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Disordered zonal and cellular CYP11B2 enzyme expression in familial hyperaldosteronism type 3

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ABSTRACT

Three forms of familial primary aldosteronism have been recognized. Familial Hyperaldosteronism type 1 (FH1) or dexamethasone suppressible hyperaldosteronism, FH2, the most common form of as yet unknown cause(s), and FH3. FH3 is due to activating mutations of the potassium channel gene *KCNJ5* that increase constitutive and angiotensin II-induced aldosterone synthesis. In this study we examined the cellular distribution of CYP11B2, CYP11B1, CYP17A1 and KCNJ5 in adrenals from two FH3 siblings using immunohistochemistry and immunofluorescence and obtained unexpected results. The adrenals were markedly enlarged with loss of zonation. CYP11B2 was expressed sporadically throughout the adrenal cortex. CYP11B2 was most often expressed by itself, relatively frequently with CYP17A1, and less frequently with CYP11B1. KCNJ5 was co-expressed with CYP11B2 and in some cells with CYP11B1. This aberrant co-expression of enzymes likely explains the abnormally high secretion rate of the hybrid steroid, 18-oxocortisol.

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1. Introduction

In 1953 the structure of a highly active mineralocorticoid isolated from the amorphous fraction of beef adrenal extracts (Grundy et al., 1952) was elucidated and named electrocortin (Simpson et al., 1953a). It was soon renamed aldosterone by the group of Simpson and Tait in an academic-industry collaboration (Simpson et al., 1953b). Just 2 years later Jerome Conn from the University of Michigan described the first clinical case of mineralocorticoid excess due to an aldosterone-producing adenoma (Conn, 1955) and the first description of primary hyperaldosteronism as hypertension, hypokalemia due to potassium wasting in the urine, and hypomagnesemia caused by an aldosterone-producing adrenal adenoma. Primary hyperaldosteronism is now known to have several additional causes including bilateral adrenal hyperplasia, unilateral adrenal hyperplasia, adrenal carcinoma, rare extra-adrenal tumors producing aldosterone, and three familial forms of primary aldosteronism (Mulatero et al., 2005). Familial Hyperaldosteronism type 1 (FH1) or Glucocorticoid-Remediable Aldosteronism, the best characterized, is due to a gene duplication resulting from the crossover recombination of the promoter region and first 4 exons of the 11β-hydroxylase (*Cyp11b1*) and the last exons of the aldosterone synthase (*Cyp11b2*) genes, resulting in a chimeric gene expressed in the zona fasciculata and regulated by ACTH that produces an enzyme that synthesizes aldosterone (Lifton et al., 1992). Familial hyperaldosteronism type 2 (FH2) is the most common FH. While the genetic basis remains unknown, many cases are in linkage with chromosome 7p22 (Stowasser et al., 1992; Lafferty et al., 2000). FH3 has been ascribed to an inherited mutation of the KCNJ5 gene disrupting the selectivity filter of the G-protein activated inward rectifying potassium channel Kir3.4 (Therien et al., 1959).

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1.1. Case reports

In 1959, a 10 year old boy was described with severe hypertension, hypokalemia, polyuria and marked increase in the urinary excretion of aldosterone (Therien et al., 1959). His severe hypertension could not be controlled with the anti-hypertensive medications available in that era, so he was subjected to bilateral adrenalectomy, resulting in marked improvement of the hypertension, hypokalemia and associated symptoms. His adrenal glands were very large, the right and left adrenals weighing 8.0 and 9.0 g, respectively, with focal nodular hyperplasia and cells of a zona fasciculata phenotype laden with lipid vacuoles (Therien et al., 1959). Twenty-six years later two of his daughters, ages 7 and 4 years, were found to have severe hypertension, hypokalemic alkalosis, suppressed renin activity, and marked serum aldosterone elevation (Geller et al., 2008). Administration of dexamethasone resulted in an increase in aldosterone levels, ruling out glucocorticoid-remediable aldosteronism. The patients were lost to follow up for 8 years and presented again with the same clinical manifestations and elevated blood pressure in spite of intensive antihypertensive therapy including spironolactone and/or amiloride and potassium supplements (Geller et al., 2008). The striking biochemical findings in addition to severe hyperaldosteronism, were marked elevations in the urinary excretion of the hybrid steroids 18-hydroxycortisol and 18-oxo-tetrahydrocortisol, with normal levels of other conventional cortisol metabolites (Geller et al., 2008). Because the patients' hypertension could not be controlled pharmacologically, including with spironolactone, they underwent bilateral adrenalectomy with normalization of the BP and serum potassium within 2 weeks. The adrenal glands were markedly enlarged with a combined left + right adrenal weight of 81 and 39 g (normal <12 g) and diffuse cortical hyperplasia (Geller et al., 2008).

1.2. Pathogenesis

A significant advance in our understanding of the pathogenesis of aldosterone-producing adenomas came from the identification of somatic mutations in or near the selectivity filter of the G-protein coupled potassium channel, Kir3.4 coded by the *KCNJ5* gene (Choi et al., 2011). Two mutations are most common, G151R and L168R. Sequencing of the *KCNJ5* gene in the family described above (Geller et al., 2008) revealed a heterozygous T158A mutation in the three affected members (Choi et al., 2011). This mutation lies between the selectivity filter and the second transmembrane domain of the Kir3.4 potassium channel and results in the loss of potassium selectivity and depolarization of the cell membrane (Choi et al., 2011). The latter initiates the signals that increase aldosterone synthesis.

We recently described the production of a highly selective mouse monoclonal antibody against the human CYP11B2 and a rat monoclonal antibody against the CYP11B1 enzyme. In this report we describe the adrenal distribution of staining of the CYP11B2 enzyme in the adrenals of the two patients described above and have done triple immunofluorescence of the CYP11B2, CYP11B1 and Kir3.4 potassium channel and triple immunofluorescence of the CYP11B1, CYP11B2 and 17α-hydroxylase enzyme.

2. Materials and methods

2.1. Immunohistochemistry

Slides were deparaffinized, subjected to antigen retrieval using a solution of EDTA 1 mM and SDS 0.05% pH 9 for 45 min in a steamer, blocked with 5% goat serum in Tris 0.1M and 0.5% SDS at pH 7.4, and immunostained with the mouse monoclonal anti-human CYP11B2-41-17C antibody (1/1000), CYB5 monoclonal antibody (Acris, San Diego) (in tris 0.1 M, tween 20 0.05% with goat serum 5%, as previously reported (Gomez-Sanchez et al., 2014). A slide was also stained with a specific monoclonal antibody against human HSD3B2 developed in our laboratory (unpublished). The slides were counterstained with hematoxylin.

2.2. Triple immunofluorescence

After deparaffinizing, antigen retrieval and blocking was done as described above, the slides were incubated overnight at 4C with a mixture of rat monoclonal anti human CYP11B1-80-7 (1/200), mouse monoclonal anti-human CYP11B2-41-17 (1/1000) (as previously documented (Gomez-Sanchez et al., 2014)), and rabbit anti-17α-hydroxylase (1/400) (Gell et al., 1998). The secondary antibodies were goat anti-mouse IgG H&L-Alexa 488, goat anti-rat IgG H&L-Alexa 594 and goat anti-rabbit-Alexa 647 (Jackson Immunoresearch Inc, Allentown, PA,USA) that have minimal cross-reactivity to mouse, human and rat immunoglobulins. Coverslips were mounted using Vector Laboratories Vectashield mounting media with DAPI (Vector Labs, Burlingame, CA, USA). Similar triple immunofluorescence was done with a KCNJ5 antibody instead of the 17α-hydroxylase antibody using a sheep antibody from EMD-Millipore (AB9808)(1/10,000) (Oki et al., 2012a) and processed as above. Immunofluorescence for the CYP17A1 (IgG2b), CyB5 (IgG1) and CYP11B2 (IgG1-biotin labeled) were done by incubating for the first two antibodies overnight, washing and incubating with specific goat anti-mouse IgG1-Alexa 488, goat anti-mouse IgG2b-Alexa 594 followed by washing and incubating for 30 min with mouse IgG, washing followed by incubation with mouse monoclonal CYP11B2-41-13B labeled with biotin for 1 h, washed and incubated with avidin-Oyster 650 for 30 min. After washing they were mounted with Vectashield with DAPI (Vector Laboratories, Burlingame, CA).

3. Results

Similar to the description of their father's adrenal (Geller et al., 2008), the adrenals of patients 1 (Fig. 1A) and 2 (Fig. 1B) stained with hematoxylin and eosin exhibited marked enlargement of the adrenal gland with complete loss of normal zonation composed primarily of lipid laden cells throughout the cortex and no clearly discernable zona glomerulosa type cells. The adrenals of patient 1, Fig. 1C, E, G, and that of patient 2, Fig. 1D, F, H have CYP11B2 immunoreactivity throughout the cortex in an irregular pattern. CYP11B2 immunoreactive cells are interspersed with cells not expressing the CYP11B2.

Fig. 2 shows immunofluorescence of the CYP11B1, CYP11B2 and KCNJ5: overlapped pictures show that cells immunoreactive for CYP11B1 and CYP11B2 are not only interspersed, but both enzymes are co-expressed in some cells. KCNJ5 was co-expressed with CYP11B2 and, in many cells, also with CYP11B1 in contrast to normal adrenals where KCNJ5 is expressed only in cells of the zona glomerulosa (Choi et al., 2011). Patient 2 showed similar co-expression and mingling of cells expressing CYP11B1 and CYP11B2, however in some areas there was greater co-expression of CYP11B1 with KCNJ5 than CYP11B2 with KCNJ5 (data not shown).

17α-Hydroxylase was expressed throughout the adrenal. As expected from normal adrenal zona fasciculata cells, it was expressed in all cells expressing CYP11B1, however, contrary to its normal expression pattern, 17α-hydroxylase was also found in a significant number of CYP11B2 expressing cells (Fig. 3). In some areas 17α-hydroxylase seemed to be preferentially expressed with CYP11B2. In some cells there was clear expression of the three

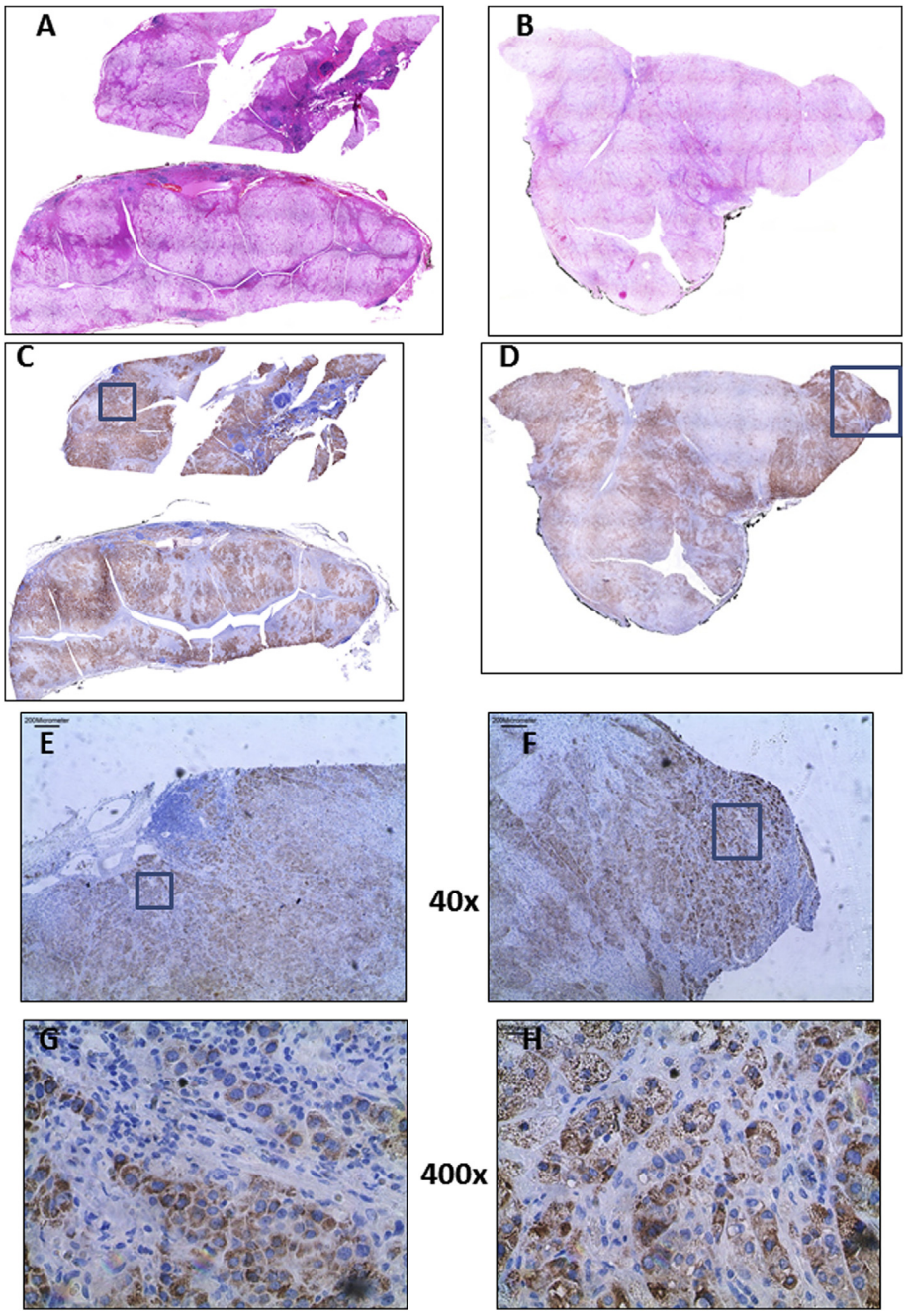

Fig. 1. Histology and immunohistochemistry of the adrenals of two patients with Familial Hyperaldosteronisms type 3 heterozygous for the KCNJ5-T158A mutation. Panels A, C, E, G: adrenal cortex from patient 1; Panels B, D, F and H adrenal cortex from patient 2. A & B: hematoxylin-eosin staining. C & D. CYP11B2 immunoreactivity throughout the cortex. E & G: sequentially higher magnifications of the boxes in panel C & E of patient 1. F & H: sequentially higher magnifications of the boxes in panel D & F of patient 2.

enzymes.

HSD3B2 was expressed strongly throughout the adrenal (Fig. 4A and C). The cytochrome B5 showed just a few small areas of staining (Fig. 4B and D). Immunofluorescence showed by in those areas CYB5 and CYP17A1 were co-expressed (Fig. 4E) and in small areas CYB5 was also co-expressed with CYP11B2 (Fig. 4F).

4. Discussion

Familial hyperaldosteronism type 3 is a rare form of hyperaldosteronism where various mutations of the potassium channel KCNJ5 cause the hypersecretion of aldosterone. The three members of the first family described had extremely severe hypertension and hypokalemia that was not controlled with high doses of spironolactone plus other antihypertensive agents and required a bilateral adrenalectomy for control of the hypertension and electrolyte abnormalities (Therien et al., 1959; Geller et al., 2008). The three patients were heterozygous for the T158A mutation of the *KCNJ5* gene.

The affected members of this family exhibited a marked increase in the secretion of aldosterone and 18-oxocortisol, but not of cortisol, and the secretion of aldosterone was not suppressed with the administration of dexamethasone (Geller et al., 2008). CYP17A1 is not normally expressed in the zona glomerulosa. It is co-

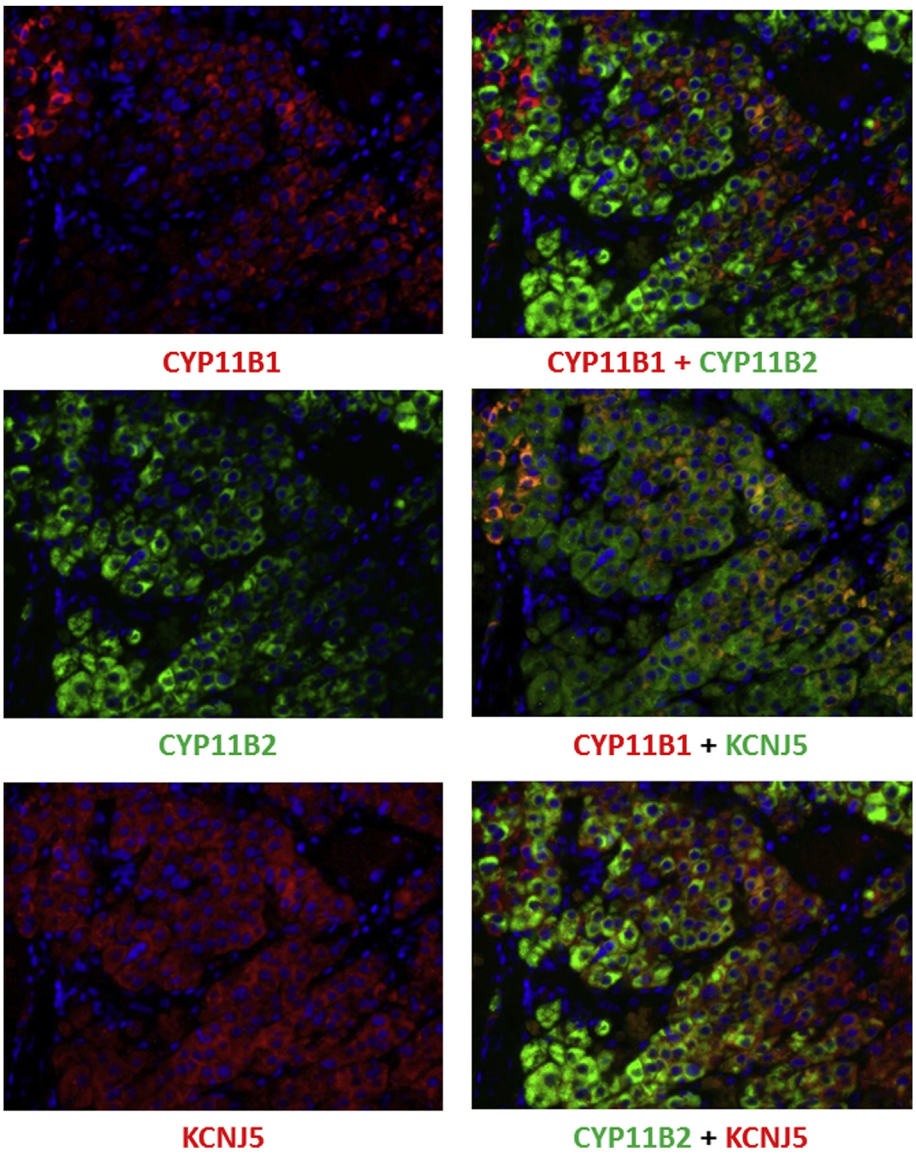

Fig. 2. Triple immunofluorescence of a section of the adrenal of patient 1 stained with the mouse monoclonal antibody against CYP11B2, rat monoclonal antibody against CYP11B1 and sheep polyclonal antibody against KCNJ5. The panels on the left show the individual staining and nuclear staining with DAPI. The right panels show simultaneous staining of nucleus with DAPI and the combination of antibodies as labeled. Co-expression of the steroidogenic enzymes is indicated by the orange color. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

expressed with CYP11B1 in normal adrenal zona fasciculata cell where their sequential action is required to synthesize cortisol. CYP11B2 is normally expressed only in the zona glomerulosa where it catalyzes the last steps in the synthesis of aldosterone (Gomez-Sanchez et al., 2014). The synthesis of the hybrid steroids 18-hydroxycortisol and 18-oxocortisol requires the sequential action of CYP17A1, then CYP11B2 (Ulick and Chu, 1982; Ulick et al., 1983). As the major blood flow in the adrenal gland is centripetal, from the zona glomerulosa, to the fasciculata, on through to the medulla, high concentrations of CYP17A1 products made downstream in the zona fasciculata do not normally become substrates for CYP11B2 directly. As in the normal adrenal there is no evidence of co-expression of these two enzymes, the synthesis of small amounts of 18-hydroxycortisol and 18-oxocortisol is thought to be by zona glomerulosa cells from circulating cortisol (Freel et al., 2004). In adrenal adenomas and in Familial Hyperaldosteronism type 1, there is a marked increase in the secretion of 18-oxocortisol (Ulick et al., 1983; Gomez-Sanchez et al., 1984; Mulatero et al., 2012a; Nakamura et al., 2011a). In the case of Familial Hyperaldosteronism type 1, the formation of the chimeric enzyme from the unequal crossing over of the promoter region and first exons of the CYP11B1 and the last exons of the CYP11B2 results in the expression of CYP11B2 in the zona fasciculata where CYP17A1 is also expressed and allows the expression of the hybrid steroids 18-hydroxycortisol and 18-oxocortisol (Lifton et al., 1992; Gomez-Sanchez et al., 1984). Co-expression of the CYP11B2, CYP11B1 and CYP17A1 enzymes, explains the increased production of the hybrid steroids in some aldosterone-producing adenomas (Nakamura et al., 2014). In this study, the CYP11B2 was widely but unevenly expressed throughout the grossly enlarged adrenal and some CYP11B2-positive cells co-expressed both the CYP11B1 and the CYP17A1 enzymes, explaining not only the marked increase in aldosterone, but the highest recorded plasma levels of 18-hydroxycortisol and 18-oxocortisol (Geller et al., 2008).

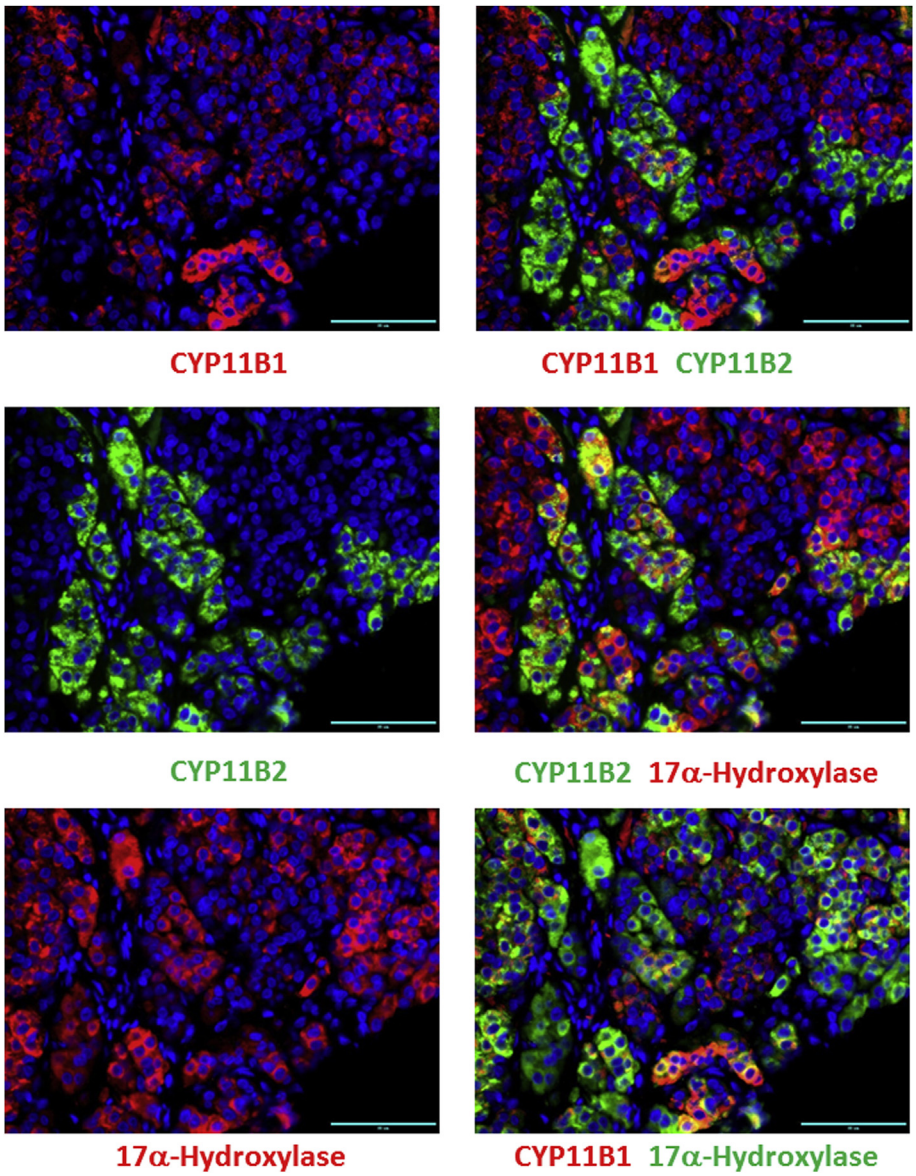

Fig. 3. Immunofluorescence staining of the adrenal of patient 1 with CYP11B1, CYP11B2 and CYP17A1. Panels on the left side are the individual immunoreactivity combined with nuclear staining with DAPI and right panels are overlap pictures of: Top right: CYP11B1 plus CYP11B2 which shows different cells staining for each enzyme and few cells showing co-expression of both enzymes. Middle right: CYP11B2 plus CYP17A1 showing separate cells staining of both enzymes in many cells and in a significant number of cells expressing both enzymes. Lower right panel: CYP11B1 and CYP17A1 showing immunoreactive for either enzyme or both in a significant number.

In vivo KCNJ5 is normally expressed only in the zona glomerulosa of the adrenal (Choi et al., 2011) The human adrenal carcinoma HAC15 cell expresses all of the enzymes required to synthesize both aldosterone and cortisol. Transduction with a lentivirus carrying the KCNJ5-T158A mutant resulted in the decrease in the selectivity of the channel, an increase in intracellular sodium, resulting in the mobilization of calcium signaling for a marked increase in the expression of the CYP11B2 enzyme and synthesis of aldosterone (Oki et al., 2012b). As HAC15 cells also express CYP11B1 and CYP17A1, it was not too surprising that the KCNJ5-T158A mutant HAC15 cells also produced significantly more of the hybrid steroid 18-oxocortisol. However the KCNJ5-T158A HAC15 cells also expressed more CYP11B1, in addition to CYP11B2, with no increase in StAR protein required to transport cholesterol in to the mitochondria, the initial step in steroidogenesis, or of other enzymes in the steroidogenic cascades to cortisol or aldosterone. CYP17A1 expression was decreased. While aldosterone production was greatly increased, cortisol was increased only slightly, albeit significantly, as was 18-oxocortisol, in the HAC15 expressing KCNJ5-T158A (Oki et al., 2012b).

KCNJ5 in the adrenals of these FH3 patients was co-expressed with CYP11B2, as expected, but was also abundantly expressed in cells expressing CYP11B1, most of which did not express detectable CYP11B2. The mechanism that normally limits KCNJ5 expression to zona glomerulosa cells is not known, but these findings suggest that it is not the same as that limiting CYP11B1 and CYP17A1 expression to the zona fasciculata. Nor does the expression of CYP11B2 appear to preclude the expression of CYP17A1. Unfortunately our studies in the HAC15 cells cannot answer these questions of zonal specificity of enzyme expression because they also co-express all of these enzymes. As these patients did not have hypercortisolism, the co-expression of the mutated KCNJ5 either had no effect on CYP11B1 expression, or, if CYP11B1 was increased in these patients' adrenal cells, their cortisol production remained

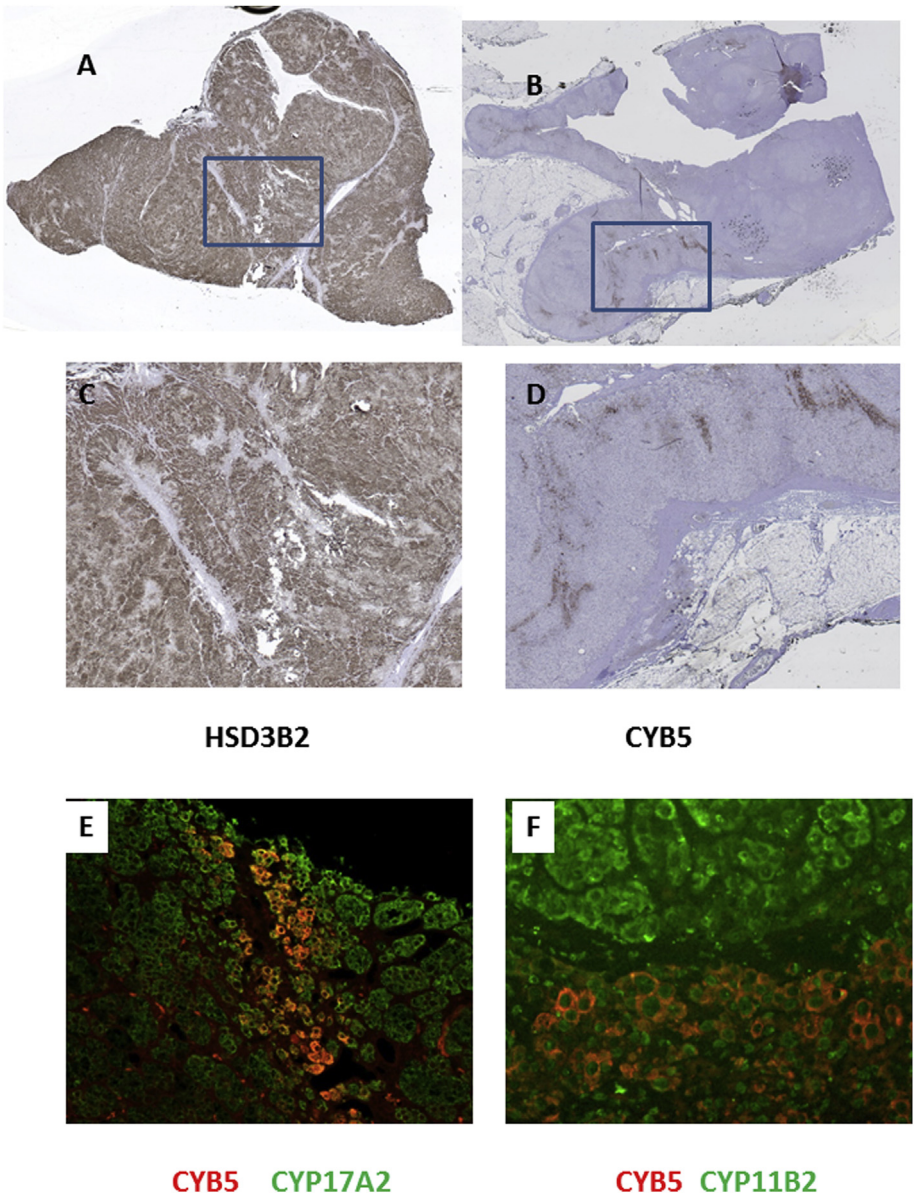

Fig. 4. A and C showed the adrenal of patient 2 stained for HSD3B2 enzyme. B and D showed the adrenal of patient 1 stained for the cytochrome B5. E shows double immunofluorescence of CYP17A1 and cytochrome B5 in the adrenal of patient 2. F shows double immunofluorescence of CYP11B2 and cytochrome B5 of the adrenal of patient 2.

normal due to the appropriate function of the HPA axis and stimulation of StAR protein by ACTH.

The KCNJ5-T158A mutation resulting in a decreased selectivity of the channel for potassium over sodium results in a very severe syndrome with a grossly enlarged adrenal (Therien et al., 1959). *In vitro* this mutation causes a slightly less alteration in the selectivity compared to other mutations found in FH3 patients including KCNJ5-G151E or G151R (Choi et al., 2011), (Scholl and Lifton, 2013; Scholl et al., 2012). However patients with these more severe KCNJ5 mutations have a milder form of primary aldosteronism probably due to a toxic effect of increased calcium mobilization resulting in an increase in adrenal cell apoptosis and lower mass of the adrenal (Scholl et al., 2012). Multiple families have now been described with germ line mutations of the *KCNJ5* gene including G151E (Scholl et al., 2012; Mulatero et al., 2012b), Y152C (Monticone et al., 2013a), I157S (Charmandari et al., 2012) and several other germ line mutations in patients with sporadic primary aldosteronism (Murthy et al., 2014). A similar picture of heterogeneous expression of the CYP11B2 in a patient with the KCNJ5 Y152C mutation was reported in the supplemental material of the paper of Monticone et al. (Monticone et al., 2013b), but with insufficient details to compare with these two patients. Patients with aldosterone-producing adenomas bearing a KCNJ5 mutation have been shown to have a high expression of CYP11B2, CYP11B1 and CYP17A1 enzymes (Azizan et al., 2012) and these patients as a group excrete high amounts of the hybrid steroids (Mulatero et al., 2012a). The co-expression of the CYP11B2 and the cytochrome B5 is very abnormal as the latter is only expressed in the zona reticularis (Nakamura et al., 2011b). It would be interesting to speculate that the co-expression of the CYP11B2, CYP17A1 (which is extensive in these adrenals) and the cytochrome *b*5 might result in the production of newer steroids that remain to be identified.

In summary, immunohistochemical analysis of the cellular distribution of steroidogenic enzymes unique to normal zona

glomerulosa or fasciculata cells in the grossly hyperplastic adrenal cortex of patients with hyperaldosteronism type 3 reveals the loss of zonation and mingling of cells that express CYP11B2 and/or CYP11B1, with and without CYP17A1, and some that express all three enzymes. KCNJ5 was abundantly expressed in almost all cells, including those that expressed CYP11B1 with no CYP11B2, suggesting that the mutation not only altered the potassium filter selectivity of the channel, but also altered a signal that designated specificity for CYP11B2-expressing cells, or that this family also has a second mutation that abolishes steroidogenic specialization by adrenal zonation.

Disclosures

The authors have nothing to disclose.

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