Disordered CYP11B2 expression in Primary Aldosteronism

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ABSTRACT

Primary aldosteronism is the most common type of secondary hypertension affecting 6-10% of patients with primary hypertension. PA is mainly caused by unilateral hyperaldosteronism due to an aldosterone-producing adenoma, unilateral hyperplasia with or without micronodules or bilateral zona glomerulosa hyperplasias with or without macro or micronodules. The development of antibodies against the terminal enzyme of aldosterone biosynthesis (CYP11B2) has permitted the further characterization of normal adrenals and resected adrenals from patients with primary aldosteronism. Normal adrenals exhibit two different patterns of cellular expression of CYP11B2: young individuals display a relatively uniform expression of the enzyme throughout the *zona glomerulosa* while the adrenals of older individuals have dispersed CYP11B2-expressing cells but have more groups of cells called aldosterone-producing cell clusters. APAs exhibit different patterns of CYP11B2 staining that vary from uniform to homogeneous. There is also a proportion of cells within the APA that co-express different enzymes that are not normally co-expressed in normal individuals. Approximately 30% of patients with unilateral hyperaldosteronism do not have an APA, but either have an increased number of CYP11B2 expressing micronodules or hyperplasia of the zona glomerulosa.

INTRODUCTION

Primary aldosteronism (PA) is the most common form of secondary hypertension with a prevalence of 5-10% of patients with primary hypertension [1] and is associated with a significant increase in morbidity and mortality [2,3]. There are multiple forms of PA that present as sporadic cases and the two most common are aldosterone-producing adenomas (APA) and bilateral *zona glomerulosa* hyperplasia with micro- or with macronodules or with both micro- and macronodules, also called idiopathic hyperaldosteronism (IHA). Less common is unilateral *zona glomerulosa* hyperplasia with micro- and/or macronodules. Familial forms are rare and there are at least 4 different familial types of hyperaldosteronism which include Type 1 (also called glucocorticoid-suppressible aldosteronism) due to a crossover recombination of the promoter region and first exons of the *CYP11B1* gene and the late exons of the *CYP11B2* gene resulting in the production of aldosterone in the *zona fasciculata* (ZF) under ACTH control [4]. Type 2 is the most common form, but the genetic basis has not been elucidated, although there is a linkage to chromosome 7p22 in some families [5]. Type 3 is due to mutations in the *KCNJ5* gene encoding the GIRK4 potassium channel and alter the selectivity filter of the channel pore [6]. Type 4 is due to mutations in the *CACNA1H* gene encoding Cav3.2 a voltage activated calcium channel subunit [7].

The biosynthesis of aldosterone occurs in the adrenal *zona glomerulosa* (ZG) through a series of enzymatic reactions starting from cholesterol. Most of the enzymes involved in aldosterone biosynthesis are also expressed in the ZF but the terminal enzyme, CYP11B2, is only expressed in the ZG and CYP11B1 is only expressed in the ZF [8,9]. The expression and distinct distribution of these two enzymes is shared by multiple species including humans, rats, mice, hamsters and guinea pigs [8,10]. Some species, such as cows, sheep, pigs, dogs and bullfrogs, express only a single CYP11B enzyme [8,11]; despite this,, aldosterone biosynthesis is restricted to the ZG as in species with two distinctly distributed enzymes [12]. The

mechanisms by which aldosterone production is suppressed in the ZF in species with a single CYP11B enzyme is unclear. The human CYP11B2 and CYP11B1 are highly homologous at the DNA (95% in the coding region) and at the protein level (93%) [13]. The presence of CYP11B2 identifies the cells of the adrenal that produce aldosterone. In the human adrenal the ZF has two unique enzymes, the CYP17A1 and the CYP11B1 which are responsible for the synthesis of cortisol [8]. The first specific polyclonal antibodies against CYP11B1 and CYP11B2 were described by Nishimoto and were more suitable for low amplification immunohistochemistry [14]. Highly specific monoclonal antibodies were then described [15] and have been extensively used to define the immunohistochemistry of normal adrenals and of resected adrenals from patients with PA [16-19].

Zona glomerulosa in normal adrenals from rodents and humans. The rat adrenal has a clearly delineated zonation. The ZG (with CYP11B2 expression) comprises 4-6 layers of cells underneath the outer capsule that are separated from the ZF by a layer that comprises progenitor cells called the undifferentiated cell zone (without CYP11B1 or CYP11B2 expression) [20]. The number of cells in the ZG that express CYP11B2 depends on the degree and duration of stimulation by the renin-angiotensin system and on a normal salt diet about half the cells express CYP11B2 and they are scattered throughout the ZG [21]; a chronic low sodium diet increases the layers of cells and most cells express CYP11B2 [21]. The human adrenal does not have a similar clear-cut separation of the ZG and ZF and cells with CYP11B2 expression are present in scattered cells in the subcapsular region (Fig 1A) [22] and in clusters that have been called variously as aldosterone-producing cell clusters (APCC) [14,15], foci and megafoci depending on the size of the cell cluster [23] (Fig 1B). These clusters show strong, uniform immunoreactivity for CYP11B2 with a ZG morphology that extends into the ZF, with no expression of CYP11B1. Adrenals from individuals from 0-11 years show a clear layered zonation with CYP11B2 expression that occupies a significant portion of the ZG and in some

cases there is an unstained layer between ZG labeled CYP11B2 and ZF labeled CYP11B1 and no APCC are found [22]. This layered arrangement remodels with age with significant portions of the ZG displaying low CYP11B2 expression while the APCC numbers increase [22,24]. In rare cases a portion of the APCC toward the ZF show an apparent remodeling to CYP11B2 expressing cells with ZF phenotype [22]. This pattern has been found in some patients with PA [25], but no clinical data was available in this study of supposed normal individuals [22]. In addition, cells expressing CYP11B1, which define the ZF can reach the capsule in some areas [15].

Aldosterone-producing adenomas. A significant advance in the pathogenesis of APAs was the discovery of somatic mutations in the selectivity filter of the G protein activated inward rectifier potassium channel GIRK4 coded by the *KCNJ5* gene [6], which has been shown to be present in 35-70% of patients [26-28]. The higher percentage was found in individuals of east Asia [26,28,29]. Mutations in pumps including the sodium potassium ATPase alpha subunit 1 (*ATP1A1* gene), membrane calcium ATPase (*ATP2B3* gene) and the calcium channel subunit $Ca_v1.3$ (*CACNA1D* gene) were then described in other cases [7,30-32] and all together these mutations explain approximately 50-80% of cases of APA. Some cases of unilateral aldosterone hyperproduction have multiple nodules that express the CYP11B2 enzyme and can have different mutated channels or pumps within the same gland [16,33,34]. APCCs from normal adrenals have an incidence of *CACNA1D* and *ATP1A1* mutations as high as 30%, but APCCs with *KCNJ5* mutations have not been detected [17]. It is unclear if APCCs harboring the mutations can develop into an aldosterone-producing adenoma.

Immunohistochemistry in primary aldosteronism. Adrenal vein catheterization is used to determine which is the abnormal adrenal producing the excessive amount of aldosterone. In most cases, unilateral aldosterone production is produced by an APA usually greater than 0.7 cm in diameter that is visible by a computerized tomography scan. Many adrenals with a clear

adenoma frequently also have APCCs present in the hyperplastic ZG [35,36].In 30% of cases a microadenoma which is not visible by imaging techniques [37], unilateral ZG hyperplasia with or without micro- or macro-nodules [37] and rare cases of aldosterone-producing carcinomas which are of larger size can occur [16].

Large or small APAs can have two different phenotypic cell characteristics, more common are those with clear cells containing lipid droplets similar to ZF-type cells whereas other have more compact cells similar to ZG-type cells and a mixture of both types [38]. Some studies have correlated the cell type with the somatic mutation present in the APA and those with clear ZF cells tend to have *KCNJ5* mutations while those with *ATP1A1*, *ATP2B3* and *CACNA1D* mutations tend to be of the ZG type phenotype [31,38,39]. However other studies have not confirmed these results and although many APA carrying a *KCNJ5* mutation have a ZF cell phenotype almost an equal number have a mixture of ZG and ZF type cells [27,38].

Aldosterone production in patients with larger adenomas is generally higher than in those patients with smaller adenomas [40]. In the study by Ono et al [40], the tumor area of the group of larger adenomas was 9 times greater than the group of smaller adenomas but plasma aldosterone concentrations were only 2.0-2.5 times increased in the group of patients with the larger APA. This indicated that aldosterone production per cell was much greater from smaller adenomas, a suggestion that was supported by the higher immunoreactivity of CYP11B2 observed in the smaller group of tumors[40] . The number of CYP11B2 immunoreactive cells in the larger adenomas was highly variable with some adenomas displaying a relatively uniform expression of CYP11B2 (Fig 1C) compared to a heterogeneous expression of the enzyme in others with many cells that were immunoreactive negative (Fig 1D). The immunoreactivity of other enzymes including the CYP17A1 was lower in the smaller adenomas [40]. Outcomes after adrenalectomy for patients with smaller or larger APAs were similar between the two groups [40].

Many APAs exhibit an intratumoral heterogeneity of expressed enzymes that are normally specific to a distinct zone of the adrenal. In a recent study using double and triple immunofluorescence staining of APAs with antibodies against CYP11B2, CYP11B1 and CYP17A1, Nakamura *et al* [41] demonstrated that there are cells co-expressing the CYP11B2 and CYP11B1 (2.1%), CYP11B2 and CYP17A1 (0.6%), CYP11B1 and CYP17A1 (0.6%) and a smaller number of triple immunoreactive stained cells (0.14%). However, the proportions of the different immunofluorescent mixed cells were highly variable between adenomas. The presence of cells that co-express the CYP11B2 and CYP17A1 probably explains the increased secretion of the hybrid steroids 18-hydroxycortisol and 18-oxocortisol [42].

The increased use of AVS has enabled the diagnosis of unilateral aldosterone production in image negative patients that occurs in about 30% of patients with unilateral PA [43,44]. Immunohistochemistry studies of the resected adrenals from 32 patients with PA operated due to unilateral (or in 6 cases bilateral) production of aldosterone studied using CYP11B2 staining showed that 19 of those with an adenoma showed CYP11B2 staining in the adenoma, 1 patient with an adenoma and 3 cases of bilateral production of aldosterone with a unilateral adenoma, the adenoma did not stained for the CYP11B2 but had had multiple APCCs and 2 specimens had multiple micronodules with diffuse ZG hyperplasia staining for the CYP11B2 enzyme [35]. Of the 9 patients without a tumor on CT, 6 had unilateral aldosterone production and 3 were bilateral. Of the unilateral aldosterone producers, 3 had a microadenoma and 1 had multiple micronodules staining for the CYP11B2 [35]. Of the 3 showing no tumors, but bilateral production of aldosterone 2 had multiple APCCs and 1 diffuse hyperplasia [35]. In a recent study of 25 adrenals with histopathology of cross-sectional image negative hyperaldosteronism they were classified into two types 13 had multiple adrenocortical micronodules and 12 had diffuse hyperplasia of the zona glomerulosa [16]. Somatic mutations of aldosterone-driver genes were detected in 81% of CYP11B2-positive micronodules with 65% had mutations of the

CACNA1D gene, 8% in the *KCNJ5* gene and 4% in the *ATP1A1* and *ATP2B3* genes, but no mutations were found in the CYP11B2-positive non-nodular areas [16].

The possibility that the origin of an APA is from further differentiation of an APCC was recently postulated through the finding of cases where cells from an APCC expressing the CYP11B2 changed morphologically from a compact cell phenotype characteristic of the ZG to a clear cell phenotype resembling ZF cells and these have been called possible APCC-to-APA transitional lesions some of which had *KCNJ5* mutations [25].

Patients with APA or those with unilateral production of aldosterone have been the treated by unilateral adrenalectomy of the involved site with either cure or significant improvement of the hypertension and biochemical abnormalities of the PA and in fewer cases resulting in no improvement. As no standard criterial for defining surgical outcomes was accepted, a recent study aimed to create a consensus criteria for outcomes [45]. The PASO study involved an international panel of 31 experts from 28 centers using the Delphi method to reach consensus. Complete clinical success correcting the hypertensionwas obtained in 37% of 705 patients with wide variance (17-62%) and partial success in 47% (range of 35-66%). Complete biochemical success was seen in 94% of patients [45]. A distinction between an adenoma and a nodule is difficult histopathologically. The frequent presence of APCCs and complete contralateral suppression of aldosterone production in the contralateral adrenal let us before to postulate that many cases are of bilateral asymmetric hyperplasia with many of the ones described as an adenoma being instead a hyperplastic steroidogenically active nodule [46]. In summary, immunohistochemistry of the CYP11B2 enzyme that catalyzes last steps of aldosterone biosynthesis, has helped uncover a significant complexity in the histological features of the adrenals of patients with unilateral production of aldosterone. Whereas the normal adrenal has a very distinctive pattern of expression of steroidogenic enzymes in the different zones, many adenomas undergo a disordered expression of the various steroidogenic enzymes with the

appearance of hybrid cells expressing a mixture of these enzymes. The wide variation in histopathological features of the adenomas and concurrent presence of APCCs raise the possibility that most cases of unilateral production of aldosterone actually might represent bilateral asymmetric hyperplasia with nodules frequently due to the development of somatic aldosterone-driving mutations.

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Legend:

Figure 1. Immunohistochemical staining of adrenals with the CYP11B2 antibody. A. Normal adrenal from a young individual showing diffuse staining in the subcapsular area. B. Normal adrenal of an older individual showing an aldosterone-producing cell cluster. C. APA showing fairly uniform staining of the whole adenoma. C. APA showing uneven staining of the adenoma. D. Case of unilateral primary aldosteronism with multiple APCCs.

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Figure 1