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 Ca2+ 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


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

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++/calmodulin-dependent protein kinase (CAMK) and the activation of transcription factors, such as NURR1/NGFI-B, 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).

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++ channels, and increased intracellular Ca++ 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 mutation, 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).

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 of ATP1A1 gene encoding Na+/K+ ATPase α subunit and the ATP2B3 gene encoding Ca++ 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 tumorigensis, 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 α1 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).](image_url)

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 immuno-localized throughout the tumor area (B, D).
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has remarkable intratumoral heterogeneity in cell morphology and molecular physiology. Interestingly, somatic mutations of \((CA\mathit{nA1D}:F747C, K\mathit{CN}J3: L168R \text{ and } A\mathit{TP1A1}:L104R)\) 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 (Nurr-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 Nur1 and NGFIB 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 \(K\mathit{CN}J5\) 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 (\(P\mathit{CP}4\)) is one of the upregulated genes (8.9-fold increase) in APA compared with in normal adrenal cortex. \(P\mathit{CP}4\) 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 \(P\mathit{CP}4\) 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 11C-metomidate; in comparison with the current gold standard of adrenal venous sampling, the sensitivity and specificity of 11C-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, 18F-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.”](image)

![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).](image)
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 later-alized 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.

**References**


