with and without KCNJ5 mutations
Since we found a significant difference in clinicopathological features between APAs with and without mutations in the KCNJ5 gene, we next compared mRNA levels for steroidogenic enzymes between these two groups. As shown in Fig. 2A, as expected, the expression of CYP11B2 mRNA was significantly higher in APAs with mutations than in those without (median, 4.5 vs. 1.7, n=29 and 9, p<0.01). In contrast, CYP11B1 mRNA levels were significantly lower in APAs with mutations (0.5 vs. 1.3, p<0.05, Fig. 2B). Similarly, although CYP17A1 and CYP11A1 mRNA levels in APAs with mutations were similar to those in normal adrenal glands, they were significantly lower than in APAs without mutations (0.7 vs. 2.8, p<0.01, and 0.9 vs. 1.5, p<0.05, Fig. 2C and 2D). Furthermore, no significant changes were observed in HSD3B2 or CYP21A2 mRNA levels (Fig. 2E and 2F).
Fig. 1  Clinical characteristics of patients with APAs with and without KCNJ5 mutations

A) No significant difference was observed in the prevalence of KCNJ5 mutations between males and females. Approximately 80% of patients in both sexes showed mutations and p.L168R was not found in males. Red bars represent the value for APAs with L168R; blue, G151R, and white, wild-type. B) Patients with KCNJ5 mutations were younger than those without. C) No changes in the maximum diameter of the tumor on CT were observed between the two groups. Bars show the median. D) Plasma aldosterone levels were higher in patients with mutations than in those without. E) Serum potassium levels were lower in patients with mutations. F) Serum chloride levels were lower in patients with mutations. G) Diastolic blood pressure was higher in patients with mutations. Wt represents patients with wild-type alleles in the KCNJ5 gene and Mut, patients with mutations. Bars indicate the median. Red dots represent the value for APAs with L168R; blue, G151R, and black, wild-type. * p<0.05, ** p<0.01, n.s., not significant.
Table 1 Summary of studies regarding somatic mutations in the KCNJ5 gene in APAs of Asian countries

<table>
<thead>
<tr>
<th>Country</th>
<th>No.</th>
<th>Prevalence of mutations (%)</th>
<th>Mean age (years) Mut vs. Wt</th>
<th>Median PAC (ng/dL) Mut vs. Wt</th>
<th>Mean tumor size (mm) Mut vs. Wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kitamoto et al.</td>
<td>Japan 108</td>
<td>69.4% (50% in males, 50% in females)</td>
<td>48.2 ± 11 vs. 55.9 ± 9.1</td>
<td>43.6 ± 24.7</td>
<td>15.0 ± 6.9 vs. 12.4 ± 5.4</td>
</tr>
<tr>
<td>Zheng et al.</td>
<td>China 168</td>
<td>76.8% (43.6% in males, 56.6% in females)</td>
<td>47 ± 11 vs. 51 ± 11</td>
<td>36.5 ± 31.5</td>
<td>15 vs. 11</td>
</tr>
<tr>
<td>Wu et al.</td>
<td>Taiwan 148</td>
<td>59.5% (43.6% in males, 47.3% in females)</td>
<td>45.1 ± 9.9 vs. 55.3 ± 12.1</td>
<td>59.7 vs. 40.7</td>
<td>16.8 vs. 17.3</td>
</tr>
<tr>
<td>Hong et al.</td>
<td>Korea 66</td>
<td>71.2% (33% in males, 66% in females)</td>
<td>46.6 ± 12.7 vs. 48.5 ± 9.6</td>
<td>41.3 vs. 48.2</td>
<td>15 vs. 18</td>
</tr>
<tr>
<td>Present study</td>
<td>Japan 47</td>
<td>78.7% (43.2% in males, 56.8% in females)</td>
<td>48 ± 12 vs. 64 ± 9</td>
<td>56.1 vs. 26.7</td>
<td>17 ± 7 vs. 15 ± 7</td>
</tr>
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</table>

PAC, plasma aldosterone concentration. Mut indicates patients with KCNJ5 mutations and Wt, patients without. Includes the 3 patients with ATP1A1 mutations and one patients with CACNA1D mutation.

Fig. 2 Steroidogenic enzyme mRNA levels in APAs with and without mutations in the KCNJ5 gene. A) CYP11B2 mRNA levels were significantly higher in APAs with mutations in KCNJ5. B) C) D) CYP11B1, CYP17A1, and CYP11A1 mRNA levels were significantly lower in APAs with KCNJ5 mutations than in those without. E) F) No significant changes were observed in HSD3B2 or CYP21A2 mRNA levels between the two groups. Wt represents patients with wild-type alleles in the KCNJ5 gene and Mut, patients with mutations. Red dots represent the value for APAs with L168R; blue, G151R, and black, wild-type. Bars indicate the median. * p<0.05, ** p<0.01, n.s., not significant.
Immunohistochemistry for KCNJ5 in APAs

Fig. 3A and 3B show the results of staining with hematoxylin-eosin and an antibody against KCNJ5 in a normal adrenal gland adjusted to an APA. Consistent with previous findings by Choi et al., the significant staining of KCNJ5-positive cells was observed, particularly in the zona glomerulosa. No staining was observed in the same section with the non-specific antibody (inside the panel of Fig. 3A). Strong and significant staining was observed in cell membranes in an APA with the KCNJ5 mutation (Fig. 3C).

Discussion

Somatic mutations in the KCNJ5 gene in adrenal APAs were first reported in early 2011 by Choi et al [6]. Several investigators including us have since confirmed these mutations in different countries [8, 9, 11-13, 22]. Choi et al. originally detected these mutations in 8 out of 22 (approximately 36%) patients with APAs in Sweden. Akersrom et al. then reported an incidence of 47% among 348 cases in Europe [10], and Fernandes-Rosa et al. an incidence of 38% among 474 cases in Europe [32]. Therefore, the overall prevalence of KCNJ5 mutations in APAs in Western countries is approximately 35~45%. As noticed, the prevalence of KCNJ5 mutations is higher in Asia than in Western countries. We first confirmed previous findings showing a high prevalence of the KCNJ5 gene (76.3%) in Japanese patients with APAs after the addition of recent cases [7, 11-13]. In addition, Kitamoto et al. reported a prevalence of 69.4% in Japanese [31], 59.5% in Taiwanese [29], 71.2% in Korean [30], and 76.8% in Chinese patients with APAs [28]. Although the reason for the higher prevalence in Japan remains unclear, a recent meta-analysis of somatic KCNJ5 mutations in APAs suggested that the apparent higher rate of KCNJ5 mutations in Japan and China might be related to the different protocols employed for patient selection or sodium intake [27].

A marked gender-difference in the frequency of KCN5 mutations in APAs has been reported in Western countries, particularly in Europe. Boulkroun et al. showed prevalences of 49% in females and 19% in males, respectively, and Akersrom et al. reported prevalences of 63% and 24%, respectively [9, 10]. In Asian countries, Hong et al. reported prevalences of 66% in females and 33% in males, respectively, in Korea [30], while Zheng et al. found prevalences of 56.6% in females and 43.3% in males in China [28]. However, we observed a similar prevalence, approximately 50%, in both sexes. Kitamoto et al. also reported a prevalence of 50% in both sexes in Japan [31], as did Wu et al. in both sexes in Taiwan [29]. Furthermore, in the gender-wise comparison, while the p.L168R mutation was identified in 11 females, the same mutation was
only detected in 1 male in the present study (Fig. 1A). Since the mechanism responsible for the gender-difference in the frequency of KCNJ5 mutations in Europe remains unclear, the reason for the discrepancy between Japan and Western countries is of great interest.

Several characteristic phenotypes of APA patients with KCNJ5 mutations have been reported. Patients with these mutations were younger than those without, and had higher plasma aldosterone levels [9, 10]. These phenotypes were also observed in Japanese patients in the present study. In addition, we observed lower serum potassium levels and chloride levels in patients with these mutations in Japan. This may be due to ethnic differences; the average salt intake in Gunma prefecture, Japan is 13.6 g/day (2001 National Nutrition Survey of Japan), which is significantly higher than that in the USA (7.8 g/day, FDA 2007). This large salt intake in Japan may also induce hypokalemia. Reflecting these phenotypes, CYP11B2 mRNA levels were significantly higher in APAs with KCNJ5 mutations than in those without. Although Boulkroun et al. found no specific molecular phenotype for APAs with KCNJ5 mutations by the hierarchical clustering analysis by Boulkroun et al. and the results of the present study may be due to ethnic differences and the preciseness of the microarray and qPCR techniques used. Furthermore, Oki et al. demonstrated that the expression of the mutant KCNJ5, T158A, induced a significant increase in CYP11B2, resulting in the increased secretion of aldosterone from the adrenal cortical carcinoma cell line, HAC15 [35]. In addition, Konosu-Fukaya et al. recently reported that APAs with the KCNJ5 mutation more strongly expressed CYP11B2 mRNA than the wild-type [36].

In Japan, APA is frequently associated with the mild co-secretion of cortisol [12, 13, 19-21]. We previously reported three PA cases that strongly secreted cortisol and found KCNJ5 mutations in 2 cases, suggesting that these tumors are derived from APA because no mutations have been identified in cortisol-producing adenomas [11]. In the present study, we found that among the 32 patients administered 1 mg DST, 10 (31.3%) had a morning cortisol level that was not suppressed to less than 3.0 μg/dL. Of these 10 patients, 6 had a mutation in the KCNJ5 gene, indicating that the mutation does not affect autonomous cortisol secretion assessed by 1 mg DST. Furthermore, no significant difference was observed in the mRNA levels of CYP11B1, which is responsible for the production of cortisol, between APAs with and without a positive result in the 1 mg DST (≥3.0 μg/dL, 0.9 ± 0.3 vs. <3.0 μg/dL, 1.2 ± 1.3). Therefore, factors other than KCNJ5 mutations and the expression of CYP11B1 may play roles in mild autonomous cortisol secretion.

Another important result of the present study is that mutations in KCNJ5 mRNA were frequently identified in APAs with low CYP17A1, CYP11B1, and CYP11A1 mRNA levels. Azizan et al. reported that APAs with KCNJ5 mutations showed higher mRNA levels of CYP17A1 than those without, and concluded that KCNJ5 mutations are common in APAs, particularly those arising from the zona fasciculata (ZF) [37]. However, the present study showed that although the mRNA levels of CYP17A1 in APAs without KCNJ5 mutations have a broad spectrum, those with mutations were significantly lower. We speculate that Japanese APA patients may have different characteristics from those in Western countries, similar to the characteristics of the zona glomerulosa (ZG) with the weak expression of CYP17A1 and CYP11B1. Regarding the expression of HSDB2 mRNA, no significant differences were observed between APAs with and without the KCNJ5 mutation; both showed a wide spectrum of expression. Doi et al. established antibodies specific for the HSDB3 isoforms, 1 and 2, and found that HSDB3 was confined to the ZG and HSDB3 to the zona fasciculata (ZF) in the non-tumoral adrenal cortex, and HSDB3 immunoreactivity was more intense in the tumor cells of APAs than that of HSDB3 [38]. In addition, Konosu-Fukaya et al. recently demonstrated that HSDB3, despite its lower levels of expression than HSDB2, correlated with CYP11B2 at both the protein and mRNA levels in APA tissues, suggesting the involvement of the HSDB1 isoform in the production of aldosterone [36]. Furthermore, in the present study, we demonstrated significant KCNJ5 immunoreactivity in the peritumoral adjacent tissues, particularly in the ZG (Fig. 3B). Taken together, Japanese APAs, particularly those with the KCNJ5 mutation, may possess the characteristics of the ZG. Regional differences in the expression of steroidogenic enzymes in APAs between Japan and Western countries is an interesting issue that warrants further study.
Acknowledgments

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Disclosure Summary

All authors have nothing to disclose.

References

KCNJ5 mutations in Japanese APAs