Phosphodiesterase 2A and 3B variants are associated with primary aldosteronism

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Abstract

Familial primary aldosteronism (PA) is rare and mostly diagnosed in early-onset hypertension (HT). However, ‘sporadic’ bilateral adrenal hyperplasia (BAH) is the most frequent cause of PA and remains without genetic etiology in most cases. Our aim was to investigate new genetic defects associated with BAH and PA. We performed whole-exome sequencing (paired blood and adrenal tissue) in six patients with PA caused by BAH that underwent unilateral adrenalectomy. Additionally, we conducted functional studies in adrenal hyperplastic tissue and transfected cells to confirm the pathogenicity of the identified genetic variants. Rare germline variants in phosphodiesterase 2A (PDE2A) and 3B (PDE3B) genes were identified in three patients. The PDE2A heterozygous variant (p.Ile629Val) was identified in a patient with BAH and early-onset HT at 13 years of age. Two PDE3B heterozygous variants (p.Arg217Gln and p.Gly392Val) were identified in patients with BAH and HT diagnosed at 18 and 33 years of age, respectively. A strong PDE2A staining was found in all cases of BAH in zona glomerulosa and/or micronodules (that were also positive for CYP11B2). PKA activity in frozen tissue was significantly higher in BAH from patients harboring PDE2A and PDE3B variants. PDE2A and PDE3B variants significantly reduced protein expression in mutant transfected cells compared to WT. Interestingly, PDE2A and PDE3B variants increased SGK1 and SCNN1G/ENaCg at mRNA or protein levels. In conclusion, PDE2A and PDE3B variants were associated with PA caused by BAH. These novel genetic findings expand the spectrum of genetic etiologies of PA.
Introduction

Primary aldosteronism (PA) is characterized by an autonomous aldosterone production, leading to increased sodium reabsorption, hypertension (HT), renin suppression and variable degrees of hypokalemia (Funder et al. 2016). PA prevalence is around 10% in referred patients with HT and 15–20% in those with resistant hypertension (Douma et al. 2008, Hannemann & Wallaschofski 2012). The most common causes of PA are aldosterone-producing adenomas (APAs) and bilateral adrenal hyperplasia (BAH), also called idiopathic hyperaldosteronism (Funder et al. 2016). Recently, aldosterone-producing cell clusters (APCCs) have been suggested as a precursor lesion for APAs and possibly other histologic forms of PA (Nishimoto et al. 2015, Omata et al. 2017).

BAH can be diagnosed in the context of familial forms of PA, which account for <5% of cases and are transmitted as an autosomal dominant trait (Fernandes-Rosa et al. 2017). Familial hyperaldosteronism type I (FHI) or glucocorticoid-remediable aldosteronism is caused by a chimeric gene with the CYP11B1 promoter and CYP11B2 coding sequences (Sutherland et al. 1966, Lifton et al. 1992). FH-III is caused by germline mutations in the KCNJ5 gene, encoding the potassium channel Kir 3.4. Most of FH-III cases have early-onset HT and macronodular BAH (Geller et al. 2008, Scholl et al. 2012). FH-IV was identified in several unrelated individuals with PA and HT (before 10 years of age) harboring CACNA1H germline mutations (Scholl et al. 2015). Additionally, de novo germline CACNA1D mutations have been described in two children with PA and neuromuscular abnormalities (Scholl et al. 2013).

FH-II is clinically and biochemically indistinguishable from sporadic forms of PA. The prevalence of FH-II was reported to be 6% in a large PA cohort (Pallau et al. 2012). FH-II is diagnosed when at least two first-degree members of the same family have confirmed PA (APA or BAH). Linkage was established with the chromosomal region 7p22 in some kindreds with FH-II (Lifton et al. 1992). FH-III is caused by germline mutations in the KCNJ5 gene, encoding the potassium channel Kir 3.4. Most of FH-III cases have early-onset HT and macronodular BAH (Geller et al. 2008, Scholl et al. 2012). FH-IV was identified in several unrelated individuals with PA and HT (before 10 years of age) harboring CACNA1H germline mutations (Scholl et al. 2015). Additionally, de novo germline CACNA1D mutations have been described in two children with PA and neuromuscular abnormalities (Scholl et al. 2013).

Materials and methods

The study was approved by the Ethics Committees of the Hospital das Clínicas, University of São Paulo and informed written consent was obtained from all patients. Since we were interested in performing paired (blood and adrenal tissue) exome sequencing analysis, we selected six patients (four females and two males) with PA caused by BAH that underwent unilateral adrenalectomy (Table 1). PA screening and confirmatory testing followed the 2016 Endocrine Society Guideline for PA management (Funder et al. 2016). In five patients, unilateral adrenalectomy was guided by CT findings. None of the six patients had PA biochemical cure or HT remission after unilateral adrenalectomy. BAH was defined by the absence of PA cure after unilateral adrenalectomy and by the findings of adrenal hyperplasia on histopathology analysis with a positive CYP11B2 staining in all cases (Table 1). Except for case 3, the nodules observed on CT imaging (with at least 1 cm diameter) were not identified in the histopathological analysis.

Besides familial cases, germline defects have been also described in sporadic PA. Three different germline KCNJ5 mutations were identified in individuals with sporadic PA (Murthy et al. 2014, Daniil et al. 2016). In addition, a germline CACNA1H mutation was described in a patient with PA without familial history and HT diagnosed at the age of 48 years (Daniil et al. 2016). Interestingly, ARMCS variants, predicted to be damaged in silico, were identified in African Americans with sporadic PA (Zilbermint et al. 2015). This link between ARMCS and PA increased the spectrum of molecular alterations of PA in a specific population. Based on this finding, we can speculate that genetic defects associated with other forms of BAH can be a clue to define new etiologies for PA.

A high prevalence of somatic mutations in the CACNA1D were identified in APCCs from BAH (Omata et al. 2018). A KCNJ5 somatic mutation was identified in only one APCC (Omata et al. 2018). However, since BAH affects both adrenals, it is reasonable to speculate the presence of germline susceptibility defects in what has been considered so far as ‘sporadic BAH causing PA’. Therefore, we analyzed here a cohort of PA patients with BAH by exome sequencing to investigate new genetic defects associated with bilateral aldosterone excess.

Materials and methods

The study was approved by the Ethics Committees of the Hospital das Clínicas, University of São Paulo and informed written consent was obtained from all patients. Since we were interested in performing paired (blood and adrenal tissue) exome sequencing analysis, we selected six patients (four females and two males) with PA caused by BAH that underwent unilateral adrenalectomy (Table 1). PA screening and confirmatory testing followed the 2016 Endocrine Society Guideline for PA management (Funder et al. 2016). In five patients, unilateral adrenalectomy was guided by CT findings. None of the six patients had PA biochemical cure or HT remission after unilateral adrenalectomy. BAH was defined by the absence of PA cure after unilateral adrenalectomy and by the findings of adrenal hyperplasia on histopathology analysis with a positive CYP11B2 staining in all cases (Table 1). Except for case 3, the nodules observed on CT imaging (with at least 1 cm diameter) were not identified in the histopathological analysis.
Table 1 Clinical and biochemical data of patients with primary aldosteronism and bilateral adrenal hyperplasia.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age at HT diagnosis (year)</th>
<th>Hypokalemia</th>
<th>A/PRA ratio</th>
<th>CT-Right adrenal</th>
<th>CT-Left adrenal</th>
<th>AVS</th>
<th>Autonomous cortisol secretion</th>
<th>Adrenalec­tomy</th>
<th>PA biochemical cure</th>
<th>HT remission</th>
<th>Histopathology (H&amp;E)</th>
<th>CYP11B2 staining (positive areas)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>13</td>
<td>Y</td>
<td>30.2</td>
<td>Normal</td>
<td>1.2 cm nodule</td>
<td>-</td>
<td>N</td>
<td>L</td>
<td>N</td>
<td>N</td>
<td>Micro-nodular hyperplasia</td>
<td>Micro-nodules</td>
</tr>
<tr>
<td>1</td>
<td>M</td>
<td>18</td>
<td>Y</td>
<td>116.5</td>
<td>1.8 cm nodule</td>
<td>Thickening</td>
<td>Li 3.6³</td>
<td>N</td>
<td>L</td>
<td>N</td>
<td>N</td>
<td>Micro-nodular hyperplasia</td>
<td>Micro-nodules</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>33</td>
<td>Y</td>
<td>232.5</td>
<td>1.4 cm nodule</td>
<td>Thickening</td>
<td>Y</td>
<td>N</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Micronodules + Nodule</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>43</td>
<td>Y</td>
<td>40</td>
<td>Normal</td>
<td>1 cm nodule</td>
<td>Y</td>
<td>Y</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Micronodular hyperplasia + dominant macronodule</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>30</td>
<td>Y</td>
<td>34.3</td>
<td>Normal</td>
<td>1.1 cm nodule</td>
<td>Y</td>
<td>Y</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Micronodular hyperplasia + dominant macronodule</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>45</td>
<td>Y</td>
<td>91</td>
<td>1.2 cm nodule</td>
<td>2.0 cm nodule</td>
<td>Y</td>
<td>Y</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Micronodules</td>
<td>-</td>
</tr>
</tbody>
</table>

³Li was inconclusive (between 3 and 4), but it indicated a higher aldosterone secretion in the left adrenal.
A/APR, aldosterone/plasma renin activity; AVS, adrenal venous sampling; ; F, female; H&E, hematoxylin and eosin; HT, hypertension; L, left adrenal; Li, lateralization index; M, male; N, no; PA, primary aldosteronism; R, right; Y, yes.

**DNA and RNA extraction**

Genomic DNA was extracted from peripheral blood leukocytes by the proteinase K–SDS salting out method (Miller et al. 1988). After surgical resection, representative areas of tumor or hyperplastic tissue were macrodissected by a pathologist. Tumor fragments were immediately frozen in liquid nitrogen and stored at −80°C until total RNA and DNA extraction using the AllPrep DNA/RNA Mini Kit (Qiagen). Before tumor RNA and DNA extraction, tumor fragment was cut in a cryostat and slides stained by hematoxylin and eosin to confirm the representativeness of hyperplastic tissue.

**Whole exome sequencing**

Exons were captured with a SureSelect Human All Exon V6 Kit (Agilent Technology) and sequenced on a HiSeq500 system (Illumina, San Diego, CA). Alignment of raw data and variant calling were performed according to steps previously described (da Silva et al. 2019). Briefly, the FASTQ files were aligned to human reference genome GRCh37/hg19 with a Burrows–Wheeler alignment tool (Li & Durbin 2010). Variant calling was performed with Freebayes (germline) and Lancet (somatic) in all BAM files, and the variants were annotated with ANNOVAR (Wang et al. 2010).

The exome and the targeted panel sequencing data were screened for rare variants with minor allele frequency <0.1% in the public databases: Genome Aggregation Database (gnomAD) (Brownstein et al. 2014), 1000 Genomes (Genomes Project et al. 2015), and the Brazilian population database (ABraOM) (Naslavsky et al. 2017). Then, we selected rare variants located in exonic and consensus splice-site regions. Subsequently, the filtration pipeline prioritized potentially pathogenic candidate variants (loss-of-function variants and variants classified as pathogenic by multiple *in silico* programs). The allelic variants were classified according to The American College of Medical Genetics and Genomics classification (Richards et al. 2015).

Sanger sequencing was used to confirm the potentially pathogenic variants identified by massively parallel sequencing and for segregation analysis. PCR products were sequenced with a BigDye® Terminator version 3.1 cycle sequencing kit followed by automated sequencing on an ABI PRISM® 3130xl genetic analyzer (Applied Biosystems). KCNJ5 somatic mutation was confirmed by Sanger sequencing as previously described (Vilela et al. 2019).

**Immunohistochemistry**

An immunoperoxidase immunohistochemical modified method with humid heat antigen retrieval was used as
previously described (Shi et al. 1991). Staining with anti-PDE2A rat MAB (sc-271394, Santa Cruz Biotechnology) and anti-PDE3B rat MAB (sc-376823, Santa Cruz Biotechnology) were performed in 13 adrenal lesions (six APAs and seven BHs). CYP11B2 (rat monoclonal, clone 41-17B, Merck MAB125) staining was also analyzed as previously described (Gomez-Sanchez et al. 2014).

Immunohistochemistry was evaluated by an experienced pathologist (M.C.N. Zerbini). Staining was evaluated according to the intensity as negative (0), low (1), medium (2), or strong (3). The percentage of positive tumor cells was visually scored as: 0 if 0% of tumor cells were positive; 1 if 1–25%; 2 if 26–50%, 3 if 51–75% and 4 if 76–100% (de Sousa et al. 2015). Protein expression was evaluated in zona glomerulosa, hyperplasia, micronodules and adenomas.

Quantitative real-time RT-PCR (qRT-PCR)

cDNA was generated from 1 μg of total RNA using the commercial kit Superscript III First Strand S (Invitrogen). Quantitative real-time PCR was performed in the ABI Prism 7000 sequence detector using TaqMan gene expression assays according to the manufacturer’s instructions (Applied Biosystems). The PCR cycling conditions were as follows: 2 min at 95°C, 40 cycles of 95°C for 15 s and 60°C for 30 s, and a final step at 72°C for 30 s. The assays for target genes were SGK1 (Hs00178612_m1), β-actin (ACTB, 4310881E) and β-glucoronidase (GUSB, 4310888E). The relative expression levels were analyzed using the 2^ΔΔCT method (Livak & Schmittgen 2001). A commercial pool of normal human adrenal cortex from autopsies was used as reference sample (Clontech).

PKA enzymatic activity in frozen tissue

PKA activity was analyzed in five frozen tissues from two APAs and two BAHs (case 1 and 3 from Table 1) and one normal adrenal (obtained from a normotensive individual that underwent radical nephrectomy). PepTag Assay for non-radioactive detection c-AMP-dependent Protein Kinase (Promega) was employed following the manufacturer’s protocol to measure PKA activity in frozen tissues. All experiments were performed in triplicate.

Mutagenesis

The human PDE2A WT (NM_002599.5) coding sequence was cloned into the pCMV6-AC-GFP vector (RG207219, Origene, Rockville, MD, USA). The human PDE3B WT (NM_000922.4) coding sequence was cloned into the pCMV6-Entry vector with C-terminal Myc-DDK tag (PS10001, Origene). The p.Ile629Val variant was introduced into the human PDE2A WT template and the p.Arg217Gln or p.Gly392Val variants were introduced into the human PDE3B WT using the QuikChange Lightning Site-directed Mutagenesis Kit (210518-5, Agilent Technologies), following the manufacturer’s protocol. The following mutagenic primers were used: PDE2A_Ile629Val_MUT_F: CAGTCAAATTTTGAAGTGTGAGC1AAATTCA TGTCCTGACGATG and PDE2A_Ile629Val_MUT_R: CATGTGCAGCGGACATGA ATTTGGCTCAAAACTACAAAA TTGACTG; PDE3B_Arg217Gln_MUT_F: AGAAG CGCAGTGCTGAGGACCCGACCG and PDE3B_Arg217 Gln_MUT_R: CGCTGCGGCC TACGGACTGGTCTT; PDE3B_Gly392Val_MUT_F: TCTTGGCCTACAGGA ACTGAAAGACCCACATT and PDE3B_Gly392Val_MUT_R: AATGGGTGCTTT CTCAGTTTCCTGTAGGCCAAAGA.

Cell culture

Human Embryonic Kidney 293T cells (HEK 293T) were grown in Dulbecco’s modified Eagle’s medium (DMEM, high glucose, pyruvate, no glutamine; 10313, Gibco) supplemented with 10% fetal bovine serum (100-106, Gemini Bio Products, West Sacramento, CA) and 1% antibiotic (penicillin-streptomycin – 15140-148, Gibco) in a humidified atmosphere at 37°C with 5% CO2.

Analysis of protein expression

HEK 293T cells were seeded into six-well plates at a density of 3 x 10^4 cells per well. After 24 h of incubation, cells were transfected with Lipofectamine 2000 (11668030, Invitrogen) according to the manufacturer's protocol, using Opti-MEM I Reduced Serum Medium (31985-070, Gibco) and 500 ng of each vector (human WT PDE2A, p.Ile629Val PDE2A, human WT PDE3B, p.Arg217Gln PDE3B and p.Gly392Val PDE3B) alone. The empty pCMV6-Entry vector was used as a negative control. After 24 h of transfection, cells were washed with PBS and resuspended in 50 μL of ice-cold lysis buffer (Tris-HCl 10 mM, pH 7.5, NaCl 150 mM, EDTA 1 mM, EGTA 1 mM, SDS 0.1%, Nonidet P-40 1%) containing a cocktail of protease and phosphatase inhibitors (Sigma-Aldrich PPC101). The collected cells were incubated for 30 min on ice and then centrifuged for 15 min at 4°C, 19,000 g. Total protein concentration of supernatant was determined by Pierce™

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BCA Protein Assay (23227, Thermo Scientific), following the manufacturer’s protocol. After quantification of protein extracts, 50 µg of total proteins of each sample were separated by electrophoresis in 10% polyacrylamide gel under denaturing conditions (SDS-PAGE). Proteins were then transferred to a nitrocellulose membrane (BioRad) and Western Blot was performed using antibodies against DDK (TA 50011-100, Origene) diluted 1:1000, GFP (TA 15004, Origene) diluted 1:1000 and GAPDH (SC-32233, Santa Cruz Biotechnology) diluted 1:2000. Fluorescent secondary antibodies (827-08364 IRDye 800CW Goat anti-Mouse and 926-68073 IRDye 680RD Donkey anti Rabbit, LI-COR Biosciences, Lincoln, NE) diluted 1:20000 and Odyssey CLx Imaging System (LI-COR Biosciences) were used to acquire the signal of the bands.

Primary antibodies against SGK1 (cat# sc-28338, Santa Cruz Biotechnology; mouse, 1:500), SCNN1G (13943-1-AP, Proteintech; rabbit, 1:1000) and GAPDH (cat# sc-25778, Santa Cruz Biotechnology; rabbit, 1:1000) as a loading control were used. Membranes were then incubated in monkey anti-rabbit or donkey anti-mouse secondary antibodies for 1 h at room temperature before visualizing the membranes with SuperSignal West Pico chemiluminescent substrate (Thermo Scientific). Ratio of densitometry volumes for the proteins of interest to GAPDH were used for quantification (Image Lab 5.2.1 software, Biorad). All experiments were performed in triplicate.

PKA enzymatic activity assay

HEK 293T cells transfected with PDE2A and PDE3B variants as previously described were homogenized in ice-cold lysis buffer (20 mM Tris (pH 7.5), 0.1 mM sodium EDTA, 1 mM dithiothreitol) with 1:100 protease inhibitor cocktail (EMD Biosciences, La Jolla, CA) and 0.5 mM PMSF. Total protein was quantified by BCA assay.

PKA enzymatic activity was measured with a previously described method that utilizes P32-labeled ATP and kemptide substrate with or without added cAMP (5 µM) (Nesterova et al. 1996). Each reaction (50 µL) was performed in duplicate and contained 10 µg total protein. Basal and total kinase activities were calculated as pmol kinase activity per minute per milligram of protein without or with a saturating concentration of cAMP (5 µM), respectively. Activity values were adjusted by subtracting non-specific kinase activity that was assessed by performing replicate reactions in the presence of a specific PKA inhibitor (PKI, 5 µM). All experiments were performed in triplicate.

Statistical analysis

Statistical analysis was performed using IBM SPSS Software (25.0; SPSS Inc., Chicago, IL) and GraphPad Prism (version 8.4.2; San Diego, CA). Data are expressed as mean ± S.E.M. Comparisons were carried out via unpaired two-tailed t-test. P-value <0.05 was considered significant.

Results

Whole exome sequencing

All cases underwent unilateral adrenalectomy guided by CT or because of inconclusive lateralization index at AVS. Then, bilateral aldosterone excess was defined by the absence of PA biochemical and clinical cure after surgery and by the presence of adrenal hyperplasia with positive CYP11B2 areas on histopathology analysis (Table 1). Since BAH with PA are rarely treated surgically, our study brings a unique opportunity to study germline variants in patients with available hyperplastic tissue for staining and molecular analysis using frozen adrenal tissue.

Regarding population frequency, we excluded all variants present with a minor allele frequency >0.1% in the 1000 Genomes, Genome Aggregation Database (gnomAD), 1000 Genomes, and the Brazilian population database (ABraOM). Of these, only missense, nonsense, frameshift variants in coding regions, and splice sites were included. Next, we searched for rare germline variants, predicted to be deleterious in at least three in silico tools, in genes encoding ion channels or in genes previously associated with forms of adrenal hyperplasias (Table 2).

In three subjects with BAH associated with PA, we identified rare germline variants in phosphodiesterase 2A (PDE2A) and 3B (PDE3B) genes. The PDE2A heterozygous variant (p.Ile629Val) was identified in a patient with PA and early-onset HT diagnosed at 13 years of age (Case 1, Table 1). Genetic analysis and subsequent clinical investigation showed that her mother carried the PDE2A variant and has HT, but not PA (Fig. 1A). Alignment of amino acid residues encoded by PDE2A showing that the 629 residue is conserved across human, mouse, and rat.

The PDE3B heterozygous variant (p.Arg217Gln) was identified in a patient with PA caused by BAH and early-onset HT diagnosed at 13 years of age (Case 2, Table 2). Familial segregation analysis was not possible due to lack of DNA from relatives (Fig. 1B). In addition, a second PDE3B heterozygous variant (p.Gly392Val) was identified in a patient with PA and BAH with HT diagnosed at 33 years of age (Case 3, Table 1). Parents’ DNA samples were not
available for segregation (Fig. 1C). Alignment of residues encoded by PDE3B showing that the 217 and 392 residues are conserved across human, mouse, and rat. All PDE2A and PDE3B germline variants were classified as variants of uncertain significance according to The American College of Medical Genetics and Genomics classification (Table 2).

A somatic KCNJ5 mutation (p.Gly151Arg) was found in the hyperplastic tissue from case 3, harboring the PDE3B p.Gly392Val germline heterozygous variant. In the hyperplastic adrenal tissue of the patients with PDE2A and PDE3B variants, there was no evidence of loss of heterozygosity. Among all adrenal hyperplastic tissues, we did not identify any somatic rare and in silico deleterious variant (loss- or gain-of-function) in driver genes related to hyperplasia.

Autonomous cortisol secretion was investigated in four out of six patients. Case 1 (harboring the PDE2A p.Ile629Val variant) and case 2 (harboring the PDE3B p.Arg217Gln) had a negative hormonal screening for hypercortisolism. On the other hand, the patient with PDE3B p.Gly392Val had an abnormal cortisol levels after an overnight 1 mg dexamethasone suppression test (varying from 3.5 to 5.6 μg/dL in different occasions). ACTH, urinary and midnight salivary cortisol were normal.

**Functional analysis with hyperplastic tissue from bilateral adrenal hyperplasias**

Next, we investigated PDE2A and PDE3B staining in hyperplastic tissue from BAH associated with PA (Table 3). Normal adrenal gland tissue displayed a strong PDE2A expression in zona glomerulosa (Fig. 2A). Interestingly, PDE2A immunoreactivity was present in zona glomerulosa and hyperplastic areas (Fig. 2B, C and D). A strong positive

### Table 2  Germline variants selected in whole exome sequencing after filtration pipeline.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Variant</th>
<th>Type</th>
<th>MAF (%)</th>
<th>MAF (%)</th>
<th>Conservative in silico</th>
<th>Deleterious in silico</th>
<th>ACMG classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDE2A (Case 1)</td>
<td>p.Ile629Val (ENST00000334456)</td>
<td>Missense</td>
<td>0</td>
<td>0</td>
<td>Yes</td>
<td>Yes</td>
<td>VUS</td>
</tr>
<tr>
<td>PDE3B (Case 2)</td>
<td>p.Arg217Gln (ENST00000282096)</td>
<td>Missense</td>
<td>0.0006</td>
<td>0</td>
<td>Yes</td>
<td>No</td>
<td>VUS</td>
</tr>
<tr>
<td>PDE3B (Case 3)</td>
<td>p.Gly392Val (ENST00000282096)</td>
<td>Missense</td>
<td>0.001</td>
<td>0.08</td>
<td>Yes</td>
<td>Yes</td>
<td>VUS</td>
</tr>
</tbody>
</table>

*Somatic KCNJ5 mutation (p.Gly151Arg). ≥3 in silico tools. Although PDE3B p.Arg217Gln variant was not deleterious in ≥3 in silico tools, we selected this variant to perform functional studies.

AA, aminoacid; ABraOM, Brazilian population database; ACMG, American College of Medical Genetics and Genomics classification; MAF, minor allele frequency; VUS, variant of uncertain significance.

**Figure 1**

(A) A PDE2A heterozygous variant (p.Ile629Val) was identified in a patient with primary aldosteronism (PA) and early-onset hypertension (HT). Pedigree showed that her mother carries the PDE2A variant and has HT, but not PA. Sanger sequencing chromatograms showing the WT PDE2A sequence of an unaffected sister and the PDE2A p.Ile629Val variant of the index case. (B) The PDE3B heterozygous variant (p.Arg217Gln) was identified in a patient with PA and early-onset HT. Familial segregation was not possible due to lack of DNA from relatives. Sanger sequencing chromatograms showing the PDE3B p.Arg217Gln variant of the index case. (C) A PDE3B heterozygous variant (p.Gly392Val) was identified in a patient with PA. Pedigree showed that only the index case harbored the PDE3B variant (Parent's DNAs not available). Subjects with PA are shown with a black-filled symbol, those with HT (without PA) are represented by gray-filled symbol and non-affected subjects are shown with unfilled symbols. Index case is shown by an arrow. A full color version of this figure is available at https://doi.org/10.1530/ERC-20-0384.
PDE2A staining was detected in all six cases of BAH in subcapsular hyperplasia and/or micronodules (positive for CYP11B2 staining) (Table 3) (Fig. 2C and D). Additionally, we analyzed PDE2A staining in five APAs. PDE2A protein expression was strong and homogeneous in four APAs. In one APA, PDE2A staining was strong but only in the peripheral tumor area. PDE3B expression was mainly diffuse in the hyperplastic adrenal with a moderate/strong immunoreactivity in three out of six cases of BAH (Table 3).

PKA activity was investigated in frozen tissue from a normal adrenal, two APAs and two BAHs (from case 1 with germline PDE2A and case 3 with PDE3B p.Gly392Val variants). Frozen tissue from the BAH patient harboring PDE3B p.Arg217Gln was no longer available for this study. PKA activity in frozen tissue was significantly higher in BAH harboring the germline PDE2A p.Ile629Val variant compared to cells transfected with WT PDE2A (Fig. 3A). Similarly, PDE3B mutants (p.Arg217Gln and p.Gly392Val) lead to a significant reduction in PDE3B expression when compared to WT PDE3B in transfected cells (Fig. 4B). Although PDE2A and PDE3B variants lead to a decrease in protein expression, these variants did not increase cAMP stimulated PKA activity in HEK 293T cells (Fig. 4C and D).

Next, we analyzed expression of SGK1 and sodium channel epithelial 1 subunit gamma (SCNN1G or ENaCg) proteins; the latter is the final aldosterone target to increase renal sodium reabsorption. The PDE2A p.Ile629Val variant did not change SGK1 expression in HEK 293T transfected cells, whereas the PDE3B p.Arg217Gln variant significantly increased SGK1 expression. The PDE3B p.Gly392Val variant also increased SGK1 expression but did not reach statistical significance (Fig. 5A and B).

### Table 3

<table>
<thead>
<tr>
<th>Case</th>
<th>PDE2A</th>
<th>PDE3B</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+++ (ZG) ++ (micronodules)</td>
<td>+ (Diffuse)</td>
</tr>
<tr>
<td>2</td>
<td>++++ (ZG)</td>
<td>+ (Diffuse)</td>
</tr>
<tr>
<td>3</td>
<td>++++ (ZG and micronodules)</td>
<td>++++ (Diffuse)</td>
</tr>
<tr>
<td>4</td>
<td>+++ (Subcapsular hyperplasia and micronodules)</td>
<td>++++ (ZG)</td>
</tr>
<tr>
<td>5</td>
<td>++ (ZG and micronodules)</td>
<td>+ (Diffuse)</td>
</tr>
<tr>
<td>6</td>
<td>++ (ZG)</td>
<td>+ (Diffuse)</td>
</tr>
</tbody>
</table>

ZG, zone glomerulosa.

SGK1 gene expression was significantly higher in BAH from PA patients with PDE2A (p.Ile629Val) and PDE3B (p.Arg217Gln and p.Gly392Val) variants than in APAs without PDEs variants (Fig. 3B).

### In vitro functional studies

PDE2A and PDE3B variants were generated by site-directed PCR mutagenesis and their protein expression levels were studied in HEK 293T cells after transfection with WT or mutants. We found a significant decrease in the expression of PDE2A in cells transfected with the PDE2A p.Ile629Val mutant compared to cells transfected with WT PDE2A (Fig. 4A). Similarly, PDE3B mutants (p.Arg217Gln and p.Gly392Val) lead to a significant reduction in PDE3B expression when compared to WT PDE3B in transfected cells (Fig. 4B). Although PDE2A and PDE3B variants lead to a decrease in protein expression, these variants did not increase cAMP stimulated PKA activity in HEK 293T cells (Fig. 4C and D).
The \textit{PDE2A} p.Ile629Val variant did not significantly increase SCNN1G expression, although we cannot rule out a biological effect based on mRNA expression data (Fig. 5C). The \textit{PDE3B} p.Arg217Gln variant significantly increased SCNN1G expression. On the other hand, the \textit{PDE3B} p.Gly392Val variant also increased SCNN1G expression but did not reach statistical significance (Fig. 5D).

\section*{Discussion}

In this study, we demonstrate that loss-of-function \textit{PDE2A} and \textit{PDE3B} variants might be associated with PA caused by BAH. PDEs constitute a large and complex superfamily that contains 11 PDE gene families comprising 21 genes; PDEs are critical regulators of the intracellular concentrations of cAMP and cGMP as well as of their signaling pathways and downstream biological effects (Azevedo \textit{et al.} 2014). Germline inactivating \textit{PDE11A} and \textit{PDE8B} sequencing defects were identified by genome-wide studies in patients with isolated micronodular adrenocortical disease (iMAD) and Cushing syndrome (Horvath \textit{et al.} 2006, 2008). \textit{PDE11A} defects may also predispose to primary macronodular adrenocortical hyperplasias, a disorder mostly associated with hypercortisolism (Vezzosi \textit{et al.} 2012). In addition, \textit{PDE8B} and \textit{PDE11A} variants might contribute to predisposition of adult and pediatric adrenocortical tumors (Libe \textit{et al.} 2008, Rothenbuhler \textit{et al.} 2012, Pinto \textit{et al.} 2020). Besides cortisol-producing adrenocortical hyperplasias and tumors, inactivating \textit{PDE11A} variants were also associated with testicular germ cell tumors (Azevedo \textit{et al.} 2013). To date, pathogenic variants in \textit{PDEs} have not been associated with BAH and PA.

\textit{PDE2A} has higher affinity for cGMP than cAMP and its catalytic activity is allosterically stimulated by cGMP binding to the PDE2 GAF-B domain (Erneux \textit{et al.} 1981). Activation of \textit{PDE2A} decreases the cAMP level and thereby inhibits ACTH-stimulated aldosterone secretion in mice (Spisserberger \textit{et al.} 2009). ANP upregulates \textit{PDE2A} activity via cGMP levels and decreases aldosterone secretion (Nikolaev \textit{et al.} 2005). Interestingly, stabilization of \(\beta\)-catenin in zona glomerulosa cells resulted in expansion of the zona glomerulosa by directly stimulating the expression of \textit{Pde2a} (Pignatti \textit{et al.} 2020). Here, we identified a rare \textit{PDE2A} variant in a patient with PA caused by BAH and very early-onset HT. The \textit{PDE2A} p.Ile629Val variant lead to an important decrease in \textit{PDE2A} expression. In addition, PKA activity was markedly increased in the adrenal hyperplastic tissue from this patient, but not in APAs.

\textit{PDE2A} is expressed in zona glomerulosa of the murine adrenal cortex (Spisserberger \textit{et al.} 2009). \textit{PDE2A} expression was also found to be prominent in zona glomerulosa of rats, mice, cynomolgus, monkeys, dogs, and humans (Stephenson \textit{et al.} 2009). In our study, we demonstrated that \textit{PDEA2} was strongly expressed in normal zona glomerulosa of adrenal cortex, subcapsular hyperplasia and micronodules from BAH associated with PA. Some micronodules from BAH were positive for both \textit{PDE2A} and \textit{CYP11B2}. Therefore, \textit{PDE2A} represents a marker for zona glomerulosa and aldosterone-producing lesions.

\textit{PDE3A} and \textit{PDE3B} constitute the \textit{PDE3B} family and display high structural homology. \textit{PDE3A} and B affinity for cAMP is higher than for cGMP (Azevedo \textit{et al.} 2014). In the current study, we identified two rare germline variants in \textit{PDE3B} (p.Arg217Gln and p.Gly392Val) gene in two patients with BAH and PA. A significant reduction in \textit{PDE3B} expression was evidenced in transfected cells with \textit{PDE3B} mutants (p.Arg217Gln and p.Gly392Val).

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure3}
\caption{(A) PKA activity in frozen tissue was significantly higher in BAH from PA patients harboring the germline \textit{PDE2A} p.Ile629Val and \textit{PDE3B} p.Gly392Val variants when compared to APAs and normal adrenal. (B) SGK1 gene expression was significantly higher in BAH from PA patients with \textit{PDE2A} (p.Ile629Val) and \textit{PDE3B} (Arg217Gln and p.Gly392Val) variants than in APAs without PDEs variants. APA, aldosterone-producing adenomas; BAH, bilateral adrenal hyperplasia; PA, primary aldosteronism; PDE, phosphodiesterase. All experiments were performed in triplicate.

\textit{Figure 3}

(A) PKA activity in frozen tissue was significantly higher in BAH from PA patients harboring the germline \textit{PDE2A} p.Ile629Val and \textit{PDE3B} p.Gly392Val variants when compared to APAs and normal adrenal. (B) SGK1 gene expression was significantly higher in BAH from PA patients with \textit{PDE2A} (p.Ile629Val) and \textit{PDE3B} (Arg217Gln and p.Gly392Val) variants than in APAs without PDEs variants. APA, aldosterone-producing adenomas; BAH, bilateral adrenal hyperplasia; PA, primary aldosteronism; PDE, phosphodiesterase. All experiments were performed in triplicate.

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Furthermore, adrenal hyperplasia from one of these patients exhibited a significant increase in PKA activity when compared to normal adrenal and APAs. Although PDE3B defects have not been previously associated with any form of HT, germline PDE3A missense mutations were associated with autosomal dominant HT and brachydactyly type E (Maass et al., 2015). This particular genetic syndrome includes beyond brachydactyly type E, severe salt-independent HT, neurovascular contact at the rostral-ventrolateral medulla, altered baroreflex

Figure 4
(A) PDE2A expression was markedly reduced in HEK 293T cells transfected with p.Ile629Val PDE2A mutant when compared to those transfected with WT PDE2A. (B) The Arg217Gln and p.Gly392Val PDE3B variants significantly decreased PDE3B expression in HEK 293T cells in comparison with HEK 293T cells transfected with WT PDE3B. (C and D) PDE2A (p.Ile629Val) and PDE3B (Arg217Gln and p.Gly392Val) variants did not increase in vitro PKA activity after cAMP induction in HEK 293T cells. All experiments were performed in triplicate. A full color version of this figure is available at https://doi.org/10.1530/ERC-20-0384.

Figure 5
(A and B) The p.Ile629Val PDE2A variant did not modify SGK1 expression in HEK 293T cells (A), whereas both p.Arg217Gln and p.Gly392Val PDE3B variants increased SGK1 expression when compared to WT PDE3B (B). (C) SCNN1G protein expression did not change after transfection with the p.Ile629Val PDE2A mutant. (D) PDE3B (p.Arg217Gln and p.Gly392Val) variants increased SCNN1G expression in HEK 293T transfected cells. All experiments were performed in triplicate. In blots shown in B and D, the intensity of the bands for GAPDH differs somewhat across lanes despite the fact that equal amount was loaded; nevertheless, data were corrected for density on the same lane and not across lanes.
blood pressure regulation and stroke before age 50 years if untreated HT (Schuster et al. 1998). Very recently, a germline nonsense variant (p.Arg783*) in PDE3B gene was identified in a child with adrenocortical tumor without TP53 mutation (Pinto et al. 2020).

Aldosterone increases the transcription of the basolateral Na+/K+-ATPase and the apical ENaC (Valinsky et al. 2018). After binding to the cytosolic mineralocorticoid receptor, aldosterone promotes the transcription of aldosterone-regulated genes, including SGK1. SGK1 increases ENaC activity by reducing its ubiquitination and receptor internalization (Staub et al. 1996). In our study, PDE2A and PDE3B variants increased the expression of SGK1 and/or SCNN1G (ENaCg) at mRNA or protein level. Our findings connect PDE2A and PDE3B with aldosterone signaling thorough SGK1 and ENaC regulation.

Besides sodium reabsorption, an increase in SGK1 activity exacerbates diet-induced obesity, metabolic syndrome and HT (Sierra-Ramos et al. 2020). In addition, SGK1 activation stimulates hypercoagulability, fibrosis and inflammation (Lang & Voelkl 2013). Aldosterone promotes fibrosis and inflammation via activation of SGK1 and NF-kB, which were inhibited by eplerenone (Terada et al. 2008). Interestingly, a SGK1 inhibitor reversed the increase of blood pressure caused by hyperinsulinism and salt excess in mice (Ackermann et al. 2011).

Defects in PDEs (PDE11A and PDE8B) have been associated with cortisol-producing micro- and macronodular adrenal hyperplasias, but not with PA (Hannah-Shmouni & Stratakis 2020). Among the three patients with germline PDE2A or PDE3B germline variants, only one harboring the PDE3B p.Gly392Val variant had autonomous cortisol secretion. A steroid metabolism analysis revealed that glucocorticoid excess is frequent in PA (Arlt et al. 2017). However, glucocorticoid metabolite excretion was not different between APAs and BAHs.

The lack of AVS confirming bilateral aldosterone excess in our patients is a limitation of this study. Bilateral adrenal hyperplasia was defined by the absence of PA cure after unilateral adrenalectomy and by the findings of adrenal hyperplasia on histopathology analysis with a positive CYP11B2 staining in the resected adrenal. Despite the fact that three cases had unilateral disease based on imaging, the persistence of PA after adrenalectomy strongly indicates the presence bilateral disease. In hyperplastic adrenal lesions, we identified only one KCNJ5 somatic mutation. In a previous study, Omata et al. identified CACNA1D somatic mutations in 58% of micronodules positive for CYP11B2 from BAH (Omata et al. 2018). The absence of CACNA1D somatic mutations in our study can be explained by the fact that genetic analysis was not guided by CYPB112 staining in adrenal lesions.

Most of the cases of BAH associated with PA are considered ‘sporadic’ and remain without a genetic diagnosis. Aldosterone-producing BAH are treated with aldosterone antagonists in the great majority of the cases. Our cohort of six patients with PA and BAH treated surgically represent a unique cohort where we could investigate germline defects and conduct functional studies in tissues from adrenal hyperplasia. In this report, we add two new genes to the roster of molecules that may be involved in the pathogenesis of aldosterone production in BAH.

In conclusion, we demonstrated that PDE2A and PDE3B variants are associated with the pathogenesis of bilateral PA. PKA activity was higher in adrenal hyperplastic tissue from those patients. Additionally, PDE2A and PDE3B variants increased gene or protein expression of SGK1 and SCNN1G/ENaCg, downstream mediators of aldosterone signaling. This evidence suggests the potential pathogenicity of PDE2A and PDE3B variants and expand the spectrum of genetic etiologies for PA and FH-II, a genetically heterogeneous disorder (Lafferty et al. 2000, Elphinstone et al. 2004).

Declaration of interest
C A S holds patents on the function of the PRKAR1A, PDE11A, and GPR101 genes and related issues; his laboratory has also received research funding on GPR101, abnormal growth hormone secretion and its treatment by Pfizer, Inc; F R F holds patent on the GPR101 gene and/or its function. The other authors have nothing to disclose.

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Author contribution statement
C A S and M Q A share senior authorship.

References


Valinsky WC, Touyz RM & Shirer A 2018 Aldosterone, SGK1, and ion channels in the kidney. *Clinical Science* 132:173–183. [https://doi.org/10.1042/CS20171525](https://doi.org/10.1042/CS20171525)


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