

ORIGINAL

## Characteristics of Japanese aldosterone-producing adenomas with KCNJ5 mutations

Takashi Okamura<sup>1)</sup>\*, Yasuyo Nakajima<sup>1)</sup>\*, Akiko Katano-Toki<sup>1)</sup>, Kazuhiko Horiguchi<sup>1)</sup>, Shunichi Matsumoto<sup>1)</sup>, Satoshi Yoshino<sup>1)</sup>, Eijiro Yamada<sup>1)</sup>, Takuya Tomaru<sup>1)</sup>, Sumiyasu Ishii<sup>1)</sup>, Tsugumichi Saito<sup>1)</sup>, Atsushi Ozawa<sup>1)</sup>, Nobuyuki Shibusawa<sup>1)</sup>, Tetsuro Satoh<sup>1)</sup>, Shuichi Okada<sup>1)</sup>, Rin Nagaoka<sup>2)</sup>, Daisuke Takada<sup>2)</sup>, Jun Horiguchi<sup>2)</sup>, Tetsunari Oyama<sup>3)</sup> and Masanobu Yamada<sup>1)</sup>

<sup>1)</sup> Department of Medicine and Molecular Science, Gunma University Graduate School of Medicine, Maebashi 371-8511, Japan

<sup>2)</sup> Department of Thoracic and Visceral Organ Surgery, Gunma University Graduate School of Medicine, Maebashi 371-8511, Japan

<sup>3)</sup> Department of Diagnostic Pathology, Gunma University Graduate School of Medicine, Maebashi 371-8511, Japan

**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 Ca<sup>2+</sup> channels. Increases in intracellular Ca<sup>2+</sup> levels then promote aldosterone production, and the chronic Ca<sup>2+</sup> 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

Submitted May 14, 2016; Accepted Aug. 23, 2016 as EJ16-0243  
Released online in J-STAGE as advance publication Sep. 28, 2016  
Correspondence to: Masanobu Yamada, M.D., Ph.D., Department of Medicine and Molecular Science, Gunma University Graduate School of Medicine, 3-39-15 Showa-machi, Maebashi Gunma 371-8511, Japan. E-mail: myamada@gunma-u.ac.jp

\* These two authors contributed equally to this work.

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*, (3 $\beta$ -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  $\pm$  13 y (mean  $\pm$  SD) (20 males and 27 females). All patients had a high plasma aldosterone/ plasma renin concentration ratio (ARR) and a unilateral adrenal cortical mass evi-

dent 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  $\pm$  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  $\mu$ L 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), 17 $\beta$ -hydroxylase (*CYP17A1*) (Hs01124136\_m1), cholesterol side-chain cleavage enzyme (*CYP11A1*) (Hs00167984\_m1), *CYP21A2* (Hs00365734\_g1), 3 $\beta$ -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  $\mu$ m. 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  $\pm$  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)).

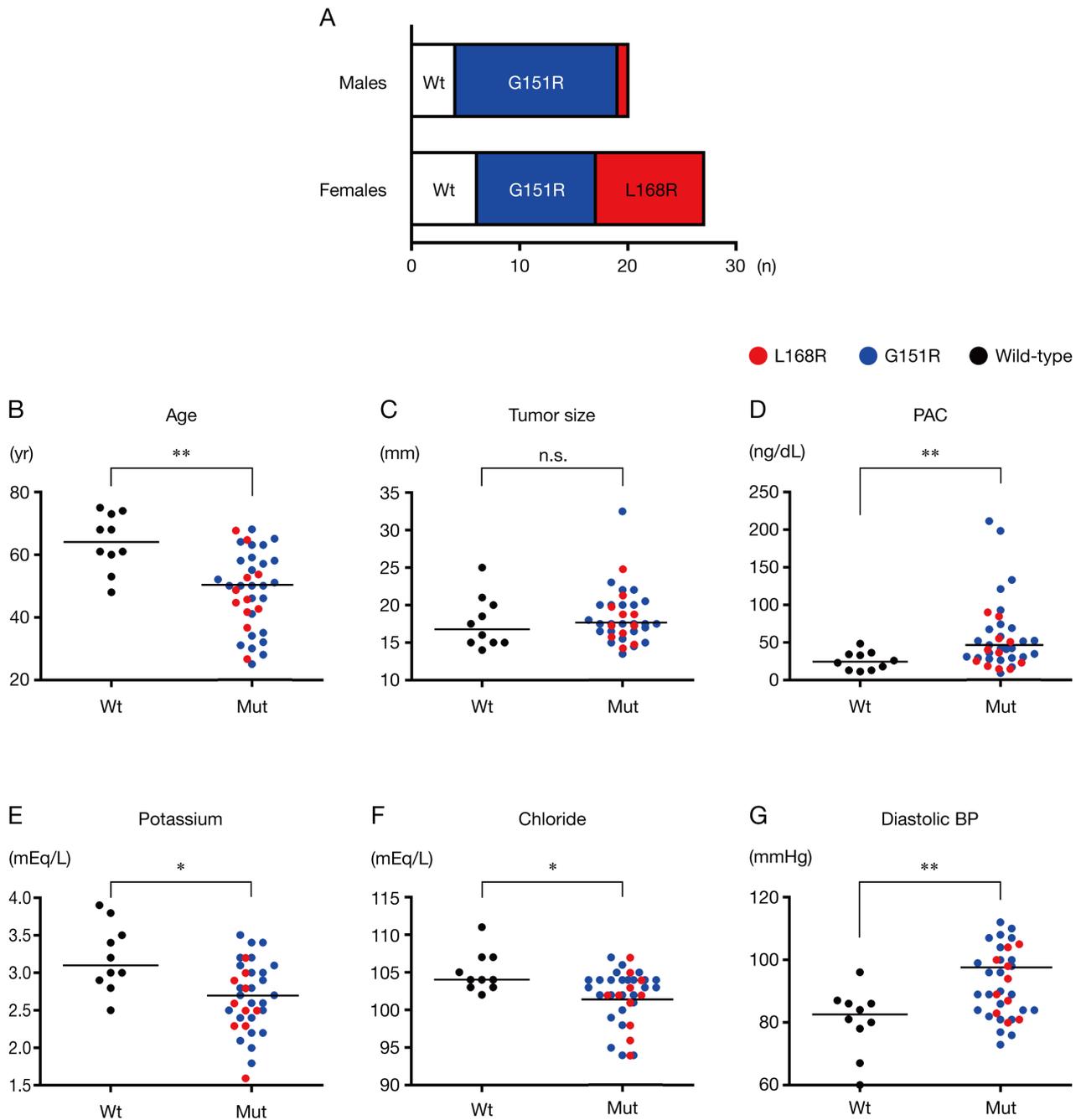
### Clinicopathological features of APA patients with and without *KCNJ5* mutations

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 ( $48 \pm 12$  years) than in those without ( $64 \pm 9$ ). No significant difference was observed in the maximum size of tumors in CT scans ( $15 \pm 7$  without mutations *vs.*  $17 \pm 7$  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).



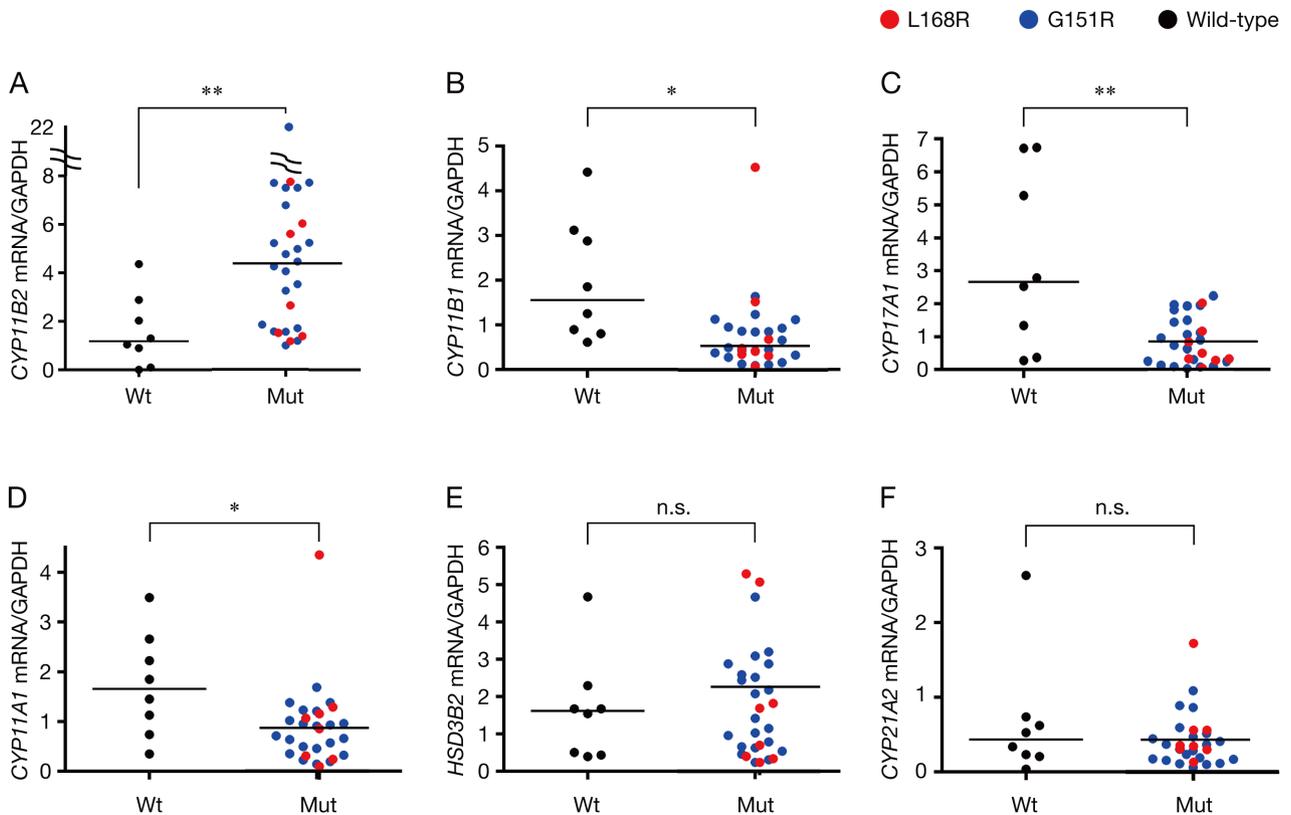
**Fig. 1** Clinical characteristics of patients with APAs with and without KCNJ5 mutations

**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. 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

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

PAC, plasma aldosterone concentration. Mut indicates patients with *KCNJ5* mutations and Wt, patients without. <sup>§</sup> 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.

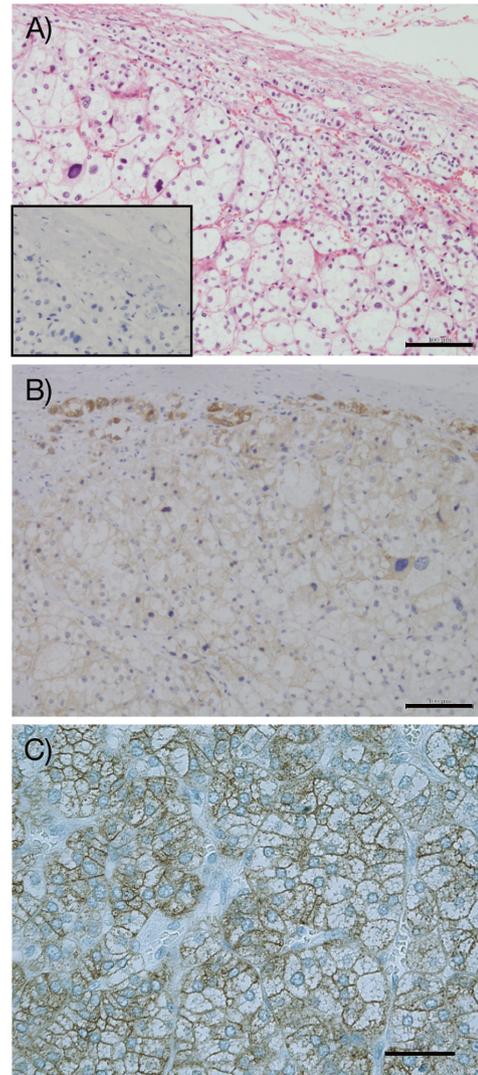
### Immunohistochemistry for *KCNJ5* in APAs

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



**Fig. 3** *KCNJ5* Immunohistochemistry

Representative immunohistochemistry for our cases is shown. **A)** Hematoxylin-eosin staining of a portion of the peritumoral tissues adjusted with an APA and no staining with a non-specific IgG and second antibody as a negative control (inside panel). **B)** A similar section was stained with a *KCNJ5* antibody. The significant staining of *KCNJ5* was observed in the zona glomerulosa. **C)** Significant staining was observed on the membranes of most cells in the APA. The bars in each panel indicate 100 μm.

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 of transcriptome profiles from 102 APAs, higher levels of *CYP11B2* mRNA in APAs with KCNJ5 mutations, similar to our results, have recently been reported by Wiilaimas *et al.* and more recently by Seccia *et al.* [9, 33, 34]. The discrepancy between the microarray analysis by Boulkroun *et al.* and the results of the present study and previous findings 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 muta-

tion 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 ( $\geq 3.0$  µg/dL,  $0.9 \pm 0.3$  vs.  $< 3.0$  µg/dL,  $1.2 \pm 1.3$ ). 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 *HSD3B2* 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 *HSD3B* isoforms, 1 and 2, and found that *HSD3B1* was confined to the ZG and *HSD3B2* to the zona fasciculata (ZF) in the non-tumoral adrenal cortex, and *HSD3B2* immunoreactivity was more intense in the tumor cells of APAs than that of *HSD3B1* [38]. In addition, Konosu-Fukaya *et al.* recently demonstrated that *HSD3B1*, despite its lower levels of expression than *HSD3B2*, correlated with *CYP11B2* at both the protein and mRNA levels in APA tissues, suggesting the involvement of the *HSD3B1* 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

We thank all the medical and co-medical staff and graduate students involved in patient care. We thank Dr. Santosh Sapkota for his assistance with the writing of this manuscript in English. This work was supported by JSPS KAKENHI Grant Numbers 15H04852 (to M.Y.) and 25893026 (to Y.N.), and was partially supported by the Advancing Care of Primary

Aldosteronism in Japan Study (to M.Y.) from the Japan Agency for Medical Research and Development, AMED, and the Research on rare and intractable disease, Health and Labour Sciences Research Grants.

## Disclosure Summary

All authors have nothing to disclose.

## References

- Funder JW, Carey RM, Fardella C, Gomez-Sanchez CE, Mantero F, *et al.* (2008) Case detection, diagnosis, and treatment of patients with primary aldosteronism: an endocrine society clinical practice guideline. *J Clin Endocrinol Metab* 93: 3266-3281.
- Douma S, Petidis K, Doumas M, Papaefthimiou P, Triantafyllou A, *et al.* (2008) Prevalence of primary hyperaldosteronism in resistant hypertension: a retrospective observational study. *Lancet* 371: 1921-1926.
- Mulatero P, Stowasser M, Loh KC, Fardella CE, Gordon RD, *et al.* (2004) Increased diagnosis of primary aldosteronism, including surgically correctable forms, in centers from five continents. *J Clin Endocrinol Metab* 89: 1045-1050.
- Rossi GP, Seccia TM, Pessina AC (2008) Primary aldosteronism - part I: prevalence, screening, and selection of cases for adrenal vein sampling. *J Nephrol* 21: 447-454.
- Young WF (2007) Primary aldosteronism: renaissance of a syndrome. *Clin Endocrinol (Oxf)* 66: 607-618.
- Choi M, Scholl UI, Yue P, Bjorklund P, Zhao B, *et al.* (2011) K<sup>+</sup> channel mutations in adrenal aldosterone-producing adenomas and hereditary hypertension. *Science* 331: 768-772.
- Taguchi R, Yamada M, Nakajima Y, Satoh T, Hashimoto K, *et al.* (2012) Expression and Mutations of KCNJ5 mRNA in Japanese Patients with Aldosterone-Producing Adenomas. *J Clin Endocrinol Metab* 97: 1311-1319.
- Azizan EA, Murthy M, Stowasser M, Gordon R, Kowalski B, *et al.* (2012) Somatic Mutations Affecting the Selectivity Filter of KCNJ5 Are Frequent in 2 Large Unselected Collections of Adrenal Aldosteronomas. *Hypertension* 59: 587-591.
- Boulikroun S, Beuschlein F, Rossi GP, Golib-Dzib JF, Fischer E, *et al.* (2012) Prevalence, Clinical, and Molecular Correlates of KCNJ5 Mutations in Primary Aldosteronism. *Hypertension* 59: 592-598.
- Cohen J (1992) A power primer. *Psychol Bull* 112: 155-159.
- Yamada M, Nakajima Y, Taguchi R, Okamura T, Ishii S, *et al.* (2012) KCNJ5 mutations in aldosterone- and cortisol-co-secreting adrenal adenomas. *Endocr J* 59: 735-741.
- Nakajima Y, Okamura T, Gohko T, Satoh T, Hashimoto K, *et al.* (2014) Somatic mutations of the catalytic subunit of cyclic AMP-dependent protein kinase (PRKACA) gene in Japanese patients with several adrenal adenomas secreting cortisol. *Endocr J* 61: 825-832.
- Nakajima Y, Okamura T, Horiguchi K, Gohko T, Miyamoto T, *et al.* (2016) GNAS mutations in adrenal aldosterone-producing adenomas. *Endocr J* 63: 199-204.
- Rossi GP, Bernini G, Caliumi C, Desideri G, Fabris B, *et al.* (2006) A prospective study of the prevalence of primary aldosteronism in 1,125 hypertensive patients. *J Am Coll Cardiol* 48: 2293-2300.
- Stowasser M, Gordon RD (2003) Primary aldosteronism. *Best Pract Res Clin Endocrinol Metab* 17: 591-605.
- Nishikawa T, Saito J, Omura M (2007) Prevalence of primary aldosteronism: should we screen for primary aldosteronism before treating hypertensive patients with medication? *Endocr J* 54: 487-495.
- Omura M, Saito J, Yamaguchi K, Kakuta Y, Nishikawa T (2004) Prospective study on the prevalence of secondary hypertension among hypertensive patients visiting a general outpatient clinic in Japan. *Hypertens Res* 27: 193-202.
- Omura M, Sasano H, Saito J, Yamaguchi K, Kakuta Y, *et al.* (2006) Clinical characteristics of aldosterone-producing microadenoma, macroadenoma, and idiopathic hyperaldosteronism in 93 patients with primary aldosteronism. *Hypertens Res* 29: 883-889.
- Nakajima Y, Yamada M, Taguchi R, Satoh T, Hashimoto K, *et al.* (2011) Cardiovascular Complications of Patients with Aldosteronism Associated with Autonomous Cortisol Secretion. *J Clin Endocrinol Metab* 96: 2512-2518.
- Hiraishi K, Yoshimoto T, Tsuchiya K, Minami I, Doi M, *et al.* (2011) Clinicopathological features of primary aldosteronism associated with subclinical Cushing's syndrome. *Endocr J* 58: 543-551.

21. Adachi J, Hirai Y, Terui K, Nakano T, Fukuda Y, *et al.* (2003) A report of 7 cases of adrenal tumors secreting both cortisol and aldosterone. *Intern Med* 42: 714-718.
22. Charmandari E, Sertedaki A, Kino T, Merakou C, Hoffman DA, *et al.* (2012) A Novel Point Mutation in the KCNJ5 Gene Causing Primary Hyperaldosteronism and Early-Onset Autosomal Dominant Hypertension. *J Clin Endocrinol Metab* 97: E1532-1539.
23. Willenberg HS, Spath M, Maser-Gluth C, Engers R, Anlauf M, *et al.* (2010) Sporadic solitary aldosterone- and cortisol-co-secreting adenomas: endocrine, histological and genetic findings in a subtype of primary aldosteronism. *Hypertens Res* 33: 467-472.
24. Spath M, Korovkin S, Antke C, Anlauf M, Willenberg HS (2011) Aldosterone- and cortisol-co-secreting adrenal tumors: the lost subtype of primary aldosteronism. *Eur J Endocrinol* 164: 447-455.
25. Ishida E, Yamada M, Horiguchi K, Taguchi R, Ozawa A, *et al.* (2011) Attenuated expression of menin and p27 (Kip1) in an aggressive case of multiple endocrine neoplasia type 1 (MEN1) associated with an atypical prolactinoma and a malignant pancreatic endocrine tumor. *Endocr J* 58: 287-296.
26. Horiguchi K, Yamada M, Satoh T, Hashimoto K, Hirato J, *et al.* (2009) Transcriptional activation of the mixed lineage leukemia-p27Kip1 pathway by a somatostatin analogue. *Clin Cancer Res* 15: 2620-2629.
27. Lenzini L, Rossitto G, Maiolino G, Letizia C, Funder JW, *et al.* (2015) A Meta-Analysis of Somatic KCNJ5 K(+) Channel Mutations In 1636 Patients With an Aldosterone-Producing Adenoma. *J Clin Endocrinol Metab* 100: E1089-1095.
28. Zheng FF, Zhu LM, Nie AF, Li XY, Lin JR, *et al.* (2015) Clinical characteristics of somatic mutations in Chinese patients with aldosterone-producing adenoma. *Hypertension* 65: 622-628.
29. Wu VC, Huang KH, Peng KY, Tsai YC, Wu CH, *et al.* (2015) Prevalence and clinical correlates of somatic mutation in aldosterone producing adenoma-Taiwanese population. *Sci Rep* 5: 11396.
30. Hong AR, Kim JH, Song YS, Lee KE, Seo SH, *et al.* (2016) Genetics of Aldosterone-Producing Adenoma in Korean Patients. *PLoS One* 11: e0147590.
31. Kitamoto T, Suematsu S, Matsuzawa Y, Saito J, Omura M, *et al.* (2015) Comparison of cardiovascular complications in patients with and without KCNJ5 gene mutations harboring aldosterone-producing adenomas. *J Atheroscler Thromb* 22: 191-200.
32. Fernandes-Rosa FL, Williams TA, Riester A, Steichen O, Beuschlein F, *et al.* (2014) Genetic spectrum and clinical correlates of somatic mutations in aldosterone-producing adenoma. *Hypertension* 64: 354-361.
33. Williams TA, Monticone S, Crudo V, Warth R, Veglio F, *et al.* (2012) Visinin-Like 1 Is Upregulated in Aldosterone-Producing Adenomas With KCNJ5 Mutations and Protects From Calcium-Induced Apoptosis. *Hypertension* 59: 833-839.
34. Monticone S, Hattangady NG, Nishimoto K, Mantero F, Rubin B, *et al.* (2012) Effect of KCNJ5 Mutations on Gene Expression in Aldosterone-Producing Adenomas and Adrenocortical Cells. *J Clin Endocrinol Metab* 97: E1567-1572.
35. Oki K, Plonczynski MW, Luis Lam M, Gomez-Sanchez EP, Gomez-Sanchez CE (2012) Potassium Channel Mutant KCNJ5 T158A Expression in HAC-15 Cells Increases Aldosterone Synthesis. *Endocrinology* 153: 1774-1782.
36. Konosu-Fukaya S, Nakamura Y, Satoh F, Felizola SJ, Maekawa T, *et al.* (2015) 3 $\beta$ -Hydroxysteroid dehydrogenase isoforms in human aldosterone-producing adenoma. *Mol Cell Endocrinol* 408: 205-212.
37. Azizan EA, Lam BY, Newhouse SJ, Zhou J, Kuc RE, *et al.* (2012) Microarray, qPCR, and KCNJ5 Sequencing of Aldosterone-Producing Adenomas Reveal Differences in Genotype and Phenotype between Zona Glomerulosa- and Zona Fasciculata-Like Tumors. *J Clin Endocrinol Metab* 97: E819-829.
38. Doi M, Satoh F, Maekawa T, Nakamura Y, Fustin JM, *et al.* (2014) Isoform-specific monoclonal antibodies against 3 $\beta$ -hydroxysteroid dehydrogenase/isomerase family provide markers for subclassification of human primary aldosteronism. *J Clin Endocrinol Metab* 99: E257-262.