RAPID COMMUNICATION

Somatic mutations of the catalytic subunit of cyclic AMP-dependent protein kinase (*PRKACA*) gene in Japanese patients with several adrenal adenomas secreting cortisol

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Abstract. Somatic mutations of the catalytic subunit of the cyclic AMP-dependent protein kinase (*PRKACA*) gene have recently been identified in about 35% of cortisol-producing adenomas (CPAs), with the affected patients showing overt Cushing's syndrome. Since we recently reported higher prevalence of mutations of the *KCNJ5* gene and associations with autonomous cortisol secretion in Japanese aldosterone-producing adenomas than in Western countries, there might be different features of CPAs between Japan and the West. We therefore investigated mutations of the *PRKACA* gene in Japanese patients with several adrenal tumors secreting cortisol, including overt Cushing's syndrome, subclinical Cushing's syndrome, and aldosterone-producing adenomas (APAs) co-secreting cortisol operated on at Gunma University Hospital. Of the 13 patients with CPA who showed overt Cushing's syndrome, 3 (23%) had recurrent somatic mutations of the *PRKACA* gene, p.L206R (c.617 T>G), and there were no mutations in subclinical Cushing's syndrome. Among 33 APAs, 24 had somatic mutations of the *KCNJ5* gene, either G151R or L168R, 11 (33%) had autonomous cortisol secretion, but there were no mutations of the *PRKACA* gene. We established a PCR-restriction fragment length polymorphism assay and revealed that the mutated allele was expressed at a similar level to the wild-type allele. These findings demonstrated that 1) the prevalence of Japanese patients with CPA who showed overt Cushing's syndrome and whose somatic mutations in the *PRKACA* gene was similar to that in Western countries, 2) the mutation might be specific for CPAs causing overt Cushing's syndrome, and 3) the mutant *PRKACA* allele was expressed appropriately in CPAs.

Key words: *PRKACA*, Cortisol-producing adenomas, Japanese

OVERPRODUCTION of adrenal cortisol induces several characteristic manifestations, including moon face, buffalo hump, striae, thin skin, and central obesity, known as Cushing's syndrome [1, 2]. Tumors secreting cortisol in patients who did not show any symptoms typical of Cushing's syndrome were described as

subclinical Cushing's syndrome [1, 3, 4], but this concept is not universal, and the definition differs among investigators and countries [5, 6]. In addition, we and others, particularly Japanese investigators, have reported an association of primary aldosteronism with mild autonomous cortisol secretion [7-9].

The mechanisms underlying the tumorigenesis of adrenal cortisol-producing adenomas (CPAs) and autonomous cortisol production are still largely unknown. However, Beuschlein *et al.* recently successfully identified the recurrent somatic mutation p.L206R (c.617T>G) by exome analysis in the gene encoding *PRKACA*, which encodes the catalytic sub-

Submitted Jun. 13, 2014; Accepted Jun. 19, 2014 as EJ14-0282
Released online in J-STAGE as advance publication Jul. 25, 2014

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unit of cyclic AMP-dependent protein kinase (protein kinase A [PKA]), in 22 of 59 (37%) patients with CPA who showed overt Cushing's syndrome [10].

Goh *et al.* also identified the *PRKACA* mutation encoding p.L206R at a similar prevalence, namely, 35% of PCAs associated with overt Cushing's syndrome [11]. Leu206 directly interacts with the regulatory subunit of PKA, PRKAR1A. The hotspot p.L206R mutation causes loss of this PRKAR1A binding, increasing the phosphorylation of downstream targets and causing constitutive activation of the PKA catalytic subunit, which induces overproduction of cortisol and cell proliferation [11].

We have recently reported high prevalence of somatic mutation in a potassium channel gene, *KCNJ5*, of approximately 70%, in Japanese patients with adrenal aldosterone-producing adenomas (APAs) and some APAs co-secreting cortisol [12, 13]. In contrast, in Western countries, the prevalence of the mutations has been reported to be approximately 35-40% of APAs, and cases of APAs co-secreting cortisol have rarely been reported [14].

Therefore, there may be differences of *PRKACA* mutations in CPAs and adrenal cortisol-secreting adenomas between Japan and Western countries. In the present study, we investigated the *PRKACA* mutations in several adrenal tumors secreting cortisol, including overt Cushing's syndrome, subclinical Cushing's syndrome, and APAs co-secreting cortisol.

Subjects and Methods

Subjects

We reviewed the medical records of 21 patients with CPA and 33 patients with APA, who were operated on at Gunma University Hospital during the period 2007-2013. Each subject provided written informed consent, and the study was approved by the ethics committee on human research of Gunma University. The diagnosis of Cushing's syndrome was made in 13 female cases (aged 54 ± 13 yr (mean $\pm$ SD)) according to guidelines published by the Endocrine Society [1]. The diagnosis of primary aldosteronism (PA) was initially performed as previously reported [7, 15]. We also examined morning and midnight cortisol levels, as well as 1 mg dexamethasone suppression test (DST) results in all the patients with suspected PA [7, 8, 16]. Failure of cortisol to suppress the level to less than $3.0 \mu\text{g/dl}$ (139.75 nmol/L) in the DST was used as a parameter

for autonomous cortisol secretion in this study.

Plasma aldosterone levels were measured with the RIA SPAC-S Aldosterone kit TFB; plasma renin activity, with the RIA Renin IRMA KIT "Daiichi" TFB; plasma cortisol, with the RIA Cortisol kit "TFB" TFB; and ACTH, with ECLIA Eclsys ACTH by Roche Diagnostics.

RNA extraction and detection of mutations of *PRKACA* cDNA by PCR and direct sequencing

All specimens of adrenal tumor were frozen in liquid nitrogen immediately after removal during operation. Total RNA was prepared from each adenoma using ISOGEN (Nippongene, Toyama, Japan) according to manufacturer's instructions. Then, cDNA was reverse-transcribed from 300 ng of total RNA (TaqMan Reverse Transcription Reagents, Applied Biosystems, Tokyo, Japan). To sequence the *PRKACA* cDNA, 1.0 μL of cDNA was used for the polymerase chain reaction (PCR), as previously reported [17]. PCR solutions were prepared according to the manual for AmpliTaq (Life Technologies, Carlsbad, CA, USA) with a final volume of 50 μL . Each primer used for PCR was set in a different exon, 5'F1, 5'-CCTGACCTTTGAGTATCTGCACT-3' (forward primer) and h3'R1, 5'-CGGCCATTTTCATAGATAAGAAC-3' (reverse primer) in putative exons 6 and 7 (ENST00000308677). After denaturing for 2 min at 94°C , amplification was performed for 35 cycles at 94°C for 30 sec, 58°C for 30 sec, and 72°C for 1 min using a GeneAmp PCR system 9700 (Applied Biosystems, Tokyo, Japan). The PCR products (244 bp) were purified with an AxyPrep PCR clean-up kit (AXYGEN Biosciences, Union City, CA, USA) for sequencing. These samples were directly sequenced using an Applied Biosystems 3730xl (Applied Biosystems, Tokyo, Japan).

In cases in which mutation of the *PRKACA* gene was identified, we sequenced the mutated nucleotide of the genomic DNA from peripheral blood using primers 5'-GTTTCTGACG GCTGGACTG-3' and 5'-CGGCCATTTTCATAGATAAGAAC-3' under the same PCR conditions as described above.

PCR-RFLP assay

We performed a polymerase chain reaction-restriction fragment length polymorphism assay (PCR-RFLP) in CPAs with p.L206R (c.617T>G), since this mutation produces a site that cannot be digested with the BciT130 I (EcoR II, Mva I) (Takara Shiga, Japan)

restriction enzyme. The 213-bp fragment encompassing the 617T>G mutation in cDNA was generated using the primers 5'-CCTGACCTTTGAGTATCTGCACT-3' and 5'-CACCAGTCCACGGCCTTGTGTGTA-3'. PCRs were performed as above. The wild-type cDNA was cut with BciT130 I into two fragments of 162 bp and 52 bp (not detected), while the mutated allele remained uncut, and similarly the wild-type genomic DNA was cut into fragments 127 bp and 147 bp in length. The fragments were resolved on a 2% agarose gel. We compared the density of the 213-bp and 162-bp fragments after digestion to evaluate the ratio of levels of the mutated and wild-type *PRKACA* mRNA. The bands were quantitatively measured using Molecular Imager FX (Bio-Rad Laboratories, Inc., Tokyo, Japan). All experiments were repeated at least twice.

Statistical analysis

All results are expressed as the mean $\pm$ SD for continuous variables and as absolute numbers. Statistical analyses were performed with ANOVA and Student's *t* test using JMP 10.0.2 (SAS Institute Inc., Cary, NC).

Results

Mutations of the *PRKACA* gene in patients with CPAs and other adrenal adenomas secreting cortisol

Table 1 shows the clinical features of patients with Cushing's syndrome. All patients showed symptoms typical of Cushing's syndrome. Mean tumor size (maximum diameter) on CT was 28 ± 4 mm. The 24-h urinary free cortisol excretion in 12 patients was over 200 $\mu\text{g/day}$, except for 1 case with 81.8 $\mu\text{g/day}$. In all patients, plasma ACTH levels were undetectable (Table 1). Furthermore, the pathology in all cases was confirmed as adrenocortical adenoma. We examined mutation of the *PRKACA* gene in these 13 patients and found that 3 CPAs had somatic mutations of the *PRKACA* gene. As shown in Fig. 1 (lower panel), mutations were concentrated at nucleotide T 617 bp downstream of the translation start site, and the conversion of T to G causing the substitution of leucine (L) by arginine (R) at residue 206 (p.L206R), which is the same substitution as reported by Beuschlein *et al.* [10]. Furthermore, we also confirmed that there were no germline mutations of *PRKACA* in DNA from

Table 1 Clinical features of 13 patients with Cushing's syndrome

Pt	Age (yr)	Sex	Specific cushingoid signs (Yes/No)	Metabolic disorders (Yes/No)				complications	ACTH (pg/mL)	Midnight serum cortisol ($\mu\text{g/dL}$)	24 h urine cortisol ($\mu\text{g/day}$)	1 mg DST ($\mu\text{g/dL}$)	Tumor Size (mm)	<i>PRKACA</i> mutation
				obesity	HT	HL	DM/IGT							
1	37	F	Yes	Yes	Yes	Yes	Yes	renal stone	1.0	13.8	295	15.4	28	c.617 T>G (p.L206R)
2	67	F	Yes	Yes	Yes	Yes	Yes		<5	25.4	249	33.0*	20	c.617 T>G (p.L206R)
3	68	F	Yes	No	Yes	Yes	Yes	osteoporosis, primary aldosteronism***	1.0	13.3	81.8	12.8	21	c.617 T>G (p.L206R)
4	71	F	Yes	Yes	Yes	Yes	Yes		<5	22.4	405	39.9	30	Wild type
5	63	F	Yes	Yes	Yes	Yes	Yes	hyperparathyroidism	1.0	17.0	446	27.2	26	Wild type
6	59	F	Yes	Yes	Yes	Yes	Yes		1.0	15.8	331	**	25	Wild type
7	54	F	Yes	Yes	Yes	Yes	Yes	stroke	<5	17.2	492	16.9	30	Wild type
8	33	F	Yes	Yes	Yes	Yes	Yes		<5	20.0	477	19.9	32	Wild type
9	41	F	Yes	Yes	Yes	No	No	small intestine tumor	1.0	13.7	205	15.7	36	Wild type
10	44	F	Yes	No	Yes	No	No	fracture, stroke, renal stone	1.0	13.4	318	13.9	30	Wild type
11	65	F	Yes	Yes	Yes	Yes	Yes	fracture	1.0	23.1	217	27.9*	26	Wild type
12	52	F	Yes	Yes	Yes	No	Yes	uterine fibroids	1.0	15.6	267	16.8	27	Wild type
13	42	F	Yes	Yes	Yes	Yes	Yes	osteoporosis, gastric ulcer	1.0	21.7	272	24.2	30	Wild type

PRKACA mutation represents a heterozygous mutation of c.617 T>G (p.L206R). M, male; F, female. Specific cushingoid signs refer to the guidelines of the Endocrine Society. HT, hypertension, The diagnosis of hypertension was made according to the 2014 guidelines of The Japanese Society of Hypertension. DM/IGT includes diabetes mellitus and impaired glucose tolerance, The diagnosis of diabetes mellitus and glucose tolerance were made according to the 2006-2007 guidelines of The Japan Diabetes Society. HL, hyperlipidemia, Hyperlipidemia was evaluated according to the 2007 guidelines of Japan Atherosclerosis Society. 1 mg DST, serum cortisol levels in the 1 mg dexamethasone suppression test. Tumor size is the maximum diameter of the tumor observed in the CT study. *Pt. 2 and Pt. 11 underwent 8 mg dexamethasone suppression test. **No data were available. ***Pt. 3 was diagnosed with primary aldosteronism at the opposite side of cortisol-producing adenoma by performing adrenal vein sampling.

Fig. 1 Sequences of mutations of the *PRKACA* gene in CPAs. Representative sequences of peripheral blood DNA (Blood) and tumor cDNA of the mutant *PRKACA* are depicted. L206R T/G represents heterozygosity for an T-to-G transition 617 bp from the translation start site in the *PRKACA* gene, resulting in a leucine-to-arginine substitution at codon 206 (c.617T>G, p.L206R). No mutations were identified in the peripheral blood DNA.

peripheral blood of the 3 patients with mutations (lower panel, Blood).

As shown in Table 2, we next examined the tumors of 8 patients who showed autonomous cortisol secretion. Serum ACTH levels in most patients were below 10 pg/mL, except for patient 2 with 14.9 pg/mL. Fractionated urine cortisol levels were within normal limits or slightly elevated, but below 150 µg/day. None of the patients showed suppression of serum cortisol level below 3 µg/dL after overnight loading of 1 mg of dexamethasone (1 mg DST). Mean tumor size was 32 ± 10 mm. We found no mutations of the *PRKACA* gene in these 8 adenomas.

Table 3 shows the clinical features of 33 cases of APAs. Among these APAs, 24 were harboring somatic *KCNJ5* mutations of either G151R or L168R, and 11 had autonomous cortisol secretion. None of the patients had any Cushingoid signs, except patient 11 in Table 3, who showed a slight moon face. However, these 11 cases showed no suppression of serum cortisol level below 3 µg/dL by 1 mg DST. As seen in tumors showing subclinical Cushing's syndrome, urine cortisol levels of these 11 patients were within the normal range or slightly high, but below 150 µg/day. We examined the mutation of the *PRKACA* gene in all of the 33 APAs, but there were no such mutations in tumors showing

Table 2 Clinical features of 8 patients with subclinical Cushing's syndrome

Pt	Age (yr)	Sex	Specific cushingoid signs (Yes/No)	Metabolic disorders (Yes/No)				complications	ACTH (pg/mL)	Midnight serum cortisol (µg/dL)	24 h urine cortisol (µg/day)	1 mg DST (µg/dL)	Tumor Size (mm)	<i>PRKACA</i> mutation
				obesity	HT	HL	DM/IGT							
1	57	F	No	Yes	Yes	Yes	Yes		8.8	5.0	25.2	3.4	35	Wild type
2	54	F	No	Yes	Yes	Yes	Yes	breast cancer	14.9	3.9	16.1	4.0	15	Wild type
3	69	F	No	Yes	Yes	Yes	Yes	primary aldosteronism *** Grave's disease	1.0	9.4	51	12.4*	30	Wild type
4	48	F	No	No	No	No	No		6	**	133	16.3	35	Wild type
5	76	F	No	No	Yes	No	No	stroke	<5	**	43.6	6.8	30	Wild type
6	66	M	No	No	Yes	No	No		7.9	8.0	94.6	5.5	22	Wild type
7	73	F	No	No	Yes	No	No		6.7	4.0	29.1	3.8	40	Wild type
8	60	F	No	Yes	Yes	Yes	Yes	arteriosclerosis obliterans	5.0	9.6	71.4	12.9	48	Wild type

PRKACA mutation represents a heterozygous mutation of c.617T>G (p.L206R). M, male; F, female. Specific cushingoid signs refer to the guidelines of the Endocrine Society. HT, hypertension. DM/IGT includes diabetes mellitus and impaired glucose tolerance. HL, hyperlipidemia. 1 mg DST, serum cortisol levels in the 1 mg dexamethasone suppression test. Tumor size is the maximum diameter of the tumor observed in the CT study. *Pt. 3 underwent 8 mg dexamethasone suppression test. **No data were available. ***Pt. 3 was diagnosed with primary aldosteronism at the opposite side of cortisol-producing adenoma by performing adrenal vein sampling.

Table 3 Clinical features of 33 patients with aldosterone-producing adenoma with or without hypercortisolism

Pt	Age (yr)	Sex	Specific cushingoid signs (Yes/No)	Mild hypercortisolism (Yes/No)	PAC (pg/mL)	PRA (ng/mL/h)	1 mg DST	ACTH (pg/mL)	Midnight serum cortisol (μg/dL)	24 h urine cortisol (μg/day)	PRKACA mutation (Yes/No)	KCNJ5 Mutation
1	30	F	No	Yes	580	0.3	Yes	16.4	6	37.1	No	c.451G>A (p.G151R)
2	53	F	No	Yes	393	0.1	Yes	34	11.4	118	No	c.503T>G (p.L168R)
3	48	F	No	Yes	888	0.4	Yes	16.7	3.4	44	No	c.503T>G (p.L168R)
4	60	F	No	Yes	114	0.4	Yes	<5	10.5	86.5	No	Wild type
5	48	F	No	Yes	231	0.4	Yes	6.8	5	35.9	No	Wild type
6	57	M	No	Yes	526	0.1	Yes	31.6	5.1	32.8	No	c.451G>A (p.G151R)
7	58	M	No	Yes	742	<0.1	Yes	14	5.4	59.1	No	c.451G>C (p.G151R)
8	75	M	No	Yes	132	0.2	Yes	13.8	5	73.3	No	Wild type
9	74	M	No	Yes	343	0.1	No	82.5	14.2	129	No	Wild type
10	53	M	No	Yes	330	0.5	Yes	22.5	9.4	63.3	No	Wild type
11	51	F	Yes	Yes	1330	0.6	Yes	<5	12.3	*	No	c.451G>A (p.G151R)
12	68	F	No	No	139	<0.1	*	22.3	3.6	21.3	No	Wild type
13	26	F	No	No	500	<0.1	No	17.1	1.7	*	No	c.503T>G (p.L168R)
14	35	F	No	No	287	0.3	No	19.7	4.7	33.3	No	c.451G>C (p.G151R)
15	61	F	No	No	366	<0.1	No	14.9	2.9	58.2	No	Wild type
16	64	M	No	No	521	<0.1	No	27.9	3.7	*	No	c.451G>A (p.G151R)
17	31	M	No	No	353	<0.1	No	29.3	2.2	39.2	No	c.451G>A (p.G151R)
18	61	M	No	No	484	0.2	No	22	1.4	102	No	Wild type
19	41	F	No	No	176	0.2	No	34.3	1.0	*	No	c.451G>A (p.G151R)
20	63	F	No	No	363	<0.1	No	29.5	2.3	30.5	No	c.451G>C (p.G151R)
21	73	F	No	No	260	<0.1	*	12.5	*	23.4	No	Wild type
22	50	M	No	No	426	0.2	No	10.8	2.5	36.2	No	c.451G>A (p.G151R)
23	55	M	No	No	299	0.1	No	28.4	1.8	52	No	c.451G>A (p.G151R)
24	36	F	No	No	542	0.2	No	11.5	5.9	49.6	No	c.503T>G (p.L168R)
25	50	M	No	No	1330	<0.1	No	17.4	29.5	29.5	No	c.451G>A (p.G151R)
26	52	M	No	No	693	0.3	No	44	4.4	132	No	c.451G>A (p.G151R)
27	42	F	No	No	358	0.9	*	15	2.4	*	No	c.503T>G (p.L168R)
28	65	M	No	No	1990	<0.1	No	43.6	3.4	69.6	No	c.451G>C (p.G151R)
29	50	F	No	No	88.9	0.3	No	20.2	1.7	74.8	No	c.451G>A (p.G151R)
30	41	F	No	No	224	0.3	No	44.1	7.4	33.1	No	c.503T>G (p.L168R)
31	32	M	No	No	1212	0.4	No	46	2.5	73.4	No	c.451G>A (p.G151R)
32	50	F	No	No	517	0.4	*	23.8	*	*	No	c.451G>C (p.G151R)
33	46	M	No	No	130	0.1	No	22.3	3.7	99.2	No	c.451G>C (p.G151R)

PRKACA mutation represents a heterozygous mutation of c.617T>G (p.L206R). KCNJ5 mutation represents a heterozygous mutation of c.451G>C (p.G151R), c.451G>A (p.G151R), or c.503T>G (p.L168R). M, male; F, female. Specific cushingoid signs refer to the guidelines of the Endocrine Society. 1 mg DST, serum cortisol levels in the 1 mg dexamethasone suppression test. Mild hypercortisolism was defined as at least one abnormal result of suppressed plasma ACTH levels below 10 pg/mL, midnight serum cortisol levels above 7.5 μg/dL, or a 1 mg dexamethasone suppression test result > 3.0 μg/dL. 1 mg DST, a 1 mg dexamethasone suppression test result > 3.0 μg/dL is indicated as Yes. * No data were available.

either pure APA or APA co-secreting cortisol.

Ratio of wild-type and mutated *PRKACA* mRNA levels examined by PCR-RFLP

To evaluate the expression of mutant mRNA, we performed a PCR-RFLP assay on the CPAs with the mutation p.L206R (c.617T>G), namely, patients 1-3 in Table 1. As shown in Fig. 2, the 213-bp band corresponded to the amplified cDNA and the 273-bp band was amplified genomic DNA that included a short intron, 148 bp in length. The complete digestion by the enzyme was confirmed by PCR fragments of the genomic DNA, and no band remained after digestion. The density of the 213-bp fragment after digestion with the BclI130 I restriction enzyme was similar to that of the 162-bp fragment (the ratio of 213-bp /162-bp fragment ($101.8 \pm 1.4\%$, $n=3$)), suggesting that the mutant mRNA c.617T>G may be expressed at a level similar to that of the wild type.

Discussion

In the present study, we identified heterozygosity for an T-to-G transition 617 bp from the translation start site in the *PRKACA* gene (c.617T>G) in 3 of 13 of the patients, which is the same mutation as reported by Beuschlein *et al.*, resulting in a leucine-to-arginine substitution at codon 206 (p.L206R) [10]. Beuschlein *et al.* reported another mutation, c.595_596insCAC, in 1 case of overt Cushing's syndrome [10]. However, we did not find the same mutation in our cases. During the preparation of this manuscript Cao *et al.* and Sato *et al.* reported a higher prevalence (approximately 67% and 52%, respectively) of the same mutation of the *PRKACA* gene in Chinese and Japanese CPAs showing overt Cushing's syndrome [18, 19]. However, we found a similar prevalence of the mutation as that in Western countries. This may be due to differences in the severity of disease or environmental factors, although this requires further study.

Although the term "preclinical" Cushing's syndrome was previously proposed, the term "subclinical" Cushing's syndrome describes this condition more accurately, as it does not imply any assumption about the further development of a clinically overt syndrome [20, 21]. Since the prevalence of overt Cushing's syndrome caused by adrenal adenoma in the general population is markedly lower than the prevalence of subclinical Cushing's syndrome in patients with clinically

Fig. 2 PCR-RFLP assay of CPAs with c.617T>G, p.L206R. The wild-type fragment had the site digested by the BclI130 I restriction enzyme. The 213-bp PCR product encompassing the 617T>G mutation was digested with BclI130 I, and the fragments were resolved on a 2% agarose gel. The wild-type allele was cut into two fragments of 162- and 52-bp, while the mutated allele remained uncut. Similarly, the wild-type genomic DNA was cut into fragments 127 bp and 147 bp in length. We compared the density of the 213-bp and 162-bp fragments after digestion to evaluate the ratio of the levels of mutated and -wild-type *PRKACA* mRNA. We found that the mutant mRNA was expressed at a level similar to that in the wild-type. Representative data are shown. 'Enzyme' refers to digestion with a restriction enzyme, BclI130 I, and lane M is a molecular marker ladder.

non-functioning adrenal adenoma, it is rather inappropriate to consider subclinical Cushing's syndrome as an early stage of the development of overt hypercortisone-mia [20, 22]. Neither we nor Beuschlein *et al.* found mutations of the *PRKACA* gene in cases that showed subclinical Cushing's syndrome, so these findings further support the evidence that the origin of the tumors showing overt Cushing's syndrome differs from that of tumors showing subclinical Cushing's syndrome [10]. Although Sato *et al.* found the mutation in one case out of 9 tumors showing subclinical Cushing's syndrome, this may be due to the difference of the definition of subclinical Cushing's syndrome used for the diagnosis [19]. Sato *et al.* diagnosed patients who had an incidentally discovered adrenocortical adenoma and no typical symptoms or signs of Cushing's syndrome.

We previously reported that some APAs showing clear autonomous cortisol secretion had mutation of the *KCNJ5* gene, suggesting that these tumors had the same features as APAs not co-secreting cortisol [13]. In a previous study, we defined the patients with all of the following criteria as having "clear cortisol secretion": 1) low ACTH level <10 pg/mL, 2) suppressed accumulation of adosterol in scintigraphy on the intact side of the adrenal gland, and 3) post-adrenal insufficiency [13]. In the present study, we used serum morning cortisol level not being suppressed below 3 μ g/dL

by 1 mg DST as a parameter of mild hypercortisolemia, but we did not find any *PRKACA* mutations in these tumors, even without *KCNJ5* mutations. Furthermore, some APAs co-secreting cortisol had *KCNJ5* mutation, suggesting that these tumors seem to be close to the features of APAs, rather than CPAs.

We established a simple assay to detect the mutation hotspot of the *PRKACA* gene using PCR-RFLP. The mutation causes loss of the enzyme site for digestion by the BclI130 I restriction enzyme. Although the sequence spike of the mutated allele seems to be lower than that of the wild-type allele in the present study and the same phenomenon has been described in reports by Beuschlein *et al.* [10] and Cao *et al.* [18], the results demonstrate that the mutated allele was expressed as was the wild-type allele.

Cao *et al.* also identified other somatic mutations co-localizing with *PRKACA* mutations in CPAs, including activating CTNNB1 mutations and an APC truncating mutation, activating GNAS (stimulatory G-protein α subunit), mutations in cancer-related genes such as *ARID1A* and *STAT3*, and recurrently mutated GPR98 [18]. Sato *et al.* also found a GNAS mutation

in some CPAs [19]. In addition, Goh *et al.* reported that some CPAs had many somatic copy number variants (CNVs), with frequent deletion of *CDC42* and *CDKN2A*, amplification of 5q31.2, mutations of TP53 or RB1, CTNNB1 (b-catenin), or GNAS [11]. In the present study, we did not find *PRKACA* mutations in any of the cases of subclinical Cushing's syndrome and APAs associated with cortisol. Therefore, the other mutations mentioned above may be involved in the pathophysiology of cortisol secretion in these tumors.

Acknowledgements

This study were supported in part by a Health and Labor Sciences Grant for Research on Measures for Intractable Disease (M.Y.) and KAKENHI (Grant-in-Aid for Research Activity Start-up 25893026) (Y.N.).

We thank all the medical and co-medical staff as well as graduate students involved in the patient care.

Disclosure Summary

All authors have nothing to disclose.

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