Novel somatic mutations and distinct molecular signature in aldosterone-producing adenomas

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Abstract

Aldosterone-producing adenomas (APAs) are found in 1.5–3.0% of hypertensive patients in primary care and can be cured by surgery. Elucidation of genetic events may improve our understanding of these tumors and ultimately improve patient care. Approximately 40% of APAs harbor a missense mutation in the *KCNJ5* gene. More recently, somatic mutations in *CACNA1D*, *ATP1A1* and *ATP2B3*, also important for membrane potential/intracellular Ca^{2+} regulation, were observed in APAs. In this study, we analyzed 165 APAs for mutations in selected regions of these genes. We then correlated mutational findings with clinical and molecular phenotype using transcriptome analysis, immunohistochemistry and semiquantitative PCR. Somatic mutations in *CACNA1D* in 3.0% (one novel mutation), *ATP1A1* in 6.1% (six novel mutations) and *ATP2B3* in 3.0% (two novel mutations) were detected. All observed mutations were located in previously described hotspot regions. Patients with tumors harboring mutations in *CACNA1D*, *ATP1A1* and *ATP2B3* were operated at an older age, were more often male and had tumors that were smaller than those in patients with *KCNJ5* mutated tumors. Microarray transcriptome analysis segregated *KCNJ5* mutated tumors from *ATP1A1/ATP2B3* mutated tumors and those without mutation. We observed significant transcription upregulation of *CYP11B2*, as well as the previously described glomerulosa-specific gene *NPNT*, in *ATP1A1/ATP2B3* mutated tumors compared to *KCNJ5* mutated tumors. In summary, we describe novel somatic mutations in proteins regulating the membrane potential/intracellular Ca^{2+} levels, and also a distinct mRNA and clinical signature, dependent on genetic alteration.

Key Words

- ▶ *ATP1A1*
- ▶ *CACNA1D*
- ▶ *KCNJ5*
- ▶ primary aldosteronism
- ▶ aldosterone-producing adenoma

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Introduction

Primary aldosteronism (PA) is the most common endocrine cause of secondary hypertension with an estimated prevalence of ~5–10% in unreferred hypertensive patients and up to 20% of those with grade III hypertension (Rossi *et al.* 2006, Fogari *et al.* 2007, Hannemann & Wallaschofski 2012). Aldosterone-producing adenomas (APAs) represent a surgically curable form of PA and occur in ~30% of PA patients (Rossi *et al.* 2006, Fogari *et al.* 2007). The aldosterone overproduction leads to increased Na⁺ and H₂O retention by the kidney, resulting in hypertension. Importantly, the excess aldosterone also promotes cardiac remodeling and vascular collagen deposition, augmenting cardiovascular disease in these patients when compared to patients with essential hypertension (Brilla & Weber 1992, Rossi *et al.* 1997, Savard *et al.* 2013).

In the normal zona glomerulosa (ZG) cell, the plasma membrane is kept at a highly hyperpolarized state (Quinn *et al.* 1987). This hyperpolarization is mainly attributed to a dominant conductance of potassium ions over the cell membrane (Spät & Hunyady 2004). Depolarizing the membrane potential increases intracellular Ca²⁺ and subsequently increases aldosterone production and secretion (Spät & Hunyady 2004).

Choi *et al.* (2011) identified mutations in the *KCNJ5* gene in both sporadic APAs and in a family with bilateral adrenal hyperplasia. Mutations affecting the selectivity filter of GIRK4 (encoded by the *KCNJ5* gene) resulted in the loss of selectivity for potassium, increased sodium influx and subsequent membrane depolarization. The membrane depolarization raises intracellular calcium concentrations, leading to increased *CYP11B2* transcription and subsequent aldosterone production (Oki *et al.* 2012). Multiple screening studies of over 1000 sporadic APAs have established a prevalence of ~40% mutations in *KCNJ5* in Western countries (Åkerström *et al.* 2012, Boulkroun *et al.* 2012, Lenzini *et al.* 2015) and even higher in Asian countries (Taguchi *et al.* 2012, Kitamoto *et al.* 2015, Wang *et al.* 2015, Zheng *et al.* 2015).

More recently, somatic mutations in genes encoding an additional ion channel (*CACNA1D*) and two ATPases (*ATP1A1* and *ATP2B3*) were discovered in APAs (Azizan *et al.* 2013, Beuschlein *et al.* 2013, Scholl *et al.* 2013). Also, germline mutations in *CACNA1D* and *CACNA1H* were described in patients with early onset PA (Scholl *et al.* 2013, Scholl *et al.* 2015). Similar to GIRK4, the protein products of these genes are ultimately involved in regulating the membrane potential and Ca²⁺ homeostasis in adrenal glomerulosa cells. To provide better knowledge

of prerequisites for potential diagnostic and therapeutic targets, we aimed to investigate the frequency of mutations in *CACNA1D*, *ATP1A1* and *ATP2B3* in 165 APAs, and explore clinical and molecular phenotype.

Materials and methods

Included patients and clinical annotation

Tumor specimens were collected from 165 patients (65 males and 100 females) with diagnosed APA from six different tertiary referral centers. The diagnosis was established by raised aldosterone/renin ratio, positive confirmatory tests, lateralization studies (AVS and/or CT/MRI) and histopathological examination, according to routine protocols at the individual centers. Patients gave written informed consent and approvals from the local ethical committees were obtained.

Cortisol-producing adenomas (*n*=4) and non-secreting adenomas (*n*=4) were included for *CYP11B2* expression analysis. Patients with cortisol-producing adenomas had elevated cortisol in serum and failure to suppress cortisol with dexamethasone and postoperative cure. Non-hormone secreting adenomas were detected incidentally during abdominal CT scan. These were deemed functionally silent after negative hormone analyses and were removed on suspicion of malignancy (size or other radiological signs).

Molecular genetic analyses

DNA/RNA was extracted using Allprep DNA/RNA mini kit, (Qiagen), AllPrep DNA/RNA FFPE Kit (Qiagen) or DNeasy Blood and tissue kit, (Qiagen). cDNA was prepared using Revertaid H-minus First strand cDNA synthesis Kit, (Fermentas, Waltham, MA, USA). In total, 90 (54.5%) *KCNJ5* mutations had previously been observed (Åkerström *et al.* 2012). Hotspot regions in *CACNA1D* exons 8A, 8B, 16 and 33, *ATP1A1* exons 4, 8 and 21 and *ATP2B3* exon 8 were analyzed in all tumors. In addition, tumors without *KCNJ5* mutation (*n*=75) were analyzed for fragments corresponding to regions in *CACNA1D* exons 5, 6, 13, 14, 23, 27, 32, 35, 36, 37, 38, 39, 40. When available, confirmatory PCR was performed on DNA from peripheral blood/surrounding normal tissue as well as in cDNA. Details on PCR conditions and primer sequences are available upon request. Sanger sequencing was conducted on amplified fragments (Beckman Coulter Genomics, Takeley, UK). Chromatograms were analyzed

using CodonCode aligner Software 4.2.4 (CodonCode Corporation, Dedham, MA, USA). NCBI Reference Sequences; NP_001122312.1 and NP_000711 (exon 8B) for *CACNA1D*, NP_001153705.1 for *ATP1A1* and NP_001001344.1 for *ATP2B3* were used for sequence alignment. Evolutionary conservation was determined using the ClustalW2 algorithm (Larkin *et al.* 2007).

Transcriptome analysis was performed on 12 APAs (six *KCNJ5* mutated, three *ATP1A1/2B3* mutated and three tumors without known mutation) using the SurePrint G3 Human Exon 4×180K Microarray (Agilent Technologies, Santa Clara, CA, USA). Data analysis was conducted using the GeneSpring Software v.13 (Agilent Technologies). Quality control of the included samples was performed in the GeneSpring v.13 Software using a flag-based method. Baseline transformation to the median of all samples was performed. Data were log-transformed and normalized using the quantile method. Unsupervised hierarchical clustering using 10% of probes with the highest variation in expression was performed in R. Using all probes, unsupervised hierarchical clustering based on Euclidean distances and complete linkage was obtained. Heat maps of the top 100 up- and downregulated transcripts in *ATP1A1/2B3* mutated tumors compared to *KCNJ5* mutated tumors were generated and *t*-tests were performed. Subsequently, the Benjamini-Hochberg method was used for multiple testing correction, and significantly differentially expressed transcripts were identified by volcano plots using fold change > 2 together with a corrected *P*-value of < 0.05.

The expression of CYP11B2 was measured using a custom assay (ThermoFisher Scientific, Waltham, MA, USA), with mRNA specific primers as previously described (Fallo *et al.* 2012). An mRNA specific GAPDH assay was used as an internal control (ThermoFisher Scientific). The relative CYP11B2 expression was compared between *KCNJ5* mutated APAs (*n*=14), *ATP1A1* (*n*=3) *ATP2B3* (*n*=2) using a calculated $2^{(-\Delta\Delta CT)}$ value (Livak & Schmittgen 2001). Non-hormone-secreting adenomas (*n*=4) and cortisol-producing adenomas (*n*=4) were used as controls.

NPNT immunohistochemistry was performed on 6 µm frozen sections from three *ATP2B3* mutated tumors, one *ATP1A1* mutated, 15 *KCNJ5* mutated tumors, one section of normal kidney tissue and one section of normal adrenal tissue next to a non-hormone-secreting adenoma. Briefly, sections were deparaffinized in xylene and rehydrated through graded ethanol. Endogenous peroxidases were inhibited by incubation in hydrogen peroxide solution. Avidin/Biotin blocking (Vector Laboratories, Burlingame, CA, USA) was applied then followed by normal goat serum. The sections were incubated with an antibody against NPNT, 1:150 (HPA003711, Sigma-Aldrich, St Louis, MI, USA) for 2 h at room temperature. For detection, a biotinylated and HRP-conjugated goat anti-rabbit secondary antibody was used, 1:200 (Vector Laboratories). The sections were developed with DAB (Vector Laboratories) and counterstained with hematoxylin.

Statistics

The overall group effect was analyzed using Oneway ANOVA for parametric data and a Kruskal-Wallis test for categorical data. If significant group effects were present, the overall analysis was followed by *post hoc* comparisons between groups using Bonferroni-corrected Mann-Whitney's *U*-test or two-tailed *T*-test. Categorical data were analyzed by χ^2 -tests. Data are expressed with either mean $\pm$ S.E.M. or median and range for non-parametric data. SPSS 22 (IBM) was used for statistical analysis.

Results

The proportion of mutations and corresponding phenotypes of the screened cohorts are summarized in Tables 1 and 2 respectively. Tumors from three of the centers contained more than 50% *KCNJ5* mutations (Table 1). These cohorts had relatively large tumors, with a mean size of 17.9 mm $\pm$ 0.92, and included many female patients (68%), and most patients were hypokalemic (96%). For the entire cohort, we observed a female gender

Table 1 Proportion of mutations in APAs stratified by cohorts

Cohort	Number of APAs	WT	<i>KCNJ5</i>	<i>ATP1A1</i>	<i>ATP2B3</i>	<i>CACNA1D</i>
Uppsala	36	8 (22.2%)	25 (69.4%)	1 (2.8%)	2 (5.6%)	0
Dusseldorf	33	17 (51.5%)	15 (45.5%)	1 (3.0%)	0	0
Halle	8	4 (50.0%)	4 (50.0%)	0	0	0
Lübeck	20	4 (20.0%)	13 (65.0%)	2 (10.0%)	1 (5.0%)	0
Sydney	34	15 (44.1%)	14 (41.2%)	2 (5.9%)	0	3 (8.8%)
Stockholm	34	7 (20.6%)	19 (55.9%)	4 (11.8%)	2 (5.9%)	2 (5.9%)
Summary	165	55 (33.3%)	90 (54.5%)	10 (6.1%)	5 (3.0%)	5 (3.0%)

overrepresentation for patients with *KCNJ5* mutated tumors compared to patients without *KCNJ5* mutated tumors (80% vs 38%, $P < 0.0001$) (Table 2). These patients were also operated at a younger age (44.9 years ± 1.23 vs 53.1 years ± 1.34 , $P < 0.0001$). No difference in adenoma size (19.4 mm ± 0.80 vs 16.5 mm ± 1.27 , $P = 0.17$) or preoperative aldosterone levels was observed (1365 pmol/l ± 219 vs 1355 pmol/l ± 145 , $P = 0.97$).

A total of 20 (12.1%) mutations in *CACNA1D*, *ATP1A1* and *ATP2B3* (Table 1) were identified. The somatic nature of the mutations was verified in all samples with available non-tumoral tissue (Supplementary Table 1, see section on supplementary data given at the end of this article). Mutations in *CACNA1D* were found in 3.0% of tumors. These mutations were located in exon 8A, 16 and 33, corresponding to segment 6 (S6) in repeats I and II, and S5 in repeat IV (Supplementary Fig. 1). We discovered a novel mutation in exon 8A (p.Val401Leu). Val401 is an evolutionary conserved amino acid located two positions upstream from the frequently mutated Gly403 residue (Supplementary Table 2). Somatic mutations in *ATP1A1* were observed in 10/165 (6.1%) and in *ATP2B3* in 5/165 (3.0%) APAs. The mutations in *ATP1A1* comprised the previously described p.Leu104Arg (c.311T>G) along with three novel deletions in the same area, as well as three novel deletions in the M9 domain (Supplementary Figs 2A and 3, and Supplementary Table 3). The mutations found in *ATP2B3* corresponded to amino acid residues located in the M4 domain of the protein. These included two previously described deletions and two novel deletions (Supplementary Figs 2B and 4). All of the observed mutations were found in non-*KCNJ5* mutated tumors.

We observed a significant difference in clinical phenotype in patients with *ATP1A1/2B3* and *CACNA1D* mutated tumors compared to patients with *KCNJ5* mutated tumors (Table 2). *ATP1A1/2B3* and *CACNA1D* mutated tumors were significantly smaller than *KCNJ5* mutated APAs (11.2 mm ± 0.94 vs 19.4 mm ± 0.80 , $P < 0.0001$). Patients were operated at an older age (53.7 years ± 2.35 vs 44.9 years ± 1.23 , $P = 0.008$), and they were more often male (65% vs 20%, $P < 0.0005$). No significant difference in preoperative aldosterone levels was observed (1671 pmol/l ± 340 vs 1365 pmol/l ± 219 , $P = 0.30$).

Transcriptome analysis and unsupervised hierarchal clustering using 10% of probes with the highest variation in expression segregated *KCNJ5* mutated tumors (Fig. 1A). Likewise, principal component analysis on all probes segregated *KCNJ5* mutated tumors (Fig. 2). Unsupervised hierarchal clustering on the complete set of probes segregated *KCNJ5* mutated tumors, except for one

Table 2 Phenotype comparison stratifying APAs according to mutations

	(A) Non- <i>KCNJ5</i> mutated (n = 75)	(B) <i>KCNJ5</i> (n = 90)	(C) <i>ATP1A1</i> (n = 10)	(D) <i>ATP2B3</i> (n = 5)	(E) <i>CACNA1D</i> (n = 5)	(F) <i>ATP1A1</i> , <i>ATP2B3</i> , <i>CACNA1D</i> (20)	Between groups comparison (B-E) (P)	A vs B (P)	B vs F (P)
Age at operation	53.1 (± 1.34)	44.9 (± 1.23)	57.5 (34–65)	60 (48–78)	45 (35–57)	53.7 (± 2.35)	0.005	<0.0001	0.008
Gender, female in %	38	80	20	60	40	35	<0.0005 ^a	<0.0001 ^b	<0.0005 ^b
Tumor size (mm)	16.5 (± 1.27)	19.4 (± 0.80)	12.9 (6–23)	10.0 (4–15)	10.0 (5–12)	11.2 (± 0.94)	<0.0001	NS	<0.0001
Aldosterone (pmol/l)	1355 (± 145)	1365 (± 219)	1018 (461–6963)	1545 (535–2240)	1140 (558–2583)	1671 (± 340)	NS	NS	NS

Values are presented as mean $\pm$ s.e.m. for parametric data and as median and range for non-parametric data. Statistical analysis was performed by between-groups tests (B, C, D and E) using One-way ANOVA.

^aKruskal-Wallis's test for categorical data. Post hoc comparison (A vs B and B vs F) using two tailed t-test with a Bonferroni corrected significance threshold ($P < 0.05/4$).

^b χ^2 -test. Non-*KCNJ5* mutated includes *ATP1A1/2B3/CACNA1D* mutated tumors and those without mutation.

Figure 1

Transcriptional signal in APA samples. (A) Unsupervised hierarchical clustering based on Euclidean distances in 12 APAs (six *KCNJ5* mutated, three *ATP1A1/2B3* mutated and three without known mutations=WT) using 10% of probes with the highest variation in expression. (B) Heat map showing the results of unsupervised hierarchical clustering based on Euclidean distances and complete linkage of all quality passed probes in the microarray.

ATP2B3 mutant (Fig. 1B). The most up- and down-regulated genes in *ATP1A1/2B3* mutated tumors compared to *KCNJ5* mutated tumors are displayed in Supplementary Table 4 and Supplementary Fig. 5 (see section on supplementary data given at the end of this article). Only two transcripts, NPNT and RBM11, satisfied greater than twofold change with a false discovery rate (FDR) of $P < 0.05$ (Fig. 3A). Comparing tumors without known mutation against *KCNJ5* mutated tumors, we observed three genes with greater than twofold change and FDR $P < 0.05$ (Fig. 3B). These included DNAH11 (FC change: -6.8 in *KCNJ5* mutated, $P = 0.013$), C1orf186 (FC change: +2.7 in *KCNJ5* mutated, $P = 0.013$) and PPP1R14A (FC change: +2.1 in *KCNJ5* mutated, $P = 0.048$). No differences in the mRNA expression of *ATP1A1*, *ATP2B3* or *KCNJ5* were observed using the microarray. One probe for *CYP11B2* was present on the microarray and showed higher expression in *ATP1A1/2B3* mutated tumors than in *KCNJ5* mutated (median signal: 0.99 vs -0.4). To further investigate *CYP11B2* expression, we conducted semiquantitative-PCR expression analysis in *ATP1A1/2B3* and those with *KCNJ5* mutation,

confirming higher expression in *ATP1A1/2B3* mutated tumors ($P < 0.05$) (Fig. 4). To verify increased NPNT expression in *ATP1A1/2B3* tumors, we analyzed protein expression using immunohistochemistry. As expected, staining of normal kidney tissue revealed selective expression in glomeruli and blood vessels (Fig. 5A). In normal adrenal tissue, expression was restricted to ZG cells (Fig. 5B). Staining of tumor tissue confirmed array findings with higher expression of NPNT in *ATP1A1/2B3* mutated APAs compared to *KCNJ5* mutated APAs (Fig. 5C and D).

Discussion

In this study, we observed somatic mutations in *CACNA1D*, *ATP1A1* and *ATP2B3* in 12.1% of APAs, including nine novel somatic mutations. *CACNA1D* mutations were detected in 3% of the tumors. *CACNA1D* encodes the alpha-1 subunit of an L-type voltage-dependent Ca^{2+} channel (VDCC) important for increasing intracellular Ca^{2+} and aldosterone production in ZG cells (Cohen et al. 1988). The observed mutations were located in segment 6 in repeats I and II, and S5 in repeat IV (Fig. 6). Interaction between S5 and S6 is important for the normal function of the channel pore (Catterall 2010). *In vitro* studies have shown that alterations in these portions of the protein lead to increased channel activity (Azizan et al. 2013, Scholl et al. 2013). We detected a novel missense mutation (p.Val401Leu) in one of the tumors. This position is located two amino acid residues upstream from the Gly403 in S6 (repeat I), one of the most frequently mutated residues in *CACNA1D* (Azizan et al. 2013, Scholl et al. 2013,

Figure 2

Principal component analysis on the transcriptional signals in APAs. Two-dimensional PCA on 12 APAs based on all probes.

Figure 3

Statistically significant expression differences in *ATP1A1/2B3/WT* compared to *KCNJ5* mutated APA. Volcano plot on all quality passed probes on the microarray. The x-axis shows the fold change ($\log_2$) and the y-axis shows corrected *P* value ($-\log_{10}$). *T*-tests were performed on greater than twofold up/down regulated probes and correction for multiple testing by the Benjamini-Hochberg method was applied. Probes with a fold change >2 and a false discovery rate of <0.05 in (A) *ATP1A1/2B3* mutated tumors compared to *KCNJ5* mutated tumors and (B) WT tumors compared to *KCNJ5* mutated tumors, displayed in red.

Fernandes-Rosa et al. 2014). Conserved status throughout evolution and its location suggest a functional consequence on protein function. Compared to previously analyzed cohorts from Western countries, we observed relatively few *CACNA1D* mutants (Azizan et al. 2013, Scholl et al. 2013, Fernandes-Rosa et al. 2014). We re-sequenced all parts of *CACNA1D* previously reported as mutated in APAs. We also expanded our analysis to areas where missense

mutations have resulted in a gain of function effect in other VDCCs (Ducros et al. 2001, Pietrobon 2002, Hoda et al. 2005, Striessnig et al. 2010, Stockner & Koschak 2013). Interestingly, in two studies conducted in Asia, only two mutations in 282 analyzed APAs were found (Wang et al. 2015, Zheng et al. 2015). They also observed a high prevalence of *KCNJ5* mutations, 76.2% (215/282). Similar to the Asian cohorts, our prevalence of *KCNJ5* mutations was relatively high. Compared to another large European cohort of 474 APAs (Fernandes-Rosa et al. 2014), our patients were more often female and had larger adenomas, a phenotype associated with *KCNJ5* mutations (Åkerström et al. 2012, Azizan et al. 2012a, Fernandes-Rosa et al. 2014). This could potentially explain our relatively low number of *CACNA1D* mutations, which seem to occur in smaller tumors in male patients (Azizan et al. 2013, Scholl et al. 2013, Fernandes-Rosa et al. 2014). This affirms the notion that aside from a possible demographic bias, the diagnostic workup and selection of patients affect the distribution of mutations.

We detected *ATP1A1* mutations in 6.1% of tumors. *ATP1A1* encodes the alpha-1 subunit of a ubiquitously expressed Na^+/K^+ ATPase, vital for establishing the membrane potential in ZG cells (Spät & Hunyady 2004). The mutations were either located close to or involving amino acid Leu104 in the M1 domain, or affecting the Glu960 and Glu961 residues of the M9 domain. Leu104 has been proposed to interact with Glu334 in the

Figure 4

mRNA expression of CYP11B2 in *ATP1A1/2B3* and *KCNJ5* mutated adenomas. Relative mRNA expression ($2^{(-\Delta\Delta\text{CT})}$) of CYP11B2 in *ATP1A1* and *ATP2B3* mutated APAs = ATPase compared to *KCNJ5* mutated, NHSA and CPA. GAPDH was used as an internal control. The relative level of CYP11B2 was compared between ATPase and *KCNJ5* mutated tumors using a Mann-Whitney's *U*-test. Median and maximum values are displayed. NHSA, non-hormone secreting adenoma; CPA, cortisol producing adenoma; $*P < 0.05$. Insert depicts the individual levels of CYP11B2 in the *ATP1A1/2B3* mutated tumors (median and range).

Figure 5

Immunohistochemistry for NPNT. Protein expression for NPNT by immunohistochemistry. (A) Normal kidney tissue. (B) Normal adrenal tissue adjacent to a non-hormone secreting adenoma. (C) Representative staining

of *KCNJ5* mutated tumor tissues. (D) Representative staining of *ATP1A1/ATP2B3* mutated tissues. G, glomeruli; C, capsule; ZG, zona glomerulosa; ZF, zona fasciculata.

transmembrane helix M4, leading to potassium binding and occlusion by the channel (Einholm *et al.* 2007). P.Leu104Arg and p.Gly99Arg mutations likely disrupt this site, and have been shown to increase depolarization, CYP11B2 expression and aldosterone production *in vitro* (Azizan *et al.* 2013, Williams *et al.* 2014). The mutations in the M9 domain were novel deletions affecting amino acids Glu960 and Glu961 in the third Na⁺ binding site of the protein. Previous *in vitro* studies of a p.Glu961Ala mutant showed reduced Na⁺ binding and a lowered affinity for K⁺ (Li *et al.* 2005). Mutations in *ATP1A1* have previously not been described in any syndromes, and no germline mutation could be detected in screened familial PA cases (Beuschlein *et al.* 2013). Given its ubiquitous expression and vital role for establishing the membrane potential, this is not surprising (Jorgensen *et al.* 2003). However, an association between *ATP1A1* haplotypes and primary hypertension has been described (Herrera *et al.* 1998, Treva Rice *et al.* 2000, Glorioso *et al.* 2007). Further investigation of the functional consequences of *ATP1A1* genotypes and haplotypes in hypertensive cohorts are therefore warranted.

ATP2B3 encodes a plasma membrane Ca²⁺ ATPase that actively transports Ca²⁺ out of the cell, resulting in low intracellular Ca²⁺ levels, thereby inhibiting aldosterone production (Spät & Hunyady 2004). We observed five APAs with *ATP2B3* mutations, including two novel deletions and two previously reported deletions (Beuschlein *et al.* 2013, Williams *et al.* 2014). The mutations span a seven amino acid region in the M4 domain, a part of the protein highly conserved among orthologous. Importantly, these amino acids are located at similar positions as the mutated residues in the M4 domain in *ATP1A1*. In contrast to *ATP1A1*, a germline *ATP2B3* mutation has been found in a family with X-linked congenital cerebellar atrophy (Zannia *et al.* 2012). Functional studies showed

that this mutation (p.Gly1107Asp) decreased the ability of the channel to extrude Ca²⁺ from the cells. To our knowledge, hyperaldosteronism has not been observed in these patients.

Previous studies have described genotype–phenotype correlations in APAs. While some have not described any gender association (Scholl *et al.* 2013, Williams *et al.* 2014), we could confirm that *ATP1A1/2B3/CACNA1D* mutated tumors are more often found in male patients, compared to a female overrepresentation for *KCNJ5* mutated tumors (Azizan *et al.* 2013, Beuschlein *et al.* 2013, Dutta *et al.* 2013, Fernandes-Rosa *et al.* 2014). We also observed an older age at the time of operation for patients with these tumors, previously reported by some (Azizan *et al.* 2013, Scholl *et al.* 2013, Fernandes-Rosa *et al.* 2014) but not by others (Beuschlein *et al.* 2013, Dutta *et al.* 2013, Williams *et al.* 2014). We could not confirm higher preoperative aldosterone levels (Beuschlein *et al.* 2013), but observed the previously reported association with smaller tumors (Azizan *et al.* 2013, Dutta *et al.* 2013, Scholl *et al.* 2013, Fernandes-Rosa *et al.* 2014). Importantly, due to their smaller size *ATP1A1/2B3/CACNA1D* mutated tumors may be missed in patients that have not undergone AVS, which may also lead to delayed diagnosis and subsequently an older age at the time of the operation. It also suggests that the use of only CT/MRI may bias the mutational spectrum towards more *KCNJ5* mutations, as previously indicated (Fernandes-Rosa *et al.* 2014).

Previous reports have described a ZG-like histological appearance in *ATP1A1/2B3/CACNA1D* mutated tumors compared to a more ZF-like composition in *KCNJ5* mutated tumors (Azizan *et al.* 2013, Dekkers *et al.* 2014, Monticone *et al.* 2015). Also, high CYP11B2 and low CYP11B1 protein expression have been observed in these APAs (Azizan *et al.* 2013, Dekkers *et al.* 2014, Monticone *et al.* 2015). However, a large transcriptomic analysis of

Figure 6

Illustration depicting mutations in different voltage-dependent calcium channels $\alpha 1$ -subunits. Color indicates different disorders associated with each isoform and mutation (Ca_v1.3 is the product of *CACNA1D*). V728I, F747V, I750M and V1353M correspond to V748I, F767V, I770M and V1373M (NP_001122312, NP_000711). P1351R, V1353M and M1369I correspond to P1336R, V1338M and M1354I (NP_001122312, NP_001122311). Orange, malignant hyperthermia susceptibility; Light blue, hypokalemic periodic paralysis type 1; Green, timothy syndrome; Red, primary aldosteronism; Dark blue, congenital stationary blindness; Black dots, familial hemiplegic migraine; A circle with an arrow indicates a gain of function mutation shown by *in vitro* studies (Pietrobon 2002, Striessnig et al. 2010, Azizan et al. 2013, Scholl et al. 2013, Stockner & Koschak 2013, Fernandes-Rosa et al. 2014, Wang et al. 2015).

92 APAs did not detect any differences in CYP11B2 and CYP11B1 expression (Fernandes-Rosa et al. 2014). Neither did it detect a specific transcriptome signature (Boukroun et al. 2012) nor a specific histological appearance (Fernandes-Rosa et al. 2014). Our transcriptomic analysis revealed differences between *KCNJ5* mutated APAs and the other tumors. However, because of the low number of samples, our results should be interpreted with some consideration. Interestingly, of the two transcripts displaying higher expression in our *ATP1A1/2B3* mutated tumors, NPNT was previously described as a ZG marker and the second most upregulated transcript in

ATP1A1/CACNA1D mutated tumors compared to *KCNJ5* mutated tumors (Azizan et al. 2013). NPNT encodes nephronectin, an extracellular matrix protein important for kidney development (Linton et al. 2007). Immunohistochemistry verified nephronectin as a possible ZG cell marker and confirmed higher expression in *ATP1A1/2B3* mutated tumors compared to *KCNJ5* mutated tumors. We also confirmed previous reports of higher CYP11B2 expression in our *ATP1A1/2B3* mutated tumors. The lower CYP11B2 expression in APAs harboring *KCNJ5* mutations may indicate a relatively lower production of aldosterone per cell, possibly compensated by their larger size, explaining the similar serum levels of aldosterone in patients with *ATP1A1/2B3/CACNA1D* mutated tumors. Our results provide additional evidence that *KCNJ5* mutated APAs do display subtle differences in their molecular phenotype compared to *ATP1A1/2B3* mutated tumors (Azizan et al. 2012b, Azizan et al. 2013, Dekkers et al. 2014, Monticone et al. 2015). Perhaps these small variances reflect a different cellular origin of *KCNJ5* mutated APAs, but because of similar intracellular events (i.e., increased Ca²⁺) these remain subtle. Of note, most *KCNJ5* mutated APAs express markers of ZG cells such as DAB2 (Boukroun et al. 2010), making the notion of different cellular origins more complex.

A limitation of this study was that we re-sequenced selected regions of these genes, possibly lowering the true prevalence of mutations. The entire coding segments of *ATP1A1* and *ATP2B3* have previously been analyzed in a large cohort of APAs (Beuschlein et al. 2013), and the results suggest that mutations outside these areas are rare. Mutations in *CACNA1D* have been found in hotspot areas in multiple exons. We chose to sequence all areas in *CACNA1D* where mutations previously have been described in APAs, and also included areas where gain of function mutations have been observed in other disorders. We could not detect any mutations outside mutational hotspot areas. If a gain of function effect is the cause of these tumors, the chance of finding other prevalent mutational areas seems less likely but cannot be excluded. Also, we did not perform functional characterization of our novel mutations; however, due to the conservation in different organisms and their location, we infer an altered protein function.

In conclusion, somatic and germline mutations in regulators of the membrane potential and/or intracellular Ca²⁺ homeostasis occur frequently in APAs. Also, non mutated tumors display a similar mRNA signature as *ATP1A1/2B3* mutated tumors. This makes it tempting to speculate that additional proteins involved in this

regulatory pathway are important for the tumorigenesis. With improved understanding of these events, the possibility of more specific treatment and better diagnostics will increase.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-15-0321>.

Declaration of interests

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Author contributions: T Åkerström performed experiments, carried out statistical analysis and drafted the manuscript. T Åkerström, S Backman, H S Willenberg, K Cupisti, J Ip, A Moser, R Maharjan, B Robinson, K A Iwen, H Dralle, C D Volpe, M Bäckdahl, J Botling, P Ståhlberg, G Westin, M K Walz, H Lehnert, S Sidhu, J Zedenius, P Björklund and P Hellman participated in either the acquisition, analysis, or interpretation of data. All authors contributed with critical revision of the manuscript and all authors performed administrative, technical or material support. P Björklund and P Hellman obtained funding. T Åkerström and P Björklund designed the study. P Björklund supervised the study.

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