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Disorganized Steroidogenesis in Adrenocortical Carcinoma, a Case Study

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Abstract

Most adrenocortical carcinomas (ACCs) produce excessive amounts of steroid hormones including aldosterone, cortisol, and steroid precursors. However, aldosterone- and cortisol-producing cells in ACCs have not yet been immunohistochemically described. We present a case of ACC causing mild primary aldosteronism and subclinical Cushing's syndrome. Removal of the tumor cured both conditions. In order to examine the expression patterns of the steroidogenic enzymes responsible for adrenocortical hormone production, 10 tumor portions were immunohistochemically analyzed for aldosterone synthase (CYP11B2), 11 β -hydroxylase (CYP11B1, cortisol-synthesizing enzyme), 3 β -hydroxysteroid dehydrogenase (3 β HSD, upstream enzyme for both CYP11B2 and CYP11B1), and 17 α -hydroxylase/C17–20 lyase (CYP17, upstream enzyme for CYP11B1, but not for CYP11B2). CYP11B2, CYP11B1, and 3 β HSD were expressed sporadically, and their expression patterns varied significantly among the different

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Compliance with Ethical Standards

Conflict of Interest

None declared.

tumor portions examined. The expression of these enzymes was random and not associated with each other. CYP17 was expressed throughout the tumor, even in CYP11B2-positive cells. Small tumor cell populations were aldosterone- or cortisol-producing cells, as judged by 3 β HSD coinciding with either CYP11B2 or CYP11B1, respectively. These results suggest that the tumor produced limited amounts of aldosterone and cortisol due to the lack of the coordinated expression of steroidogenic enzymes, which led to mild clinical expression in this case. We delineated the expression patterns of steroidogenic enzymes in ACC. The coordinated expression of steroidogenic enzymes in normal and adenoma cells was disturbed in ACC cells, resulting in the inefficient production of steroid hormones in relation to the large tumor volume.

Keywords

Adrenocortical carcinoma; Aldosterone synthase; Primary aldosteronism; 11 β -hydroxylase; Subclinical Cushing's syndrome

Introduction

Adrenocortical carcinomas (ACC) frequently produce adrenocortical hormones including cortisol, early steroid precursors and, to a lesser extent, aldosterone [1]. Nishimoto et al. previously performed immunohistochemical examinations on formalin-fixed paraffin-embedded (FFPE) adrenal sections for human 11 β -hydroxylase (CYP11B1, a cortisol-synthesizing enzyme) and aldosterone synthase (CYP11B2) [2]. The expression patterns of these enzymes in a normal adrenal gland, aldosterone-producing adenoma (APA), and cortisol-producing adenoma (CPA) were described. In the zona glomerulosa (ZG) and APA, CYP11B2-positive cells co-express 3 β -hydroxysteroid dehydrogenase (3 β HSD), an enzyme upstream of CYP11B2 in the aldosterone synthetic pathway [2]. In the zona fasciculata (ZF), APA, and CPA, CYP11B1-positive cells co-express 3 β HSD and 17 α -hydroxylase/C17–20 lyase (CYP17), both of which are enzymes upstream of CYP11B1 in the cortisol synthetic pathway [3]. Gomez-Sanchez et al. recently developed monoclonal antibodies for human CYP11B2 and CYP11B1 [4]. Despite advances in adrenocortical pathohistology, the distribution of cortisol- or aldosterone-producing cells in ACC has not yet been fully described. We herein performed immunohistochemical analyses for CYP11B1, CYP11B2, 3 β HSD, and CYP17 in a case of ACC that presented with subclinical Cushing's syndrome (SCS) and mild primary aldosteronism (PA).

Methods

Ethics

The molecular studies in the current case report were approved by the Medical Ethics Committee of the School of Medicine, Keio University (approval#: 20090018).

Immunohistochemistry for CYP11B1, CYP11B2, 3 β HSD, and CYP17

Sections from archival FFPE surgical specimens of the case were immunostained using a mouse monoclonal anti-human CYP11B2 antibody [4], rat monoclonal anti-human CYP11B1 antibody [4], mouse monoclonal anti-human CYP17 antibody which prepared as

described below, and polyclonal rabbit anti-human 3 β HSD antibody (a gift from Dr. Takeshi Yamazaki at Hiroshima University) [2]. Single staining for CYP11B2, CYP11B1, 3 β HSD, and CYP17 was performed as previously reported [2, 5], in which the nucleus was counterstained by hematoxylin. Double staining for CYP11B2 with 3,3'-diaminobenzidine (brown) and 3 β HSD with 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetra-zolium (blue) was performed as previously reported [2], and the nucleus was not counterstained.

Mouse Monoclonal Antibody Preparation Against the Human CYP17 Enzyme

Six-week-old Swiss-Webster female mice were initially immunized intraperitoneally with a mixture of 50 μ g of the plasmid pcDNA3.1-hCYP17A and 10 μ g of a poly(I:C) HMW adjuvant (Cat. Code tlr-pic, Invivogen.com, San Diego, CA), followed by subcutaneous immunization at multiple sites with 10 μ g of recombinant human CYP17 in complete AdjuLite Freund's adjuvant (catalog#: A5001, Pacific Immunology Corp, Ramona, CA) (total volume 0.1 ml) and 4 weeks later with the recombinant CYP17 enzyme in incomplete AdjuLite Freund's adjuvant (catalog#: A5002, Pacific Immunology Corp, Ramona, CA). Four weeks after the final immunization, animals were intraperitoneally injected under isoflurane anesthesia with 10 μ g of the recombinant enzyme, and blood and the spleen were obtained under isoflurane anesthesia 3 days later.

The spleen cells from the animal with the highest titer to CYP17, as determined by an enzyme-linked immunosorbent assay (ELISA) using plates coated with 20 ng/0.1 ml of recombinant enzyme/well in 1 M sodium chloride and 0.05 M sodium phosphate buffer (pH 7.4), were selected for fusion. Spleen cells were fused to mouse myeloma SP2-mIL6-hIL21 cells [SP2 cells from ATCC (Manassas, VA) were transduced with the retrovirus pMSCV-mIL6-puro (kindly provided by Dr. Scott K. Dessain from Thomas Jefferson University), selected with 5 μ g/ml, and then transduced with the lentivirus p6NST50-hIL21-IRES-GFPzeocin (created by cloning a plasmid from DNASU.org, HsCD00288055 into p6NST50-MCS-GFPzeocin, which was kindly provided by Dr. Monika Valink from the Institute of Anatomy, Medical Faculty Carl Gustav Carus in Dresden, Germany, and selected with 0.5 mg/ml of zeocin)] and cultured in Iscove's media (I7633, Sigmaaldrich.com) with 15 % Fetal Clone I sera (Hyclone, Provo, UT) with HAT (H0262, Sigmaaldrich.com) and 10 % of conditioned media from the same myeloma cell line (1). Clones were screened after 2 weeks using ELISA, and those exhibiting positivity were then subjected to a Western blot analysis using an extract from H293TN cells transfected with the plasmid pcDNA3.1-CYP17A (kindly provided by Dr. Richard Auchus at the University of Texas, Southwestern Medical School). A clone (isotype IgG2b) that gave a single band was then used for immunohistochemistry on normal human adrenal glands and stained the ZF and ZR only.

Positive Cell Area to Total Area (PCA/TA) Measurement

PCA/TA was measured as follows: (1) High resolution images (2400 dpi) of immunostained sections for CYP11B2, CYP11B1, and 3 β HSD were captured using a scanner machine. Positive cell area (PCA) was isolated using Colour Deconvolution Software [6], and PCA was measured using ImageJ software at the same threshold. Each section was traced using Adobe Photoshop CS6 Extended software and the traced areas were measured by ImageJ software (total area, TA). PCA/TA was calculated as PCA divided by the corresponding TA.

Mitotic Cell Count

Five sites of CYP11B2-positive area, CYP11B1-positive area, 3 β HSD-positive area, and area negative for these enzymes (black circles in Figs. 3, 4, 5, and 6, respectively) were selected by KN. Mitotic cells were counted in 5 microscopic high power field of each site by three pathologists (YF, MA, and TY). The average values of these counts were used for statistical analysis.

DNA and RNA Isolation from FFPE Tissues, cDNA Generation from RNA, and a Quantitative Real-Time Polymerase Chain Reaction (qPCR) Analysis Using cDNA

Whole FFPE adrenocortical tissues including connective tissue were scraped out from the glass slides. RNAs were isolated from these tissues using the Qiagen Allprep FFPE DNA/RNA kit (catalog#: 80234, Qiagen), according to the manufacturer's instructions. The isolation protocol was modified by extending the xylene incubation to 5 min, centrifugation during deparaffinization to 5 min, and eluting in a volume of 30 μ l. cDNA samples were generated from RNA using the High-Capacity cDNA Reverse Transcription Kit (catalog#: 4368814, Thermo Fisher Scientific). cDNAs were used in the qPCR analysis of CYP11B2 and the 18S ribosomal RNA gene with the primer/TaqMan probe mix for *CYP11B2* [2] and TaqMan ribosomal RNA control reagents (catalog#: 4308329, Thermo Fisher Scientific).

Statistical Analysis

Relationships between values having a non-normal distribution were analyzed by Spearman's rank-order correlation. Non-normal distribution values were compared by a Kruskal-Wallis one-way analysis of variance on ranks. In these analyses, a *p* value <0.05 was considered to be significant.

Case Report

A 37-year-old Japanese woman was referred to the Juntendo University Hospital (JUH) with a large adrenal tumor. One year before the initial visit to JUH, the tumor was detected by ultrasound and was 7.2 cm in diameter; however, she underwent no further evaluation because of her pregnancy, which ended in a normal birth. In the initial visit to JUH, although her appearance was normal with a height of 155.9 cm, weight of 56.0 kg (body mass index, 23 kg/m²), and no overt signs of Cushing's syndrome, mild hypertension (140/88 mmHg) was noted. Computed tomography (data not shown) and contrast-enhanced magnetic resonance imaging (Fig. 1) revealed an enlarged, heterogeneous adrenal tumor (12 cm in diameter) without detectable metastatic lesions. Blood tests, including her plasma cortisol concentration (PCC, 7.4 μ g/dl [normal range, 5.1–23.6 μ g/dl]), were normal, except for a low serum potassium level (3.2 [3.5–5.0] mEq/l), low serum adrenocorticotrophic hormone level (ACTH, <1.0 [7.2–63.3] pg/ml), and high plasma aldosterone concentration (PAC, 243 [29.9–159] pg/ml). The 24-h urinary free cortisol excretion was high (116 μ g/day [normal range, 11–80 μ g/day]).

Further endocrinological tests were performed based on the proposed diagnostic criteria for SCS [7] and a clinical practice guideline for PA [8]. Her PCC was high at 11:00 pm (11.2 μ g; cutoff value <5 μ g [7]). The overnight administration of low-dose dexamethasone (1 mg)

did not reduce her PCC (9.8 μ g [cutoff value <1.8] [7]). A saline infusion test did not suppress PAC (116 pg/ml [cutoff value, <100 pg/ml] [8]). A 25-mg captopril suppression test did not suppress PAC (before administration, 149 pg/ml vs. 60 min after, 151 pg/ml [8]). Based on these results, she was diagnosed with SCS and PA causing mild hypertension due to the adrenocortical tumor. She underwent right adrenalectomy, during which the tumor was removed with the surrounding lymph nodes and fat tissue (Gerota's fascia). The 410-g, 11.5 $\times$ 11.0 $\times$ 7.0-cm heterogeneous tumor and lymph nodes were fixed with 10 % formaldehyde. The tumor was subjected to a pathological analysis at 10 regions, which were arbitrary selected for regular pathological diagnosis (blocks [sections] 1–10) (Figs. 2 and 3A–J), and was diagnosed as conventional ACC by fulfilling seven out of nine Weiss criteria including 32.5 % of Ki67 positive cells, 135 mitotic cells per 50 microscopic high power field, a tumor thrombus (Fig. 3K), and atypical mitosis (Fig. 3L) [9]. No lymph node metastases were detected. Blood pressure (115/65 mmHg), ACTH (14.5 pg/ml), and PAC (16 pg/ml) normalized 4 days after surgery and plasma renin activity increased to 1.2 ng/ml/h, suggesting her SCS and PA were cured. The patient has been free of tumor recurrence for 12 months.

In order to examine the expression patterns of the steroidogenic enzymes responsible for hormone production, 10 tumor blocks were subjected to immunohistochemical analyses for CYP11B2, CYP11B1, and 3 β HSD (1st and 3rd column panels in Figs. 4, 5, and 6). CYP11B2, CYP11B1, and 3 β HSD were expressed sporadically throughout the tumor with their specific patterns not being associated with each other. The size of the stained area in each image was measured (2nd and 4th column panels in Figs. 4, 5, and 6) and expressed as PCA/TA (%; see Methods). In order to confirm the PCA/TA measurement method, CYP11B2 expression levels were evaluated at the mRNA level with qPCR, in which RNA was prepared from whole sections of blocks 1–10 (Table 1, see Methods). Of note, the *CYP11B2*-qPCR method for cDNA from FFPE tissues has been standardized in our previous study [10]; therefore, we selected *CYP11B2*-qPCR for PCA/TA confirmation. PCA/TA in CYP11B2-stained sections strongly correlated with *CYP11B2* mRNA levels among the 10 blocks ($r = 0.867$, $p = 0.0000002$, Spearman's rank-order correlation, Fig. 7), confirming that the PCA/TA measurement method is in fact quantitative. PCA/TA in 3 β HSD-stained sections was significantly higher (median value [25th percentile value–75th percentile value], 4.77 [2.45–9.66]) than those of CYP11B1 (0.69 [0.22–3.94], $p < 0.05$) and CYP11B2 (0.45 [9.13–2.40], $p < 0.05$) (Kruskal-Wallis one-way analysis of variance on ranks). Mitotic cells (median value [25th percentile value–75th percentile value]) in five CYP11B2-positive area, CYP11B1-positive area, 3 β HSD-positive area, and area negative for these enzymes (black circles in Figs. 3, 4, 5, and 6, respectively) were independently counted by three pathologists (YF, MA, and TY), and were 6.0 [3.8–10.3], 12.0 [5.0–17.5], 14.3 [6.0–19.7], and 8.6 [5.5–27.0], respectively (Fig. 8). The mitotic cell count were not significantly different between these areas ($p = 0.111$, one-way analysis of variance). No significant relationships were observed in PCA/TA of 3 β HSD, CYP11B1, and CYP11B2 between sections ($p > 0.05$ each, Spearman's rank-order correlation, Table 1), suggesting that the regulation of enzyme expression in ACC cells was disorganized.

We carefully observed the expression patterns of the enzymes in each section. Section 6 had the highest PCA/TA in CYP11B2 (18.5 %, Fig. 4K–L), low in CYP11B1 (0.2 %, Fig. 5K–

L), and relatively high in 3β HSD (10.8 %, Fig. 6K–L). CYP17, detected with a novel antibody, was expressed throughout the tumor, even in CYP11B2-positive cells (data not shown). CYP11B2 and 3β HSD were expressed sporadically, and their expression patterns were not necessarily overlapping (Fig. 9a). Thus, there were many CYP11B2-positive/ 3β HSD-negative cells (light brown cells in Fig. 9b [light brown arrowheads]) and CYP11B2-negative/ 3β HSD-positive cells (light blue cells in Fig. 9a [blue arrowheads]), both of which were unable to produce aldosterone (Fig. 8). Only a small population of cells expressed 3β HSD and CYP11B2 (dark brown cells in Fig. 9a [dark brown arrowheads]), which may be aldosterone-producing cells. Similarly, section 10 had the highest PCA/TA in CYP11B1 (10.7 %, Fig. 5S, T), low in CYP11B2 (0.6 %, Fig. 4S, T), and low in 3β HSD (2.5 %, Fig. 6S, T) (Table 1). However, only a few potential cortisol-producing cells, i.e., cells co-expressing 3β HSD and CYP11B1, were observed. These potential aldosterone-producing cells and cortisol-producing cells were rarely observed throughout 10 sections (blocks 1–10). These results suggest that the lack of the coordinated expression of steroidogenic enzymes throughout the tumor hampered the production of aldosterone and cortisol, which resulted in mild clinical expression.

Discussion

We delineate for the first time the expression patterns of steroidogenic enzymes including CYP11B2 and CYP11B1 in ACC. The coordinated expression of steroidogenic enzymes found in normal and adenoma cells was disturbed in ACC cells, resulting in the inefficient production of steroid hormones.

We have found only an old report describing ACC immunohistochemistry using steroidogenic enzyme antibodies including cholesterol side chain cleavage, 3β HSD, steroid 21-hydroxylase (CYP21), CYP17, and CYP11B1 [11]. 3β HSD and CYP21 are upstream enzymes of both CYP11B1 and CYP11B2. The antibody for CYP11B1 used in that study was targeted for bovine CYP11B1, which presumably detect both human CYP11B1 and CYP11B2 [12]. The report shows that ACC cells positive for a steroidogenic enzyme do not necessarily express other up/down-stream enzymes. For example, 3β HSD positive ACC area does not express CYP21. And they concluded the expression of steroidogenic enzymes in individual carcinoma cells was disorganized.

Approximately 60–70 % of ACC produce excessive amounts of steroid hormones and they are not clinically apparent in many cases [1]. ACC have been shown to be relatively inefficient in steroid production and the lack of clear hormonal manifestations is due to increased secretion of steroid precursors [13]. Urine steroid metabolomics measuring 32 distinct adrenal-derived steroids has revealed a pattern of predominantly immature, early stage steroidogenesis in most ACC cases, including androgen metabolites and precursors, mineralocorticoid precursor metabolites, and glucocorticoid precursor metabolites [13]. PA in the context of ACC is rare. The immunohistochemical findings in this study where there is a lack of coordination in the expression of steroidogenic enzymes explain that for such a large tumor, steroid hyper-production was disproportionate to the size of the tumor. Although further analyses are needed to contrast with benign adenoma and normal adrenal

cortex [2, 14], the steroidogenic enzyme expression may generally be disorganized in ACC causing variable phenotypes including Cushing's syndrome and androgen excess.

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Fig. 1.
Contrast-enhanced, fat-suppressed, and T1-weighted magnetic resonance image showed a 12-cm right adrenal tumor (T)

Fig. 2.
A cut surface of the formalin-fixed, adrenocortical tumor. *White frames with a number*
indicate portions of blocks 1–10

Fig. 3.

Whole H&E images of blocks 1–10 are shown in panels A–J, respectively. Each image is labeled with a block number. A tumor thrombus in block #8 (indicated by a frame) is enlarged in panel K. A tumor thrombus is observed in a vein. L, A high-magnification microscopic image of the thrombus (arrow in panel C). The bars in panels A–J, K, and L indicate 5 mm, 1 mm, and 10 μ m, respectively. Five black circles represent areas for mitotic cell count

Fig. 4. CYP11B2-immunohistochemistry. CYP11B2-immunostained images labeled with 3,3'-diaminobenzidine (*brown*) and nuclear counterstained with hematoxylin are shown on the 1st and 3rd columns. Separate positive signal images over the same threshold for CYP11B2 are shown on the 2nd and 4th columns corresponding to the immunostained images. These images were used for PCA/TA measurements. Bars, 5 mm. Five black circles represent areas for mitotic cell count

Fig. 5. CYP11B1-immunohistochemistry. CYP11B1-immunostained images labeled with 3,3'-diaminobenzidine (*brown*) and nuclear counterstained with hematoxylin are shown on the 1st and 3rd columns. Separate positive signal images over the same threshold for CYP11B1 are shown on the 2nd and 4th columns corresponding to the immunostained images. These images were used for PCA/TA measurements. Bars, 5 mm. Five black circles represent areas for mitotic cell count

Fig. 6. 3 β HSD-immunohistochemistry. 3 β HSD-immunostained images labeled with 3,3'-diaminobenzidine (*brown*) and nuclear counterstained with hematoxylin are shown on the 1st and 3rd columns. Separate positive signal images over the same threshold for 3 β HSD are shown on the 2nd and 4th columns corresponding to the immunostained images. These images were used for PCA/TA measurements. *Bars*, 5 mm. *Five black circles* represent areas for mitotic cell count

Fig. 7.

Relationship between PCA/TA of CYP11B2 immunohistochemistry (x -axis) and *CYP11B2* mRNA levels (y -axis) in each section PCA/TA of CYP11B2-stained sections strongly correlated with the expression levels of *CYP11B2* mRNA among the 10 sections blocks examined ($r = 0.867$, $p = 0.0000002$, Spearman's rank-order correlation)

Fig. 8.

Mitotic cell count per 5 microscopic high power field. The *boundary of the box* indicates the 25th percentile and 75th percentile, and a *line within the box* marks the median. There were not significant difference in mitotic cell count between CYP11B1-positive area, CYP11B2-positive area, 3βHSD-positive area, and area negative for these enzymes ($p = 0.111$). *HPF*, microscopic high power field

Fig. 9.

a A whole image of block 6, which was double-immunostained for CYP11B2 and 3βHSD. CYP11B2 and 3βHSD were labeled with (*brown*) and 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (*blue*), respectively. The nucleus was not counterstained. The *bar* indicates 1 cm. **b** A high-magnification image of CYP11B2/3βHSD double immunostaining (*arrow* in panel **a**). *Blue and light brown arrowheads* indicate 3βHSD-positive/CYP11B2-negative cells and 3βHSD-negative/CYP11B2-positive cells, which were not able to produce aldosterone. *Dark brown arrowheads* indicate double-positive cells for 3βHSD and CYP11B2, which may produce aldosterone. The *bar* indicates 10 μm

Table 1

Immunohistochemistry and qPCR results

Block (section) #	Area (mm ²)		Immunohistochemistry (%)					qPCR	
	TA	PCA	PCA/TA (%)						
			3 β HSD	CYP11B1	CYP11B2	CYP11B2			
						3 β HSD	CYP11B1		
1	485.6	11.0	4.5	0.8	2.3	0.9	0.2	32.9	(21.1–51.4)
2	298.7	16.7	19.8	0.9	5.6	6.6	0.3	8.0	(4.4–14.3)
3	552.2	51.2	1.3	0.3	9.3	0.2	0.1	8.7	(6.9–10.9)
4	385.0	15.2	11.7	0.5	3.9	3.0	0.1	1.0	(0.6–1.7)
5	261.1	44.4	2.4	3.3	17.0	0.9	1.3	70.8	(65.4–76.8)
6	482.4	52.2	1.1	89.5	10.8	0.2	18.5	414.9	(288.7–596.4)
7	531.1	43.8	2.5	15.5	8.2	0.5	2.9	154.0	(96.6–245.4)
8	432.3	13.1	0.5	9.7	3.0	0.1	2.2	134.7	(116.8–155.3)
9	397.6	8.7	0.7	0.5	2.2	0.2	0.1	6.3	(5.8–6.8)
10	248.0	6.2	26.6	1.5	2.5	10.7	0.6	20.3	(14.6–28.2)
			25th percentile value:		2.4	0.2	0.1		
			median value:		4.8	0.7	0.5		
			75th percentile value:		9.7	3.9	2.4		

TA total area, PCA positive cell area, PCA/TA positive cell area per total area, qPCR quantitative real-time polymerase chain reaction