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Invited Review

Expression of CYP11B2 in Aldosterone-Producing Adrenocortical Adenoma: Regulatory Mechanisms and Clinical Significance

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Aldosterone-producing adrenocortical adenoma (APA) is responsible for the majority of cases clinically diagnosed as primary aldosteronism. Aldosterone synthase (CYP11B2) is one of the enzymes that play essential roles in aldosterone synthesis and is involved in the pathogenesis of APA. Recent studies have demonstrated that various factors and regulators influence the expression and function of CYP11B2 in APA. In particular, somatic mutations, such as gain-of-function and loss-of-function mutations, have been identified in several genes, each of which encodes a pivotal protein that affects the calcium signaling pathway, the expression of CYP11B2, and aldosterone production. The gain-of-function mutations were reported in *KCNJ5* that encodes G-protein activated inward rectifier K^+ channel 4 (Kir3.4) and in *CACNA1D,* encoding calcium channel, voltage-dependent, L type, alpha subunit Cav1.3. The loss-of-function mutations were found in *ATP1A1* that encodes Na^+/K^+ ATPase α subunit and in *ATP2B3,* encoding Ca^{2+} ATPase. Furthermore, the aberrant expression of gonadotropin-releasing hormone receptor is associated with the overexpression of CYP11B2 and overproduction of aldosterone in APA with activating mutations in *CTNNB1* encoding β-catenin. On the other hand, CYP11B2 also catalyzes the conversion of cortisol to 18-hydroxycortisol and subsequently converts 18-hydroxycortisol to 18-oxocortisol. The recent studies have identified 18-oxocortisol as an important and distinct biomarker to diagnose primary aldosteronism. In this review, we summarize the recent findings on CYP11B2 and discuss the molecular pathogenesis of APA and the clinical significance of CYP11B2.

Keywords: aldosterone-producing adrenocortical adenoma; CYP11B2; gene mutation; transcription factor; 18-oxocortisol
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Introduction

Primary aldosteronism occurs in 6-10% of all hypertensive patients, caused by the over-secretion of aldosterone from pathological adrenal tissues including aldosterone-producing adrenocortical adenoma (APA) (Milliez et al. 2005). Recent studies have developed several analytical methods and revealed many novel and key findings regarding the molecular pathogenesis of APA, especially the upregulation system of aldosterone synthase (CYP11B2) expression in APA (Oki et al. 2012; Beuschlein et al. 2013; Scholl et al. 2013; Nakamura et al. 2014b; Hattangady et al. 2016).

CYP11B2 is known to convert deoxycorticosterone to corticosterone, corticosterone to 18OH-corticosterone, and finally 18OH-corticosterone to aldosterone (Curnow et al. 1991) (Fig. 1). Therefore, the final step in the biosynthesis of aldosterone is regulated by CYP11B2. One of the mechanisms causing the overproduction and oversecretion of aldosterone in APA may be the elevated expression of CYP11B2. Here, we review the updated literature on the molecular mechanisms, by which CYP11B2 expression is regulated in APA and the clinical significance of its expression.

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Dr. Yasuhiro Nakamura is a recipient of the 2015 Gold Prize, Tohoku University School of Medicine.

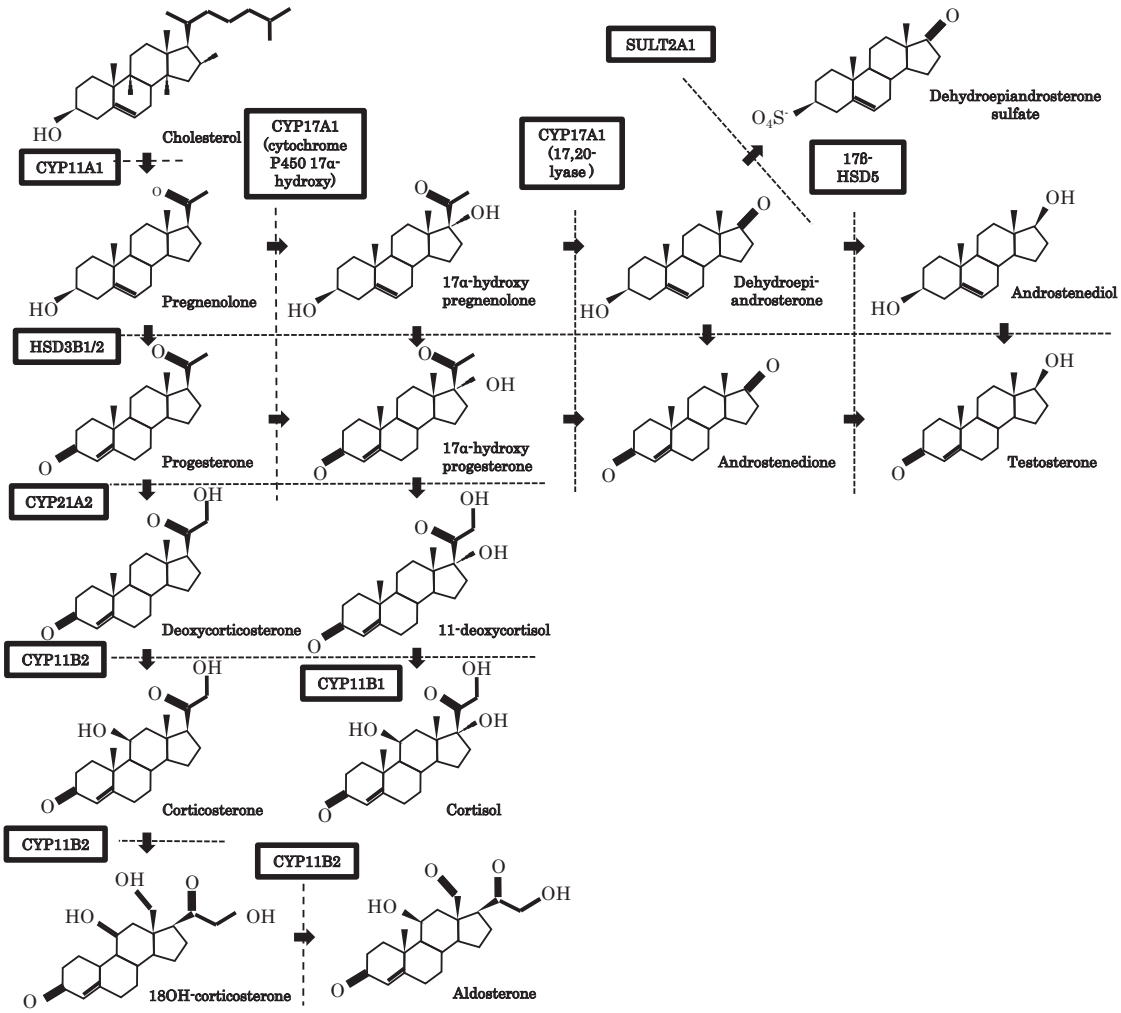

Fig. 1. General pathway of steroid hormone biosynthesis in human normal adrenal glands.

The status of CYP11B2 expression in APA

Studies by Bassett et al. (2005) using microarray and quantitative reverse-transcriptase polymerase chain reaction (qPCR) analyses showed that CYP11B2 mRNA levels are higher in APAs than in normal adrenal glands. In addition, the status of CYP11B2 protein expression in APA has recently been elucidated because of the availability of a CYP11B2-specific monoclonal antibody (Gomez-Sanchez et al. 2014).

The amino acid sequence of CYP11B2 is 93% identical to that of 11β-hydroxylase (CYP11B1) that is involved in the final step of corticosterone synthesis (Nakamura et al. 2014a). Therefore, it was challenging to produce these specific antibodies. However, Gomez-Sanchez et al. (2014) developed specific monoclonal antibodies against human CYP11B1 and CYP11B2, making it possible to analyze the expression of CYP11B1 and CYP11B2 specifically in APA tissues. By using these CYP11B1 and CYP11B2 antibodies, we have reported that the expression level of CYP11B1 was significantly lower, while that of CYP11B2 was significantly higher in APA of smaller size than those in APA of larger size (Ono et al. 2014). Moreover, the relative expression level of CYP11B1 was significantly correlated with tumor area, whereas that of CYP11B2 was inversely correlated with tumor area (Ono et al. 2014). These findings support the hypothesis that both CYP11B1 and CYP11B2 could play cooperative roles in aldosterone production in APA. In addition, we have recently identified a small number of tumor cells co-expressing CYP11B1/B2 (hybrid cell type A), CYP11B2/17 (hybrid cell type B), CYP11B1/17 (hybrid cell type C), and CYP11B1/B2/17 (triple-positive cell) in the APA area (Nakamura et al. 2016). Therefore, we have hypothesized that APA is generally composed of heterogeneous or a mixture of cortical cells from different adrenal zones. In addition, these hybrid cells may represent the origins of tumor cells forming APA.

Somatic mutations in aldosterone-driver genes and CYP11B2 expression in APA

Several somatic mutations in aldosterone-driver genes have been reported, including gain-of-function mutations in *KCNJ5* and *CACNA1D* and loss-of-function mutations in *ATP1A1* and *ATP2B3* (Choi et al. 2011; Azizan et al. 2013;

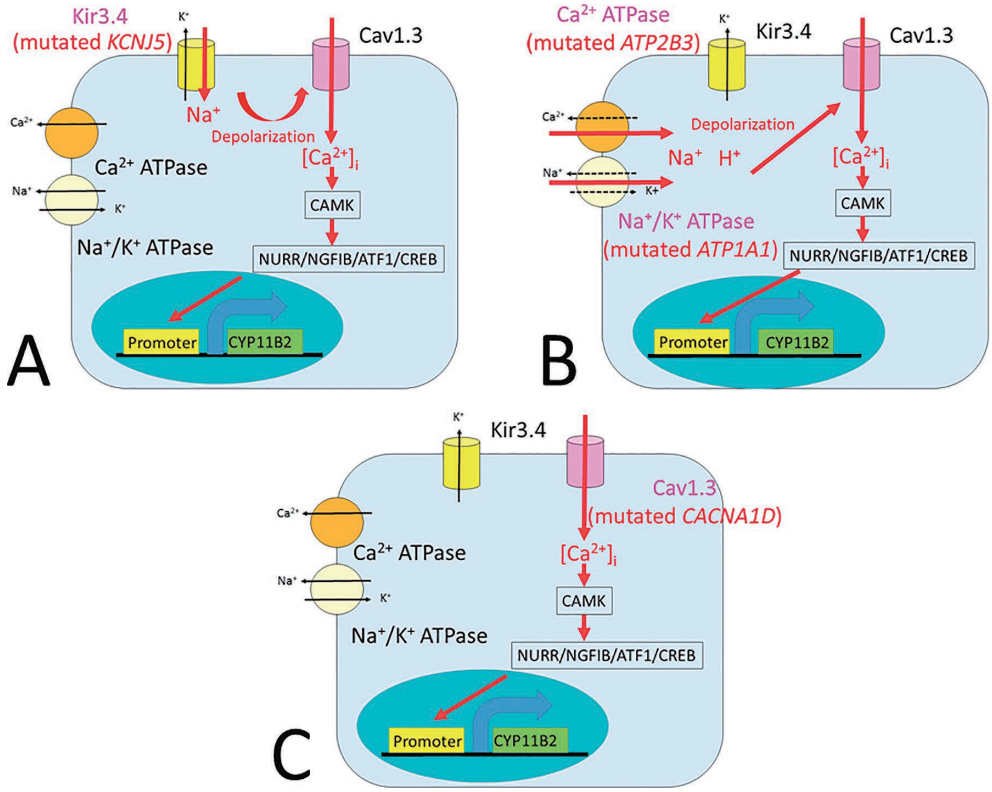

Fig. 2. The schema on signaling pathways of CYP11B2 expression associated with several gene mutations in APA. A. Mutations in *KCNJ5*. B. Mutations in *ATP1A1* or *ATP2B3*. A missense mutation in each gene causes abnormal permeability for sodium or protons, resulting in cellular depolarization, opening of voltage-gated calcium channels (Cav1.3), increased intracellular calcium, the activation of Ca^{2+}/calmodulin-dependent protein kinase (CAMK) and the activation of transcription factors, such as NURR1/NGFIB, CREB, and ATF-1. The activation of these factors can elevate expression level of CYP11B2. C. A mutation of *CACNA1D* causes increased calcium influx and similarly induces the overexpression of CYP11B2 in APA. This figure is constructed, based on previous reports (Choi et al 2011; Beuschlein et al. 2013; Seidel and Scholl 2016).

Beuschlein et al. 2013). The schema on signaling pathways of CYP11B2 expression affected by these mutations in APA is summarized in Fig. 2.

The prevalence of *KCNJ5* somatic mutations was approximately 30-40% of APA cases (Choi et al. 2011; Chen et al. 2015), and it is especially higher among Japanese, occurring in 65% of APA cases (Taguchi et al. 2012). The *KCNJ5* gene encodes G-protein activated inward rectifier K^+ channel 4 (Kir3.4), and its mutation was first detected in a case of familial hypertension III (Geller et al. 2008; Choi et al. 2011). The hot spots of the point mutation in the *KCNJ5* gene are reported as G151R and L168R (Choi et al. 2011). By using in vitro study, Oki et al. (2012) demonstrated that a mutation of *KCNJ5* (T158A) resulted in decreased ion selectivity of the channel, membrane depolarization, activation of voltage-gated Ca^{2+} channels, and increased intracellular Ca^{2+} concentration, CYP11B2 overexpression, and aldosterone production. We also have demonstrated that the relative expression levels of CYP11B2 and HSD3B1 mRNAs were significantly higher in *KCNJ5*-mutated APA (G151R or L168R) than in APA without the *KCNJ5* mutilation, while the mRNA of cytochrome P450 17α-hydroxy/17,20-lyase (CYP17A1), a microsomal enzyme essential for the biosynthesis of adrenal and gonadal steroids, was significantly higher in APA without the *KCNJ5* mutation than in *KCNJ5*-mutated APA (G151R or L168R) (Konosu-Fukaya et al. 2015).

Scholl et al. (2013) identified somatic mutations of *CACNA1D* (G403R and I770M), encoding calcium channel, voltage-dependent, L type, alpha subunit Cav1.3. The mutation results in channel activation at less depolarized potentials and impairment of channel inactivation (G403R), which cause increased Ca^{2+} influx, overexpression of CYP11B2 and over-secretion of aldosterone production

Recent studies have also revealed that somatic mutations in genes encoding ATPase are also involved in the overproduction and over-secretion of aldosterone in APA. Beuschlein et al. (2013) identified somatic mutations in the *ATP1A1* gene encoding Na^+/K^+ ATPase α subunit and the *ATP2B3* gene encoding Ca^{2+} ATPase in APA, which are genes involved in regulating sodium, potassium, and calcium ion homeostasis. They demonstrated by in vitro and ex vivo studies that *ATP1A1* mutants showed loss of pump activity and strongly reduced affinity for potassium, causing inappropriate depolarization of the cells (Beuschlein et al. 2013). In addition, these mutation cases have distinct char-

acteristics, including male dominance, increased plasma aldosterone concentration, and lower potassium concentration, compared with cases without these mutations (Beuschlein et al. 2013).

In addition, Teo et al. (2015) reported three cases of APA with activating mutations in *CTNNB1* encoding *β*-catenin, associated with aberrant *β*-catenin accumulation in the Wnt cell-differentiation pathway and overexpression of luteinizing hormone/choriogonadotropin receptor (LHCGR) and gonadotropin-releasing hormone receptor (GnRHR). Subsequently, *CTNNB1* mutations have been reported in 5.1% of APA cases, and APA tissues with *CTNNB1* mutations were demonstrated to have a significantly higher CYP11B2 mRNA expression level compared with those with *KCNJ5* mutations (Åkerström et al. 2016). However, the underlying mechanisms how *CTNNB1* mutations cause the overexpression of CYP11B2 remain unclear (Seidel and Scholl 2016).

Somatic mutation status and tumor cell morphology

Microscopically, APA is composed mainly of clear cortical cells resembling the zona fasciculata (ZF) cells with round to oval vesicular nuclei, often with a small nucleus and lipid-laden cytoplasm (Nakamura et al. 2014a). Compact cells resembling the zona glomerulosa (ZG) may also be seen in APA in various ratios among APA cases (Nakamura et al. 2014a) Azizan et al. (2013) reported that *KCNJ5* mutations are common in APAs resembling the cortisol-secreting cells of the adrenal ZF but are absent in a subset of APAs resembling the aldosterone-secreting cells of the adrenal ZG. The dimension of tumor tissues with a *KCNJ5* mutation is frequently larger than that of APA with the wild-type *KCNJ5* (Cheng et al. 2015; Åkerström et al. 2016), indicating that a *KCNJ5* mutation might be associated with APA tumorigenesis, including cell proliferation and cytoplasmic lipid depletion. Representative images of APA with a *KCNJ5* mutation are shown in Fig. 3. On the other hand, somatic mutations in either *ATP1A1*, encoding Na^+/K^+ ATPase *α*1 subunit, or *CACNA1D*, encoding calcium channel, voltage-dependent, L type, alpha subunit Cav1.3, are detected in APA cases resembling the aldosterone-secreting cells of the adrenal ZG (Azizan et al. 2013). Kitamoto et al. (2016) recently reported that APAs composed mainly of compact eosinophilic tumor cells tend to harbor *ATPase* mutations, whereas APAs composed predominantly of clear tumor cells tend to harbor *CACNA1D* mutations.

Somatic mutation status and APA intratumoral heterogeneity

Intratumoral heterogeneity in APA has been well focused and is the current topic of interest in this field. APA

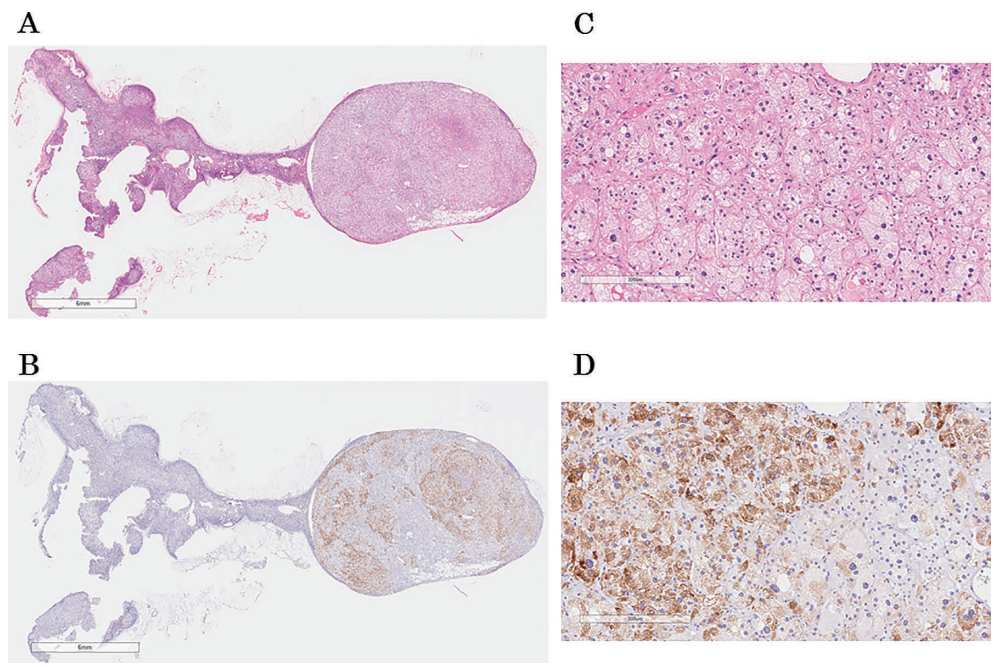

Fig. 3. Representative histological images of APA with the *KCNJ5* mutation (L168R).
The images of APA with low-power magnification (loupe) are shown at left: Hematoxylin & eosin staining (A) and CYP11B2 immunohistochemistry (B). C. Hematoxylin & eosin staining (high-power field, ×100). D. CYP11B2 immunohistochemistry (high-power field, ×100). The tumor is mainly composed of clear cells with lipid-rich cytoplasm, and the size of these neoplastic cells is relatively larger than that of non-neoplastic cells in the adjacent adrenal cortex (C). Mild nuclear atypia is observed, but mitosis and atypical mitosis are not detected. The score of the Weiss criteria, the evaluation system of pathological features commonly used to distinguish adrenocortical adenoma from adrenocortical carcinoma, for this tumor is 0 points with no histological malignancy. CYP11B2 expression is heterogeneously immunolocalized throughout the tumor area (B, D).

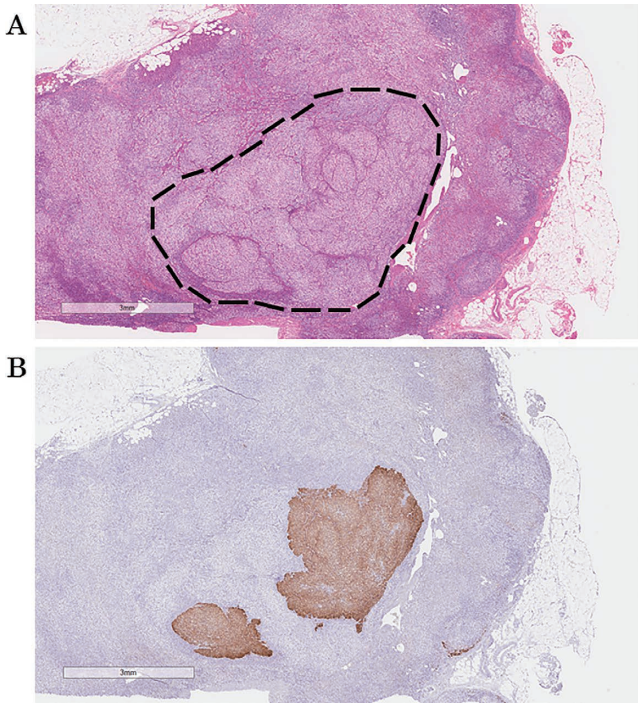

Fig. 4. Representative histological images of APA with distinct heterogeneity.
A. Hematoxylin & eosin staining. The tumor is mainly composed of clear cells mixed with a small number of compact cells. Tumor-like nodules with a fibrous capsule are detected in the tumor area. The tumor borderline is partially unclear because of the absence of a fibrocapsule around the tumor, which makes it difficult to differentiate neoplastic cells from adjacent non-neoplastic cells in the periphery of the tumor. The tumor area is surrounded by the dotted line. B. CYP11B2 immunohistochemistry. CYP11B2 immunolocalization is distinctly heterogeneous in APA and clearly differentiated between the CYP11B2-positive and negative area.

has remarkable intratumoral heterogeneity in cell morphology and molecular physiology. Interestingly, somatic mutations of (*CACNA1D*:F747C, *KCNJ5*:L168R and *ATP1A1*:L104R) were detected only in the CYP11B2-positive area but not in the CYP11B2-negative area of tumors (Nanba et al. 2016). CYP11B2 is commonly immunolocalized diffusely but heterogeneously in tumor tissues. Approximately 15% of APA was shown to have distinct heterogeneity with CYP11B2-positive and negative areas (Fig. 4). Nanba et al. (2016) reported that only one case had different somatic gene mutations detected in the same tumor at different CYP11B2-positive areas. However, it has not been confirmed whether these mutations in aldosterone-driver genes coexist in the same tumor or are exclusive to each other.

These somatic gene mutations play an essential role in autonomous aldosterone over-secretion and might be associated with APA tumorigenesis; however, their pathophysiology remains controversial and requires further investigation.

Transcription factors of CYP11B2 in APA

The transcription factors of the *CYP11B2* gene are considered to affect the pathophysiology of APA. Upregulated CYP11B2 expression and elevated aldosterone biosynthesis are caused by an increase in intracytoplasmic Ca^{2+} concentration, leading to the phosphorylation of CaMK (calmodulin-dependent kinase) and activation of several transcriptional factors of CYP11B2 (Choi et al. 2011; Zennaro et al. 2012). The NGFIB family of orphan nuclear receptors (nerve growth factor-induced clone B or NR4 subgroup) including NGFIB (NR4A1), NURR1 (Nur-related factor 1 or NR4A2), and NOR1 (neuron-derived orphan receptor 1 or NR4A3) (Hazel et al. 1988; Milbrandt 1988; Wilson et al. 1991) are considered to be involved in the regulation of CYP11B2 and APA. Bassett et al. (2004) reported that the *CYP11B2* gene has two functional NGFIB response elements (NBREs) in the promoter region, and both NGFIB (NR4A1) and NURR1 upregulated the expression level of CYP11B2 in vitro. Furthermore, Lu et al. (2004) revealed the upregulated expression of Nurr1 and NGFI-B in APA. Romero et al. (2007) demonstrated by in vitro study that NGFIB, NURR1, and NOR1 upregulated the expression of CYP11B2. Recently, Yarimizu et al. (2015) reported that in response to both angiotensin II and K^+, the phosphorylation of the CREB/ATF family transcription factor ATF2 occurs, and it binds to the *CYP11B2* promoter and activates the transcription. Subsequently, Hattangady et al. (2016) have demonstrated the mutation of *KCNJ5* also causes the activation of *CYP11B2* transcriptional regulators, NURR1 and ATF2. As a next task, it becomes important to analyze the correlation the expression level of ATF2 with clinicopathological factors in APA cases.

The neural regulators of CYP11B2 expression in APA

Certain genes that are classically expressed in the nervous system and play important roles in tissue are also expressed in APA and involved in aldosterone overproduction.

We reported the aberrant expression of GnRHR in APA and confirmed by an in vitro model that the chronic activation of GnRHR with GnRH increased CYP11B2 expression and aldosterone production via the calcium signaling pathway (Nakamura et al. 2014b). In addition, Kishimoto et al. (2016) has recently reported that patients of APA with the higher GnRHR expression showed higher GnRH-stimulated aldosterone response.

According to a previous microarray analysis, Bi et al. (2013) reported that Purkinje cell protein 4 (*PCP4*) is one of the upregulated genes (8.9-fold increase) in APA compared with in normal adrenal cortex. PCP4 is a calmodulin (CaM)-binding protein that accelerates calcium association and dissociation with CaM and has been characterized in neural tissues. We have recently reported the higher expression level of PCP4 in APA and the significant correlation

between PCP4 and CYP11B2 mRNA levels in APA (Felizola et al. 2014). In addition, the relative expression level of PCP4 mRNA was significantly higher in cases with a *KCNJ5* mutation (G151R or L168R) than in cases with the wild-type *KCNJ5* (Felizola et al. 2014). We have also confirmed that PCP4 expression is associated with CYP11B2 mRNA levels and aldosterone production in vitro (Felizola et al. 2014).

The clinical significance of CYP11B2

As mentioned above, the expression of CYP11B2 is driven by certain channel mutations and regulators that result in aldosterone hypersecretion and severe clinical symptoms such as hypertension and hypokalemia. Therefore, some researchers have taken advantage of the increased CYP11B2 in APA and developed diagnostic methods to detect APA. Metomidate, a potent inhibitor of both CYP11B1 and CYP11B2, was first used as a positron emission tomography (PET) radiotracer in the 2000s (Bergström et al. 2000). APA was demonstrated to have a high uptake of ^{11}C-metomidate; in comparison with the current gold standard of adrenal venous sampling, the sensitivity and specificity of ^{11}C-metomidate PET-CT were 76% and 87%, respectively, which were comparable with those of adrenal venous sampling (Burton et al. 2012). Moreover, Abe et al. (2016) recently synthesized a novel imaging agent for PET-CT, ^{18}F-CDP2230, which has an affinity and higher selectivity for CYP11B2 compared with previous agents. Although PET-CT with these imaging agents could be an alternative for noninvasive APA detection, further studies are required to confirm their utility and safety before use in a clinical setting.

Recent studies have also highlighted another characteristic of CYP11B2 that could convert cortisol (F) to 18-oxocortisol (18oxoF) (Freel et al. 2004) (Fig. 5). 18oxoF is a so-called "hybrid steroid" because it has the structural features of both glucocorticoid and mineralocorticoid. Ulick et al. (1983) first reported the presence of 18oxoF in adrenal tissues but in a small amount. However, patients with glucocorticoid-remediable aldosteronism (GRA), a rare variety of primary aldosteronism showing autosomal dominant inheritance, are characterized by unusual sensitivity of aldosterone secretion to ACTH and higher levels of 18oxoF because of the genetic recombination between *CYP11B1* and *CYP11B2* (chimeric *CYP11B1/CYP11B2* gene) (Dluhy and Lifton 1999; Takeda et al. 1999). In GRA, the synthesis of not only aldosterone but also 18oxoF could be suppressed by administering dexamethasone because their synthesis depends on ACTH stimulation. Furthermore, patients with primary aldosteronism, especially those with APA, had higher plasma and urinary 18oxoF levels than patients with essential hypertension (Mulatero et al. 2012). We have, therefore, developed a highly sensitive test based on liquid chromatography-tandem mass spectrometry (LC/MS/MS) to measure 18oxoF (Nakamura et al. 2011), showing that the levels of 18oxoF and the ratio of 18oxoF/F before and after ACTH stimulation were significantly higher in adrenal venous samples of APA cases compared with the levels in those of the contralateral adrenal glands of hyperaldosteronism cases. We also

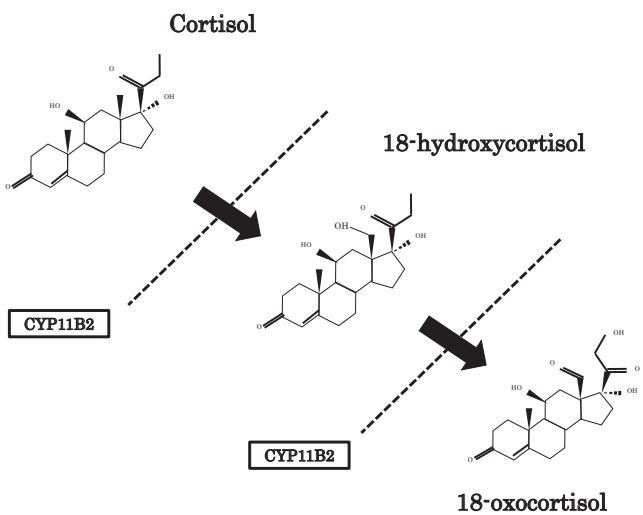

Fig. 5. The biosynthesis of 18-oxocortisol in human normal and pathological adrenal glands.
CYP11B2 converts cortisol to 18-hydroxycortisol and 18-hydroxycortisol to 18-oxocortisol, one of the "hybrid steroids."

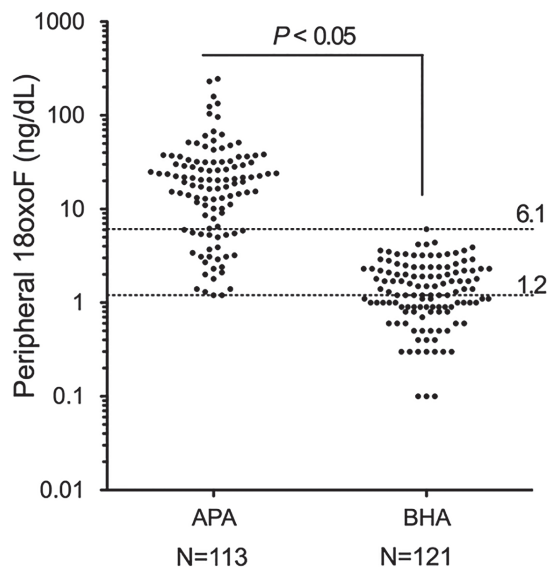

Fig. 6. The level of peripheral plasma 18-oxocortisol (18oxoF) in patients with APA and bilateral hyperaldosteronism (BHA).
The mean level of peripheral plasma 18oxoF is significantly elevated in patients with APA (23.6 ± 3.4 ng/dL), compared to those with BHA (1.89 ± 0.14 ng/dL). The minimum level of peripheral plasma 18oxoF was 1.2 ng/dL in APA cases, while the maximum level of peripheral plasma 18oxoF was 6.1 ng/dL in BHA cases. The original figure is found in Satoh et al. (2015) and is shown with the permission according to the policy of American Heart Association (AHA) Journals (http://www.ahajournals.org/site/rights/) (2015).

showed the clinical significance of peripheral plasma concentrations of 18-hydroxycortisol (18-OHF) and 18oxoF, both of which are synthesized by CYP11B1, in a subtype classification of primary aldosteronism (Satoh et al. 2015) (Fig. 6); namely, the measurement of peripheral plasma 18oxoF demonstrated high diagnostic ability with the sensitivity of 83% and specificity of 99% in the differentiation from bilateral hyperaldosteronism (BHA). These results indicate that the peripheral level of 18oxoF, which is significantly affected by APA secretion, can be a clinically useful biomarker not only to differentiate APA from BHA but also to avoid unnecessary surgery for non-functioning adrenocortical nodules concurrent with hyperplasia or microadenoma (Satoh et al. 2015). However, Williams et al. (2016) demonstrated that the 18oxoF concentration in APA cases with *KCNJ5* mutations was 18- and 16-fold higher in lateralized adrenal veins and peripheral vein plasma, respectively, compared with that in APA cases with other mutations (*ATP1A1*, *ATP2B3*, and *CACNA1D*). The cause of the upregulation of 18oxoF synthesis in APA cases remains unclear. Further research would be required to clarify the significance of 18oxoF.

Summary

CYP11B2 is a key enzyme of primary aldosteronism, and several factors are involved in the regulation of CYP11B2 expression and the overproduction of aldosterone. Somatic mutations in aldosterone-driver genes are strongly associated with CYP11B2 expression and have been only detected in the CYP11B2-positive tumor area, indicating that APA intratumoral heterogeneity corresponds to non-uniform CYP11B2 expression in neoplastic cells. In addition, CYP11B2 can contribute to the clinical diagnosis of primary aldosteronism. CYP11B2 has the potential to synthesize hybrid steroids, which is a unique and characteristic behavior of APA that is distinctive from BHA. CYP11B2 is also directly associated with disease severity and clinical complications; however, the pathophysiology of primary aldosteronism in both APA and non-neoplastic subtypes remains controversial.

Conflict of Interest

The authors declare no conflict of interest.

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