Steroidogenic Activity of Aldosterone-Producing Adenoma with and without KCNJ5 Gene Mutations, Comparing with that in Each Adherent Normal Tissue

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Abstract

Introduction: The steroidogenic activity in a KCNJ5-gene mutated aldosterone-producing adenoma (APA) was reported to be much stronger than that in an APA of the wild type, while it is still controversial.

Objectives: We examined whether the normal adrenal tissue attached to APA also possesses KCNJ5 gene mutations, and also investigated steroidogenic activity of mutated and non-mutated APA tissue cells with that of each normal tissue cell attached to APA in order to clarify the nature of steroidogenic activity.

Methods: We selected 12 patients with and without KCNJ5 gene mutation-harboring APA in whom normal adrenal tissue adhered to the APA. We analyzed the presence of KCNJ5 gene mutations by using those tissues.

Results: APA tissues of 6 patients possessed KCNJ5 gene mutations, whereas those mutations were not detected in any of the adjacent normal tissue samples as well as another 6 cases of APA. In two APA tissues with or without KCNJ5 gene mutation, we also compared the steroidogenic activity of isolated cells from each APA tissue with that from the normal adherent tissues. The cortisol production of the mutated APA cells was markedly decreased compared with that of the normal cells, suggesting that the aldosterone production pathway becomes dominant and the cortisol production pathway is downregulated in that APA tissue. Furthermore, the ratio of aldosterone to cortisol in the normal adherent tissue was about $1/4^{\text{th}}$ of that of the wild-type APA tissue, while it was about $1/10^{\text{th}}$ in the mutated APA.

Conclusion: KCNJ5 gene mutations in the normal tissue adhering to KCNJ5 gene mutation-harboring APA was not observed, suggesting that somatic mutations of the KCNJ5 gene only occur in APA tissue. It is also suggested that KCNJ5 gene mutations dominantly induce the aldosterone production pathway as well as concomitant decline in cortisol production, resulting in highly autonomous aldosterone production.

Keywords: Aldosterone-producing adenoma; KCNJ5 gene; Adherent normal tissue; Aldosterone; Cortisol

Introduction

Two recurrent somatic mutations in and near the gene for the selectivity filter of the potassium (K^+) channel KCNJ5 were recently detected in aldosterone-producing adenomas (APA). These mutations were reported to produce increased sodium (Na^+) conductance and cell depolarization resulting in autonomous aldosterone production in the APA [1]. In a recent study of Japanese patients with APA, it was reported that 15 (65.2%) of the 23 APA had two somatic mutations in their KCNJ5 genes [2], suggesting that KCNJ5 gene mutations are closely related to the pathogenesis of APA in Japanese primary aldosteronism (PA) patients [2]. Thus, we tried to compare clinical characteristics of KCNJ5 gene mutation-harboring APA, with those of APA with no mutations. Furthermore, we examined whether the normal adrenal tissue attached to APA also possesses KCNJ5 gene mutations, and also investigated steroidogenic activity of mutated and non-mutated APA tissue cells with that of each normal tissue cells attached to APA in order to directly clarify the nature of steroidogenic activity for synthesizing aldosterone and cortisol by using isolated cells prepared from each APA and normal tissue.

Subjects and Methods

Tissue specimens from 8 (Cases 1~8) and 4 patients (Cases 9~12) with APA, who underwent unilateral total and partial resection of the affected adrenal gland respectively, were analyzed (Table 1). We could use the removed adrenal tissues containing adenoma with normal adherent tissue in Cases 1~8 for genetic analysis because of unilateral total adrenalectomy, while could use only APA tissues in Cases 9~12 due to partial resection of the affected adrenal gland. Furthermore, tissue samples were too limited to prepare isolated cells for studying steroidogenesis described below. Thus, we only used tissue samples from Case 2 and 7 since those cases were female, demonstrating almost the same values of PAC and PRA as shown in Table 1. The subjects were definitively diagnosed with APA during the screening of hypertensive patients who visited our general outpatient clinic between January 2013 and June 2013 according to the

guidelines developed by the Japan Endocrine Society (JES) [3]. The clinical findings of 12 subjects were shown in Table 1, and we could definitely localize affected adrenals by adrenal vein sampling as depicted in Table 2. The diagnosis of primary aldosteronism was confirmed by endocrinological examinations such as the furosemide plus upright test, saline-loading test, and captopril-loading test [3]. Computed tomography scans and adrenocorticotrophic hormone-loading adrenal vein sampling (ACTH-AVS) were performed to determine the laterality of each patient's hyperaldosteronism, as reported previously [4,5]. All of the patients displayed obvious unilateral hyperaldosteronism and underwent unilateral total resection of the affected adrenal gland. In addition, they all exhibited postoperative improvements in their endocrinological abnormalities, such as hyporeninemic hyperaldosteronemia, and blood pressure (data not shown). The present study was approved by the research ethics committee of Yokohama Rosai Hospital, and all of the subjects understood the objectives of the study and gave their written consent.

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

The specimens of APA tissue and the normal adrenal tissue that had been separated from each APA were separately frozen in liquid nitrogen immediately after their removal during surgery and were stored at -80 °C. Total RNA was prepared from each tissue using the TriPure Isolation Reagent and High Pure RNA Isolation kit (Roche Diagnostics), as reported previously [6-8]. Then, cDNA was reverse-transcribed from the total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). To sequence the KCNJ5 cDNA, PCR was performed using 1.0 µl cDNA together with Tks Gflex™ DNA Polymerase (Takara-bio) and the following primers: forward primer: 5'-ACCTGGACCATGTTGGCGACC-3', reverse primer: 5'-TCCCGCATGGAGATGACTGCG-3'. The PCR products (286 bp., positions 664~949 NM_000890.3) were purified by

electrophoresis (2% agarose gel) and NucleoSpin Gel and PCR Clean-up (MACHEREY-NAGEL GmbH & Co.KG), before being directly sequenced by a custom service (Premix sequence, Takara-bio.). All experiments were repeated at least twice.

Preparation of isolated adrenal cells and assessment of steroidogenesis

Isolated adrenal adenoma cells or adherent normal adrenal cells were soon prepared after their removal during surgery as described previously [9,10] and then resuspended in Krebs-Ringer bicarbonate buffer (pH 7.4) containing 1.3 mM CaCl₂, 0.2% glucose, 0.1% bovine serum albumin, and 20 mM HEPES (KRBGA-HEPES). Cell numbers were determined by the CyQUANT cell proliferation assay kit (Molecular Probes, Inc., Eugene, OR, USA). More than 98% of the dispersed cells were viable according to the trypan blue dye exclusion test. Next, the adrenal cells were re-suspended at a final density of 10×10⁴ cells/ml in KRBGA-HEPES containing 1.3 mM CaCl₂ and incubated for 120 min under constant agitation at 37°C after 15 min pre-incubation with the same buffer. The aldosterone and cortisol concentrations of the medium were measured as reported previously [6,7,9,10].

Statistical analysis

All results are expressed as the mean±SD from at least three different experiments/measurements. We have used three different portions of the APA and its adherent normal adrenal tissues of Cases 2 and 7 for analyzing steroidogenesis. Student's *t*-test was used for comparisons. Differences were considered significant if the *p*-value was less than 0.05.

Results

Clinical characteristics

Age, PAC, PRA, ARR and serum potassium level in APA patients with KCNJ5 gene mutations (Cases 1~6) were 44±7 years old, 510±578 pg/ml, 0.17±0.10 ng/dl/hr, 4756±6045 and 2.8±0.5

Table 1: Clinical characteristics of subjects with aldosterone-producing adenoma.

Case#	Age	Gender	PAC	PRA	ARR	K	Dexa Suppression test (cortisol, µg/dl)	Unilateral adrenalectomy
1	56	female	120	0.1	1200	2.8	1.8	Right adrenal
2	41	female	738	<0.1	>7380	2.7	1.3	Left adrenal
3	43	male	192	0.3	640	3.5	2.1	Left adrenal
4	36	female	1590	<0.1	>15900	2.1	0.9	Right adrenal
5	46	female	303	0.1	3030	2.6	1.0	Right adrenal
6	41	female	116	0.3	386.6	3.2	0.8	Right adrenal
7	61	female	953	0.2	47650	3.5	1.7	Right adrenal
8	61	female	127	0.6	212	3.8	0.9	Right adrenal
9	62	female	235	0.1	2350	3.3	1.7	Left adrenal
10	62	female	172	<0.1	>1720	3.4	2.5	Right adrenal
11	49	female	209	0.2	1045	2.8	1.4	Left adrenal
12	29	female	213	0.2	1065	2.9	1.9	Right adrenal

Cases 1 to 6 showed KCNJ5 gene mutations in their aldosterone-producing adenomas, while cases 7 to 12 did not show any mutations.

PAC- Plasma Aldosterone Concentration; PRA- Plasma Renin Activity; ARR- Aldosterone-Renin Ratio; K- serum potassium level; Dexa suppression-overnight 1mg dexamethasone suppression test; AVS- Adrenal Vein Sampling; n.p.- not performed due to allergy against a contrast medium.

mEq/L, respectively (Table 1). Age, PAC, PRA, ARR and serum potassium level in APA patients without KCNJ5 gene mutations (Cases 7~12) were 54±13 years old, 318±313 pg/ml, 0.23±0.19 ng/dl/hr, 9007±18945 and 3.3±0.4 mEq/L, respectively. There was a significant difference in serum potassium level between two groups ($p<0.05$). PAC and PRA were higher and lower in the mutated group than the wild group, respectively, while there was statistically no significant difference between two groups. Highest value of PAC in right or left adrenal vein sampling in the mutated group and the wild type group was 67038±81269 pg/ml and 74383±45736 pg/ml, respectively without any statistically significant difference. Moreover, the ratio of PAC to cortisol in the affected adrenal vein in the mutated group and the wild type group was 117.2±170 and 96.1±43.0, respectively without any statistically significant difference (Table 2).

KCNJ5 gene mutations

No KCNJ5 gene mutations were noted in the adjacent normal tissues (N) in any of the 8 APA patients with or without KCNJ5 gene mutations, although the APA tissues in cases 1 to 6 demonstrated KCNJ5 gene mutations and those in cases 7 to 12 did not (Table 3). We could not analyze KCNJ5 gene mutations in N of cases 9 to 12, because we did not remove the adherent normal portion of adrenals by partial adrenalectomy.

Steroidogenesis

APA and adherent normal adrenal cells were isolated from two of the above patients (Cases 2 & 7) and cultured. Case 2 demonstrated that the aldosterone level of the APA culture supernatant was about 1.4 times higher than that of the normal adrenal cell culture supernatant (Figure 1A). Furthermore, the APA cells secreted 1/10th of the amount of cortisol secreted by the normal cells (Figure 1B). Thus, in case 2 the aldosterone to cortisol ratio of the normal tissue was almost 1/10th of that of

the APA tissue (Figure 1C). On the other hand, in Case 7 which do not harbor KCNJ5 gene mutation, the amount of aldosterone was almost twice higher in the APA tissue cells than that of the normal adherent tissue cells (Figure 2A). The cortisol production by the APA tissue cells of Case 7 was 6/10th of that by the normal adherent tissue cells (Figure 2B). Then, the ratio of aldosterone to cortisol in the normal adherent tissue of Case 7 was almost 1/4th of that of the APA tissue (Figure 2C). Aldosterone production by isolated cells of the adenoma tissue in Case 2 was significantly lower than that in Case 7 ($p<0.02$), while cortisol production by isolated cells of the adenoma tissue in Case 2 was not significantly differed from that in Case 7. Aldosterone production by isolated cells of the adherent normal tissue in Case 2 was significantly higher than that in Case 7 ($p<0.05$), while cortisol production by isolated cells of the adherent normal tissue in Case 2 was significantly higher than that in Case 7 ($p<0.05$). The ratio of aldosterone to cortisol production in the APA tissue of Case 2 and 7 was 0.05±0.01 and 0.08±0.01, respectively without significant difference between two cases. The ratio of aldosterone to cortisol production in the adherent normal tissue of Case 2 was significantly lower than that in Case 7 ($p<0.01$).

Discussion

In the present study, whilst KCNJ5 gene mutations were detected in the APA tissues of cases 1 to 6 they were not detected in any of the associated normal adherent adrenal tissue samples. These findings were consistent with previous studies in which KCNJ5 gene mutations were not found in the control tissue samples, which were obtained from the normal adrenal gland tissue adjacent to the APA [2]. Therefore, it seems that KCNJ5 gene mutations only occur in APA tissue, and it is unlikely that APA is caused by gene aberrations being present in all of the cells of the adrenal gland. It was also reported that significantly lower levels of the potassium channel GIRK4 were detected in KCNJ5

Table 2: Adrenal vein sampling.

Case#	Laterality of adrenal adenoma	ACTH-stimulated AVS				
		Right	Left	Right	Left	LR
1	Right	1580	637	51400	6160	3.35
2	Left	n.p.	n.p.	n.p.	n.p.	n.p.
3	Left	371*	497*	1560*	6790*	3.26
4	Right	500	491	209000	3410	60.1
5	Right	535	728	44200	2880	20.9
6	Right	607	626	23800	10000	2.45
7	Right	1000	683	117000	2690	2.97
8	Right	1070	613	26100	10900	1.37
9	Left	864	902	11500	127000	10.58
10	Right	388	881	36900	3040	27.56
11	Left	776	526	2090	37300	26.33
12	Right	796	540	102000	2030	34.09

Each value in adrenal vein sampling (AVS) was described as the value after ACTH-stimulation, while the values of PAC* in case 3 was expressed as the basal value without ACTH-stimulation. Case 2 clearly demonstrated the left adrenal tumor by CT images and left adrenalectomy was done after talking informed consent. A unilateral lesion can be detected if the lateralized ratio (L.R.) (the adrenal vein aldosterone/cortiso; (A/C) ratio of the high-value side)/(that of the low-value side) is 2.6 after ACTH stimulation or if aldosterone is more than 14000pg/ml in the affected adrenal side after ACTH stimulation, according to the guideline [3].

Table 3: KCNJ5 gene mutations in each subject.

Case#	KCNJ5 Gene analysis		
		Codon 151 (p.G151R)	Codon 168 (p.L168R)
1	A	G G G	C T / G C
	N	G G G	C T C
2	A	G G G	C T / G C
	N	G G G	C T C
3	A	G G G	C T / G C
	N	G G G	C T C
4	A	G / A G G	C T C
	N	G G G	C T C
5	A	G G G	C T / G C
	N	G G G	C T C
6	A	G / (A) G G	C T C
	N	G G G	C T C
7	A	G G G	C T C
	N	G G G	C T C
8	A	G G G	C T C
	N	G G G	C T C
9	A	G G G	C T C
	N	No data	No data
10	A	G G G	C T C
	N	No data	No data
11	A	G G G	C T C
	N	No data	No data
12	A	G G G	C T C
	N	No data	No data

Mutations of KCNJ5 gene in adrenal tissues were analyzed as described in detail in the text.

A- adenoma tissue, N- normal adrenal tissue adhered to the adenoma

Figure 1: Steroidogenic activity in the APA cells and normal adherent adrenal cells in case 2

A) Aldosterone production by APA cells and normal adherent adrenal cells that had been cultured for 2 hours as described in the text.

Results were expressed as mean±SD. p<0.003; adenoma vs. normal

B) Cortisol production by APA cells and normal adherent adrenal cells that had been cultured for 2 hours as described in the text.

Results were expressed as mean±SD. p<0.01; adenoma vs. normal

C) The ratio of aldosterone to cortisol in the APA cells and normal adherent adrenal cells after 2 hours' incubation.

Results were expressed as mean±SD. p<0.006; adenoma vs. normal

A- the APA tissue of case 2; N- the normal adherent adrenal tissue

Figure 2: Steroidogenic activity in the APA cells and normal adherent adrenal cells in case 7
A) Aldosterone production by APA cells and normal adherent adrenal cells that had been cultured for 2 hours as described in the text. Results were expressed as mean±SD. $p < 0.015$; adenoma vs. normal
B) Cortisol production by APA cells and normal adherent adrenal cells that had been cultured for 2 hours as described in the text. Results were expressed as mean±SD. $p < 0.05$; adenoma vs. normal
C) The ratio of aldosterone to cortisol in the APA cells and normal adherent adrenal cells after 2 hours' incubation. Results were expressed as mean±SD. $p < 0.04$; adenoma vs. normal
A- the APA tissue of case 7; N- the normal adherent adrenal tissue

mutation-harboring APA, although the channel was expressed in APA tissue and the zona glomerulosa of the adjacent cortex [11]. On the other hand, no previous studies have compared the steroidogenic activity of APA and normal tissue by using isolated cells prepared from the removed adrenal tissues. Thus, we compared the amounts of aldosterone and cortisol secreted from APA cells with and without harboring KCNJ5 gene mutations with those secreted by normal adrenal cells from the region adjacent to each APA tissue (Cases 2 & 7). Aldosterone production when incubated isolated cells of the adenoma tissue of Case 2 with KCNJ5 gene mutation was not higher than that when incubated isolated cells of the adenoma tissue of Case 7 with no mutation. Thus, it is suggested that the activity of basal aldosterone synthesis by the cells of the KCNJ5 gene mutated APA tissue seems to be not so strong, comparing with that in the non-mutated APA tissue. On the contrary, the present experiments clearly demonstrated that the cortisol production of the APA cells was markedly decreased compared with that of the adjacent normal cells in Case 2, suggesting that the aldosterone production pathway becomes dominant and the cortisol production pathway is downregulated in the APA tissue, and resulting in autonomous aldosterone production by the cells of the KCNJ5 gene mutated APA tissue. Case 7 was still possessing autonomous ability of aldosterone production, while the ratio of aldosterone to cortisol in the normal adherent tissue was almost 1/4th of that of the APA tissue. Thus, it is suggested that isolated cells prepared from the APA tissue harboring KCNJ5 gene mutation can synthesize more dominantly aldosterone than cortisol compared with the cells prepared from

the APA tissue without harboring KCNJ5 gene mutation. On the other hand, it was reported that the non-tumor portions of the microadenomas showed marked and sustained CYP11B2 mRNA expression under the suppressed renin-angiotensin system [12]. Thus it is suggested that the normal adrenal portion adhered to APA possesses the ability of aldosterone synthesis, and also that steroidogenic activity of cortisol and aldosterone production in the normal adherent tissue may be differently influenced by functional characteristics of steroidogenesis of each APA tissue.

This observation is consistent with our previously reported findings regarding APA tissue [8]. CYP11B2 and CYP17 are key enzymes of the aldosterone and cortisol pathways, respectively. In the abovementioned study, we detected a low CYP17/HSD3B2 ratio in APA tissue, indicating that the cortisol pathway is suppressed in APA. In addition, we also observed a high CYP11B2/CYP11B1 ratio in APA tissue, suggesting that the aldosterone pathway is accelerated in APA; however, it was unclear whether the examined APA tissues possessed KCNJ5 gene mutations. The results of the present study also agree with those reported by Stowasser M [13], who found that KCNJ5 mutations caused reduced K/Na channel selectivity and Na influx, and hence, induced increases in the cell membrane depolarization, calcium influx, aldosterone synthesis-promoting gene expression, and aldosterone production of adrenocortical cells. Moreover, it was reported that KCNJ5 mutations are associated with young age, female gender and a more florid hyperaldosteronism with higher preoperative aldosterone and/or lower plasma K levels [2,14-

17]. From the present data of clinical findings, the mutated group demonstrated significantly lower plasma K levels than the wild type group, and also the mutated group showed not significant but higher tendency of PAC than the wild type group. Thus, our clinical data are consistent with the previous findings [2,14-17]. Moreover, the present results by using isolated cells from the APA tissues with and without KCNJ5 gene mutation clearly demonstrated the higher ability of synthesizing aldosterone rather than cortisol in KCNJ5 mutation-harboring APA than that in the APA tissue of the wild type, even though the amount of basal aldosterone production was slightly lower in the mutated group than in the wild type group. Those data on *in vitro* study also are consistent with clinical findings as reported previously [2,14-17].

Conclusion

We did not detect KCNJ5 gene mutations in the normal tissue adhering to KCNJ5 gene mutation-harboring APA, suggesting that somatic mutations of the KCNJ5 gene only occur in APA tissue. We need to conduct further studies involving larger numbers of adrenal tissue samples obtained from patients with PA, although it is quite difficult to obtain sufficient amounts of APA and adherent normal tissue. On the other hand, the present results clearly demonstrate that APA cells produce markedly less cortisol than normal adrenal cells, and it is suggested that KCNJ5 gene mutations dominantly induce the aldosterone production pathway as well as a concomitant decline in cortisol production, resulting in highly autonomous aldosterone production.

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