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3β-hydroxysteroid dehydrogenase isoforms in human aldosterone-producing adenoma

Sachiko Konosu-Fukaya[a], **Yasuhiro Nakamura**[a], **Fumitoshi Satoh**[b], **Saulo J.A. Felizola**[a], **Takashi Maekawa**[a], **Yoshikiyo Ono**[b], **Ryo Morimoto**[b], **Kazue Ise**[a], **Ken-ichiro Takeda**[c], **Koshin Katsu**[c], **Fumiyoshi Fujishima**[a], **Atsuko Kasajima**[a], **Mika Watanabe**[a], **Yoichi Arai**[d], **Elise P. Gomez-Sanchez**[e,f,g], **Celso E. Gomez-Sanchez**[e,f], **Masao Doi**[h], **Hitoshi Okamura**[h], and **Hironobu Sasano**[a,*]

[a] Department of Pathology, Tohoku University Graduate School of Medicine, Sendai, Japan

[b] Division of Nephrology, Endocrinology and Vascular Medicine, Department of Medicine, Tohoku University Hospital, Sendai, Japan

[c] Tohoku University School of Medicine, Sendai, Japan

[d] Department of Urology, Tohoku University School of Medicine, Sendai, Japan

[e] Endocrine Section, G.V. (Sonny) Montgomery VA Medical Center, MS, USA

[f] Endocrinology, University of Mississippi Medical Center, Jackson, MS, USA

[g] Department of Pharmacology and Toxicology, University of Mississippi Medical Center, Jackson, MS, USA

[h] Department of Systems Biology, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan

Abstract

It has become important to evaluate the possible involvement of 3β-hydroxysteroid dehydrogenase type 1 (HSD3B1) and 2 (HSD3B2) isoforms in aldosterone-producing adenoma (APA). In this study, we studied 67 and 100 APA cases using real-time quantitative PCR (qPCR) and immunohistochemistry, respectively. Results of qPCR analysis demonstrated that *HSD3B2* mRNA was significantly more abundant than *HSD3B1* mRNA ($P < 0.0001$), but only *HSD3B1* mRNA significantly correlated with *CYP11B2* (aldosterone synthase) mRNA ($P < 0.0001$) and plasma aldosterone concentration (PAC) of the patients ($P < 0.0001$). Results of immunohistochemistry subsequently revealed that HSD3B2 immunoreactivity was detected in the great majority of APA but a significant correlation was also detected between HSD3B1 and CYP11B2 ($P < 0.0001$). In *KCNJ5* mutated APA, *CYP11B2* mRNA ($P < 0.0001$) and *HSD3B1* mRNA ($P = 0.011$) were significantly higher than those of wild type APA. These results suggest that HSD3B1 is involved in aldosterone production, despite its lower levels of expression compared with HSD3B2, and also possibly associated with *KCNJ5* mutation in APA.

* Corresponding author. Department of Pathology, Tohoku University School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan. Tel.: +81 22 717 8050; fax: +81 22 717 8051. hsasano@patholo2.med.tohoku.ac.jp (H. Sasano)..

Keywords

Aldosterone-producing adenoma (APA); 3β-hydroxysteroid dehydrogenase type 1; (HSD3B1); 3β-hydroxysteroid dehydrogenase type 2; (HSD3B2); CYP11B2 (aldosterone synthase); CYP11B1 (11β-hydroxylase) and *KCNJ5*

1. Introduction

Primary aldosteronism (PA), characterized by autonomous aldosterone production, has been reported as a cause of hypertension in 4–20% of the patients with hypertension (Funder, 2011; Rossi et al., 2006; Williams et al., 2006). In addition, these patients are also known to have a higher incidence of cardiovascular events than those with essential hypertension (Milliez et al., 2005). Aldosterone-producing adenoma (APA) is one of the principal causes of PA.

There are two rate-limiting steps in aldosterone production in the human adrenal. The first and early step is chiefly regulated by the steroidogenic acute regulatory (StAR) protein involved in cholesterol transport and the second and late step by aldosterone synthase (CYP11B2) (Bassett et al., 2004). APA generally expresses relatively high levels of CYP11B2 but *StAR* mRNA expression is usually not elevated in tumor tissues (Bassett et al., 2005). Toward explaining this discrepancy above, 3β-hydroxysteroid dehydrogenase (HSD3B) has been proposed as another rate-limiting step of aldosterone biosynthesis (Doi et al., 2010). Among HSD3B isoforms, type 2 (HSD3B2) has been considered to be specifically responsible for overall steroidogenesis in human adrenal cortex (Simard et al., 2005), while HSD3B type 1 (HSD3B1) has been recently reported exclusively in the zona glomerulosa (ZG) cells in human adrenal cortex (Doi et al., 2010). Doi et al. also demonstrated that HSD3B1 was confined to the ZG, whereas HSD3B2 was in zona fasciculata (ZF) in non-tumoral adrenal cortex using immunohistochemistry with newly developed mouse monoclonal antibodies against human HSD3B1 and HSD3B2 (Doi et al., 2010). In addition, they also reported that APA tumor cells demonstrated markedly elevated HSD3B2 immunoreactivity compared with HSD3B1, in contrast to the hyperplastic ZG cells in idiopathic hyperaldosteronism (IHA) which predominantly expressed HSD3B1 immunoreactivity (Doi et al., 2010). However, the number of the cases examined was rather limited and the expression was evaluated only by immunohistochemistry which could not necessarily detect the expression with low and diffuse expression in the cells. In addition, the status of HSD3B1/2 was not correlated with that of CYP11B1/2. Therefore, the clinical and biological significance of these two isoforms has still remained largely unknown especially in APA cases.

Therefore, in this study, we first evaluated the mRNA expression of both HSD3B1 and HSD3B2 in APA tissues using real-time quantitative PCR (qPCR) analysis and then studied their immunolocalization using immunohistochemistry analysis. We subsequently examined the correlation of their expression with other key enzymes involved in corticosteroid biosynthesis including CYP11B2, CYP11B1 (11β-hydroxylase) and CYP17A1 (17α-hydroxylase/17, 20-lase), as well as clinicopathological factors of the patients to further explore the significance of these enzymes. In addition, we also evaluated the correlation

between the expression of *HSD3B1/2* and somatic mutations in the sequences of the *KCNJ5* encoding the selectivity filter of the potassium channel, that are frequently identified in APA (Choi et al., 2011).

2. Materials and methods

2.1. Human adrenal tissues

This research protocol was approved by the Ethics Committee at Tohoku University Graduate School of Medicine (Sendai, Japan). All the patients also had read and signed informed consent documents regarding the diagnostic and scientific use of tissue specimens and clinical data.

qPCR and immunohistochemistry analysis were performed in 67 and 100 APA cases, respectively. These were all retrieved from the pathology files at Department of Pathology, Tohoku University Hospital (Sendai, Japan). The histopathological diagnosis of APA was made according to a previous report (Sasano, 1994). All APA patients were treated with spironolactone before surgery.

2.2. RNA isolation and qPCR

RNA isolation with subsequent cDNA production was performed as previously reported (Felizola et al., 2013). qPCR technique was performed using Taqman MGB-containing fluorogenic probes as previously reported by Doi et al. for *HSD3B1/2* (Doi et al., 2010). This technique allows accurate discrimination among only a few base pair mismatches, because of the high sequence similarity between human *HSD3B1* and *HSD3B2*. qPCR for *CYP11B1/2* and *CYP17A1* were performed as previously reported (Felizola et al., 2014c). The primer sequences and Taqman probes used in our present study were summarized in Table 1. The cDNA produced from a human brain specimen was used as a positive control in the *RPL13A* qPCR experiments, while the cDNA from H295R adrenocortical carcinoma cells was used as a positive control for *HSD3B1, HSD3B2, CYP11B1, CYP11B2* and *CYP17A1*.

RPL13A was used as an endogenous control gene. The relative gene expression was calculated by using each standard curve as previously reported (Felizola et al., 2013; Nakamura et al., 2006; Pfaffl, 2001; Shibahara et al., 2012).

2.3. Histological and immunohistochemical analysis

Hematoxylin and eosin (H&E) staining and immunohistochemistry were performed on the specimens fixed with 10% formalin and embedded in paraffin-wax with routine protocol. Table 2 summarized the immunohistochemistry markers of APA evaluated in this study. Antigen-antibody complexes were visualized with 3, 3′-diaminobenzidine (DAB) using a peroxidase-based Histofine SABPO Kit (Nichirei, Tokyo, Japan) or ImmPRESS REAGENT (VECTOR, Burlingame, CA, USA).

After completely reviewing the slides, relative immunoreactivity of each enzyme in tumor specimens was evaluated using H-score of cytoplasm, which consists of a sum of the percentages of positively stained cells multiplied by a weighted immunointensity of staining.

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). The evaluations were independently and blindly carried out by two of the authors (S K F and T M). Serial tissue sections on slides in a paired flip-flopped orientation and the exposed surfaces of each pair of sections allowed the evaluation of two immunoreactivity in the same cells through mirror images of one another ($n = 20$). Subsequently, the number of CYP11B2-positive cells and negative cells were counted in HSD3B1-positive tumor cells in APA in order to further examine the correlation of HSD3B1 with CYP11B2 in the same tumor cells of APA. Double immunostaining of HSD3B1 and CYP11B2 was also performed as follows ($n = 5$); after first staining of HSD3B1 visualized by DAB, antigen retrieval was performed by heating the slides in an autoclave for 5 minutes in EDTA buffer (pH 9.0). Blocking was performed for 1 hour using blocking buffer (normal rabbit serum 10% with SDS 0.5%) at room temperature. After drainage of blocking agents, the reacted sections were incubated with anti-human CYP11B2 overnight at 4 °C. Slides were then incubated with secondary antibody (Nichirei, Tokyo, Japan) at room temperature for 30 minutes and were incubated with alkaline phosphatase conjugated streptavidin (Nichirei) at room temperature for 30 minutes. Slides were subsequently developed with Vector Blue Kit III (Vector Laboratories, Burlingame, CA).

2.4. DNA sequencing in KCNJ5

KCNJ5 primer sequences used in our study were forward, 5′-CGA CCA AGA GTG GAT TCC TT-3′ and reverse, 5′-AGG GTC TCC GCT CTC TTC TT-3′. Annealing temperature at 65 °C, after cooling, all the samples were submitted to electrophoresis in agarose gel 0.1% and DNA purification with a QIAquick gel extraction kit (Qiagen). Analysis of the purified DNA was carried out with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems), and mutations at the G151R and L168R codons of the *KCNJ5* gene were analyzed as described (Choi et al., 2011).

2.5. Statistical analysis

The values for all results in our present study were demonstrated as mean ± SE for quantification of each steroidogenic enzyme and SD for clinicopathological factors. We used Mann–Whitney multiple comparison tests with significance level less than 0.01 among relative mRNA levels and to compare relative mRNA levels of various steroidogenic enzymes in *KCNJ5* mutated APA. Data from qPCR and immunohistochemistry of APA samples were evaluated using Pearson correlation analysis and Spearman's rank-order tests, respectively. A correlation coefficient of R (ρ) more than 0.4 and P value of less than 0.01 was considered significant in this study.

3. Results

3.1. Cases of APA

APA cases examined by qPCR analysis were composed of 67 patients including 42 men and 25 women (men; 62.69%) with the age of 51.86 ± 11.47 years. Systolic and diastolic blood pressure (SBP, DBP) were 153.45 ± 22.86 and 91.38 ± 13.71 mmHg, respectively. Plasma aldosterone concentration (PAC), urine aldosterone, plasma renin activity (PRA), cortisol

and serum potassium (s-K^+) were 48.87 ± 79.07 ng/dl, 25.39 ± 20.47 μg/day, 0.61 ± 1.86 ng/ml/h, 8.83 ± 3.69 ng/dl and 3.41 ± 0.76 mmol/l, respectively.

Those evaluated by immunohistochemistry included 100 patients (61 men and 39 women (men; 61%)), with the mean age of 52.51 ± 11.60 years. All of the APA cases examined were predominantly composed of clear cortical cells and in part compact cells with the mean ratio of compact cells 14.1 ± 0.20% following careful histological evaluation. Spironolactone bodies were detected in 18 of 100 cases (18%). The histological score discerning malignancy proposed by Weiss was 0.25 ± 0.5 (Weiss, 1984). SBP and DBP were 154.54 ± 21.02 and 94.35 ± 13.61 mmHg, respectively. PAC, urine aldosterone, PRA, cortisol and s-K^+ were 28.24 ± 17.39 ng/dl, 22.30 ± 16.36 μg/day, 0.80 ± 1.89 ng/ml/h, 8.73 ± 3.48 ng/dl and 3.49 ± 0.66 mmol/l, respectively.

All were unilateral APA with the tumor diameter as follows, qPCR; 16.03 ± 5.84 mm, immunohistochemistry; 16.47 ± 8.45 mm.

3.2. qPCR analysis

HSD3B1, HSD3B2, CYP11B1, CYP11B2 and *CYP17A1* mRNAs were detected in all the cases of APA examined in this study; the relative mRNA levels were 0.22 ± 0.06, 83.65 ± 9.19, 0.62 ± 0.07, 0.78 ± 0.12 and 1.00 ± 0.12, respectively. The amount of *HSD3B2* mRNA was significantly higher than that of *HSD3B1* in APA cases examined ($P < 0.0001$) (Fig. 1A). The amount of *HSD3B1* mRNA was positively correlated with that of *CYP11B2* ($P < 0.0001$; $R = 0.52$) (Fig. 1B). In addition, the amount of *HSD3B1* mRNA was significantly correlated with PAC ($P < 0.0001$; $R = 0.63$) (Fig. 1C), although not between *HSD3B2* and any of other clinicopathological factors examined in this study (data not shown). No significant correlation was detected between the relative expression levels of *HSD3B1* or *CYP17* mRNA and any of the clinicopathological factors of the patients examined; gender, age, SBP, DBP, urine aldosterone, PRA, s-K^+, cortisol, or PAC (data not shown).

KCNJ5 mutations were detected in 47/67APA cases examined (70.15%). They were significantly more prevalent in females (88.0%) than males (59.52%; $P = 0.0099$). *CYP11B2* mRNA levels were significantly higher in *KCNJ5* mutated APA ($P < 0.0001$) (Fig. 1D). *HSD3B1* mRNA was also significantly higher in *KCNJ5* mutated APA than wild type (WT) cases ($P = 0.011$) (Fig. 1E) but no significant difference was detected in the status of *HSD3B2* between *KCNJ5* mutated and WT APA cases (Fig. 1F). *CYP17A1* mRNA was also significantly lower in *KCNJ5* mutated than WT APA ($P = 0.012$) (Fig. 1G). No significant differences were detected in the status of *CYP11B1* between *KCNJ5* mutated and WT APA (Fig. 1H).

3.3. Immunohistochemistry analysis

The great majority of APA cases were diffusely positive for HSD3B2, while HSD3B1 immunoreactivity detected rather focally in clusters of cells (Fig. 2B and C). HSD3B2 immunoreactivity was detected in all APA cases and its H-scores were 147.16 ± 45.14 (95% confidence interval: 138.21–156.12), significantly higher than those of HSD3B1 (Fig. 3A). HSD3B1 immunoreactivity was also detected in 97 APA and its H-scores were 72.69

± 38.54 (95% CI: 65.04–80.34). CYP11B2 and CYP11B1 immunoreactivity was also detected with their H-scores 50.91 ± 39.06 (95% CI: 43.16–58.66) and 60.12 ±54.40 (95% CI: 49.33–70.91), respectively (Fig. 2D and E). CYP11B2 positive tumor cells were also focally detected in clusters, whereas CYP11B1 positive tumor cells were rather extensively detected in APA tumor tissues. Immunolocalization patterns of CYP11B2 were similar to those of HSD3B1 in some cases (Fig. 2C and D). A significant positive correlation was detected between H-scores of HSD3B1 and CYP11B2 ($P < 0.0001$; $R = 0.49$) (Fig. 3B). The flip-flopped mirror-image tissue sections stained with HSD3B1 and HSD3B2 demonstrated that tumor cells in APA were diffusely positive for HSD3B2, and the major part of them was also positive for HSD3B1 (Fig. 4A and B). That of HSD3B1 and CYP11B2 also revealed that almost all CYP11B2 positive tumor cells were positive for HSD3B1, but not vice versa (Fig. 4C and D). The double positive (HSD3B1 and CYP11B2) tumor cells were detected as illustrated in Fig. 4E. In addition, double positive tumor cells for both HSD3B1 and CYP11B2 were also clearly identified in double immunostaining analysis (Fig. 4F). CYP17A1 immunoreactivity was also detected in tumor cells (Fig. 2F) with its H-scores of 51.61 ± 44.45 (95% CI: 42.79–60.43). A significant positive correlation was also detected between CYP11B1 and CYP17A1 H-scores ($P < 0.0001$; $R = 0.50$) (Fig. 3C). HSD3B1, HSD3B2, CYP11B1, CYP11B2 and CYP17A1 immunoreactivities were all detected in both clear and compact cells. No significant correlation between these enzymes and cell types, tumor size and clinicopathological factors of the APA patients examined (data not shown).

4. Discussion

The enzyme HSD3B plays pivotal roles in the biosynthesis of all hormonal steroids. The human genome contains two HSD3B enzymes sharing 94% sequence homology, HSD3B1 and HSD3B2 (Dupont et al., 1991). Results of our qPCR analysis of APAs demonstrated that mRNA levels of *HSD3B2* were much higher than those of *HSD3B1* (Fig. 1A). In addition, HSD3B2 immunoreactivity was rather diffuse, marked and homogeneous in the tumor cells (Fig. 2B) and had significantly higher H-score compared with that of HSD3B1 in APA (Fig. 3A), while HSD3B1 immunoreactivity was detected in clusters of tumor cells with relatively weaker immunointensity compared with that of HSD3B2 (Fig. 2C). These findings indicated that the HSD3B2 expression was induced in the neoplastic process of aldosterone producing cells in human adrenal glands. However, results of our present study in APA did reveal the absence of significant correlation of mRNA levels of *HSD3B2* with those of other steroidogenic enzymes including *CYP11B2* or with any of clinicopathlogical features related to aldosterone excess of the patients.

On the other hand, HSD3B1 was significantly correlated with CYP11B2 at both protein and mRNA levels in APA tissues. It then becomes important to evaluate whether the same APA tumor cells express these enzymes. We evaluated this by using two different approaches, one double immunostaining and the other immunohistochemistry performed in a pair of flip-flopped mirror image serial tissue sections. Almost all CYP11B2 positive cells were also positive for HSD3B1 but many HSD3B1 positive cells were not CYP11B2 positive. These findings all indicated that CYP11B2 was closely related to HSD3B1 in APA tumor cell. HSD3B1 was reported to have markedly higher enzymatic activity than HSD3B2, by using substrates as pregnenolone, 17α-hydroxypregnenolone and dehydroepiandrosterone

(Thomas et al., 2002). Therefore, the co-localization of HSD3B1 and CYP11B2 in the very same APA tumor cells could play important roles in autonomous production of aldosterone. Results of our present study also suggest the potential therapy of APA patients with the HSD3B1 inhibitors, such as trilostane and epostane, which competitively inhibit purified human HSD3B1 with 12 to 16-fold higher affinities compared with the noncompetitive inhibition of human HSD3B2 (Thomas et al., 2002, 2011) but it awaits further investigations for clinical feasibility.

Somatic mutations in *KCNJ5* have been reported to cause a loss of ion selectivity and cell depolarization in adrenal ZG cells, which result in the opening of voltage-gated calcium channels. The calcium influx stimulates *CYP11B2* gene expression, and subsequently aldosterone secretion is increased (Beuschlein et al., 2013; Oki et al., 2012). Patients with *KCNJ5* mutations were more frequently female (Boulkroun et al., 2012), as demonstrated in our present study. In addition, APA cases with *KCNJ5* mutations have been reported to display higher levels of *CYP11B2* expression compared with those with WT *KCNJ5* (Williams et al., 2012, 2014). In our present study, *CYP11B2* mRNA expression was significantly increased as previously reported in the literature (Fig. 1D). In HAC15 adrenocortical carcinoma cells with T158A mutation of *KCNJ5* gene, no significant differences were reported in mRNA level of *HSD3B2* (Oki et al., 2012). Results of our present study revealed higher expression of *HSD3B1* mRNA in APAs with mutated compared with WT *KCNJ5*, whereas no significant difference of *HSD3B2* mRNA in APAs with mutated and WT *KCNJ5*. This finding is also consistent with the study that angiotensin II-stimulated human adrenocortical H295R cells demonstrated a rapid increase in expression of HSD3B1, but not HSD3B2 (Ota et al., 2014). *HSD3B1* and *HSD3B2* share their coding sequences and promoters, suggestive of the presence of some common regulatory mechanism (Simard et al., 2005). Calcium signaling was reported to be partly involved in the regulation of both HSD3B1 and HSD3B2 expression (Beaudoin et al., 1997; Nogueira et al., 2009). Further possible analyses of whether and to what extent HSD3B1 and HSD3B2 are differently regulated through intracellular calcium signaling are therefore considered important to understand the different degrees of associations detected between HSD3B isoforms and *KCNJ5* mutation. CYP17A1 have been also reported more abundant in APA cases with *KCNJ5* mutated than in WT *KCNJ5* on gene expression profiling (Azizan et al., 2012). However, in our present study, *CYP17A1* mRNA expression was also significantly decreased in APA cases with *KCNJ5* mutated than WT *KCNJ5*. The discrepancy may be due to the difference of ethnicity or race (Japanese versus Caucasian patients) or the rather limited number of the cases examined in our present study but it awaits further investigation for clarification of this interesting difference.

5. Conclusion

In conclusion, we demonstrated for the first time that HSD3B1 may contribute to autonomous over secretion of aldosterone in APA possibly in conjunction with *KCNJ5* mutations.

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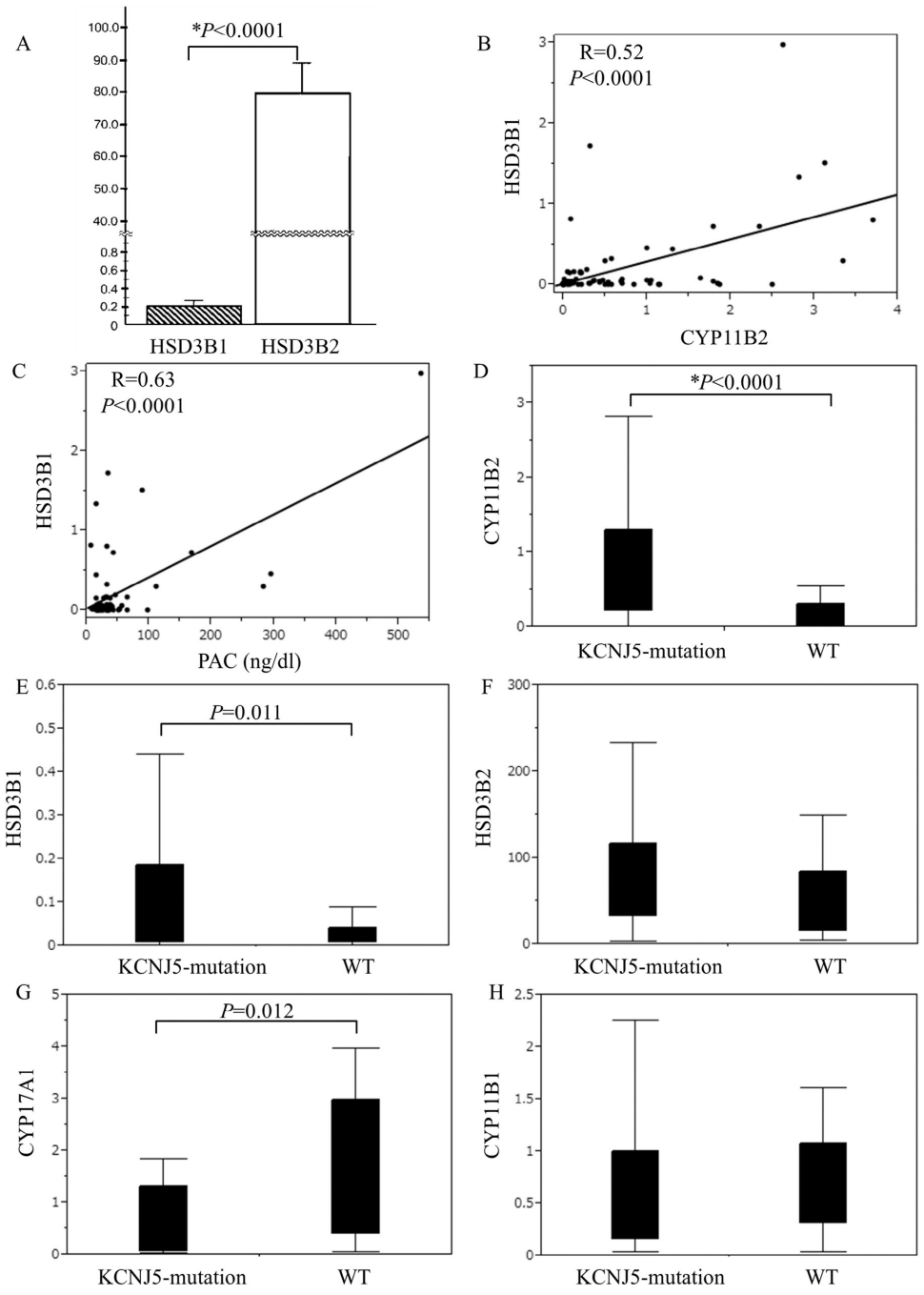

Fig. 1.

Summary of real-time quantitative PCR (qPCR) analysis of *HSD3B1, HSD3B2, CYP11B1, CYP11B2, CYP17A1* and DNA sequencing analysis in *KCNJ5* of 67 aldosterone-producing adenoma (APA) cases. Relative mRNA levels of *HSD3B1/2* (A). A statistically significant positive correlation was detected in their relative mRNA levels between *HSD3B1* and *CYP11B2* ($P < 0.0001$; R = 0.52) (B), *HSD3B1* and plasma aldosterone concentration (PAC) ($P < 0.0001$; R = 0.63) (C), qPCR analysis of *CYP11B2, HSD3B1, HSD3B2, CYP17A1* and *CYP11B1*, comparing the results between *KCNJ5* mutated and wild type

(WT) APA. *KCNJ5* mutations were detected in 47 of 67 APA cases (70.15%). A significantly high level of mRNA of *CYP11B2* ($P < 0.0001$) were detected in the *KCNJ5* mutated APA (D). *HSD3B1* clearly showed tendency of high expression in *KCNJ5* mutated APA ($P = 0.011$) (E), while no significant differences were detected in the status of *HSD3B2* between *KCNJ5* mutated and WT APA (F). *CYP17A1* mRNA expression was also lower in *KCNJ5* mutated APA cases ($P = 0.012$) (G). No significant differences were detected in the status of *CYP11B1* between *KCNJ5* mutated and WT APA (H). PAC, plasma aldosterone concentration; KCNJ5-mutation, *KCNJ5* mutated APA; WT, wild type APA.

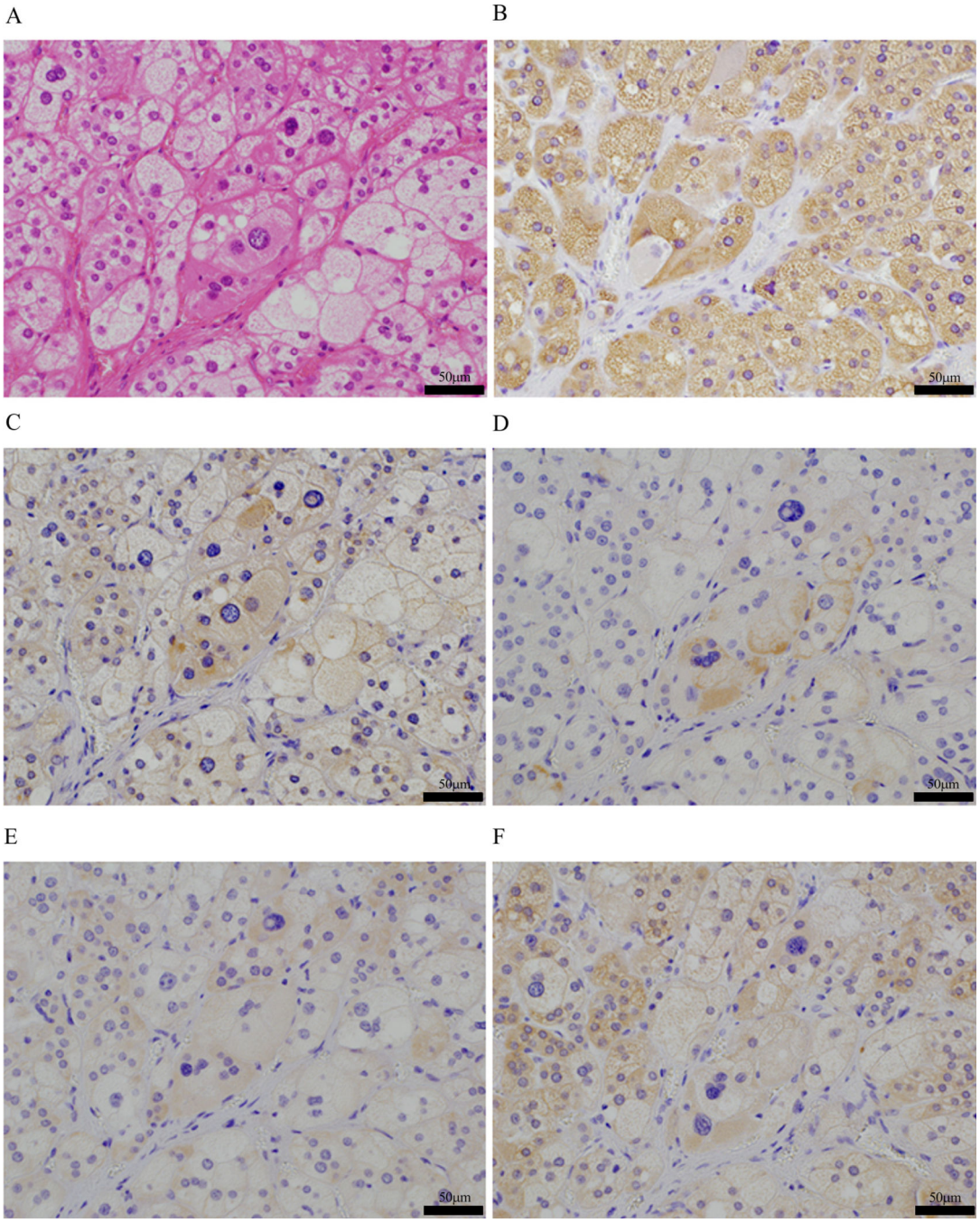

Fig. 2.

Histologically, aldosterone-producing adenoma (APA) are predominantly composed of clear tumor cells (A). APA cells were diffusely positive for HSD3B2 immunohistochemically (B), whereas sporadically or focally positive for HSD3B1 (C). APA cells were also positive for CYP11B2 (D), CYP11B1 (E) and CYP17A1 (F). Distribution of CYP11B2 positive cells was mostly similar to that of HSD3B1 (C and D).

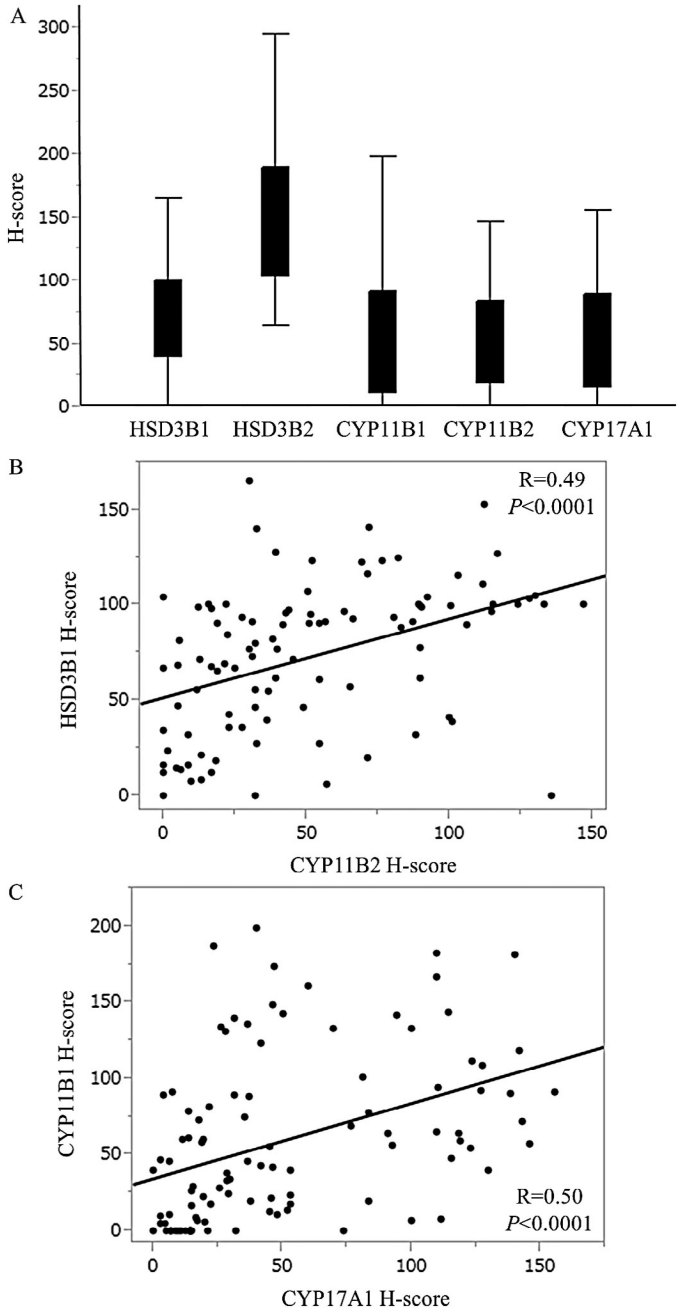

Fig. 3.

Immunohistochemistry analysis of HSD3B1, HSD3B2, CYP11B1, CYP11B2 and CYP17A1 in 100 APA cases. H-scores of each enzyme (A). A statistically significant correlation was detected between HSD3B1 and CYP11B2 ($P < 0.0001$; R = 0.49) (B), CYP11B1 and CYP17A1 ($P < 0.0001$; R = 0.50) (C).

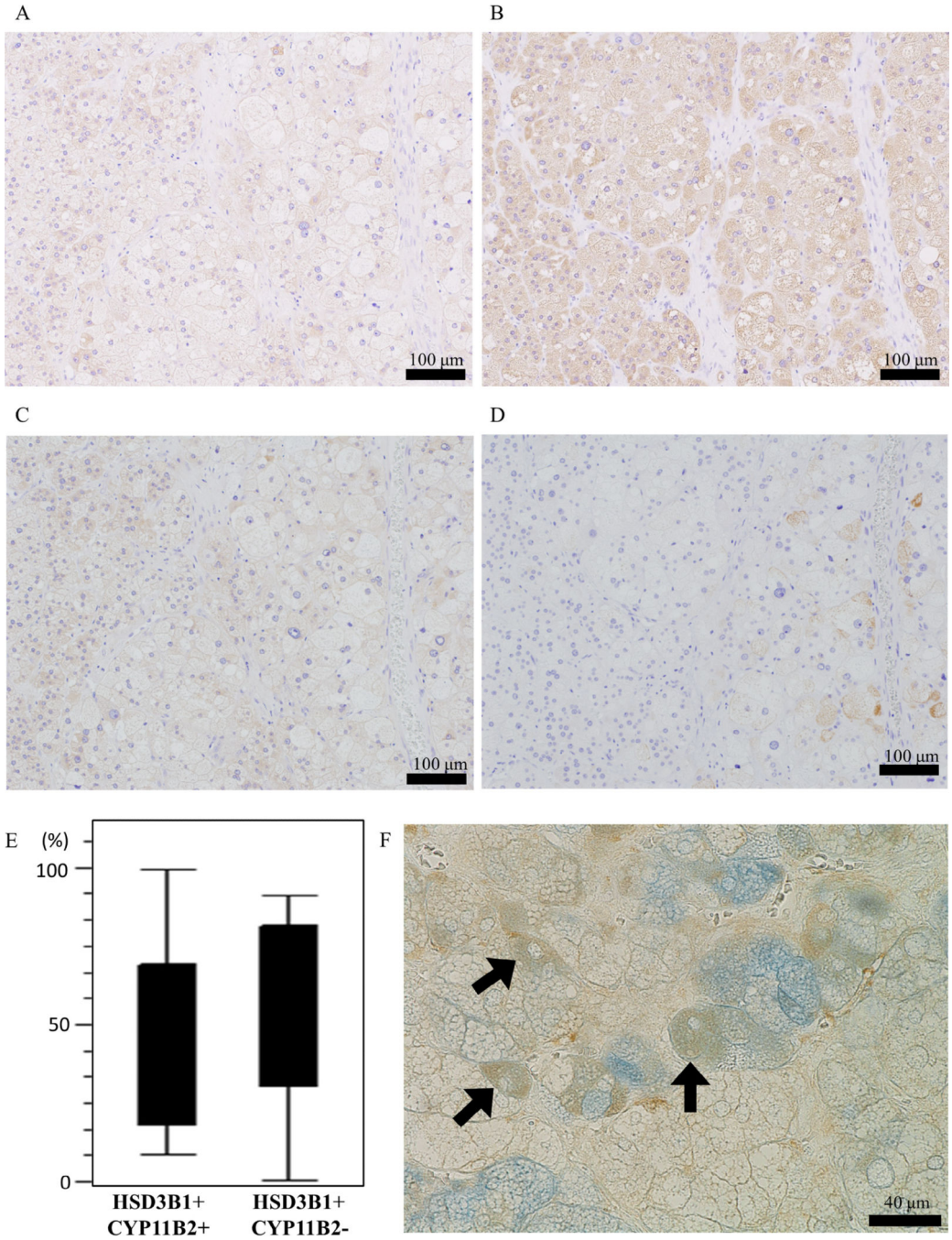

Fig. 4.

A pair of flip-flopped mirror-image tissue sections immunostained with HSD3B1 and HSD3B2, HSD3B1 and CYP11B2 in APA cases (A–E) in order to explore whether these enzymes present in the same tumor cells or not. HSD3B1/2 positive cells were detected in a rather diffuse fashion (A, B and C). CYP11B2 positive tumor cells were focally present (D) in the lesion with relatively wider distribution of HSD3B1 (C). The double positive (HSD3B1/CYP11B2) tumor cells were detected (E). Double immunostaining of HSD3B1 and CYP11B2 visualized by 3, 3′-diaminobenzidine (DAB) and Vector Blue, respectively

(F). The double positive (HSD3B1/CYP11B2) tumor cells were detected (designated by arrows).

Table 1

Sequences of specific primers and Taqman probes for each steroidogenic enzyme used in real-time quantitative PCR (qPCR) study.

Gene symbols	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Reference
HSD3B1	AGA AGA GCC TCT GGA AAA CAC ATG	TAA GGC ACA AGT GTA CAG GGT GC	Doi et al., 2010
			Azmahani et al., 2014
HSD3B2	AGA AGA GCC TCT GGA AAA CAC ATG	CGC ACA AGT CAA GGT ATC A	Doi et al., 2010
			Azmahani et al., 2014
CYP11B1	CCC AGC ACA AAT GGA ACT CCC GA	CCG CTT AAT GAC TCT GAC AGT CTG CG	Felizola et al., 2014a, 2014b, 2014c
CYP11B2	TCC AGG TGT GTT CAG TAG TTC C	GAA GCC ATC TCT GAG GTC TGT G	Felizola et al., 2014a, 2014b, 2014c
CYP17A1	TGA GTT TGC TGT GGA CAA GG	TCC GAA GGG CAA ATA GCT TA	Felizola et al., 2014c
			Azmahani et al., 2014
RPL13A	CCT GGA GGA GAA GAG GAA AG	TTG AGG ACC TCT GTG TAT TT	Felizola et al., 2013
			Nakamura et al., 2006
			Shibahara et al., 2012

Gene symbols	Taqman probes (5′ to 3′)	Reference
HSD3B1	FAM-CCA TAC CCA CAC AGC-MGB	Doi et al., 2010
		Azmahani et al., 2014
HSD3B2	VIC-TCC ATA CCC GTA CAG CA-MGB	Doi et al., 2010
		Azmahani et al., 2014

Table 2

Antibodies used for immunohistochemical studies.

Antibody		Clone	Source/Reference	Dilution
HSD3B1	Mouse monoclonal	37-2	Santa Cruz Biotechnology, Inc. (TX, USA)	1:500
HSD3B2	Mouse monoclonal	12E4	Doi et al., 2014	1:1500
CYP11B1	Rat monoclonal	80.22	Gomez-Sanchez et al., 2014	1:200
CYP11B2	Mouse monoclonal	41.17	Gomez-Sanchez et al., 2014	1:750
CYP17A1	Rabbit polyclonal	–	BEX (Tokyo, Japan) (Nakamura et al., 2014)	1:500