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# Adrenal CYP11B1/2 expression in primary aldosteronism: Immunohistochemical analysis using novel monoclonal antibodies

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ABSTRACT

CYP11B1 and CYP11B2 play pivotal roles in adrenocorticosteroids synthesis. We performed semi-quantitative immunohistochemical analysis in adrenals from patients with primary aldosteronism using these novel monoclonal antibodies. Clusters of cortical cells positive for CYP11B2 were detected in the zona glomerulosa (ZG) of normal adrenal gland (NA), idiopathic hyperaldosteronism (IHA) and the adjacent adrenal of aldosterone-producing adenoma (APA). In APA, heterogenous immunolocalization of CYP11B2 and diffuse immunoreactivity of CYP11B1were detected in tumor cells, respectively. The relative immunoreactivity of CYP11B2 in the ZG of adjacent adrenal of APA was significantly lower than that of NA, IHA and APA tumor cells, suggestive of suppressed aldosterone biosynthesis in these cells. These findings did indicate the regulatory mechanisms of aldosterone biosynthesis were different between normal/hyperplastic and neoplastic aldosterone producing cells in human adrenals. CYP11B2 immunoreactivity in the ZG could also serve as a potential immunohistochemical marker differentiating morphologically hyperplastic ZG of IHA and APA adjacent adrenal.



## 1. Introduction

Increasing number of the patients with primary aldosteronism (PA) has been recently detected using advanced diagnostic techniques and 6–10% of all hypertensive patients are currently considered to harbor PA (Funder et al., 2008). PA patients have an increased risk of developing cardiovascular events compared to matched patients with essential hypertension (Milliez et al., 2005). There are two main causes of PA: aldosterone-producing adenomas (APA) in 30–60% of patients and idiopathic hyperaldosteronism (IHA) in 30–70% of patients (Young, 2007). It has become pivotal to examine the regulation of aldosterone mechanism in these adrenal tissues because of different clinical approaches in these two conditions (Young, 2007).

The final steps in glucocorticoids and mineralocorticoids biosynthesis are catalyzed by two closely related mitochondrial enzymes: aldosterone synthase (CYP11B2) and 11β-hydroxylase (CYP11B1) (Fardella et al., 1996). In the normal adrenal gland (NA), CYP11B1 is the classical 11β-hydroxylase which converts 11-deoxycortisol to cortisol and deoxycorticosterone to corticosterone and is expressed only in the zona fasciculata (ZF) and zona reticularis (ZR) (Nishimoto et al., 2010). CYP11B2 is present only in the ZG, where its 11β-hydroxylase, 18-hydroxylase and

*Abbreviations:* CYP11B1, Cytochrome P450, family 11, subfamily B, polypeptide 1; CYP11B2, Cytochrome P450, family 11, subfamily B, polypeptide 2; CYP17, 17α-hydroxylase; NA, normal adrenal gland; IHA, idiopathic hyperaldosteronism; APA, aldosterone-producing adenoma; ZG, zona glomerulosa; ZF, zona fasciculate; ZR, zona reticularis; HSD3B, 3 beta hydroxysteroid dehydrogenase; PA, primary aldosteronism.

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18-methyl oxidase activities are required to convert deoxycorticosterone to aldosterone (Ogishima et al., 1991; Curnow et al., 1991; Kawamoto et al., 1992). Although CYP11B2 expression appears to be high in IHA and APA (Takeda et al., 1999; Miyamori et al., 2000; Fallo et al., 2002; Bassett et al., 2005), only descriptive low resolution studies using polyclonal antibody have been published (Nishimoto et al., 2010; Nanba et al., 2013).

We recently developed monoclonal antibodies against these proteins and reported their expression in human normal adrenal gland (Gomez-Sanchez et al., 2013). In this study, we used these novel rat anti-human CYP11B1 and mouse anti-human CYP11B2 monoclonal antibodies to quantify the expression of these steroidogenic enzymes in adrenals associated with PA.

## 2. Materials and methods

### 2.1. Human adrenals

Fifty cases of adrenocortical surgical pathology materials (10 NA, 9 IHA and 31 APA) were retrieved from surgical pathology files of Tohoku University Hospital (Sendai, Japan). The specimens had been fixed in 10% formalin and embedded in paraffin. The research protocol was approved by the ethics committee at Tohoku University Graduate School of Medicine (Sendai, Japan) (No. 2011-544).

### 2.2. Antibodies

Mouse monoclonal antibody for CYP11B2 and rat monoclonal antibody for CYP11B1 were recently developed in the laboratory of Dr. Gomez Sanchez (University of Mississippi Medical Center, Jackson, MS, USA) (Gomez-Sanchez et al., 2013). Rabbit polyclonal antibody against 3β-hydroxysteroid dehydrogenase/$\Delta^5$–$\Delta^4$ isomerase (HSD3B) was kindly provided by Dr. Mason (University of Edinburgh, Edinburgh, UK) (Suzuki et al., 2000). Rabbit polyclonal antibody against 17α-hydroxylase (CYP17) was previously described (Gell et al., 1998).

### 2.3. Triple immunofluorescence analysis

The slides were thoroughly de-paraffinized and placed in Trilogy™ solution (Cell Marque Corporation, Rocklin, CA, USA) in a steamer for 45 min. Nonspecific staining was blocked with Tris 0.1 M pH 7.4 with 5% goat serum and 0.5% SDS for 1 h. The slides were then incubated with mixture of mouse monoclonal anti-hCYP11B2-41-17B (1:100), rat monoclonal anti-hCYP11B1-80-2-2 (1:10) and rabbit polyclonal anti CYP17 antibody (1:600) overnight in Tris 0.1 M, 5% goat serum and 0.1% tween-20. Samples were thin incubated with second antibody: goat anti-mouse IgG-Alexa 488, goat anti-rat IgG Alexa 594 and goat anti-rabbit IgG Alexa 647 (Jackson Immunoresearch Inc. Allentown, PA, USA) at 1:500 each for an hour using the same buffer as above. Coverslips were mounted using Vector Laboratories Vectashield mounting media with DAPI (Vector Labs, Burlingame, CA, USA).

### 2.4. Single immunohistochemical staining analysis

For CYP11B1 and CYP11B2, immunostaining was performed using the ImmPRESS REAGENT (VECTOR, Burlingame, CA, USA). Antigen retrieval was performed by heating the glue-coated slides in an autoclave for 5 min in EDTA buffer (pH 9.0). Blocking was performed for 1 h using Blocking buffer (normal horse serum 2.5% with SDS 0.5% for CYP11B2 and normal goat serum 2.5% with SDS 0.5% for CYP11B1) at room temperature. For HSD3B, immunohistochemical analysis was performed employing the streptavidin–biotin amplification method using a Histofine Kit (Nichirei, Tokyo, Japan) without antigen retrieval. The dilutions of the primary antibodies used in this study were summarized as follows: 1:750 (CYP11B2), 1:200 (CYP11B1) and 1:2500 (HSD3B), respectively. The antigen–antibody complex was visualized with 3,3′-diaminobenzidine solution [1 mM 3,3′-diaminobenzidine, 50 mM Tris–HCl buffer (pH 7.6), and 0.006% $H_2O_2$] and counterstained with hematoxylin.

### 2.5. Immunohistochemical evaluation in single immunohistochemistry and statistical analysis

Five hundred parenchymal cells were evaluated in each corresponding region and the ratio of positive cells was subsequently obtained in relevant areas of the specimens, i.e., the ZG, ZF and ZR of NA, the ZG of IHA, the ZG of adjacent adrenal gland of APA and tumor lesion of APA.

Immunoreactivity was semi-quantitatively assessed using McCarty's H-scoring system, in which the percentage of stained cells is multiplied by a number, 0–3, reflecting the intensity of their immunopositivities (McCarty et al., 1985). The relative immunointensity of specific immunoreactivity was characterized as not present (0), weak but detectable above control (1+), distinct (2+), and very strong (3+) (Budwit-Novotny et al., 1986).

Statistical analysis was performed using R software version 3.0.2. and data from the quantification of CYP11B2 and HSD3B protein levels were evaluated in groups of 4 (NA, IHA, APA-adjacent and APA-tumor) using Mann–Whitney multiple comparison tests with significance level set to $\alpha = 0.05$; Bonferroni inequality was used to correct multiple comparison, with $0.05/6 = 0.0083$, which determined $P < 0.0083$ as the statistically significant value, as previously described by our group (Felizola et al., 2014a,b).

## 3. Results

### 3.1. Localization of CYP11B2 and CYP11B1 in triple immunofluorescence analysis

In NA, IHA and adjacent adrenal of APA cases, CYP11B1 immunoreactivity was diffusely detected in the ZF and ZR (data not shown) but not in the ZG, compatible with our recent report (Gomez-Sanchez et al., in press) (Fig. 1). The clusters of cortical cells positive for CYP11B2 were sporadically detected in the ZG of NA, IHA and adjacent adrenal of APA, where CYP17 and CYP11B1 were absent (Gomez-Sanchez et al., 2013) (Fig. 2). CYP11B2 immunoreactivity was relatively abundant in the ZG of IHA, while barely detectable in the histologically hyperplastic ZG of the adjacent adrenal of APA (Fig. 2).

In APA, CYP11B1 immunoreactivity was diffusely detected, while CYP11B2 immunoreactivity was heterogeneously present (Fig. 3A and B). In these areas, CYP11B1 and CYP17 were co-localized in the great majority of the tumor cells of APA, while CYP11B2 immunoreactivity focally present in the tumor cells double positive for CYP11B1 and CYP17 (Fig. 3A–C).

### 3.2. Immunohistochemical evaluation of adrenocortical tissues using H-scoring system

The representative immunohistochemical finding of NA, IHA, adjacent adrenal gland of APA and APA tumorous lesion were illustrated in Fig. 4A. Results were all consistent with those in triple immunofluorescence analysis. Semi-quantitative results using H-scoring system above were also summarized in Fig. 4B, C and Table 1. The range of relative levels for CYP11B2 and HSD3B immunoreactivity was identified in the ZG of NA, IHA and adjacent adrenal of APA, respectively (Table 1). In tumor cells of APA cases, the wide



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