

## Disordered CYP11B2 expression in Primary Aldosteronism

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## ABSTRACT

1  
2 Primary aldosteronism is the most common type of secondary hypertension affecting 6-10% of  
3 patients with primary hypertension. PA is mainly caused by unilateral hyperaldosteronism due  
4 to an aldosterone-producing adenoma, unilateral hyperplasia with or without micronodules or  
5 bilateral zona glomerulosa hyperplasias with or without macro or micronodules. The  
6 development of antibodies against the terminal enzyme of aldosterone biosynthesis (CYP11B2)  
7 has permitted the further characterization of normal adrenals and resected adrenals from  
8 patients with primary aldosteronism. Normal adrenals exhibit two different patterns of cellular  
9 expression of CYP11B2: young individuals display a relatively uniform expression of the  
10 enzyme throughout the *zona glomerulosa* while the adrenals of older individuals have dispersed  
11 CYP11B2-expressing cells but have more groups of cells called aldosterone-producing cell  
12 clusters. APAs exhibit different patterns of CYP11B2 staining that vary from uniform to  
13 homogeneous. There is also a proportion of cells within the APA that co-express different  
14 enzymes that are not normally co-expressed in normal individuals. Approximately 30% of  
15 patients with unilateral hyperaldosteronism do not have an APA, but either have an increased  
16 number of CYP11B2 expressing micronodules or hyperplasia of the zona glomerulosa.

17

## INTRODUCTION

18 Primary aldosteronism (PA) is the most common form of secondary hypertension with a  
19 prevalence of 5-10% of patients with primary hypertension [1] and is associated with a  
20 significant increase in morbidity and mortality [2,3]. There are multiple forms of PA that present  
21 as sporadic cases and the two most common are aldosterone-producing adenomas (APA) and  
22 bilateral *zona glomerulosa* hyperplasia with micro- or with macronodules or with both micro- and  
23 macronodules, also called idiopathic hyperaldosteronism (IHA). Less common is unilateral *zona*  
24 *glomerulosa* hyperplasia with micro- and/or macronodules. Familial forms are rare and there are  
25 at least 4 different familial types of hyperaldosteronism which include Type 1 (also called  
26 glucocorticoid-suppressible aldosteronism) due to a crossover recombination of the promoter  
27 region and first exons of the *CYP11B1* gene and the late exons of the *CYP11B2* gene resulting  
28 in the production of aldosterone in the *zona fasciculata* (ZF) under ACTH control [4]. Type 2 is  
29 the most common form, but the genetic basis has not been elucidated, although there is a  
30 linkage to chromosome 7p22 in some families [5]. Type 3 is due to mutations in the *KCNJ5*  
31 gene encoding the GIRK4 potassium channel and alter the selectivity filter of the channel pore  
32 [6]. Type 4 is due to mutations in the *CACNA1H* gene encoding Cav3.2 a voltage activated  
33 calcium channel subunit [7].

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

42 mechanisms by which aldosterone production is suppressed in the ZF in species with a single  
43 CYP11B enzyme is unclear. The human CYP11B2 and CYP11B1 are highly homologous at the  
44 DNA (95% in the coding region) and at the protein level (93%) [13]. The presence of CYP11B2  
45 identifies the cells of the adrenal that produce aldosterone. In the human adrenal the ZF has  
46 two unique enzymes, the CYP17A1 and the CYP11B1 which are responsible for the synthesis  
47 of cortisol [8]. The first specific polyclonal antibodies against CYP11B1 and CYP11B2 were  
48 described by Nishimoto and were more suitable for low amplification immunohistochemistry  
49 [14]. Highly specific monoclonal antibodies were then described [15] and have been extensively  
50 used to define the immunohistochemistry of normal adrenals and of resected adrenals from  
51 patients with PA [16-19].

52 *Zona glomerulosa* in normal adrenals from rodents and humans. The rat adrenal has a clearly  
53 delineated zonation. The ZG (with CYP11B2 expression) comprises 4-6 layers of cells  
54 underneath the outer capsule that are separated from the ZF by a layer that comprises  
55 progenitor cells called the undifferentiated cell zone (without CYP11B1 or CYP11B2 expression)  
56 [20]. The number of cells in the ZG that express CYP11B2 depends on the degree and duration  
57 of stimulation by the renin-angiotensin system and on a normal salt diet about half the cells  
58 express CYP11B2 and they are scattered throughout the ZG [21]; a chronic low sodium diet  
59 increases the layers of cells and most cells express CYP11B2 [21]. The human adrenal does  
60 not have a similar clear-cut separation of the ZG and ZF and cells with CYP11B2 expression  
61 are present in scattered cells in the subcapsular region (Fig 1A) [22] and in clusters that have  
62 been called variously as aldosterone-producing cell clusters (APCC) [14,15], foci and megafoci  
63 depending on the size of the cell cluster [23] (Fig 1B). These clusters show strong, uniform  
64 immunoreactivity for CYP11B2 with a ZG morphology that extends into the ZF, with no  
65 expression of CYP11B1. Adrenals from individuals from 0-11 years show a clear layered  
66 zonation with CYP11B2 expression that occupies a significant portion of the ZG and in some

67 cases there is an unstained layer between ZG labeled CYP11B2 and ZF labeled CYP11B1 and  
68 no APCC are found [22]. This layered arrangement remodels with age with significant portions  
69 of the ZG displaying low CYP11B2 expression while the APCC numbers increase [22,24]. In  
70 rare cases a portion of the APCC toward the ZF show an apparent remodeling to CYP11B2  
71 expressing cells with ZF phenotype [22]. This pattern has been found in some patients with PA  
72 [25], but no clinical data was available in this study of supposed normal individuals [22]. In  
73 addition, cells expressing CYP11B1, which define the ZF can reach the capsule in some areas  
74 [15].

75 Aldosterone-producing adenomas. A significant advance in the pathogenesis of APAs was the  
76 discovery of somatic mutations in the selectivity filter of the G protein activated inward rectifier  
77 potassium channel GIRK4 coded by the *KCNJ5* gene [6], which has been shown to be present  
78 in 35-70% of patients [26-28]. The higher percentage was found in individuals of east Asia  
79 [26,28,29]. Mutations in pumps including the sodium potassium ATPase alpha subunit 1  
80 (*ATP1A1* gene), membrane calcium ATPase (*ATP2B3* gene) and the calcium channel subunit  
81  $Ca_v1.3$  (*CACNA1D* gene) were then described in other cases [7,30-32] and all together these  
82 mutations explain approximately 50-80% of cases of APA. Some cases of unilateral aldosterone  
83 hyperproduction have multiple nodules that express the CYP11B2 enzyme and can have  
84 different mutated channels or pumps within the same gland [16,33,34]. APCCs from normal  
85 adrenals have an incidence of *CACNA1D* and *ATP1A1* mutations as high as 30%, but APCCs  
86 with *KCNJ5* mutations have not been detected [17]. It is unclear if APCCs harboring the  
87 mutations can develop into an aldosterone-producing adenoma.

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

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

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

104 Aldosterone production in patients with larger adenomas is generally higher than in those  
105 patients with smaller adenomas [40]. In the study by Ono et al [40], the tumor area of the group  
106 of larger adenomas was 9 times greater than the group of smaller adenomas but plasma  
107 aldosterone concentrations were only 2.0-2.5 times increased in the group of patients with the  
108 larger APA. This indicated that aldosterone production per cell was much greater from smaller  
109 adenomas, a suggestion that was supported by the higher immunoreactivity of CYP11B2  
110 observed in the smaller group of tumors [40]. The number of CYP11B2 immunoreactive cells in  
111 the larger adenomas was highly variable with some adenomas displaying a relatively uniform  
112 expression of CYP11B2 (Fig 1C) compared to a heterogeneous expression of the enzyme in  
113 others with many cells that were immunoreactive negative (Fig 1D). The immunoreactivity of  
114 other enzymes including the CYP17A1 was lower in the smaller adenomas [40]. Outcomes after  
115 adrenalectomy for patients with smaller or larger APAs were similar between the two groups  
116 [40].

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

126 The increased use of AVS has enabled the diagnosis of unilateral aldosterone production in  
127 image negative patients that occurs in about 30% of patients with unilateral PA [43,44].

128 Immunohistochemistry studies of the resected adrenals from 32 patients with PA operated due  
129 to unilateral (or in 6 cases bilateral) production of aldosterone studied using CYP11B2 staining  
130 showed that 19 of those with an adenoma showed CYP11B2 staining in the adenoma, 1 patient  
131 with an adenoma and 3 cases of bilateral production of aldosterone with a unilateral adenoma,  
132 the adenoma did not stained for the CYP11B2 but had had multiple APCCs and 2 specimens  
133 had multiple micronodules with diffuse ZG hyperplasia staining for the CYP11B2 enzyme [35].

134 Of the 9 patients without a tumor on CT, 6 had unilateral aldosterone production and 3 were  
135 bilateral. Of the unilateral aldosterone producers, 3 had a microadenoma and 1 had multiple  
136 micronodules staining for the CYP11B2 [35]. Of the 3 showing no tumors, but bilateral  
137 production of aldosterone 2 had multiple APCCs and 1 diffuse hyperplasia [35]. In a recent  
138 study of 25 adrenals with histopathology of cross-sectional image negative hyperaldosteronism  
139 they were classified into two types 13 had multiple adrenocortical micronodules and 12 had  
140 diffuse hyperplasia of the zona glomerulosa [16]. Somatic mutations of aldosterone-driver  
141 genes were detected in 81% of CYP11B2-positive micronodules with 65% had mutations of the

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

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

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



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

172

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185 Legend:

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

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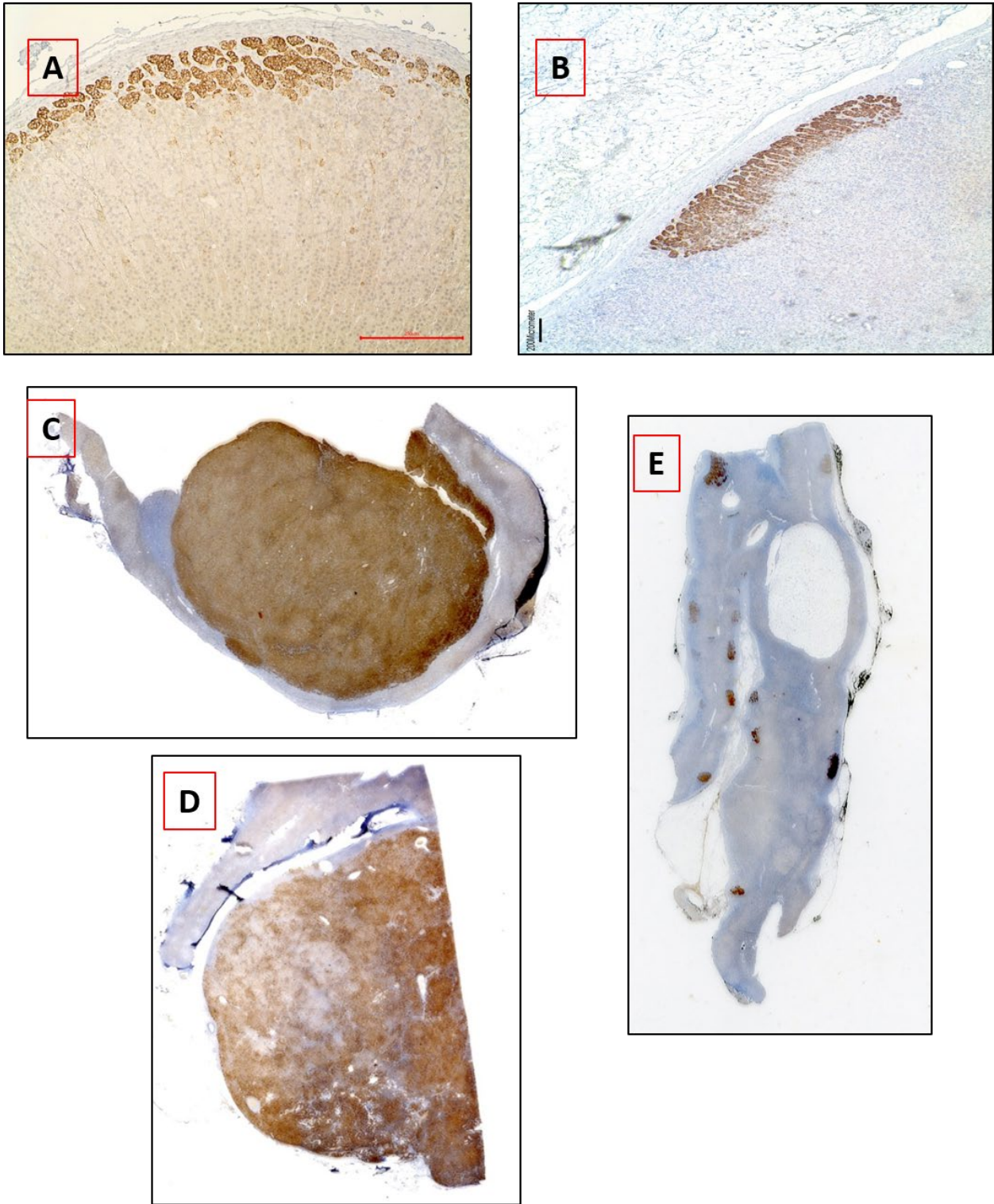
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369 **Figure 1**



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