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Original Article

Variegated distribution of CYP11B2 contributes to complex aldosterone production in aldosterone-producing adenoma

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Abstract: CYP11B2 is expressed in the zona glomerulosa (ZG) of the adrenal cortex. However, only a small portion of the ZG in the normal adrenal gland contributes to aldosterone production. To explore the remodeling of the adrenal cortex and the sources of aldosterone in aldosterone-producing adenoma (APA), we analyzed the expression of CYP11B1 and CYP11B2 in adenoma and paired peritumoral tissue by RT-q-PCR. We also examined distribution of CYP11B2 protein and transcript in the adrenal cortex by immunohistochemistry (IHC) and RNA *in situ* hybridization, respectively. Four cases were identified that showed lower transcriptional levels of CYP11B2 in APA compared to normal adjacent tissue. Two different types of cells, namely, zona glomerulosa-like (ZG-like) cells and zona fasciculate-like (ZF-like) cells, were identified in APA by hematoxylin-eosin-safran (HES) staining. IHC results showed that the proportions of CYP11B2-positive cells in APA ranged from 20.6% to 90.3%. Results from *in situ* hybridization were concordant with IHC. There were one or more CYP11B2-positive clusters or large nodules. These aldosterone-producing cell clusters (APCCs) were observed in the adrenal cortex of eight APAs. Among them, CYP11B2 expression in APA was lower than in adjacent adenoma tissue in 4 cases. This contributed to the higher CYP11B2 expression levels found in adjacent tissue. APCCs were observed in 8 out of 13 cases in the present study, suggesting that adrenal cortex remodeling based on the variegated distribution of CYP11B2 may be a common occurrence in APA. ZG-like cells in APA and subcapsule APCCs are the major source of aldosterone for APAs.

Keywords: *Primary aldosteronism*, aldosterone-producing adenoma, *aldosterone, CYP11B2*

Introduction

Histological examination and biochemical studies on steroids produced from isolated tissue fragments [1, 2] have shown that the adrenal cortex is divided into three zones-the zona glomerulosa (ZG), the zona fasciculata (ZF), and the zona reticularis (ZR).

In humans, the biosynthesis of cortisol and aldosterone depends on two key enzymes: 11 p-hydroxylase (CYP11B1) and aldosterone synthase (CYP11B2) [1, 3]. Initial studies suggested that CYP11B2 is expressed in the ZG of the adrenal cortex and that CYP11B1 is expressed in both the ZF and ZR [3, 4]. However, the exact distribution of CYP11B2 was unclear until the recent application of a human CYP11B2 antibody. In contrast to the continuous distribution of CYP11B2 in the ZG of rodents, Nishimoto *et al* [5] performed IHC using a novel human CYP11B2 antibody and showed that CYP11B2 is only sporadically detected in the ZG of humans. Consistent with Nishimoto, Aiba *et al* [6] also identified that the human ZG is morphologically atrophic in the normal adrenal cortex compared to that of other mammals, such as rats. In fact, only a small portion of the ZG in the normal adrenal gland contributes to aldosterone production [7].

In addition to conventional zonation, Nishimoto *et al* [5] described a variegated zonation consisting of subcapsular cell clusters expressing CYP11B2 instead of CYP17; they termed these aldosterone-producing cell clusters (APCCs). They also reported that the non-tumor region of

Table 1. Clinical data and genes expression of 13cases of APA

Case	Gender	Age (years)	Follow up (months)	Clinical outcome	APCCs (n)	CYP11B2 (+) area in APA (%)	Relative expression of genes in adenoma compared with adjacent tissue (folds = $2^{-\Delta\Delta ct}$)	
							CYP11B1	CYP11B2
1	F	37	4	Cure	0	90.3	0.082	518.396
2	M	55	6	Cure	1	85.2	0.778	8.566
3	M	40	6	Improvement	1	40.5	0.464	0.014
4	M	39	3	Cure	0	60.7	122.648	1476.066
5	F	27	4	Cure	1	50.8	1.052	2.044
6	F	50	12	Cure	1	75.1	0 .590	6.562
7	F	58	6	Uncontroll	3	25.9	0.080	0.120
8	F	59	11	Improvement	0	30.2	123.097	77.876
9	M	52	10	Cure	0	80.3	0.758	2.641
10	M	41	12	Improvement	1	70.8	0.390	1.467
11	F	54	9	Improvement	2	20.6	2.017	0.358
12	F	42	8	Uncontroll	2	40.9	4.903	0.057
13	F	57	10	Improvement	0	30.3	3.378	4.876

NOTES: APA: aldosterone-producing adenoma; APCCs: aldosterone-producing cell clunsters.

APA frequently contained one or more APCCs. Compared with normal adrenal tissue, the authors found that APCCs also exist at the ZG of the normal adrenal cortex [5].

Adrenal cortex remodeling associated with variegated expression of CYP11B2 [5] is not well characterized. Since APCCs are positive for CYP11B2, they may provide another source of aldosterone. Therefore, adrenal cortical remodeling makes the source of adrenal aldosterone under both physiological and pathological conditions more complex. In this study, we analyzed adrenal cortex remodeling and explored the main source of aldosterone for primary aldosteronism (PA).

Materials and methods

Patients and specimens

Data from thirteen patients diagnosed with PA with a supposed aldosterone-producing adenoma (APA) who underwent adrenalectomy at Xiamen University First Affiliated Hospital between January 2012 and October 2012 were retrospectively reviewed (**Table 1**).

The clinical diagnosis of PA was established by a history of hypertension with an abnormal screening test of an elevated plasma aldosterone to renin ratio (ARR), followed by one or two confirmatory tests [8]. Patients with evidence of adenoma confirmed pre-operatively by CT scan or by surgery and pathology were diagnosed as having APA [9]. Two normal adrenal glands harvested from two adult renal cell carcinoma patients that had undergone radical nephrectomy without a history of hypertension were also included in this study. The research protocol was approved by the ethics committee at Xiamen University First Affiliated Hospital.

Adrenal adenoma sections (1.0 cm×1.0 cm×0.3 cm), including paired adjacent tissue, were formalin-fixed and paraffin-embedded for immunohistochemistry and View RNA *in situ* hybridization analysis. After the adrenal medulla was dissected with a fine scalpel, all freshly harvested tissues were snap-frozen in liquid nitrogen and stored immediately at -70°C until further analysis.

Histological examination

For histological examination, hematoxylin-eosin-safran (HES) staining was performed, and all adrenal specimens were evaluated by two experienced pathologists. An adenoma was defined as a solitary nodule with a capsule or a well-demarcated border to the surrounding tissue. The cellular composition of APA was determined by HES staining.

Immunohistochemistry

Immunohistochemical staining was performed using the EnVision System according to the

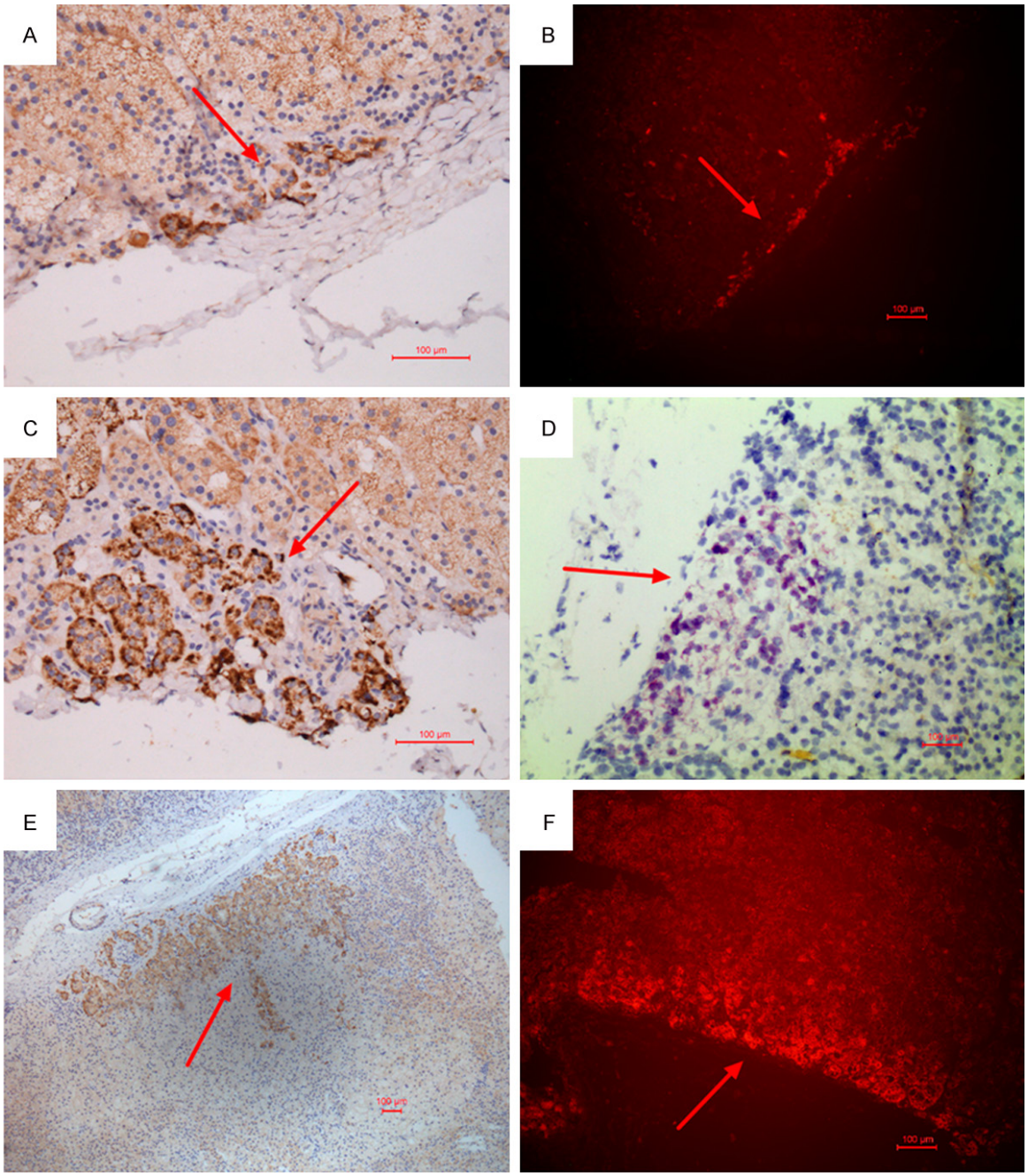

Figure 1. Distribution expression of CYP11B2. A: Segmental expression of CYP11B2 at normal adrenal zona glomerulosa (IHC 200×) (arrow); B: Segmental expression of CYP11B2 at normal adrenal zona glomerulosa (View RNA ISH 200× with fluorescence microscope) (arrow); C: Aldosterone-producing cells cluster (APCC) (IHC 200×) (arrow); D: Aldosterone-producing cells cluster (APCC) (View RNA ISH 200× with ordinary microscope, nucleus was light stained) (arrow); E: Aldosterone-producing cells cluster (APCC) (IHC 100×) (arrow); F: Aldosterone-producing cells cluster (APCC) (View RNA ISH 200×, with fluorescence microscope) (arrow).

manufacturer's instructions (Maxin Biotech, Fuzhou, China). In brief, tissue sections were deparaffinized in dimethyl benzene and dehydrated through graded alcohols (100%, 90%, 70%, and 50% alcohol; 5 min each). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 min. For antigen retrieval, sections were incubated in sodium citrate buffer (0.01 mol/L, pH 6.0) for 20 min in a household microwave oven. Next, endogenous peroxidases were inhibited by incubation in 3% hydrogen peroxide in water for 15 min, and nonspe-

cific staining was blocked using normal goat serum (Maxin, Fuzhou, China). Primary CYP-11B2 antibody (Catalog # AP11213c, Abgent, China) was diluted 1:150 and incubated overnight at 4°C. Secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG) was added according to the manufacturer's instructions. Detection was achieved by colorimetric reaction using diaminobenzidine chromogen solution (Maxin Biotech, Fuzhou, China), and all slides were counterstained with hematoxylin.

View RNA in situ hybridization (View RNA ISH)

CYP11B2 RNA was detected using the QuantiGene View RNA ISH Tissue Assay Kit (qvt0002) with specific FISH probes designed by Affymetrix. Microscopy and image acquisition were performed on a LEICA DM2500 microscope (LEICA, Germany) with 10, 20, or 40 × HCX PL FLUOTAR objective lenses and using a LEICA DFC420C camera and acquisition software (Leica Application Suite V3.5.0).

Quantitative real-time PCR

Expression levels of CYP11B1 and CYP11B2 were analyzed by real-time, quantitative PCR. Reverse transcription was performed with the PrimeScriptTM RT reagent Kit, using total RNA extracted with TaKaRa RNA isoReagent.

All real-time PCR reactions were performed using the Applied Biosystems Step-One Real-Time PCR System (Applied Biosystems, USA). Amplifications were performed using the SYBR Green PCR Master Mix (Applied Biosystems). PCR cycles were performed as follows: initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 10 s, and extension at 72°C for 30 s, and a final extension at 72°C for 5 min.

Real-time PCR primers for CYP11B1 were forward 5'-ctgggacattggtgcgc-3' and reverse 5'-gtgtttcagcacatggt-3'; for CYP11B2 were forward 5'-gggacattggtacagg-3' and reverse 5'-agttaatcgctctgaaagt-3'; for β-Actin were forward 5'-acaccccagccatgtacg-3' and reverse 5'-tggtggtgaagctgtagcc-3'; each primer pair has been previously described [10].

Statistical analysis

In all experiments, the relative gene expression was calculated by the *Ct* method. Briefly, the resultant mRNA was normalized to a calibrator; in each case, the calibrator chosen was the basal sample. Final results were expressed as n-fold difference in gene expression relative to β-Actin and calibrator as follows: n-fold = $2^{-(Ct\ \text{sample}-Ct\ \text{calibrator})}$, where *Ct* values of the sample and calibrator were determined by subtracting the average *Ct* value of the β-Actin gene from the average *Ct* value of the transcript under investigation for each sample.

Enumeration data were analyzed with Fisher's exact test to a significance level of P value < 0.05 using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, California, USA, www.graphpad.com).

Area Fraction analysis of CYP11B2-positive cells within APAs was carried out with Image J software from the National Institutes of Health (NIH; http://rsb.info.nih.gov/ij).

Results

Histological examination results

HES staining showed that APA was comprised of two different cell types: ZF-like and ZG-like cells (**Figure 2A**, **2B**).

Immunohistochemical results

Expression of CYP11B2 in normal ZG: CYP11B2 was expressed in the normal adrenal ZG in a sporadic, segmented manner (**Figure 1A**).

Expression of CYP11B2 in APA: Expression of CYP11B2 in APA was heterogeneous. Immunohistochemistry confirmed that ZG-like cells were positive for CYP11B2 expression, and that these were aldosterone-secreting cells; in contrast, ZF-like cells did not express CYP11B2 (**Figure 2C**, **2D**).

Expression of CYP11B2 in adenoma adjacent tissue: Clusters of CYP11B2-positive cells were identified by IHC in the subcapsule of the adrenal cortex (**Figure 1C**, **1E**). These cell clusters were ZG-like cells that were present as clusters or larger nodules. This was distinct from the pattern of segmental expression of CYP11B2 in the normal adrenal cortex (**Figure 1A**). Therefore, we hypothesized that these cell clusters are the same aldosterone-producing cell clusters (APCCs) described by Nishimoto *et al* [5]. Eight out of 13 cases in our group had one or more APCCs (**Table 1**).

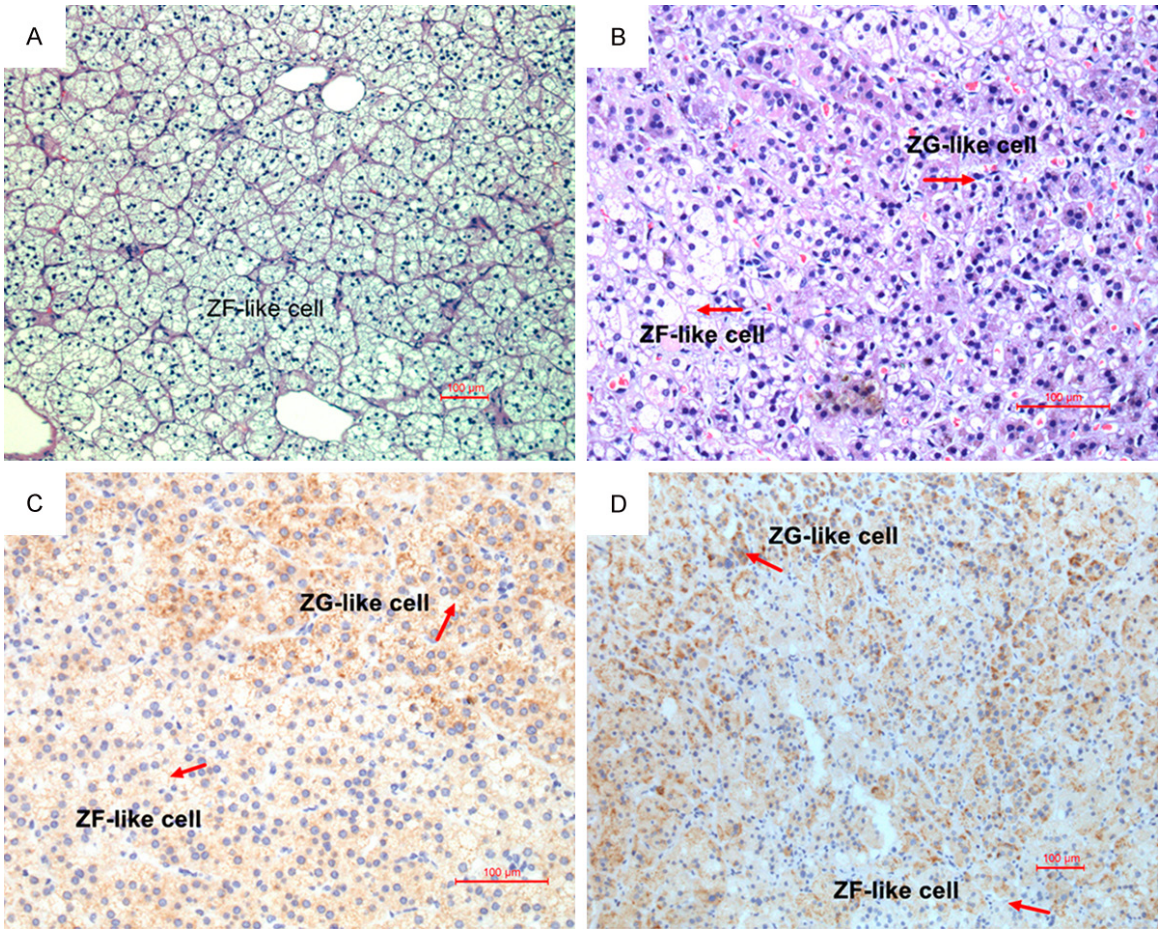

Figure 2. Cell composition of APA. A, B: ZG-like cells/ZF-like cells (HES 200×); C: ZG-like cells (40.5%), with CYP11B2 (+); ZF-like cells (59.5%), with CYP11B2 (-) (IHC 200×); D: ZG-like cells (80.3%), with CYP11B2 (+); ZF-like cells (19.7%), with CYP11B2 (-) (IHC 200×).

View RNA ISH results

Expression of CYP11B2 in normal ZG: Consistent with the IHC results, CYP11B2 transcript was expressed in the normal adrenal ZG in a sporadic, segmented fashion (**Figure 1B**).

Expression of CYP11B2 in adenoma adjacent tissue: Consistent with IHC data, clusters of CYP11B2-positive cells were identified by View RNA ISH in the subcapsule of the adrenal cortex (**Figure 1D**, **1F**).

Quantitative real-time PCR results

Heterogeneous expression of CYP11B1 and CYP11B2 was identified in APA compared to adjacent tissue (**Table 1**). We identified 4 cases with lower expression of CYP11B2 in adenoma compared to adjacent tissue. Five cases displayed CYP11B1 overexpression in APA.

Discussion

In the present study, we examined RNA and protein expression of CYP11B2 in the subcapsule of the adrenal cortex (**Figure 1C**, **1D**). The cell clusters we identified in this region were comprised of ZG-like cells. They were present as clusters or large nodules, which was in contrast to the segmented expression of CYP11B2 in the normal adrenal cortex (**Figure 1A**, **1B**). Therefore, we hypothesized that these cell clusters are the same aldosterone-producing cell clusters (APCCs) previously described by Nishimoto *et al.* [5]. Consistent with Nishimoto's results [5], eight out of thirteen cases in our group had one or more APCCs, suggesting that the occurrence of APCCs is a common phenomenon.

Relative expression of CYP11B2 was lower in APA than in adjacent tissue for 4 cases includ-

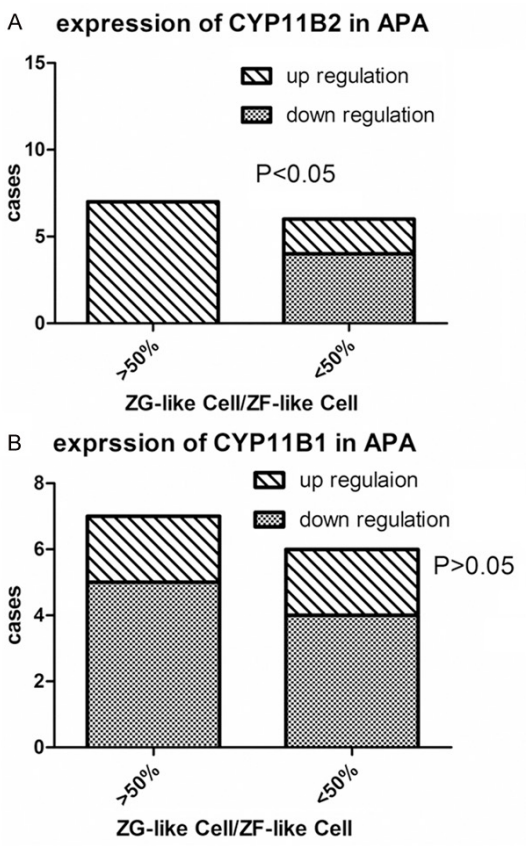

Figure 3. A: Association between proportion of ZG-like/ZF-like cells and the mRNA expression of CYP11B2 in APA. B: Association between proportion of ZG-like/ZF-like cells and the mRNA expression of CYP11B1 in APA.

Table 2. Phenotypes of APA

TYPE	CYP11B2 relative expression (APA/ adjacent tissue)	APCCs (No.)	Percentage
I	++	0	5/13 (38.4%)
II	+	1	4/13 (30.8%)
III	–	1~3	4/13 (30.8%)

ed in this study (cases 3, 7, 11, and 12; **Table 1**). Consistent with this, Lenzini *et al* [9] performed whole transcription comparative analysis of APA and found significantly decreased expression of CYP11B2 in 4 out of 16 APAs compared with the control group (normal adrenal cortex). The authors considered that despite the decreased expression of CYP11B2 in APA, the number of cells capable of aldosterone secretion in APA was actually more than in normal tissue [9]. It should be noted that in the Lenzini study, the authors compared CYP11B2 expression between APA and normal adrenal cortex tissue. In contrast, we compared expression of CYP11B2 between APA and its paired adjacent tissue. Therefore, it is likely that our results more accurately reflect the functional status of APA.

Factors leading to subtle changes in CYP11B2 expression in APA should be better characterized. We hypothesize that the decreased CYP11B2 expression in APA compared to adjacent tissue is due to APCCs contained within the adjacent tissue, which continuously express high levels of CYP11B2. This, in turn, contributes to lower expression of CYP11B2 in APA. These findings question the conventional view of aldosterone production from adenomas in APA.

We found that, in addition to CYP11B2, APAs also overexpress CYP11B1 (cases 4, 8, 11, 12, and 13; **Table 1**), which is consistent with earlier reports [9]. APAs rarely contain pure ZG-like cells. Instead, they often consist of both ZF- like and ZG-like cells [11-13] (**Figure 2A**, **2B**). Immunohistochemistry confirmed that ZG-like cells positively express CYP11B2 (**Figure 2C**, **2D**), and that these are the aldosterone-secreting cells. In contrast, ZF-like cells do not express CYP11B2. We speculate that ZF-like cells in APAs share the same function of cortisol secretion as ZF cells in the normal adrenal cortex. The relative abundance of ZF-like and ZG-like cells in APA contributes to the decreased expression of CYP11B2 in APA compared with adjacent tissue.

The proportion of the CYP11B2 (+) area was likely associated with the different expression of CYP11B1 and CYP11B2 in APA identified by RT-q-PCR. Overexpression of CYP11B2 was more apparent when ZG-like cells were the dominant cell type in APA (percentage of CYP11B2 (+) area >50%) ($P < 0.05$) (**Figure 3A**). In contrast, expression of CYP11B1 in APA was not affected by the proportion of ZG-like to ZF-like cells ($P>0.05$) (**Figure 3B**), suggesting a less important role for CYP11B1 (+) cells in APA.

We identified 3 phenotypes of APA based on the different expression of CYP11B2 in APA and the number of APCCs (**Table 2**). Type I exhibited significant overexpression of CYP11B2 in APA compared with adjacent tissue that did not con-

tain any APCCs; This is representative of the conventional view of APA. Type II displayed slight overexpression of CYP11B2 in APA compared with adjacent tissue containing only a single APCC. Type III displayed lower expression of CYP11B2 in APA compared to adjacent tissue with more than one APCC. In this study, 30.8% of cases (Type III) had a lower expression of CYP11B2, indicating that this was the most common phenotype.

Based on our findings, we propose that some APAs might actually be nonfunctional adenomas or adenomas with subclinical Cushing's syndrome (SCS). In fact, nonfunctional adenomas are quite common in adults [14, 15]. Individuals with SCS tend to express slightly increased levels of CYP11B1 [16]. Moreover, aldosterone-and cortisol-co-secreting adenomas are becoming more and more of a concern in patients with PA [17]. Para-adenoma tissues with prominent expression of CYP11B2 (either due to adrenal hyperplasia or APCCs) promote extra aldosterone secretion, resulting in a clinical diagnosis of PA. We believe that this phenotype of PA is actually more in line with primary aldosteronism caused by bilateral adrenal hyperplasia (BAH).

Interestingly, patients with lower expression of CYP11B2 had a longer duration of hypertension as well as decreased efficacy after surgical treatment [9]. These findings support the hypothesis that cases with lower expression of CYP11B2 in APA are more similar to another subtype of primary aldosteronism, namely, BAH. Two patients (cases 7 and 12; **Table 1**) with poor blood pressure control also had lower expression of CYP11B2 in their adenomas.

The main drawbacks of the present study include its small sample size and its lack of adrenal vein sampling tests (AVS) to diagnose APA. However, the diagnosis of APA was supported by both clinical characteristics and pathological results. Additionally, adrenal vein sampling tests may be misinterpreted due to cortisol production of APA; as such, caution must be exercised when interpreting results of AVS [18-20].

To the best of our knowledge, this is the first study to directly compare mRNA and protein expression of CYP11B2 between APA and paired adjacent tissue. We find that only a small portion of the ZG in the normal adrenal gland was CYP11B2-positive, which contributes to aldosterone production. Adrenal cortex remodeling based on the variegated distribution of CYP11B2 is common in PA. APA is heterogeneous and comprised of two main cell types: ZG-like cells and ZF-like cells. ZG-like cells are found in APAs and in APCCs with strong expression of CYP11B2; they are the major source of aldosterone for aldosterone-producing adenomas. The presence of APCCs and the diverse composition of ZF-like and ZG-like cells in APAs contribute to the complexity of the source of aldosterone.

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Disclosure of conflict of interest

None.

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