Isoform-specific monoclonal antibodies against 3β-hydroxysteroid dehydrogenase/isomerase family provide markers for subclassification of human primary aldosteronism

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Short title: HSD3B isoforms in primary aldosteronism

Abstract

Context: Therapeutic management of primary aldosteronism (PA) requires accurate differentiation between aldosterone-producing adenoma (APA) and idiopathic hyperaldosteronism (IHA). However, little is known about the molecular features that delineate the difference between APA and IHA. Two different isoforms of 3β-hydroxysteroid dehydrogenase (HSD3B1 and HSD3B2) are thought to be expressed in human adrenal gland, but the lack of isoform-specific antibody has so far hampered mapping of these isoforms in APA and IHA.

Objectives: The aim of our study is to develop and characterize isoform-specific monoclonal antibodies against HSD3B1 and HSD3B2. Using these antibodies, we determined for the first time the immuno-localization of HSD3B1 and HSD3B2 in normal human adrenal cortex as well as in adrenal specimens from APA and IHA.

Results: Immunohistochemical analysis with isoform-specific antibodies revealed zone-specific expression of HSD3B1 and HSD3B2 in the adrenal cortex. HSD3B1 immunoreactivities were essentially confined to the zona glomerulosa (ZG), where aldosterone is produced. In contrast, HSD3B2 was not confined to ZG but was found across the zona fasciculata (ZF), which is where cortisol is produced. Moreover, immunohistopathological analysis of PA revealed a previously uncharacterized difference between APA and IHA. Notably, hyperplasia of ZG seen for IHA was accompanied by a robust expression of ZG isoform HSD3B1. In contrast, tumor cells in APA were not immunopositive to HSD3B1. Rather, a strong and dominant expression of HSD3B2 characterized APA. Moreover, perhaps due to compensatory responses to excess aldosterone, APA had an adjacent ZG whose immunoreactivities to HSD3B1 and HSD3B2 were profoundly reduced.

Conclusions: Isoform-specific monoclonal antibodies against HSD3B1 and HSD3B2 may be of great value for immunohistochemical differentiation between APA and IHA.

Introduction

The enzyme 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD) is essential for the biosynthesis of all active steroid hormones, such as those secreted from the adrenal gland, testis, ovary, skin and placenta (1). The 3β-HSD enzymes exist in multiple isoforms in humans and rodents with different tissue-specificity. We previously showed that in humans, the two distinct isoforms *HSD3B1* and *HSD3B2* are expressed in the adrenal gland (2), providing a reason to speculate that these isoforms may play a different role in adrenal physiology and pathophysiology.

APA (unilateral benign tumor) and IHA (bilateral hyperplasia) are the two principal causes of PA (3). Because optimal treatment is different between the two modalities, accurate subtyping of PA is crucial (4). Unfortunately, however, little is known about the molecular features (or markers) that separately illustrate APA and IHA. Autonomous overproduction of aldosterone by the adrenal gland is a common feature of PA. Based on this definition, both APA and IHA express aldosterone synthase. On the other hand, we speculated that the 3β-HSD family enzymes, encoded by the two separate genes (*HSD3B1* and *HSD3B2*), may provide differential markers that delineate APA and IHA. However, testing this hypothesis has been precluded so far by the lack of isoform-specific antibody. Because of a high degree of sequence similarity between the two isoforms (93.5% identity at the amino acid level), none of the antibodies so far developed for 3β-HSDs could differentiate between HSD3B1 and HSD3B2.

In the present study, we characterized newly generated mouse monoclonal antibodies against human HSD3B1 and HSD3B2. Notably, both antibodies were highly isoform-specific, allowing the two structurally similar isoforms to be distinguished by a single amino acid difference between them. Taking advantage of these antibodies, we compared for the first time the immunolocalization of HSD3B1 and HSD3B2 in human adrenal glands. The results clearly show that expression patterns of HSD3B1 and HSD3B2 are different between APA and IHA.

Materials and Methods

Antibodies

HSD3B2 monoclonal antibody was developed by using GANP/Balb mice (5). The antigen (amino acids 8-48; Figure 1A) was produced as a GST-fused protein and injected into GANP/Balb mice. Hybridomas were obtained with a standard method (6), and HSD3B2-positive clones were screened by ELISA. To qualify the subtype specificity, we excluded the monoclonal antibodies that cross-react with GST-fused HSD3B1 (amino acids 9-49). This study also utilized the antibodies to HSD3B1 (mouse monoclonal, Abnova, #3C11-D4), Pan-HSD3B (non-isoform-selective, rabbit polyclonal (7)), CYP11B2 (rabbit polyclonal, Aviva Systems Biology, #ARP41750_P050), CYP17A1 (rabbit polyclonal (8)), Dab2 (rabbit polyclonal, Santa Cruz, H-110) and HA (rabbit polyclonal, MBL, #561).

Immunoblot and Immunocytochemistry

For ectopic expression of human HSD3Bs, full-length protein coding clones were obtained from GeneCopoeia (HSD3B1/pEZ and HSD3B2/pEZ), and an HA epitope tag sequence was introduced into their C-terminal ends. Single amino acid point mutations were generated with a standard sequential PCR method (9). The resultant constructs (HSD3B1-HA/pEZ, HSD3B2- HA/pEZ, and their mutants) and pEZ empty vector were separately introduced into COS-1 cells by lipofection, and the cells were either harvested in Laemmli buffer for immunoblot or fixed with 4% formaldehyde in phosphate buffer for double-label immunofluoresce (Alexa594 for αHSD3B1 and αHSD3B2; Alexa488 for αHA) with our standard protocol (10).

Specimens for immunohistochemistry

Adrenal specimens obtained from patients with unilateral APA (8 cases) or bilateral IHA (7 cases) were examined immunohistochemically. Diagnosis of PA was established as described previously (11, 12). In all APA cases, the lesions were diagnosed as benign adenoma predominantly formed by clear cells (11). Thus, the specimens used in this study do not include “ZG-like” APA (13). In IHA, hyperplasia of ZG was observed for all cases (11). In our IHA cases, surgical treatments were performed for the following reasons that we had already clarified in our previous report (11). All possibilities to treat these patients with anti-hypertensive drugs (including mineralocorticoid receptor antagonists) were undertaken before surgery, but these medications were not sufficiently efficacious for them. Moreover, the patients were relatively young, and to prevent progression of organ damage in the future, unilateral adrenalectomy was performed to lower circulating aldosterone levels (see also online Supplemental Information for more details). For comparison, we used adrenals in surgical specimens from patients with renal cancer, as normal adrenal (NA) control. For distribution analysis in steroidogenic tissues, we used specimens from non-pathological human placenta, skin, ovary, and testis retrieved from adults at autopsy. These studies were all approved by the Institutional Review Board of Tohoku University School of Medicine.

Immunohistochemistry

The specimens fixed with 10% formalin were embedded in paraffin-wax with routine protocol. For immunodetection of HSD3B1 and HSD3B2, the sections (5 μm thick) were antigen-retrieved with microwave (15 min in citric acid buffer, pH 6.0) and incubated with either αHSD3B1 (final IgG concentration: 0.15μg/mL) or αHSD3B2 (0.05μg/mL) in phosphate-buffered saline for 18 h at 4°C. The immunoreactivities to αHSD3B1 and αHSD3B2 were visualized with 3,3-diaminobenzidine (DAB) using a peroxidase-based Histofine Simple Stain Kit (MAX PO M, Nichirei). Methods for immuno-staining of CYP17A1, CYP11B2, and Dab2 are described in online Supplemental Information.

Results

Generation and characterization of isoform-selective 3β-HSD antibodies

Because amino acid sequences of HSD3B1 and HSD3B2 are highly homologous (Figure 1A: 93.5% identity), we first determined the specificity of antibodies that we used for HSD3B1 (αHSD3B1) and HSD3B2 (αHSD3B2): the former was obtained from Abnova (Taipei, Taiwan) and the latter was developed by ourselves using immunoproficient GANP mice, a transgenic mouse line genetically modified to enhance somatic mutation at the variable regions of immunoglobulin (5). To test for the specificity, HA-tagged HSD3B1 (HSD3B1-HA) and HSD3B2 (HAS3B2-HA) were separately expressed in COS-1 cells, and immunoreactivities to αHSD3B1 and αHSD3B2 were examined by Western blot. We found that both αHSD3B1 and αHSD3B2 show a strong and highly specific immunoreactivity to the corresponding enzyme without any cross-reaction (Figure 1B). Moreover, site-directed mutagenesis (Figure 1C) further demonstrates that the subtype selectivity arises from a distinctive amino acid difference between HSD3B1 and HSD3B2: Gly to Arg substitution at position 40 (G40R) of HSD3B1 abolished immunoreactivity to αHSD3B1 but produced a high affinity for αHSD3B2. Conversely, HSD3B2 protein with a R39G mutation changed its preferential immunoreactivity from αHSD3B2 to αHSD3B1. These results provide evidence that αHSD3B1 and αHSD3B2 detect a single amino acid difference between the two isozymes.

The specificity of antibody was also examined immunocytochemically (Figure 1D). Double-label immunostaining showed that the cells expressing HSD3B1-HA were indeed immunopositive to αHSD3B1 (red) and αHA (green) but not for αHSD3B2. On the contrary, the cells expressing HSD3B2-HA were immunopositive to αHSD3B2 (red) and αHA (green) but not for αHSD3B1, providing additional evidence for the specificity of antibody against HSD3B1 and HSD3B2. Immunohistochemistry using human placenta, testis, ovary, and skin (Supplemental Figure 1) further demonstrated that immuno-labeling with αHSD3B1 and αHSD3B2 could faithfully recapitulate the reported tissue specificity of *HSD3B1* (skin and placenta) and *HSD3B2* (testis and ovary) (1). Moreover, cell-type specificities seen for HSD3B1 (sebaceous gland in skin and fetal villi in placenta) and HSD3B2 (Leydig cells in testis and theca cells in ovary) (Supplemental Figure 1) were in excellent agreement with those previously observed for Pan-HSD3B antibody (1), which is the gold standard antibody so far available for the immunohistochemistry of 3β-HSDs (7, 11, 14). We thus concluded that αHSD3B1 and αHSD3B2 are subtype-selective antibodies that can be used for immunohistochemical investigation of human tissues.

Layer-specific localization of HSD3B1 and HSD3B2 in normal adrenal gland

First, immunolocalization of HSD3B1 and HSD3B2 was determined in normal human adrenal gland (Figure 2A, NA). Remarkably, immunoreactivities to HSD3B1 were found exclusively in the ZG, the outermost cortical layer of the cortex. On the other hand, immunoreactivities to HSD3B2 were not

confined to ZG but distributed widely across the ZF, the middle layer of the cortex. To further specify the immunolocalization of HSD3B1, we performed dual-label immunohistochemistry with CYP17A1, an enzyme involved in cortisol production within ZF cells. The results (Figure 2B) clearly show that immunolabeling of HSD3B1 (brown) and CYP17A1 (blue) are mutually exclusive, providing evidence that HSD3B1 is not expressed in ZF cells. In addition, we noticed that HSD3B1 is widespread in ZG: nearly all CYP17A1-negative ZG cells were immunopositive to HSD3B1, with diffuse cytoplasmic staining (Figure 2B and Supplemental Figure 2). This markedly differs from sporadic and granular staining of ZG cells with anti-CYP11B2 (aldosterone synthase) antibody (15) (Supplemental Figure 3). Thus, these data illustrate that HSD3B1 is a definitive marker for ZG cells, which is reminiscent of Dab2, a marker protein that has been used for ZG cells (16, 17) (Supplemental Figure 4).

Robust expression of ZG-specific HSD3B1 characterizes IHA

Next, immunohistopathological examination of IHA was performed (Figure 2A). We confirmed that all IHA patients examined have no adenoma and exhibit hyperplasia of ZG cells (11). Notably, in all cases tested (n=7), we found a robust expression of HSD3B1 within the hyperplastic ZG cells (Figure 2A and Supplemental Figure 5). Dual-label immunohistochemistry of HSD3B1 and CYP17A1 (Figure 2B) further demonstrated that HSD3B1 is not expressed in ZF but is widely expressed throughout the CYP17A1-negative, hyperplastic ZG cells. These data illustrate that HSD3B1 is a prominent marker for assessing the hyperplasia of ZG. In contrast, immunostaining of HSD3B2 was found almost equally in ZF and ZG (Figure 2A and Supplemental Figure 5), indicating that HSD3B2 is not ZG-specific.

Dominant expression of HSD3B2 characterizes APA

We next examined APA (Figure 2C and Supplemental Figure 6). In all cases tested (n=8), APA expressed almost exclusively HSD3B2. Differently from IHA, HSD3B1 was low in the tumor cells. Rather, the cells that were strongly stained with αHSD3B2 were found across the tumor. Such a strong and dominant expression of HSD3B2 was not seen for normal (non-neoplastic) ZG cells (they are dual positive to HSD3B1 and HSD3B2). Thus, the tumor cells of APA appear to have (or gain) a characteristic different from that of native ZG cells.

In addition, we also observed that non-secretory nodules that are often found in the APA-adjacent adrenal cortex were also characterized by a predominant expression of HSD3B2 (Supplemental Figure 7).

Suppressed expression of HSD3B1 and HSD3B2 in APA-associated ZG cells

Of note, perhaps due to compensatory responses to excess aldosterone, the ZG cells adjacent to APA had remarkably reduced immunoreactivity to HSD3B1 and HSD3B2 (Figure 2C and Supplemental Figure 8). Under the same condition CYP11B2 (aldosterone synthase) showed sustained expression,

as was reported (15) (Supplemental Figure 9). These observations therefore provide evidence to speculate that the two isoforms of 3β-HSD (but not CYP11B2) play a key role in limiting the capacity of steroid production in non-neoplastic ZG cells in the ipsilateral adrenal gland.

Discussion

The 3β-HSD enzyme family is comprised of multiple, structurally similar isozymes that are encoded by different genes. It has been demonstrated that at least at the mRNA level, two distinct isoforms (*HSD3B1* and *HSD3B2*) are expressed in the human adrenal gland (2). However, due to the lack of isoform-specific antibody, (patho)physiological roles of them have remained unexplored. In the present study, we characterized newly generated mouse monoclonal antibodies against human HSD3B1 and HSD3B2. These antibodies allowed us to map HSD3B1 and HSD3B2 in the normal human adrenal gland as well as in the pathological lesions of APA and IHA.

Immunohistochemical examination of normal adrenal gland revealed non-identical zonation of HSD3B1 and HSD3B2. HSD3B2 is expressed in both ZG and ZF, but HSD3B1 is essentially confined to ZG. Interestingly, the mouse also has two isoforms in the adrenal: one (Hsd3b1) is ubiquitous in the cortex, but the other (Hsd3b6) is ZG-specific (2, 18). These results indicate that the zone specificities observed for the two isozymes are the feature that is evolutionally conserved in humans and mice.

Immunohistological analysis of PA also revealed a previously uncharacterized difference between APA and IHA. We found that tumor cells in APA are not immunolabeled for HSD3B1, the enzyme that we identified as a marker for ZG in the normal adrenal gland. Instead, APA was characterized by a robust expression of the alternative enzyme, HSD3B2. Tumor cells in APA thus appear to have a different characteristic from ZG cells. In contrast, hyperplastic ZG cells in IHA were all immunolabeled for HSD3B1. Such features are reminiscent of what we observed in *Cry*-null mice (2), the adrenals of which also manifest bilateral hyperplasia of ZG with a strong expression of ZG-specific isoform, Hsd3b6. It is therefore likely that hyperplastic ZG cells (which are non-neoplastic) can be characterized by a robust expression of ZG-specific 3β-HSD isozyme, a feature that was not observed for APA. It is not known why these isoforms are differently expressed between APA and IHA. Different gene transcription mechanisms appear to allow them to be expressed in a different set of tissues and cell types (1, 2, 18).

In the present study, all APA specimens were diagnosed as a benign adenoma predominantly formed by large clear cells. These patients showed abundant expression of HSD3B2. Considering that APA is a heterogeneous group of clinical disorders with different responsiveness to ACTH and angiotensin II (13, 19), it will be worth testing whether the expression patterns of HSD3B1 and

HSD3B2 are different between these groups of APA patients.

Finally, we analyzed non-tumor portions of APA-containing adrenal gland. Interestingly, the ZG cells located in this region were characterized by a profound suppression of HSD3B1 and HSD3B2. This suggests a role for these enzymes in limiting the capacity of aldosterone synthesis from this region. Although underlying mechanism is not known, one possible explanation is that a negative feedback regulation occurs on these enzymes to counteract the excess of aldosterone caused by APA.

In conclusions, we provide a pair of isoform-specific antibodies against HSD3B1 and HSD3B2. These offer unique tools for zonation of the adrenal cortex as well as for immunohistopathological differentiation of PA.

Acknowledgments

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Figure Legend

Figure 1. Characterization of monoclonal antibodies against HSD3B1 and HSD3B2. (A) Aligned protein sequences of HSD3B1 and HSD3B2. The conserved amino acid residues are shown on yellow backgrounds. The horizontal line indicates the antigen region. Asterisk, the position of key residues found to be crucial for subtype differentiation by αHSD3B1 and αHSD3B2. (B) Immunoblots showing the specificity of antibodies. Either HSD3B1-HA or HSD3B2-HA was ectopically expressed in COS-1 cells and immunoblotted with αHSD3B1 and αHSD3B2. Anti-HA (αHA) and Pan-HSD3B antibodies were used as a non-selective control. pEZ, a plasmid vector. (C) Immunoblots of HSD3B1-HA, HSD3B2-HA, and their mutant proteins carrying a single amino acid substitution at the indicated positions. (D) Double-label immunofluorescence of cells expressing HSD3B1-HA or HSD3B2-HA. Transfected COS-1 cells were fixed and stained with either αHSD3B1 or αHSD3B2, together with αHA. Merge shows combined images for HSD3Bs (red), HA (green), and DAPI staining (blue).

Figure 2. Differential mapping of HSD3B1 and HSD3B2 in normal adrenal gland, IHA, and APA. (A) Representative images of immunohistochemistry for HSD3B1 and HSD3B2 in serial sections of normal adrenal gland (NA) and IHA. Vertical lines indicate the positions of the cortical layers: ZG (zona glomerulosa), ZF (zona fasciculata) and ZR (zona reticularis). (B) Representative images for single and double immunostaining of HSD3B1 (brown) and CYP17A1 (blue) in serial adrenal sections of NA and IHA. (C) Representative staining of APA with αHSD3B1 and αHSD3B2. Shown are images of tumor and non-tumor portions of APA-containing adrenal. Note that the ZG cells adjacent to APA are immuno-negative to HSD3B1 and HSD3B2 and exhibit paradoxical hyperplasia. In contrast, the ZF cells adjacent to APA exhibit sustained expression of HSD3B2, suggesting that functional suppression occurs only in the ZG cells. Scale bars in (A)-(C), 100 μm.

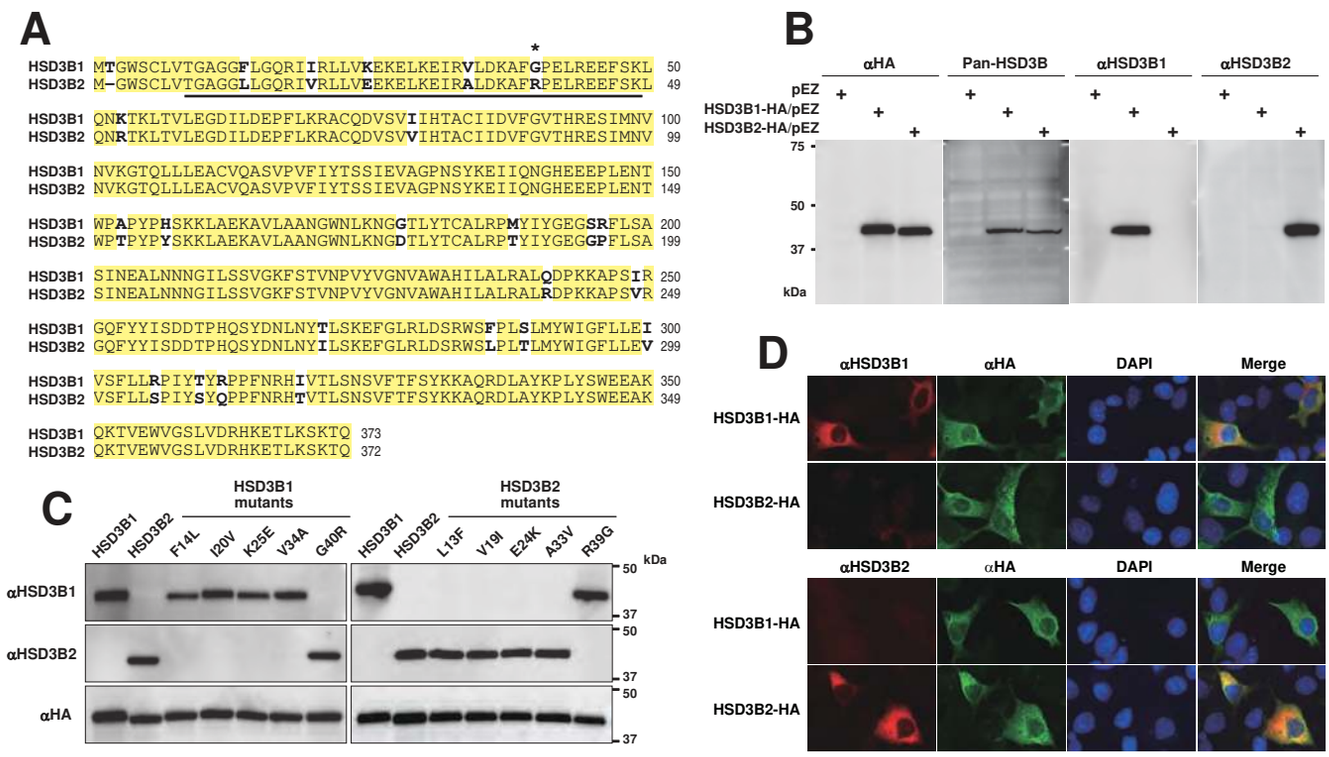
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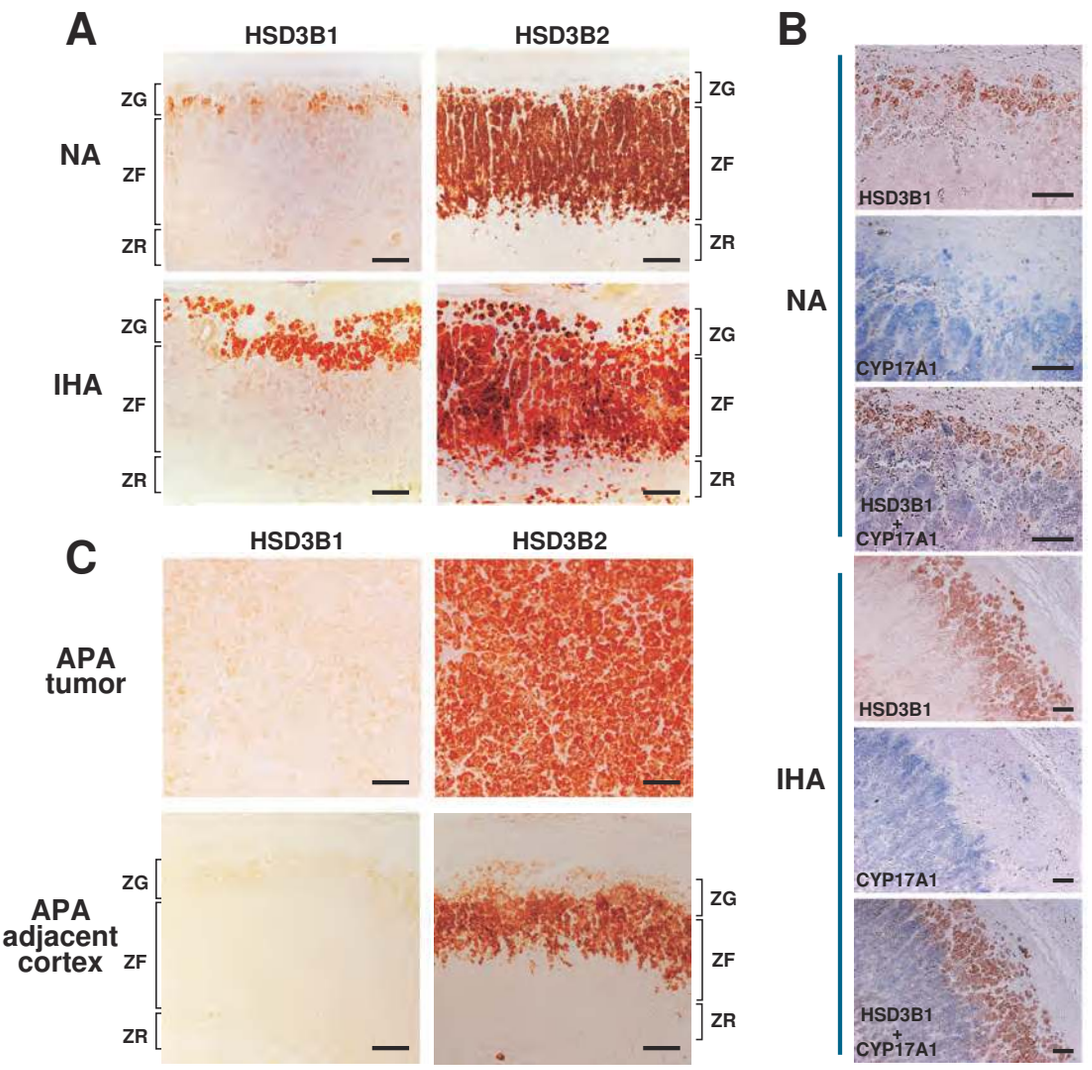
Figure 1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HSD3B1
HSD3B2
NA
IHA
ZG
ZF
ZR
B
NA
IHA
HSD3B1
CYP17A1
HSD3B1 + CYP17A1
C
APA tumor
APA adjacent cortex

Figure 2

Supplemental Information

Isoform-specific monoclonal antibodies against 3β-hydroxysteroid dehydrogenase/isomerase family provide markers for subclassification of human primary aldosteronism

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Supplemental Materials and Methods:

Supplemental Figures:

Supplemental Materials and Methods

Adrenal specimens for immunohistochemistry

Differential diagnosis of PA was established by measuring the plasma aldosterone concentration/ plasma renin activity ratio, which was greater than 20 at both 1 and 2 h after oral administration of 50 mg captopril, and overnight 1mg dexamethasone suppression test was performed in all patients to exclude PA patients co-localized with cortisol-producing adenomas. The plasma cortisol concentration following dexamethasone suppression was below 1.8 μg/dL in all cases with APA and IHA in the present study. Finally, lateralization was determined by adrenal computed tomography scan and ACTH-loading adrenal venous sampling as described (1, 2). Bilateral adrenal veins were simultaneously catheterized in all patients (1, 2). After baseline samples were simultaneously obtained from both adrenal veins, a second set of blood samples was collected from the same sites 15 min after iv bolus injection of 0.25 mg (10 IU) of ACTH (1-3). Successful adrenal venous cannulation was based on an AVS cortisol level that was greater than 5-fold compared with that in the iliac vein sample after ACTH stimulation (1-3)

Histopathological examinations were performed with resected adrenal specimens from APA (8 cases) and IHA (7 cases). The entire specimen of each resected adrenal was examined histopathologically, and all patients were diagnosed as either APA or IHA as previously described (1, 2, 4, 5). In all APA cases, the lesions were diagnosed as benign adenoma predominantly formed by clear cells, as well as having an adjacent zona glomerulosa (ZG) that had negative immunoreactivity for Pan-HSD3B antibody (4, 5). In all IHA cases, immunoreactivity for Pan-HSD3B antibody was positive in the hyperplastic ZG cells (5).

Surgical treatment is generally recommended for APA, whereas for IHA patients more conservative medical treatment is suggested to avoid possible progression of cardiac disease caused by aldosterone excess (6). However, in our IHA cases, surgical treatments were performed for the following reasons that we had already clarified in our previous report (1). All possibilities to treat these patients with anti-hypertensive drugs (including mineralocorticoid receptor antagonists) were undertaken before surgery, but these medications were not sufficiently efficacious for them. Moreover, the patients were relatively young, and to prevent progression of organ damage in the future, unilateral adrenalectomy was performed to lower circulating aldosterone levels. Unilateral adrenalectomy has been proposed to be of benefit in some patients with apparent bilateral PA (7). Besides, we could not rule out the possibilities of APA in a couple of IHA cases based on the measurement of aldosterone/cortisol ratio and aldosterone level before ACTH stimulation, although lateralization ratio became suppressed after ACTH administration (8). Therefore, all IHA patients in this study agreed to surgical treatment and the subsequent immunohistochemical diagnostic examination. These studies were all approved by the Institutional Review Board of Tohoku University School of Medicine.

Immunohistochemistry of CYP17A1, CYP11B2, and Dab2

For detecting Dab2, sections were antigen-retrieved with autoclave (5 min at 121°C in citric acid

buffer, pH 6.0), and treated with blocking reagent (Histofine, Nichirei) for 30 min at 20°C. Sections were incubated with αDab2 (1:400) overnight at 4°C, and the immunoreactivity was visualized with DAB (brown staining) with a peroxidase-based Histofine Simple Stain Kit (MAX PO R, Nichirei). Immunoreactivity to αCYP17A1 (1:500, 18 h, 4°C) was visualized with Vector Blue substrate (Vector Laboratories) using an alkaline phosphatase-based Histofine Simple Stain Kit (AP R, Nichirei). Sequential double immunolabeling analysis of CYP17A1 followed by HSD3B1 was also performed for differential mapping of the enzymes. For detection of CYP11B2, sections were pretreated with peroxidase blocking reagent (Dako) to block endogenous peroxidase activity. Immunoreactivity to αCYP11B2 (1:1000, 60 min, 20°C) was visualized with DAB using the EnVision FLEX+ Rabbit (LINKER) detection kit (Dako).

References

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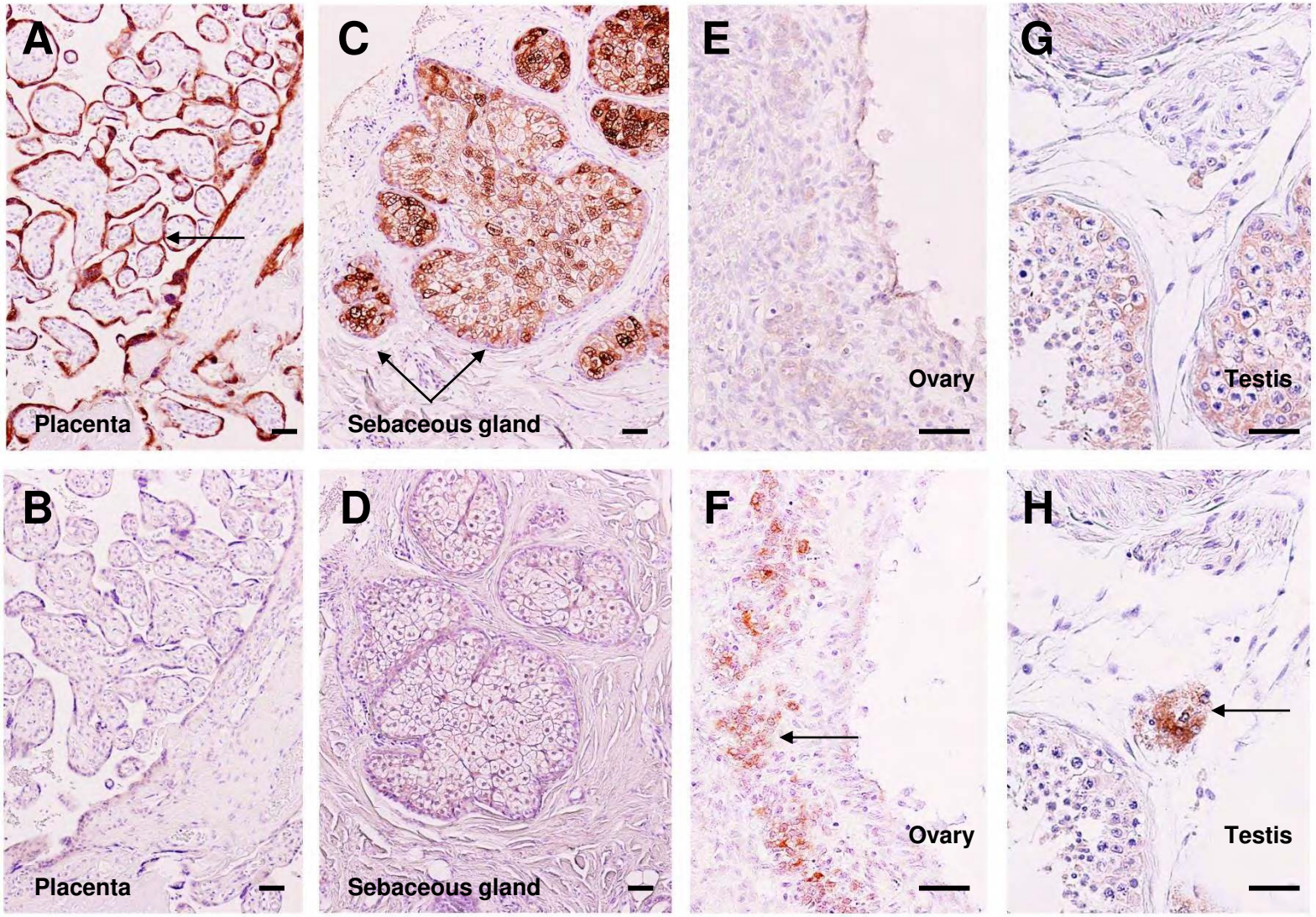
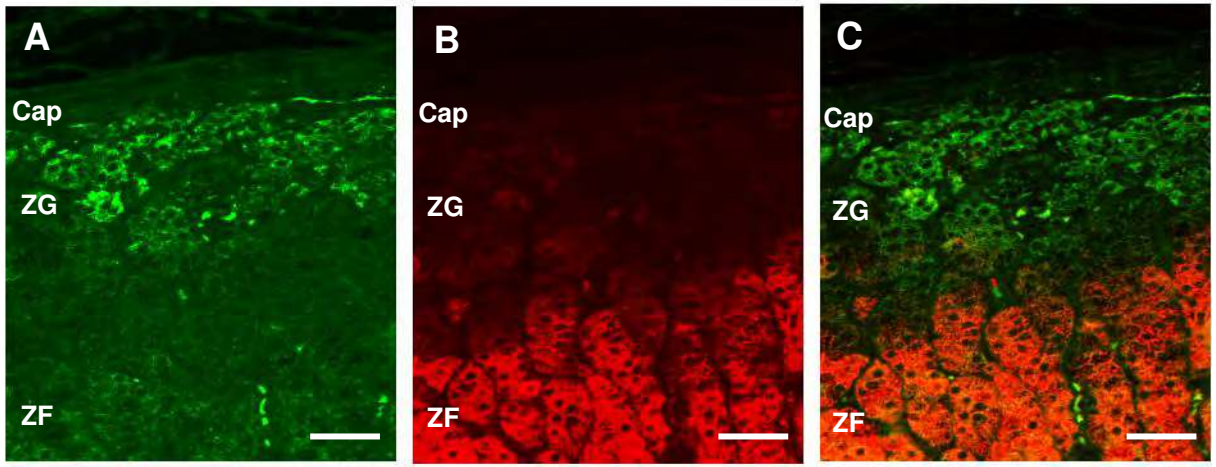
Supplemental Figure 1



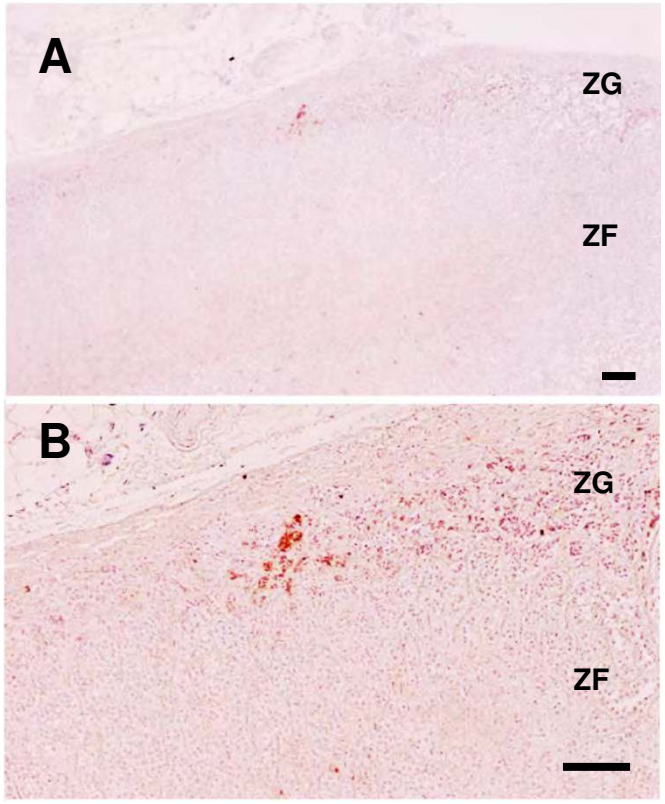
Supplemental Figure 1 Immunohistochemical analysis of HSD3B1 and HSD3B2 in human placenta (**A**, **B**), skin (**C**, **D**), ovary (**E**, **F**) and testis (**G**, **H**). The sections were stained with either αHSD3B1 (**A**, **C**, **E**, **G**) or αHSD3B2 (**B**, **D**, **F**, **H**). Note that syncytiotrophoblasts in placenta (**A**, arrow) and cells composing the sebaceous glands in skin (**B**, arrows) were HSD3B1-immunopositive, while theca cells in the ovary (**F**, arrow) and interstitial Leydig cells in testis (**H**, arrow) were HSD3B2-positive. Bars, 50 µm.

Supplemental Figure 2



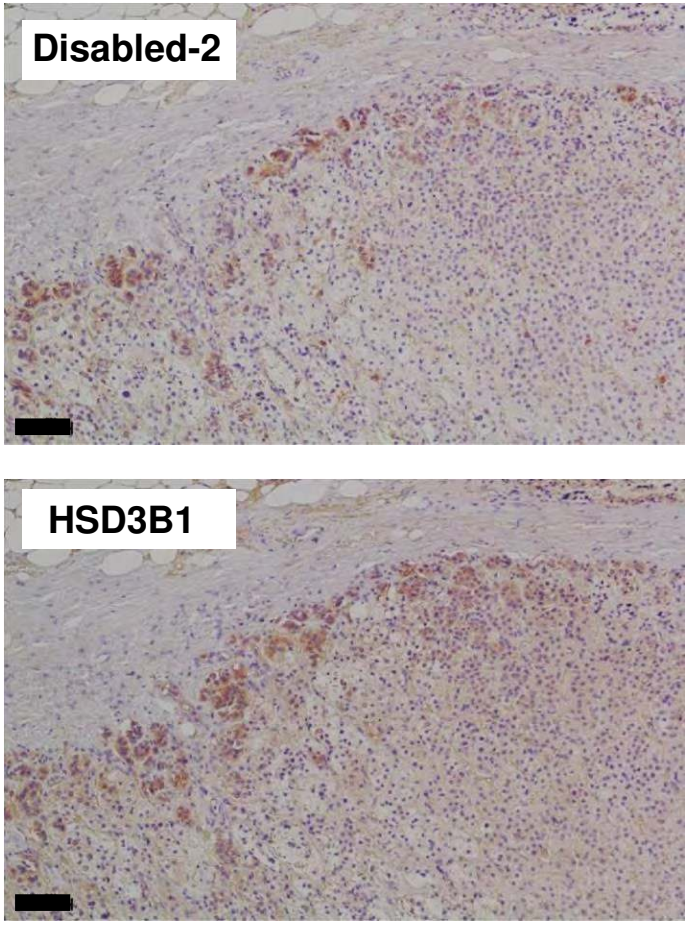
Supplemental Figure 2 Immunohistochemistry of HSD3B1 (**A**), CYP17A1 (**B**) and their merged image (**C**) in the adrenal cortex of IHA. The section was double stained with αHSD3B1 (mouse monoclonal) and αCYP17A1 (rabbit polyclonal), and their immunoreactivities were detected with species-specific donkey anti-mouse IgG labeled with Alexa488 (green) and donkey anti-rabbit IgG labeled with Alexa594 (red). Merge shows mutually exclusive expression of HSD3B1 and CYP117A1. Cap, capsule; ZG, zona glomerulosa; ZF, zona fasciculata. Bars, 50 µm.

Supplemental Figure 3



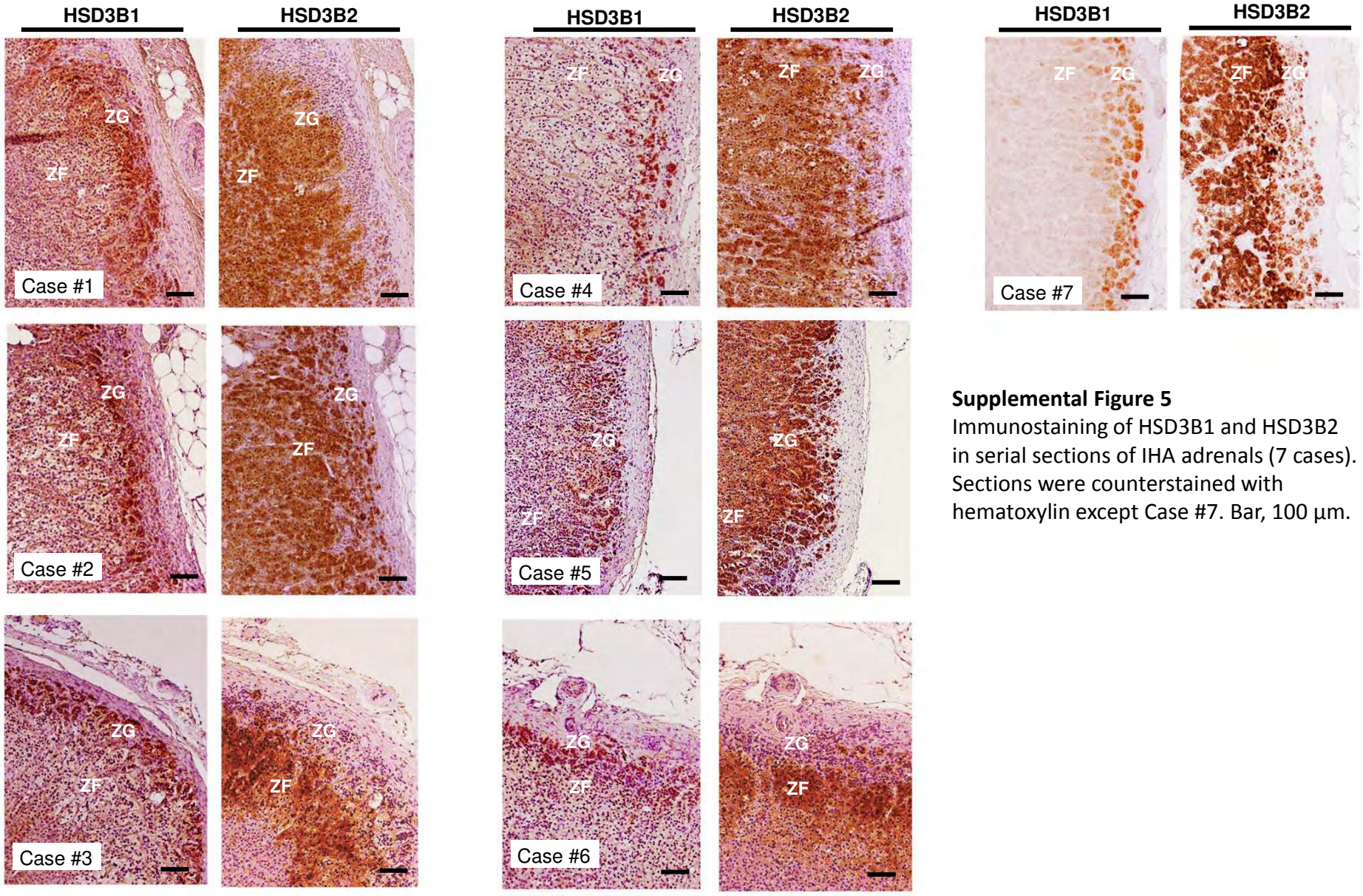
Supplemental Figure 3 Representative images of immunohistochemistry for CYP11B2 in normal adrenal cortex at low-power (**A**) and high-power (**B**) fields. Bars, 100 µm. Immunoreactivities to CYP11B2 were only sporadically detected in the zona glomerulosa (ZG).

Supplemental Figure 4



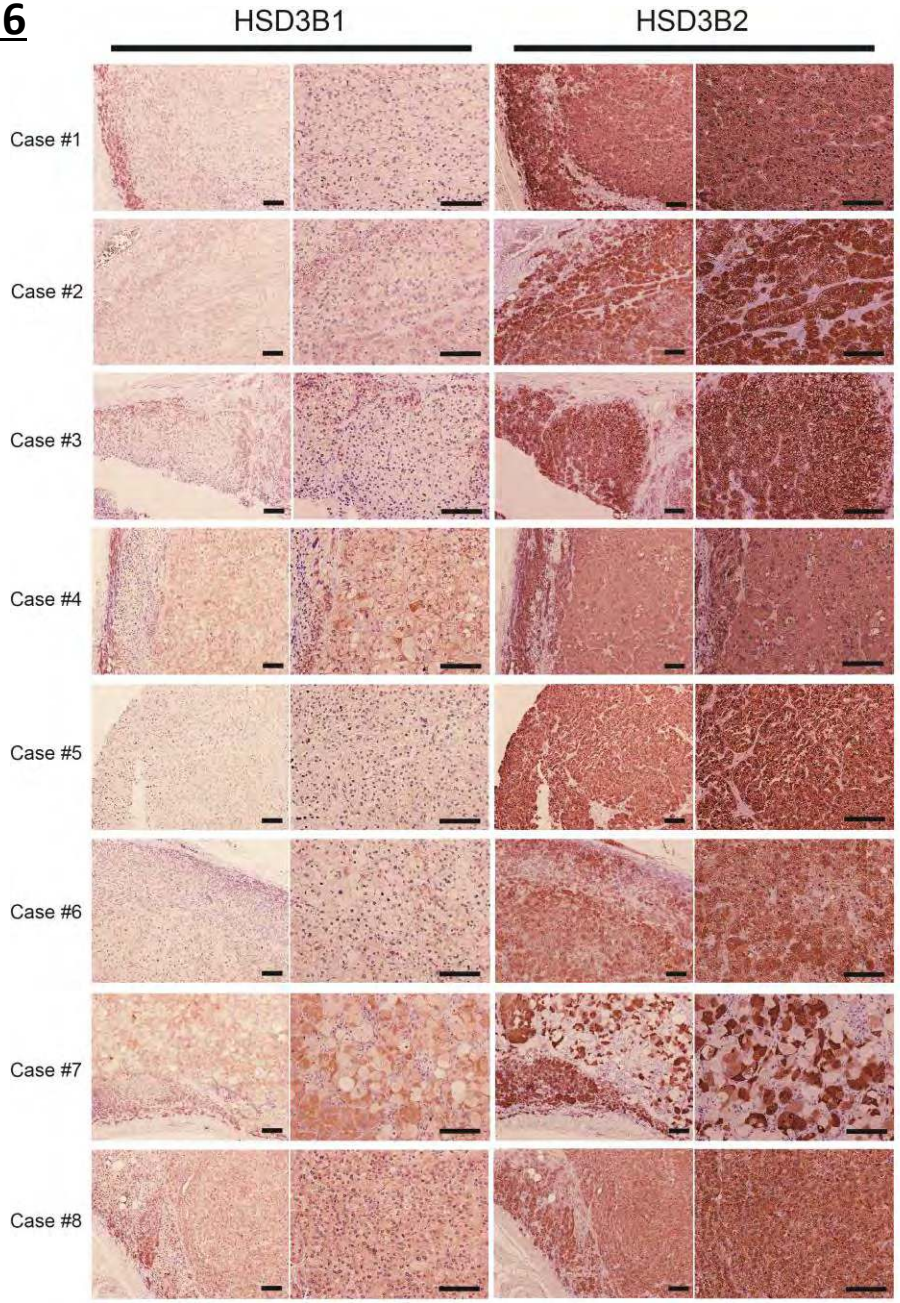
Supplemental Figure 4
Immunostaining of Disabled-2 (Dab2) and HSD3B1 in serial sections of normal adrenal gland. Sections were counterstained with hematoxylin. Bar, 100 µm.

Supplemental Figure 5



Supplemental Figure 5
Immunostaining of HSD3B1 and HSD3B2 in serial sections of IHA adrenals (7 cases). Sections were counterstained with hematoxylin except Case #7. Bar, 100 µm.

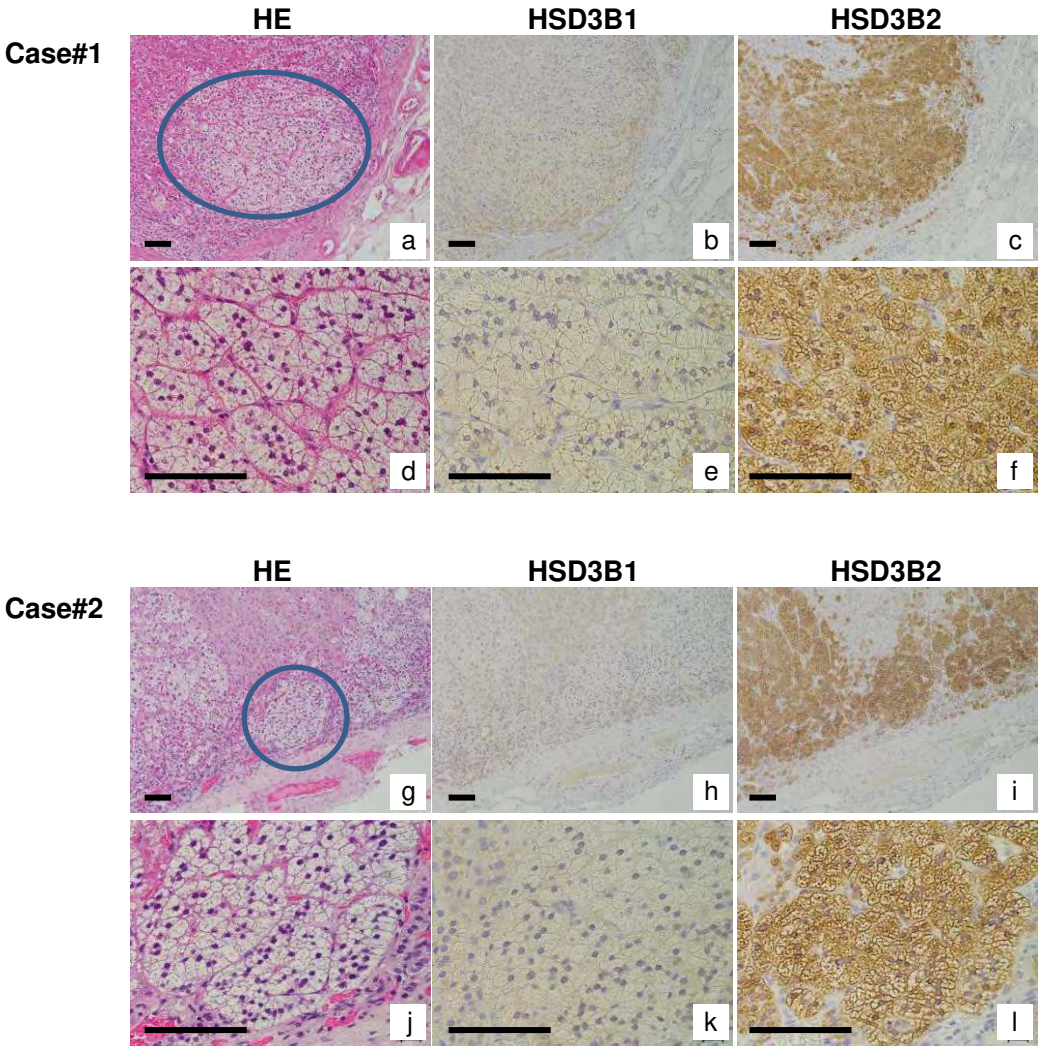
Supplemental Figure 6



Supplemental Figure 6

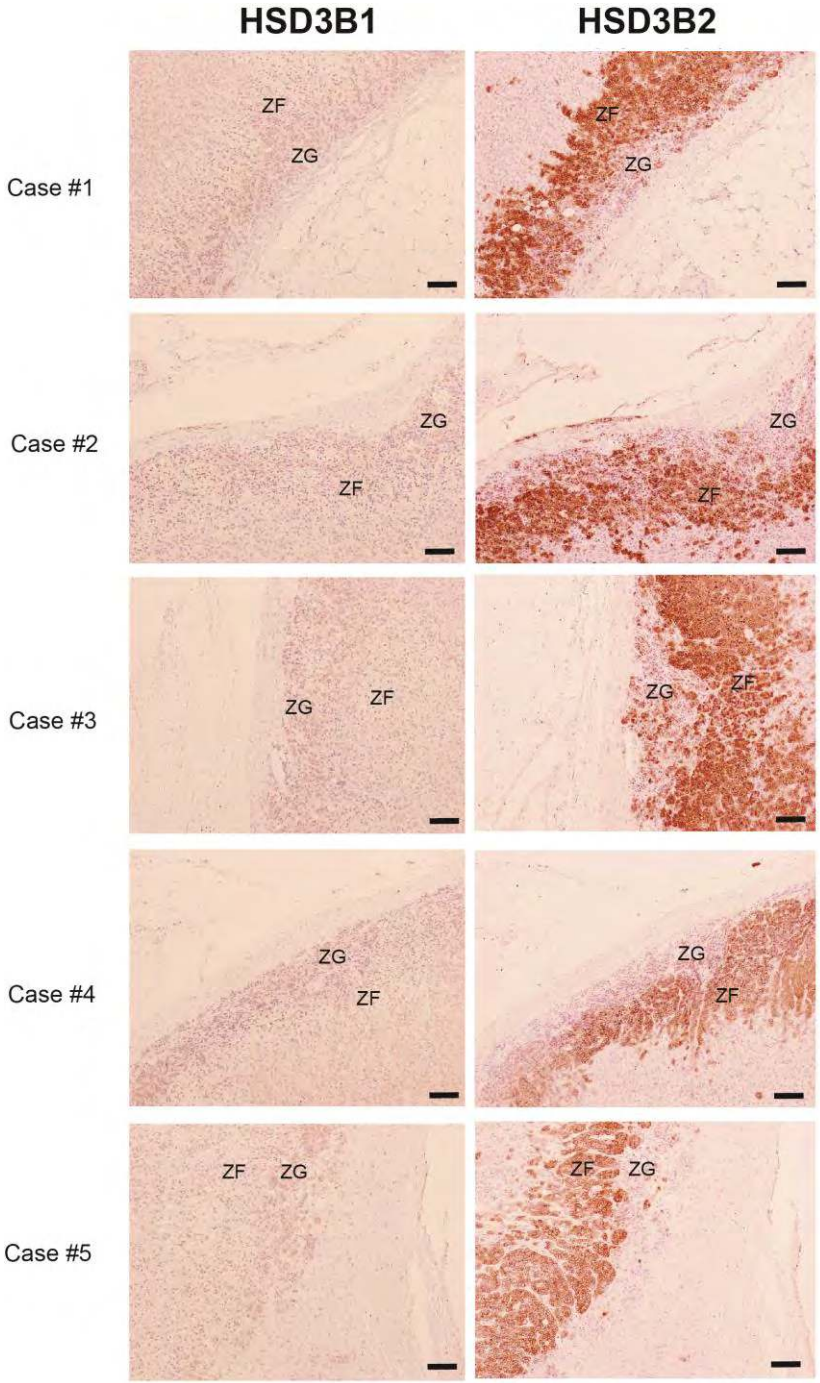
Immunohistochemistry of HSD3B1 and HSD3B2 in serial sections of tumors from the patients with APA (8 cases). Pictures show high and low power microscopic images of APA. Sections were counterstained with hematoxylin. Note that the tumor cells in APA express massively and almost predominantly HSD3B2. Bars, 100 µm.

Supplemental Figure 7



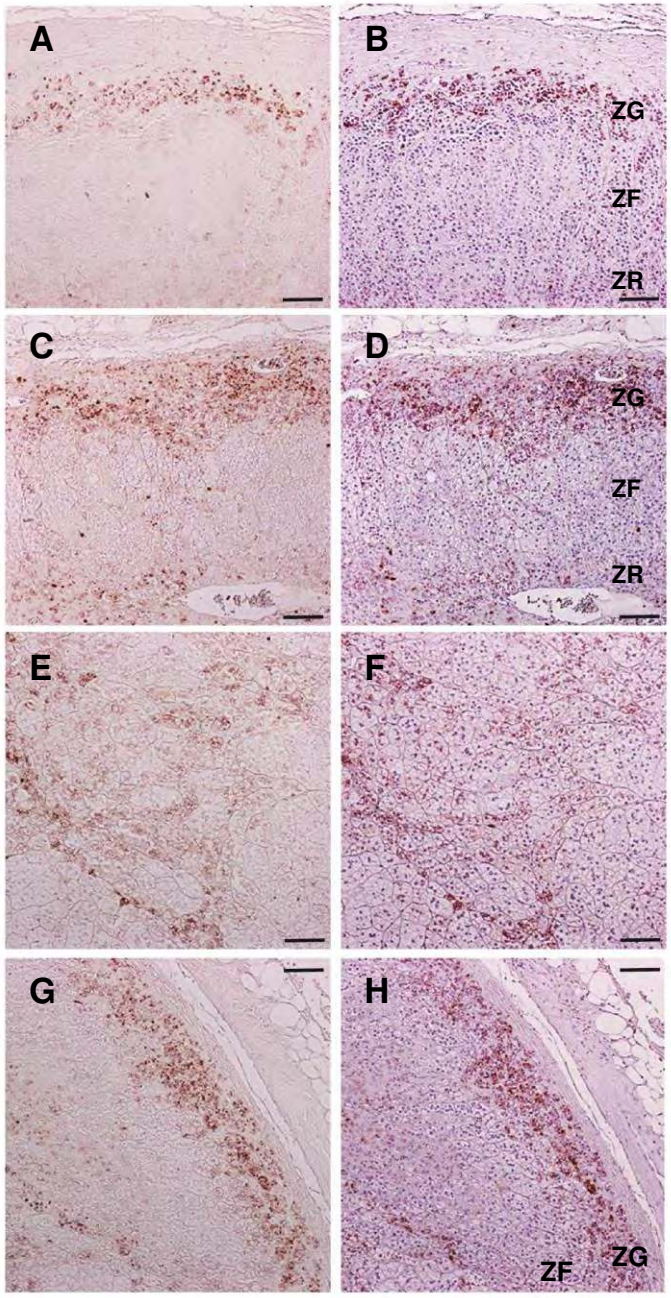
Supplemental Figure 7
Immunohistochemistry of HSD3B1 and HSD3B2 in serial sections of non-secretory nodules of APA-adjacent adrenal cortex (2 cases). Shown are the sections stained with hematoxylin-eosin (HE) (a, d, g, f), αHSD3B1(b, e, h, k), or αHSD3B2 (c, f, i, l). Circles indicate the nodules. Images shown in d, e, f, j, k, and l are high-power photomicrographs of a, b, c, g, h, and i, respectively. The sections were counterstained with hematoxylin. Bar, 100 µm.

Supplemental Figure 8



Supplemental Figure 8
Immunohistochemistry of HSD3B1 and HSD3B2 in serial sections of non-tumor portions of APA-bearing adrenal glands (5 cases). Note that the levels of 3β-HSDs were profoundly suppressed in peritumoral ZG cells. Bars, 100 μm.

Supplemental Figure 9



Supplemental Figure 9 Immunohistochemistry of CYP11B2 in normal adrenal cortex (**A**, **B**), IHA (**C**, **D**), tumor of APA (**E**, **F**), and tumor-associated adrenal cortex (**G**, **H**). Shown are serial sections with (**A**, **C**, **E**, **G**) or without (**B**, **D**, **F**, **H**) hematoxylin counterstaining. As reported, in normal adrenal gland, immunoreactivities to CYP11B2 are detected only in ZG cells, with varied cellular intensities. Shown in **A** and **B** are the ZG regions expressing relatively high amount of CYP11B2, but in different regions of ZG, CYP11B2-positive cells are present only sparsely (Supplemental Figure 3) (ref. 9). In IHA, the hyperplastic ZG region harbors an increased number of CYP11B2-positive cells, albeit with increased variability of cellular intensity (**C**, **D**): about half of the hyperplastic ZG cells are immunopositive to CYP11B2. In APA, a small fraction of tumor cells (3-30%; here only 5%) has positive immunoreactivities (**E**, **F**): most cells are immunonegative to CYP11B2. Note that in tumor-associated adrenal cortex, the expression of CYP11B2 is not suppressed (**G**, **H**), which differs from the lowered expression of 3β-HSDs in peritumoral ZG cells (Figure 2C). It is also interesting to note that immunoreactive substances to CYP11B2 are coarse-grained, probably reflecting the mitochondrial localization of this enzyme. This sharply contrasts with cytoplasmic diffuse staining of HSD3B1 and HSD3B2 (Figure 2): these are the enzymes residing in endoplasmic reticulum. Bars, 100 μm.