GENETICS IN ENDOCRINOLOGY

The expanding genetic horizon of primary aldosteronism

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Abstract

Aldosterone is the main mineralocorticoid hormone in humans and plays a key role in maintaining water and electrolyte homeostasis. Primary aldosteronism (PA), characterized by autonomous aldosterone overproduction by the adrenal glands, affects 6% of the general hypertensive population and can be either sporadic or familial. Aldosterone-producing adenoma (APA) and bilateral adrenal hyperplasia (BAH) are the two most frequent subtypes of sporadic PA and 4 forms of familial hyperaldosteronism (FH-I to FH-IV) have been identified. Over the last six years, the introduction of next-generation sequencing has significantly improved our understanding of the molecular mechanisms responsible for autonomous aldosterone overproduction in both sporadic and familial PA. Somatic mutations in four genes (KCNJ5, ATP1A1, ATP2B3 and CACNA1D), differently implicated in intracellular ion homeostasis, have been identified in nearly 60% of the sporadic APAs. Germline mutations in KCNJ5 and CACNA1H cause FH-III and FH-IV, respectively, while germline mutations in CACNA1D cause the rare PASNA syndrome, featuring primary aldosteronism seizures and neurological abnormalities. Further studies are warranted to identify the molecular mechanisms underlying BAH and FH-II, the most common forms of sporadic and familial PA whose molecular basis is yet to be uncovered.

Introduction

Aldosterone is the main mineralocorticoid hormone in humans and, under physiological conditions, its secretion is tightly regulated by angiotensin II, extracellular potassium and adrenocorticotropic hormone (ACTH) (1). Its principal site of action is the distal nephron, where it promotes sodium retention and potassium excretion, playing a key role in maintaining water and electrolyte homeostasis. The autonomous aldosterone overproduction by one or both adrenal glands is a clinical syndrome known as primary aldosteronism (PA), that can affect up to 6% of the general hypertensive population (2).
The molecular basis of GRA was elucidated by Lifton and coworkers in 1992 (9) and resides in the chimeric CYP11B1/CYP11B2 gene, resulting from a non-homologous crossing over on chromosome 8q24.3 between CYP11B1 gene encoding 11beta-hydroxylase and CYP11B2, encoding aldosterone synthase. The result is a chimeric enzyme, that can synthesize aldosterone under ACTH control, since it contains CYP11B1 regulatory sequences at 5′ and coding sequences from CYP11B2 at the 3′ (Fig. 1). Two residues (Gly288 and Ala320, Fig. 1) are necessary to retain aldosterone synthase activity and therefore in all the chimeric genes causing FH-I reported so far, the recombination break-point is comprised between CYP11B2 intron 2 and exon 4 (13). While aldosterone synthase expression is limited to the outer zona glomerulosa, the chimeric enzyme is expressed throughout the entire adrenal cortex. In the adrenal zona fasciculata, cortisol is available as substrate and the chimeric enzyme can catalyse its C-18 hydroxylation and C-18 oxidation, resulting in the production of the so-called ‘hybrid steroids’, 18OH-cortisol and 18-oxo cortisol (11). The hybrid steroids display weak mineralocorticoid activity (14, 15) but are a hallmark of the disease and, until the description of the chimeric gene, have been regarded as an essential diagnostic feature.

After the identification of the chimeric gene, and the subsequent introduction of the long-range polymerase chain reaction strategy for the amplification of the hybrid gene (16), the clinical spectrum of FH-I dramatically changed (Supplementary Table 1). Large kindreds were available for the easy and relatively inexpensive genetic testing, allowing to diagnose the disease in patients with mild hypertension or even in normotensive subjects (17) indicating that the disease can display a variable clinical phenotype. Moreover, a retrospective report from the International Registry for GRA showed that hypokalaemia is infrequent, and affected patients display an elevated prevalence of cerebrovascular events at young age (mainly haemorrhagic stroke, as a result of intracranial aneurysms rupture) (18) (Supplementary Table 1).

According to the Endocrine Society guideline, genetic testing for FH-I is appropriate in PA patients with a family history and/or early onset (<20 years) of PA or in case of strokes at a young age (19). Once the diagnosis has been established, a therapy with low doses of an exogenous glucocorticoid (such as dexamethasone 0.125–0.25 mg/day) should be started to suppress ACTH secretion and to avoid glucocorticoid-related adverse effects, adding a mineralocorticoid receptor antagonist should be considered (19).
Familial hyperaldosteronism type II

Familial hyperaldosteronism type II was reported for the first time as a novel, not glucocorticoid remediable, form of PA in Australia in 1991 (20). It is transmitted with an autosomal dominant pattern in most of the families, but the mode of inheritance is less certain in others. It is the most common form of FH (with a prevalence of 5% among patients with PA) (4) and can be due to either an APA or a BAH. There are no clinical or biochemical characteristics that allow to distinguish patients affected by FH-II from the ones affected by a sporadic form of PA (4). A linkage with 7p22 was reported in some kindreds from 3 continents (21), but notably, some families thought to be affected by FH-II where subsequently re-classified as FH-III (22). There is now general agreement that FH-II might be a heterogeneous group of genetic forms of PA whose molecular basis is yet to be elucidated.

The diagnosis is made when at least two first-degree members of the same family are affected by PA, and the other forms of FH have been excluded through available genetic tests (19). Due to its relatively high prevalence, the Endocrine Society guideline recommends that all hypertensive first-degree relatives of patients with PA should undergo screening test (19).

Familial hyperaldosteronism type III

The first case of FH-III dates back to 1959 (23), but the disorder was recognized as a distinct clinical entity with a peculiar clinical and biochemical phenotype solely in 2008 (24). The index case was a young boy affected by polyuria, polydipsia, nicturia, headache and severe hypertension (known since the age of 5) (23). PA was diagnosed on the basis of hypertension (maximum recorded reading of 300/190 mmHg), hypokalaemia (2.1–3.0 mEq/L), metabolic alkalosis and elevated average urinary aldosterone (67 μg per day, normal range 1–8 μg per day) (23) and at the age of 9 years, he underwent bilateral adrenalectomy. At histopathological examination, the removed adrenals were bilaterally enlarged, with nodular hyperplasia mainly of the zona fasciculata. The two daughters of the index case presented, at the age of 7 and 4 years, with a similar clinical and biochemical phenotype characterized by resistant hypertension, severe hypokalaemia (2.1–3.0 mEq/L), metabolic alkalosis and elevated plasma aldosterone levels (67 μg per day, normal range 1–8 μg per day) (23) and at the age of 9 years, he underwent bilateral adrenalectomy. At histopathological examination, the removed adrenals were bilaterally enlarged, with nodular hyperplasia mainly of the zona fasciculata. The two daughters of the index case presented, at the age of 7 and 4 years, with a similar clinical and biochemical phenotype characterized by resistant hypertension, severe hypokalaemia (1.8–1.9 mEq/L) and elevated plasma aldosterone levels (137–185 ng/dL) despite suppressed plasma renin activity (0.2–0.3 ng/ml/h) (24). Notably, both girls displayed extremely elevated levels of urinary hybrid steroids and FH-I was suspected, but two dexamethasone suppression tests did not confirm the
diagnosis. Surprisingly, blood pressure and aldosterone not only failed to be suppressed by dexamethasone administration, but also showed an unexpected and paradoxical increase (24). Similarly, in both patients, cortisol levels were not suppressed after dexamethasone administration, indicating a complete deregulation of adrenal cortex functioning. As for the father, bilateral adrenalectomy was required to obtain normalization of blood pressure and plasma potassium. In both cases, adrenal glands were markedly enlarged, with a complete loss of normal zonation (24, 25). Immunohistochemical staining and immunofluorescence studies for the main enzymes involved in cortisol and aldosterone biosynthesis revealed that aldosterone synthase is expressed throughout the entire adrenal cortex and frequently co-expressed with CYP17 (17α-hydroxylase), explaining the abnormally high production of hybrid steroids in these patients (25).

FH-III is transmitted as an autosomal dominant disease and its molecular basis was uncovered by Choi and coworkers in 2011 (26). Through NGS technology, the authors identified 2 recurrent heterozygous KCNJ5 somatic mutations (p.Gly151Arg and p.Leu168Arg) in a cohort of 22 sporadic APAs (26). The experimental evidences obtained from sporadic adenomas suggested that inherited mutations in KCNJ5 could cause FH-III and targeted sequencing of the gene revealed a germline p.Thr158Ala mutation that co-segregated with the disease (26). KCNJ5 is located on chromosome 11q24 and encodes the G protein-activated inward rectifier potassium channel 4, (GIRK4), which is expressed in adrenal zona glomerulosa (26, 27), where it contributes to maintain the cell membrane in a hyperpolarized state. The mutations disrupt the selectivity filter of the channel and are responsible for loss of ion selectivity, Na⁺ entry and cell membrane depolarization with subsequent opening of the voltage-gated Ca²⁺ channels (26, 28). The increase in intracellular Ca²⁺ activates the signalling cascade that leads to CYP11B2 expression and autonomous aldosterone overproduction (27).

FH-III (OMIM # 613677) is a rare condition, affecting <1% of patients with PA (7). To date, 6 KCNJ5 germline mutations associated with FH-III have been reported (Fig. 2), for a total of 12 families and 22 affected family members (29). Notably, none of the further reported cases displayed the peculiar hormonal phenotype described by Geller and coworkers (24). The majority of the patients presented with an early onset and severe form of PA, requiring bilateral adrenalectomy to control hypertension and hypokalaemia. However, the carriers of the p.Gly151Glu mutation (7 patients form 3 different families) (22, 30) displayed a favourable disease progress: only two of them underwent adrenalectomy (one had bilateral adrenalectomy and the other had 90% left adrenalectomy); none of the patients displayed adrenal hyperplasia at imaging. Similarly, the patient carrying the p.Tyr152Cys mutation displayed a less severe phenotype (31). Interestingly, a case of FH-III (due to the KCNJ5 p.Glu145Gln mutation) presenting with severe PA and typical Cushing’s syndrome has recently been reported in a Chinese boy (32). In vitro electrophysiological studies showed that the p.Gly151Glu substitution was associated with a particularly severe impairment of the channel functioning, with massive Na⁺ entry, osmotic shock and cell death. It has been postulated that this could at least partially account for the mild clinical presentation and the lack of adrenal hyperplasia observed in these patients (30).

According to the Endocrine Society guideline, testing for germline mutations in KCNJ5 causing FH-III is appropriate in very young patients with PA (19).

Familial hyperaldosteronism type IV

FH-IV is a familial form of primary aldosteronism (OMIM #617027) caused by germline mutations in the CACNA1H gene located on chromosome 16p13 (33) (Fig. 3, panel A) encoding the pore-forming α subunit of a T-type calcium channel, Cav3.2. CACNA1H is the second most expressed Ca²⁺ channel gene in the adrenal zona glomerulosa (8, 26), where it is activated at small depolarizing potentials (34). Through NGS analysis, a novel germline CACNA1H

![Germline mutations in GIRK4 associated with FH-III. FH-III-causing KCNJ5 germline mutations (black dots) are located near or within the selectivity filter of the GIRK4 channel. N indicates the N-terminus and C indicates the C-terminus.](Image 315x545 to 553x714)
mutation (p.Met1549Val) was identified in 5 out of 40 unrelated patients affected by hypertension and PA in childhood (33). The clinical presentation of the index cases was uniform, without any distinctive clinical or biochemical feature and normal appearing adrenal glands at CT scanning (33). Target sequencing of the CACNA1H gene in the family members of the index cases, allowed to identify 5 additional subjects carrying the p.Met1549Val mutation. Of note, two mutation carriers did not receive a diagnosis of early-onset hypertension and were normotensives as adults, suggesting an incomplete penetrance (33).

The in vitro electrophysiological characterization revealed that the p.Met1549Val CACNA1H displays loss of normal inactivation together with a shift of activation to more hyperpolarized potentials (33), alterations that are very likely to cause an increase in intracellular Ca²⁺ concentration in adrenal zona glomerulosa cells.
To elucidate the role of the mutation in autonomous aldosterone overproduction, the p.Met1549Val mutant channel was expressed in HAC15 adrenocortical cells, resulting in a 7.1-fold increase in CYP11B2 transcription and a 3.7-fold increase in aldosterone production compared to the cells expressing the wild-type channel (35).

Subsequently, four additional germline CACNA1D mutations were identified in patients with PA (p.Met1549Ile, p.Ser196Leu, p.Pro2083Leu and p.Val1951Glu) (36). The p.Met1549Ile substitution was a de novo event identified in a sporadic PA patient and both p.Ser196Leu and p.Pro2083Leu mutations were detected in pairs of brothers/sisters affected by PA. Interestingly, the p.Val1951Glu was identified in a patient affected by apparently sporadic APA who was cured by unilateral adrenalectomy (36). These data indicate that CACNA1H might represent a susceptibility gene for PA development that could present with a wide range of clinical phenotypes (36).

**PASNA syndrome**

PASNA (primary aldosteronism with seizures and neurologic abnormalities, OMIM #615474) is a clinical syndrome characterized by primary aldosteronism and neurological symptoms. It is caused by gain-of-function mutations in the CACNA1D gene located on the chromosome 3p14.3 (8) (Fig. 3, panel B), coding for the α1D subunit of a L-type voltage-gated calcium channel (Cav 1.3), which is expressed in adrenal zona glomerulosa cells. Electrophysiological in vitro studies showed that the mutations cause channel activation at less depolarized potentials and altered channel inactivation, with subsequent abnormal calcium signalling (8).

Two paediatric patients affected by PASNA syndrome due to de novo CACNA1D germline mutations (p.Gly403Asp and p.Ile770Met) have been reported (8). The index cases presented with early-onset severe hypertension, hypokalaemia and neurological manifestations, including seizures and cerebral palsy. In one of the two patients, blood pressure was successfully controlled by the calcium channel blocker amlodipine, raising the possibility that calcium channel blockers might represent a specific treatment for individuals affected by APAs carrying a CACNA1D somatic mutation.

Interestingly, a new missense CACNA1D germline mutation (p.Val104Leu) was identified in a patient affected by autism and epilepsy with a phenotype partially overlapping with that observed in patients with PASNA syndrome (37).

**Genetic of sporadic primary aldosteronism**

Until recently, genetic studies on sporadic PA were mainly focused on genetic variants potentially able to increase the susceptibility to the disease or affect the clinical phenotype, including CYP11B2, α-adducin and bradykinin B2 receptor polymorphisms (7). The introduction of NGS technology allowed the identification of aldosterone-stimulating somatic mutations in a significant proportion of sporadic APAs (7).

**Germline mutations in sporadic PA**

While the molecular determinants of autonomous aldosterone overproduction have been at least partially unravelled, the molecular basis of bilateral hyperaldosteronism and adrenal cell proliferation (in both APA and BAH) are still poorly elucidated. It was postulated by Choi and coworkers (26) that the intracellular calcium influx induced by KCNJ5 mutations, other than driving aldosterone secretion, might promote cell proliferation. However, subsequent studies demonstrated that the expression of mutant GIRK4 has a negative effect on HAC15 adrenocortical cells growth (28) suggesting that a second hit might be necessary for APA formation (38).

KCNJ5 sequencing in peripheral blood DNA from 251 patients affected by sporadic bilateral hyperaldosteronism revealed three heterozygous missense germline mutations. The mutations (p. p.Arg52His, p.Glu246Lys and p.Gly247Arg) are not associated with FH-III and are not located in proximity of the selectivity filter of the channel (39). Electrophysiological studies conducted in Xenopus oocytes showed that the expression of both the p.Arg52His and p.Glu246Lys substitutions resulted in cell membrane depolarization, while the p.Gly247Arg mutation did not alter the resting potential (39).

ARMC5 gene maps on 16p11 and encodes the armadillo repeat containing 5, whose function is currently unknown, but is likely to act as a tumour-suppressor gene. Somatic and germline mutations in ARMC5 are frequently found in macronodular adrenal hyperplasia and Cushing syndrome (40) and in a significant proportion of PA patients of African American descent (41). However, another study failed to confirm this association in patients of European ancestry (42). Similarly, a potential role of ARMC5 in FH-II has been recently ruled out (43).
**KCNJ5 somatic mutations**

Following the seminal report by Choi and coworkers (26), several centres from four continents investigated the prevalence of KCNJ5 somatic mutations in APAs. In the largest study conducted in a Western population, comprising 474 adrenal adenomas collected through the European Network for the Study of Adrenal Tumours (ENS@T), the prevalence of KCNJ5 mutations resulted to be 38% (44), while the largest study conducted in East Asia reported, in a cohort of 168 samples, a 78% prevalence (45). According to a recent meta-analysis including 13 studies for a total of 1,636 patients, the overall prevalence of KCNJ5 mutations is 43%, with wide variation across centres (46). The prevalence appears to be consistently higher in East Asian populations compared to Western populations, and also in those centres where strict criteria for adrenal vein sampling interpretation were used (47). Sequencing analysis allowed to identify 15 further KCNJ5 somatic mutation associated with sporadic unilateral PA (48).

Comprehensive clinical, biochemical and histopathological studies showed that the adenomas carrying KCNJ5 mutations are more prevalent in females than in males (46) and are associated with younger age at diagnosis and higher preoperative aldosterone levels (46). Moreover, adenomas carrying KCNJ5 mutations express lower levels of aldosterone synthase compared to APAs carrying mutations in ATP1A1, ATP2B3 or CACNA1D and are composed mainly of zona fasciculata-like cells (49) expressing CYP17A1 (50). These characteristics might at least partially account for the high amount of hybrid steroids detected in APA patients carrying KCNJ5 mutations (51) and, considering the high prevalence of KCNJ5 mutations in the East Asian patients, explain the potential diagnostic value of 18-oxocortisol in subtype differentiation in this specific subpopulation (52).

In vitro pharmacological studies showed that mutated GIRK4 exhibited a different pharmacological profile compared to the wild type, in particular, the calcium channel blocker verapamil strongly inhibits the p.Leu168Arg mutant channel, suggesting a potential therapeutic use of this drug (53). Even more surprisingly, mutant GIRK4, but not the wild-type channel, is effectively inhibited by a series of molecules belonging to the macrolide class of antibiotics and by synthetic derivatives lacking the antibiotic activity (54). In light of this recent finding, a murine model of PA due to a germline KCNJ5 mutation would be an extremely valuable tool for further pharmacological studies.

**ATP1A1 and ATP2B3 somatic mutations**

The application of the NGS technology to a series of sporadic KCNJ5 wild-type APAs led to the identification of four different somatic mutations affecting ATP1A1 (55, 56) and two different in-frame deletions of ATP2B3 (56). ATP1A1 is located on chromosome 1p21 and encodes the sodium/potassium-transporting ATPase subunit alpha-1, while the ATP2B3 gene is located on chromosome Xq28 and encodes the plasma membrane calcium-transporting ATPase 3. The sodium/potassium ATPase exchanges three cytoplasmic sodium ions for two extracellular potassium ions against the concentration gradient thus maintaining the resting membrane potential and the cellular excitability; Ca\(^{2+}\)-ATPase3 removes one cytosolic Ca\(^{2+}\) in exchange for two H\(^+\) and plays a key role in calcium homeostasis. In vitro studies showed that the mutant Na\(^+\)/K\(^+\) ATPase exhibits reduced K\(^+\) affinity and disturbed gating properties, resulting in lowered intracellular pH, but, surprisingly not in cell membrane depolarization (55, 57). On the contrary, the mutant Ca\(^{2+}\)-ATPase3 strongly depolarized the plasma membrane, as a consequence of a complete loss of its physiological pump function, that become permeable to cations, permitting Na\(^+\) and Ca\(^{2+}\) influx (58).

A total of 13 different ATP1A1 and 9 ATPB3 somatic mutations have been reported so far (48). ATP1A1 somatic mutations account for 5.3% of the sporadic APAs while ATP2B3 mutations have been identified in 1.7% of the samples (44).

**CACNA1D somatic mutations**

Since its original description (8, 56), a total of 31 different CACNA1D mutations have been reported (48), accounting for 9.3% of the sporadic APAs (44). APAs carrying CACNA1D mutations are composed mainly of zona-glomerulosa-like cells (49, 50) and are smaller compared with those with KCNJ5 or no mutations (44, 49). Accordingly, somatic CACNA1D mutations are the most frequent genetic alteration in CYP11B2-positive cortical micro-nodules in cross-sectional imagine-negative PA (59).

**CTNNB1 somatic mutations**

CTNNB1 gene is located on chromosome 3 and encodes β-catenin, which is part of a complex of proteins that constitute adherens junctions. β-catenin plays a key role in adrenocortical function: inactivation of β-catenin, using the Cre-loxP transgenic strategy, causes adrenal aplasia...
in newborn mice (60) while its constitutive activation in murine adrenal cortex results in increased aldosterone production (61). Similarly, mice lacking the WNT inhibitor SFRP2 display increased aldosterone production, supporting the evidence that SFRP2 downregulation in APAs is likely to cause WNT/β-catenin constitutive activation (62). Activating CTNNB1 mutations have been detected in both benign and malignant adrenocortical tumours (63). Somatic mutations in CTNNB1 have been identified in around 3% of sporadic APAs (64, 65) and have been associated to female gender and relatively large adenomas. APAs-associated CTNNB1 mutations are located on exon 3 (64, 65) and result in aberrant activation of Wnt signalling, by altering specific residues that are involved in β-catenin degradation.

Somatic mutations in aldosterone-producing cell clusters

The adrenal zona glomerulosa, composed of compact cells forming nests, is the exclusive site of CYP11B2 expression and aldosterone production (1); however, the APAs are composed mainly of zona-fasciculata-like cells (large cells with lipid-laden cytoplasm) and only less frequently display a zona-glomerulosa-like phenotype (49). Moreover, histological examination of the removed adrenal glands, following a diagnosis of unilateral PA, revealed significant heterogeneity in both the nodules and the adjacent adrenal cortex (49, 66).

The recent development of specific monoclonal antibodies able to distinguish between the highly homologous CYP11B1 and CYP11B2 allowed a more specific characterization of both normal adrenals and unilateral PA, opening a new scenario that goes beyond the classical view of adrenocortical zonation (10). Histological examination and immunohistochemical staining of adrenal specimens revealed the presence of subcapsular nests of adrenocortical cells extending in the zona fasciculata and strongly expressing CYP11B2, named aldosterone-producing cell clusters (APCCs) (67). The APCCs were found in both normal adrenals and in the cortex adjacent to an APA (68) and a significant proportion of them carries somatic mutations in ATP1A1 and CACNA1D genes (but not in KCNJ5), supporting the hypothesis that they might display autonomous aldosterone overproduction and progress to overt PA over time (68). In particular, APCCs are likely to progress to CT-negative PA, as suggested by the elevated prevalence of CACNA1D mutations in this particular subtype of PA (59), but less likely to CT-detectable adenomas (which more frequently harbour somatic mutations in KCNJ5).

Clinical correlates indicated that APCCs number and size increase with age, (69) paralleled by a progressive transition towards a discontinuous CYP11B2 expression pattern in older-age adrenal glands, which might account for the age-related changes in renin and aldosterone physiology (70).

Conclusions

The last six years have witnessed major advances in the field of both sporadic and familial PA. Three novel familial forms have been characterized and somatic mutations, altering intracellular ion homeostasis, drive aldosterone overproduction in around 60% of sporadic APAs. Notably, some of the somatic mutations have also been detected in APCCs, which might represent the precursors of CT-undetectable PA. In the near future, steroid profiling and targeted inhibition of mutated GIRK4 are very likely to change the classical clinical approach to patients affected by PA due to an aldosterone-producing adenoma.

References


48 Prada ETA, Burrello J, Reinecke M & Williams TA. Old and new concepts in the molecular pathogenesis of primary aldosteronism. Hypertension 2017 70 875–881. (https://doi.org/10.1161/HYPERTENSIONAHA.117.10111)


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Genetics of primary aldosteronism

S Monticone and others

European Journal of Endocrinology 2018

R111

Received 11 November 2017
Revised version received 16 December 2017
Accepted 18 January 2018


