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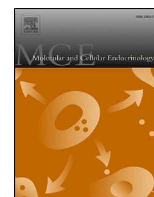

Genotype-specific cortisol production associated with Cushing's syndrome adenoma with *PRKACA* mutations

Ryuta Baba [a], Kenji Oki [a,*], Celso E. Gomez-Sanchez [b,c], Yu Otagaki [a], Kiyotaka Itcho [a], Kazuhiro Kobuke [a], Takaya Kodama [a], Gaku Nagano [a], Haruya Ohno [a], Masayasu Yoneda [a], Noboru Hattori [a]

[a] *Department of Molecular and Internal Medicine, Graduate School of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan*
[b] *Division of Endocrinology, G.V. (Sonny) Montgomery VA Medical Center and University of Mississippi Medical Center, Jackson, MS, USA*
[c] *Department of Pharmacology & Toxicology, University of Mississippi Medical Center, Jackson, MS, USA*

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ABSTRACT

The intracellular molecular mechanisms underlying the genotype of cortisol-producing adenoma (CPA) have not been fully determined. We analyzed gene expressions in CPA and the human adrenocortical cell line (HAC15 cells) with *PRKACA* mutation. Clustering analysis using a gene set associated with responses to cAMP revealed the possible differences between *PRKACA* mutant CPAs and *GNAS* and *CTNNB1* mutant CPAs. The levels of *STAR*, *CYP11A1*, *CYP17A1*, *CYP21A2,* and *FDX1* transcripts and cortisol levels per unit area in *PRKACA* mutant CPAs were significantly higher than those in *GNAS* mutant CPAs. *PRKACA* mutations led to an increase in steroidogenic enzyme expression and cortisol production in HAC15 cells. Transcriptome analysis revealed differences between *PRKACA* mutant CPAs and *GNAS* and *CTNNB1* mutant CPAs. Cortisol production in *PRKACA* mutant CPAs is increased by the cAMP-PKA signaling pathway-mediated upregulation of steroidogenic enzymes transcription. The intracellular molecular mechanisms underlying these processes would be notably important in *PRKACA* mutant CPAs.

1. Introduction

Cushing's syndrome is classified as adrenocorticotropic hormone (ACTH)-independent or ACTH-dependent cortisol overproduction. ACTH-independent Cushing's syndrome is most often due to a unilateral cortisol-producing adenoma. Cortisol excess leads to Cushingoid features such as central obesity, moon face, purple striae, buffalo hump and others. Patients with Cushing's syndrome often present with diabetes mellitus, hypertension, atherosclerosis, osteoporosis and mental disorders (Newell-Price et al., 2006,Nieman et al., 2008).

The etiology of isolated adrenal cortisol-producing adenoma (CPA) has long remained unclear, but recent studies have demonstrated that many harbors somatic mutations in *PRKACA*, *GNAS*, or *CTNNB1* (Beuschlein et al., 2014,Calebiro et al., 2014,Cao et al., 2014,Goh et al., 2014,Sato et al., 2014). The *PRKACA* is the most commonly somatic mutated gene, and occurring in 35–65% of CPA (Beuschlein, 2014,Thiel et al., 2015,Zhou et al., 2016). *PRKACA* encodes a catalytic subunit of protein kinase A (PKA) with a hotspot mutation in the *PRKACA* gene at L206R. In the absence of cAMP, the PRKACA is bound to the regulatory subunit rendering inactive. The *PRKACA* mutant does not bind to the regulatory subunit of PKA, allowing the PKA's catalytic activity to be constitutively active in cAMP-PKA signaling (Beuschlein, 2014; Calebiro, 2014, 2017,Cao et al., 2014,Goh et al., 2014,Sato et al., 2014). Additionally, the *PRKACA* mutation-mediated suppression of the cAMP-dependent PKA regulatory subunit IIβ (RIIβ) further contributes to the stimulation of cortisol secretion in adrenocortical cells (Calebiro et al., 2017; Weigand et al., 2017, 2021). *GNAS* and *CTNNB1* encode guanine nucleotide-binding protein subunit alpha (Gsα) and β-catenin, respectively. These mutations stimulate different intracellular signaling pathways, including mutation-mediated tumorigenesis and/or cortisol production. In this study, we focused on *PRKACA* mutation-mediated molecular mechanisms underlying the pathogenesis of CPAs, since *PRKACA* mutations are the most frequent somatic mutations in CPAs.

Recently, the relationship between genotype and clinical or pathological phenotypes was reported in patients with adrenal Cushing's syndrome. The clinical features of patients with *PRKACA* mutant CPA

* Corresponding author. 1-2-3 Kasumi, Minami-ku, Hiroshima, 734-8551, Japan.
E-mail address: kenjioki@hiroshima-u.ac.jp (K. Oki).

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presented at a younger age, with higher cortisol levels compared to the cases in patients with non-*PRKACA* mutant CPA (Di Dalmazi et al., 2014, Li et al., 2016,Thiel et al., 2015). Pathological features based on the CPA genotype demonstrated that *PRKACA* mutant cortisol-producing adenomas had higher CYP17A1 and 3βHSD immunoreactivities than wild-type cortisol-producing adenomas (Gao et al., 2020). *PRKACA* mutant CPAs have high *STAR* mRNA expression (Cao et al., 2014), and CPAs with mutations in *PRKACA* and *GNAS* exhibited strong immune-positive staining for STAR (Zhou et al., 2016). Clustering analysis using RNA sequencing data revealed that *PRKACA* mutant CPAs tended to have different gene expression profiles from adrenocortical carcinomas, subclinical Cushing's adenomas, nonfunctioning adrenocortical adenomas (NFAs) and CPAs with *CTNNB1* mutations (Di Dalmazi et al., 2020).

cAMP-PKA signaling activation potentiates the transcription of gene encoding steroidogenic enzymes that are needed in cortisol production, including *STAR*, *CYP11A1*, *CYP17A1*, *CYP21A2*, and *CYP11B1* (Aumo et al., 2010). *FDX1* which encodes ferredoxin 1 (also called adrenodoxin) is also stimulated by cAMP-PKA signaling (Imamichi et al., 2013). Ferredoxin 1 plays a pivotal role in transferring electrons in the mitochondria and is essential for the synthesis of adrenal steroid hormones (Muller et al., 2001,Sheftel et al., 2010). Upregulation of ferredoxin 1 facilitates the action of CYP11A1 and participates in the molecular mechanisms underlying cortisol overproduction. Although transcriptome data of CPAs with several mutants have been reported (Di Dalmazi et al., 2020), the differences in steroidogenic enzyme and *FDX1* expressions between *PRKACA* mutant CPAs and other genotypes of CPAs has not yet been fully studied. Additionally, the effects of *PRKACA* mutations on steroidogenic enzymes and *FDX1* have not been investigated *in vitro*.

The available data regarding the structural differences between each gene mutation and the differences in clinical and pathological findings among various CPA genotypes support the hypothesis that *PRKACA* mutations are associated with different gene expression profiles compared to those in CPAs with other mutations such as those in *GNAS* and *CTNNB1*. We first investigated whether gene expression in *PRKACA* mutant CPAs was different compared to that in other CPA genotypes. Next, we focused on the association between the genotypes and expression of cAMP-PKA signaling-associated genes in CPAs with *PRKACA* mutations. In addition, we modulated a *PRKACA* mutation in HAC15 cells and investigated the effects of *PRKACA* mutations on the transcription of steroidogenic enzyme-encoding genes.

2. Material and methods

2.1. Patients and tissue collection

Cushing's syndrome was diagnosed based on the existing guidelines (Nieman et al., 2008). Briefly, the patients presenting with Cushingoid features and adrenal tumors were detected using computed tomography. Suppressed basal ACTH levels, unsuppressed serum cortisol levels by overnight 1-mg dexamethasone suppression test (DST), and high midnight serum cortisol levels were observed in all patients. The patients without Cushingoid features, who were diagnosed as having subclinical Cushing's syndrome, were excluded. NFA was diagnosed based on radiological findings showing lipid-containing adenomas obtained by computed tomography or magnetic resonance imaging, and by endocrinological findings that did not show cortisol or aldosterone excess, as reported previously (Baba et al., 2018,Oki et al., 2012).

Twenty-five adrenal CPA and six NFA samples were obtained by adrenalectomy at Hiroshima University Hospital between August 2007 and May 2019. The details of tissue collection and storage were mentioned, as reported previously (Kishimoto et al., 2016). This study was approved by the ethics committee of Hiroshima University, and written informed consent was obtained from all the patients.

2.2. Clinical measurements

Serum cortisol levels in patients were determined using the ECLusys 2010 cortisol assay (Roche Diagnostics Co., Germany). Tumor sizes were measured as the major and minor axes in the computed tomography images. The tumor area was calculated as half the length of the major axis × that of the minor axis × π as an ellipse. Cortisol levels per unit area were obtained by dividing the cortisol levels, as determined by overnight 1-mg DST, by the tumor area.

2.3. Cell culture and materials

HAC15 human adrenocortical carcinoma cells were provided by Professor WE Rainey (University of Michigan). The cells were cultured in DMEM/F12 with 10% Cosmic Calf serum (HyClone, Logan, UT) as described previously (Kishimoto et al., 2016,Kobuke et al., 2018). Incubation with forskolin in HAC15 cells were performed as described previously (Itcho et al., 2019). Forskolin was purchased from Sigma Aldrich Co.Ltd. (St. Louis, MO, USA).

2.4. Lentiviral production and infection

The open reading frame of the *PRKACA* gene with or without the L206R (c.620T > G) mutant was obtained from GenScript (Piscataway, NJ). They were ligated into the multiple cloning site of the lentiviral plasmid pCDH-CMV-MCS-EF1-Puro (System Bioscience, Palo Alto, CA). The empty plasmid was used for control. Lentivirus production and infection in HAC15 cells were performed as described previously (Itcho et al., 2020,Kishimoto et al., 2016).

2.5. DNA and RNA extraction and DNA genotyping

Genomic DNA and total RNA from a piece of adrenal frozen tissues and HAC15 cells were isolated using the AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany). Genotyping of the adenomas for *PRKACA*, *GNAS* and *CTNNB1* was performed using targeted next-generation sequencing (NGS). The primers for *PRKACA* were 5′-TGCCAACTGCCTGTTCTTGTGC-3′ and 5′-GGAGGCTCCTACTTTGCT-CAGG-3'. The primers for *GNAS* were 5′-CTTTGGTGA-GATCCATTGACCTC-3′ and 5′-ACTGGGGTGAATGTCAAGAAACC-3'. The primers for *CTNNB1* were 5′-TGATTTGATGGAGTTGGACATGG-3′ and 5′-TTGGGAGGTATCCACATCCTCTTC-3'. The PCR products were created using the Multiplex PCR Assay Kit Version 2 (TaKaRa Bio Inc. Shiga, Japan). The libraries were prepared for sequencing using the Nextera XT DNA Library Preparation Kit (Illumina. San Diego, CA). Amplicon-based NGS was performed using MiSeq platform (Illumina, San Diego, CA).

2.6. RNA sequencing analysis

Total RNA extracted from the NFA and CPA specimens and HAC15 cells (control cells and cells with *PRKACA* mutations) were used for RNA sequencing analysis. RNA integrity and quantitation were checked by Agilent 2100 bioanalyzer (Agilent Technologies Inc., Santa Clara, CA). Preparation of a paired-end RNA library and sequencing analysis using the Illumina HiSeq 2500 platform were performed as described previously (Itcho et al., 2020).

For the clustering analysis of all genes among the various genotypes, the single linkage method was performed using the R software package (https://www.r-project.org/). Ninety-four genes related to the response to cAMP in humans were retrieved using the AmiGO 2 website (http://amigo2.geneontology.org/amigo). Genes not expressed in adrenal cells were excluded from the analysis. Fifty-eight genes were used for the heatmap and clustering analyses. Gene ontology (GO) and pathway analyses using RNA sequencing data were performed using R software package. Bioinformatics data, such as those obtained from the GO (http:

//geneontology.org/), Kyoto Encyclopedia of Genes and Genomes (https://www.genome.jp/kegg/), and REACTOME databases (https://reactome.org/), were collected. Transcription factors which may regulate genes in each genotype of CPA were analyzed using Enrichr website (https://maayanlab.cloud/Enrichr/) with TRRUST data base (https://www.grnpedia.org/trrust/).

2.7. Cortisol and protein assays in vitro study

Cortisol levels in the cell culture and cellular protein levels were measured using an ELISA kit and a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA), respectively, as described previously (Itcho et al., 2020). The cortisol levels were determined as arbitrary units normalized to protein levels.

2.8. Statistical analyses

Results of the clinical characteristics and basic research are expressed as the mean ± SD and mean ± SE, respectively. Differences between two groups were analyzed by the *t*-test, and those between multiple groups were analyzed by one-way ANOVA followed by Bonferroni comparisons. Differences were considered significant at $P < 0.05$. Analyses were performed using SPSS for Windows (version 27.0; SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Clinical characteristics

Sequencing analysis using targeted NGS demonstrated that p.L206R (c.620T > G) in *PRKACA* gene was detected in 15 samples of CPA. In the *GNAS* gene, two samples were found to have p.R201H (c.602G > A) and two other samples were found to have p.R201C (c.601C > T) (Table 1). p.S45P (c.133T > C) in the *CTNNB1* gene was found in one sample. One sample had a multiple mutations, R201S (c.601C > A) mutation in the *GNAS* gene and P44A (c.130C > G) and p.S45P (c.133T > C) in the *CTNNB1* gene (Table 1). No mutations were detected in four CPA or the six NFA samples. Basal and midnight serum cortisol levels or serum cortisol levels by 1-mg DST were not different among Cushing's syndrome patients separated based on the genotype (Table 1). Basal plasma ACTH levels were undetectable in all patients with CPAs.

3.2. RNA sequencing and clustering analysis

RNA sequencing analysis detected 40,600 genes in each sample. Genes whose expression levels in CPAs and NFAs showed significant and more than 2-fold differences were enrolled in the clustering analysis. As shown in Fig. 1A, NFAs were markedly differentiated from CPAs. Furthermore, the gene expression in CPAs with *PRKACA* mutations differed from that of CPAs with *GNAS* mutations.

Both *PRKACA* mutations and *GNAS* mutations stimulate the cAMP-PKA signaling pathway (Beuschlein, 2014), and thus the gene set associated with responses to cAMP was used for subsequent comparison. The analysis revealed that the gene expression profiles of *PRKACA* mutant CPAs were different from those of *GNAS* mutant CPAs and that the gene expression profiles of *CTNNB1* mutant CPAs were similar to those of *GNAS* mutant CPAs (Fig. 1B and Table S1). Genes related to the response to cAMP were listed in Table S1, and the highest expression in PRKACA mutant CPAs was *STAR*, followed by *FDX1*.

3.3. Expression of steroidogenic enzymes and FDX1 among various genotype of CPAs

For the analyses of gene expressions, one case of CPA with *CTNNB1* mutation was excluded, and one case of CPA with *GNAS* and *CTNNB1* mutation was included in *GNAS* mutation group. Thus, we compared the gene expression profiles between CPAs with *PRKACA* mutations and CPAs with *GNAS* mutations. *PRKACA* mutant CPAs had higher *FDX1* expression than *GNAS* mutant CPAs (Fig. 2). The expression levels of *STAR*, *CYP11A1*, *CYP17A1*, and *CYP21A2* in *PRKACA* mutant CPAs were higher than those in *GNAS* mutant CPAs, whereas the expression of *CYP11B1* in *PRKACA* mutant CPAs was lower than that in *GNAS* mutant CPAs (Fig. 2).

Presumed transcription factors in CPAs with *PRKACA* mutations and CPAs with *GNAS* mutations were detected by Enrichr using genes whose expression levels showed significant and more than 2-fold differences compared to NFAs (Table 2). Two transcription factors, ATF3 and CREB1, related with cAMP-PKA signaling were found in *PRKACA* mutant CPAs, but not in *GNAS* mutant CPAs (Table 2).

3.4. Cortisol levels in patients with CPA

PRKACA mutant CPAs had higher levels of some steroidogenic enzymes. Thus, cortisol levels in patients with *PRKACA* mutant CPAs were compared with those in patients with *GNAS* mutant CPAs (Table S2). Two patients with bilateral CPAs were excluded for the analysis. Basal and midnight serum cortisol levels or serum cortisol levels by 1-mg DST were not different between them, whereas the tumor areas of CPAs with *GNAS* mutations were significantly larger than those of CPAs with *PRKACA* mutations (Table S2). Cortisol levels per unit area were significantly higher in *PRKACA* mutant CPAs than in *GNAS* mutant CPAs (Fig. 3).

3.5. Effects of PRKACA mutation in HAC15 cells

We first compared cortisol levels in the media among control, *PRKACA* wild type and *PRKACA* mutation in HAC15 cells. Cortisol levels in *PRKACA* mutation cells was significantly higher than the others (Figure S1), and thus subsequent comparisons for gene expression were performed in control and *PRKACA* mutation cells. Cortisol levels in the media of HAC15 cells transduced with the *PRKACA* mutant were 5.5-fold higher than those in the control cells ($P < 0.01$) (Fig. 4). Forskolin (10 μM) had no effects on cortisol production in HAC15 cells with *PRKACA* mutation (Fig. 4).

Table 1
Clinical characteristics of the patients with Cushing's syndrome and NFA.

Diagnosis (number of patients)	Cushing's syndrome (n = 25)					NFA (n = 6)
Mutation (number of patients)	No mutation(n = 4)	*PRKACA*(n = 15)	*GNAS*n = 4)	*CTNNB1*(n = 1)	*GNAS/CTNNB1*(n = 1)	–
Male/Female	2/2	3/12	0/4	1/0	0/1	1/5
Age (years)	54.8 ± 4.1	44.7 ± 11.5	51.5 ± 15.9	71	69	47.0 ± 11.5
Body mass index	22.0 ± 2.1	24.1 ± 2.9	23.6 ± 4.6	26.1	29.4	29.3 ± 7.5
Basal serum cortisol level (μg/dl)	16.9 ± 9.9	17.9 ± 4.2	14.8 ± 5.6	12.6	10.0	13.1 ± 6.6
Midnight serum cortisol level(μg/dl)	17.4 ± 10.1	16.7 ± 4.1	16.6 ± 6.0	13.2	9.9	2.2 ± 1.2
Serum cortisol level by 1-mg DST(μg/dl)	13.7 ± 8.3	17.2 ± 3.9	17.3 ± 3.1	10.5	16.0	0.7 ± 0.2
Tumor area (cm^2)	4.4 ± 1.5	4.9 ± 1.6	8.0 ± 3.6	4.9	7.3	3.4 ± 1.7

NFA, nonfunctioning adenoma; DST, dexamethasone suppression test. Data presented as mean ± standard deviation or number.

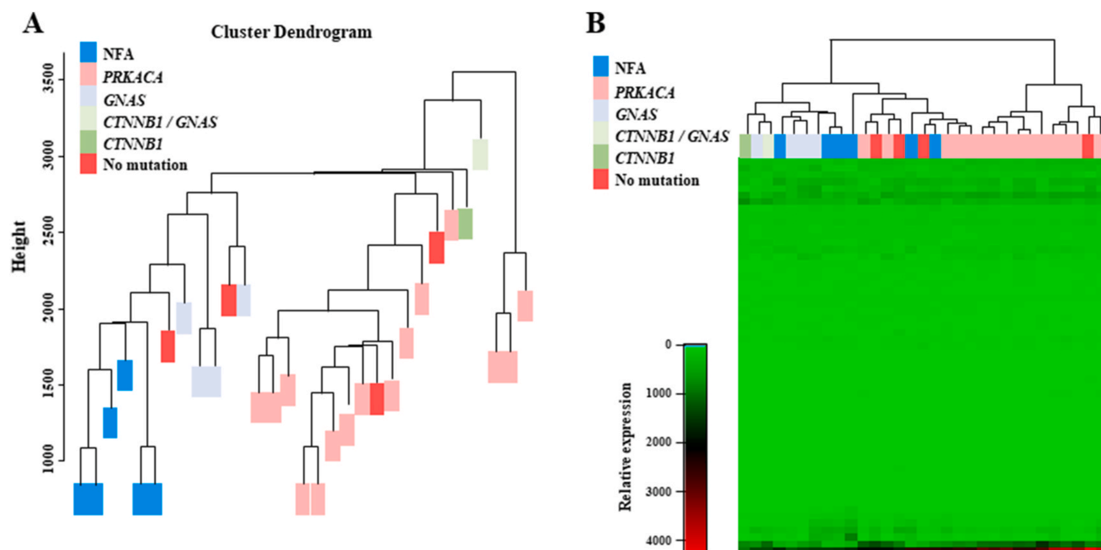

Fig. 1. Clustering analysis of cortisol-producing adenomas (CPAs) and nonfunctioning adrenocortical adenomas (NFAs). (A) Genes whose expression levels show significant and more than 2-fold differences between NFAs and CPAs were applied for clustering analysis using the R software package. The single-linkage statistical method was used. The figure shows the single-linkage dendrogram and the sequence of combinations of the clusters. The distances of merging between clusters, called heights, are illustrated on the y-axis. (B) Fifty-eight genes from the gene associated with responses to cAMP were used for clustering analysis using the R software package. Hierarchical clustering was performed for both the rows and columns of the data matrix. The columns of the data matrix, such as those showing gene expression, are re-ordered according to the hierarchical clustering results and are visualized by a heatmap with a color scheme.

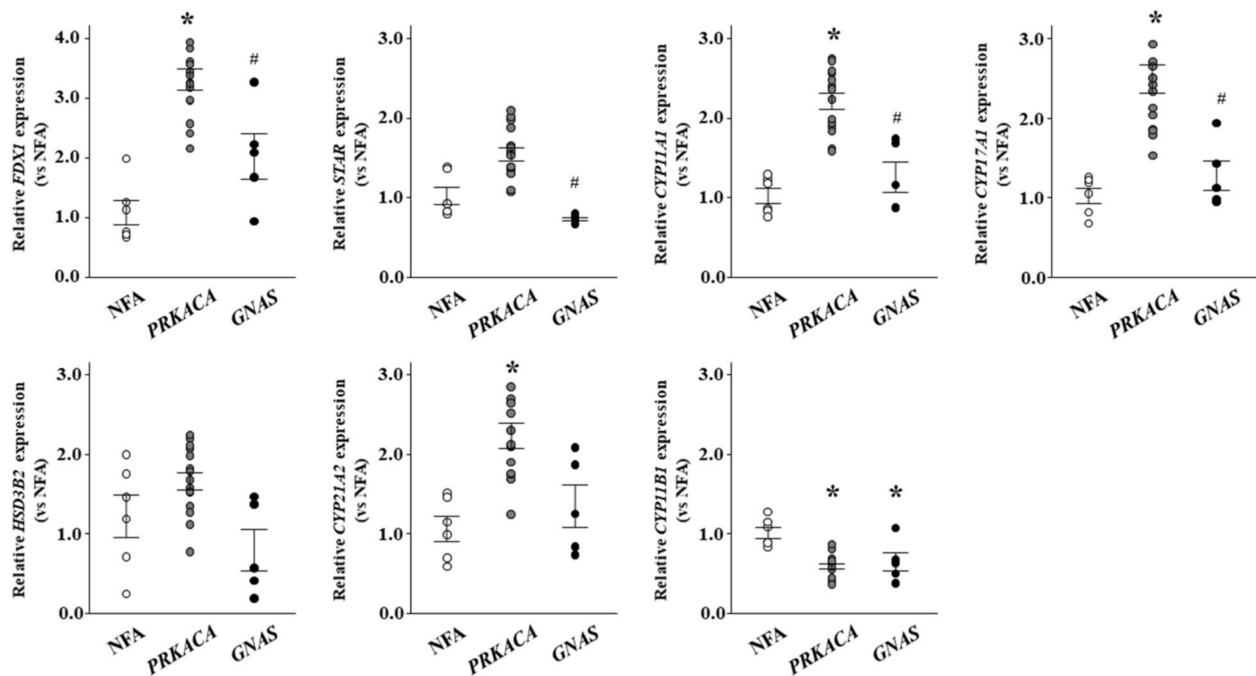

Fig. 2. The mRNA expression levels of *FDX1*, *STAR,* and adrenal steroidogenic enzymes in nonfunctioning adrenocortical adenomas (NFAs) and cortisol-producing adenomas (CPAs) with *PRKACA* and *GNAS* mutations. The mRNA expression levels were detected by RNA-sequencing analysis. *, $P < 0.01$ *vs*. NFAs; #, $P < 0.01$ *vs*. CPAs with *PRKACA* mutation.

According to the results of RNA sequencing, average transduction efficacy was 26.0% in *PRKACA* mutant cells (Figure S2). GO and pathway analyses using RNA sequencing data revealed that steroid hormone biosynthesis was the most enriched pathway in *PRKACA* mutant cells (Table S3). The *FDX1* expression in *PRKACA* mutant cells was significantly increased by 2.7-fold compared to that in control cells ($P < 0.01$) (Fig. 5). The expression of all genes encoding steroidogenic enzymes associated with cortisol production was markedly higher in *PRKACA* mutant cells than in the control cells.

4. Discussion

We demonstrated that *PRKACA* mutant CPAs differed from *GNAS* mutant CPAs, as shown by the clustering analysis using a gene set associated with responses to cAMP. *FDX1* was the second most active gene among the gene set associated with responses to cAMP in *PRKACA* mutant CPAs. The levels of *STAR*, *CYP11A1*, *CYP17A1*, and *CYP21A2* transcripts in *PRKACA* mutant CPAs were higher than those in CPAs with other mutations. Cortisol levels per unit area were higher in

Table 2
Top 10 transcription factors were extracted by TRRUST data base using RNA sequencing results in each genotype of Cushing's adenoma.

Rank	*PRKACA*		*GNAS*
1	*GATA6*		*MSX1*
2	*SP1*		*HOXA9*
3	*ATF3*	#	*POU2F2*
4	*PPARG*		*ZBTB16*
5	*NR5A1*		*HOXA10*
6	*CREB1*	#	*TP63*
7	*ERG*		*NFE2L2*
8	*SMAD3*		*FOXM1*
9	*CDX2*		*ELK1*
10	*REST*		*POUF5F1*

Transcription factors which may regulate genes in each genotype of cortisol-producing adenomas (CPAs) were analyzed by Enrichr website (https://maayanlab.cloud/Enrichr/) using TRRUST data base (https://www.grnpedia.org/trrust/). Genes with more than 2-fold and significant difference between NFAs and genotypes with CPAs were enrolled. # indicates transcription factors related with cAMP-protein kinase A (PKA) signaling.

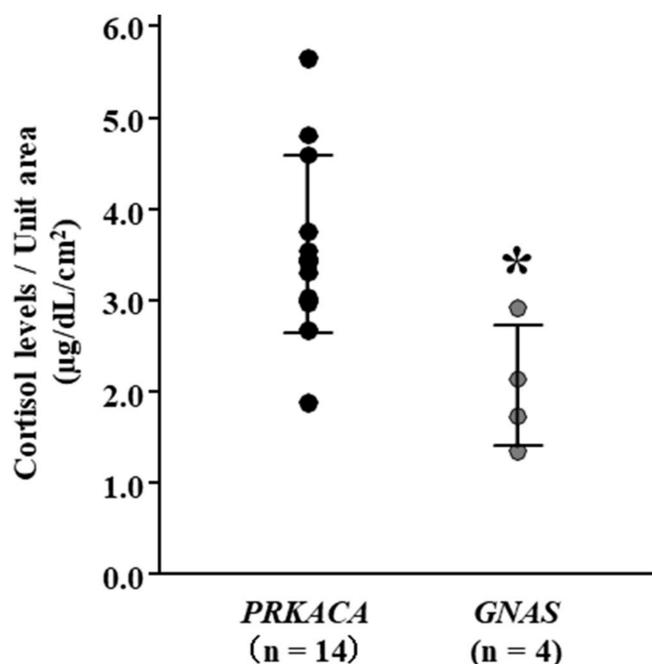

Fig. 3. Cortisol levels per unit area in cortisol-producing adenomas (CPAs) with *PRKACA* and *GNAS* mutations. The tumor area was calculated as half the length of the major axis × that of the minor axis × π as ellipse. Cortisol levels per unit area were obtained by dividing the cortisol levels of overnight 1-mg dexamethasone suppression test by the tumor area. *, $P < 0.01$ *vs*. *PRKACA* mutation group.

PRKACA mutant CPAs than in *GNAS* mutant CPAs. *In vitro*, HAC15 cells transduced with the *PRKACA* mutation showed increased expression levels of steroidogenic enzymes and cortisol production.

The gene expression profiles of CPAs with *PRKACA* mutations were different from those of CPAs with other mutations, as demonstrated by the clustering analysis using RNA sequencing. Although the clustering analysis of adrenal tumors including CPAs has already showed that *PRKACA* mutant CPAs tended to have different gene expression profiles from others (Di Dalmazi et al., 2020), especially when utilizing a gene set associated with responses to cAMP, gene expression in the *PRKACA* mutant CPAs was clearly distinguished from that in the *GNAS* and/or *CTNNB1* mutant CPAs. Remarkably, the expression of adrenal steroidogenic enzyme-associated genes in the *PRKACA* mutant CPAs was

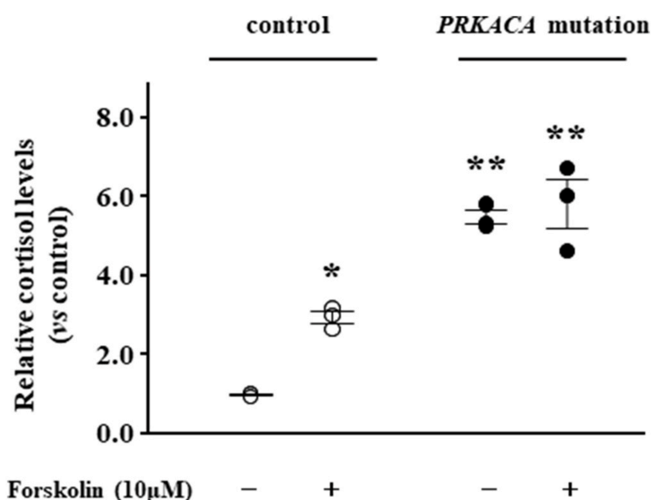

Fig. 4. Basal cortisol production in control HAC15 cells or cells with *PRKACA* mutations. Forty-eight hours after *PRKACA* mutation transduction using lentivirus, the cells were serum-starved by treatment with DMEM-F12 containing 0.1% cosmic calf serum for 24 h. The cells were then incubated in fresh medium containing 0.1% serum with or without forskolin (10 μM). Media were collected to measure the cortisol levels. Relative cortisol levels were compared between the control HAC15 cells and those with *PRKACA* mutations. *, $P < 0.05$ *vs*. control; **, $P < 0.01$ *vs*. control (n = 3).

higher than that in the *GNAS* mutant CPAs. Gao X et al. reported that CYP17A1 immunoreactivities were higher in the *PRKACA* mutant CPAs than in the other CPAs (Gao et al., 2020); our results are consistent with the reports. ATF3 and CREB1, which are known to be activated by cAMP-PKA signaling (Tao et al., 2019), were candidate transcription factors to regulate genes in *PRKACA* mutant CPAs, but not in *GNAS* mutant CPAs (Table 2). Taken together, the cAMP-PKA signaling pathway is activated to a greater degree in *PRKACA* mutant CPAs among the various types of CPAs.

This is the first study to show the transcriptome data including entire steroidogenic enzymes for the transduction of the *PRKACA* mutation in HAC15 cells *in vitro*. Bioinformatics analysis revealed that the pathway whose activity was enhanced to the greatest degree by the *PRKACA* mutation was steroid biosynthesis (Table S3). The expression of steroidogenic enzymes and cortisol production were potentiated by *PRKACA* mutation in HAC15 cells (Figs. 4 and 5). These results support prior findings that *PRKACA* mutations drive cortisol production in CPAs and cAMP-PKA signaling activation stimulated steroidogenic enzymes in H295R cells (Beuschlein, 2014,Cao et al., 2014,Di Dalmazi et al., 2014, Goh et al., 2014,Rizk-Rabin et al., 2020,Sato et al., 2014,Weigand et al., 2021). Calcium signaling pathway activation also induced STAR expression and cortisol production *in vitro* (Nishikawa, 1996), however *PRKACA* mutation mediated calcium signal activation was not found by our bioinformatics analysis (Table 2 and Table S3). Previous reports demonstrated that patients with *PRKACA* mutant CPA had higher cortisol levels and smaller adenoma size compared with those without any mutations, and higher cortisol levels compared with those without *PRKACA* mutation (Beuschlein, 2014,Di Dalmazi et al., 2014). These evidences also support our clinical results that the cortisol levels per unit area in CPAs with *PRKACA* mutation were higher than those with *GNAS* mutation.

Steroidogenic factor 1 (SF-1), also known as adrenal 4-binding protein (Ad4BP), is encoded by NR5A1, and regulates development of adrenal gland and the expression of steroidogenesis-related genes (Lala et al., 1992,Morohashi et al., 1992). According to the predicting transcription factor analysis, NR5A1 may participate the regulation of gene expressions in *PRKACA* mutant CPA (Table 2). NR5A1 expression was stimulated by cAMP-PKA signaling activation in Y1 cells (Kulcenty et al., 2015). mRNA expression of *NR5A1* was correlated with *CYP17A1* and

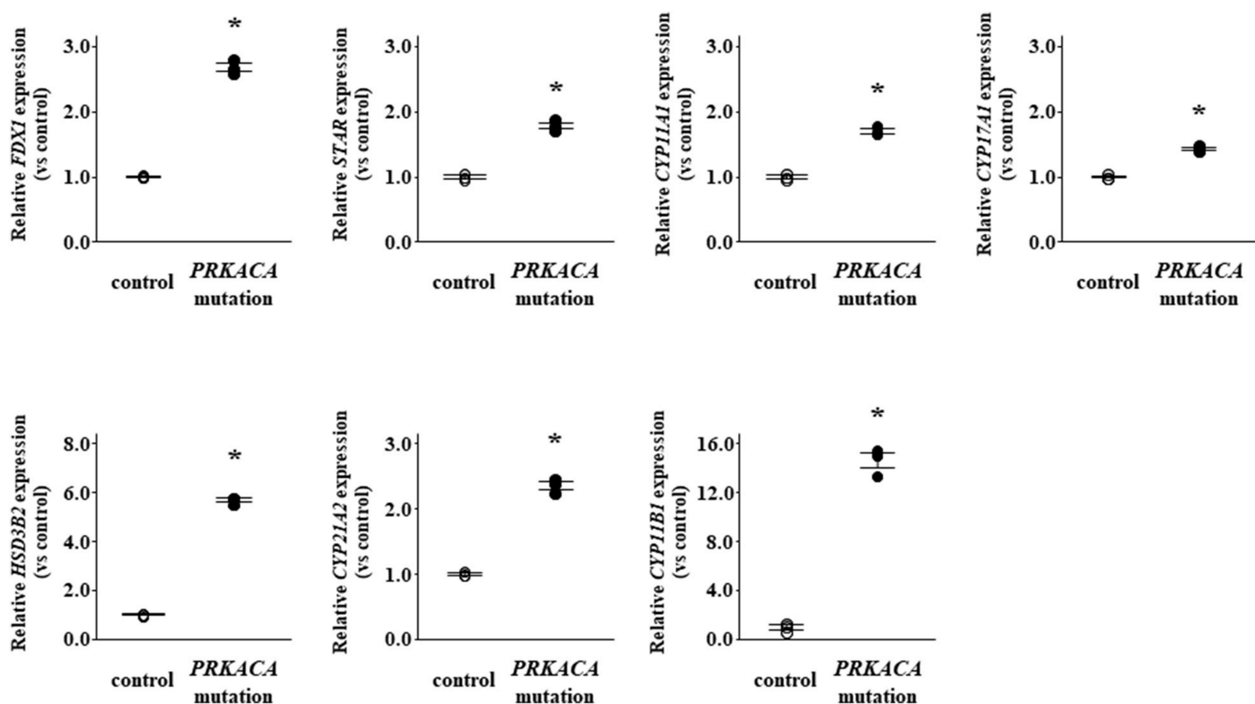

Fig. 5. The effects of *PRKACA* mutation on the mRNA expression of *FDX1*, *STAR,* and adrenal steroidogenic enzymes in HAC15 cells. Forty-eight hours after *PRKACA* mutation transduction using lentivirus, the cells were serum starved by treatment with DMEM-F12 containing 0.1% cosmic calf serum for 24 h, and then incubated with fresh medium containing 0.1% serum for 24 h. After aspiration of medium, the cells were collected for RNA extraction and RNA sequencing. *, $P < 0.01$ *vs.* control (n = 3).

CYP11B1 mRNA expression in CPA (Kubota-Nakayama et al., 2016). Taken together, NR5A1 would play a pivotal role in the regulation of gene expressions including steroidogenic enzymes in *PRKACA* mutant CPA.

Although *PRKACA* mutation stimulated the transcription of *CYP11B1* in HAC15 cells, *CYP11B1* expressions in CPAs with *PRKACA* and *GNAS* mutations was lower than that in NFAs. A previous study also showed that *CYP11B1* transcription was not stimulated in CPAs (Sakuma et al., 2013). CYP11B1 immunoreactivity is detected in CPAs, although the expression was not compared between CPAs and NFAs (Gomez-Sanchez et al., 2014,Kubota-Nakayama et al., 2016). It has been suggested that CYP11B1 expression in *PRKACA* mutant adenomas is not a rate-limiting factor in the synthesis of cortisol.

Somatic mutations of *CTNNB1* causes loss of β-catenin phosphorylation, and it prevents the ubiquitination of β-catenin and leads to the constitutive activation of the Wnt/β-catenin signaling pathway (Kikuchi, 2003). *CTNNB1* mutations have been detected in several human cancers, and the molecular mechanisms support their role in the causation and progression of tumors (Kikuchi, 2003; Polakis, 2000). Clustering analysis using RNA sequencing has shown that CPAs with *CTNNB1* mutations are similar to adrenocortical carcinomas (Di Dalmazi et al., 2020). It is suggested that *CTNNB1* mutations induce the proliferation of zona fasciculata cells and lead to the development of CPAs.

In this study, the results obtained for the *GNAS* mutant CPAs following the clustering analysis of the gene set associated with responses to cAMP were similar to those obtained for the *CTNNB1* mutants (Fig. 1B). Previous reports regarding the intracellular molecular mechanisms underlying the role of *GNAS* mutations in several tumors or cancers state that these mutations activate not only the cAMP-PKA signaling pathway, but also the Wnt/β-catenin and RAS-MAPK pathways, to stimulate gene transcription (Goh et al., 2014,Lin et al., 2020). The cAMP-PKA signaling pathway may be less important in *GNAS* mutant CPAs than in *PRKACA* mutant CPAs. Both the Wnt/β-catenin and RAS-MAPK pathways were well known to stimulate cell proliferation and tumor progression (Kikuchi, 2003; Pereira et al., 2019; Polakis, 2000), and thus *GNAS* mutations as well as *CTNNB1* mutations would induce the zona fasciculata cells proliferation and the development of CPAs. Further basic experiments are needed to clarify the molecular mechanisms underlying tumorigenesis and cortisol production in *GNAS* mutant CPAs.

A limitation of present study is small numbers of *GNAS* and *CTNNB1* mutations, because the frequency of those mutations in CPAs is small. Although a large sample size is needed to investigate whether similar results would be obtained, the current study would be helpful to reveal gene expression profile and clinical findings in CPAs with *PRKACA* mutation.

In conclusion, it is suggested that the cAMP-PKA signaling pathway is activated mainly in *PRKACA* mutant CPAs, resulting in cortisol overproduction via the increase in the expression of *FDX1* and steroidogenic enzymes. *PRKACA* mutations led to increased cortisol production and increased steroidogenic enzymes activities in HAC15 cells. Collectively, these findings indicate that cortisol production may be activated by the cAMP-PKA signaling pathway mainly in *PRKACA* mutant CPAs.

CRediT authorship contribution statement

Ryuta Baba: Methodology, Data acquisition, Data analysis and interpretation, writing—original draft preparation. **Kenji Oki:** Conceptualization, and, and, Funding acquisition. **Celso E. Gomez-Sanchez:** Conceptualization, writing—review and editing, Funding acquisition. **Yu Otagaki:** Methodology, Data acquisition. **Kiyotaka Itcho:** Methodology, Data acquisition, Funding acquisition. **Kazuhiro Kobuke:** and, Data acquisition, and. **Takaya Kodama:** and. **Gaku Nagano:** writing—review and editing. **Haruya Ohno:** writing—review and editing. **Masayasu Yoneda:** and, Supervision. **Noboru Hattori:** and.

Declaration of competing interest

The authors have nothing to disclose.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.mce.2021.111456.

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