The landscape of molecular mechanism for aldosterone production in aldosterone-producing adenoma

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Abstract. Primary aldosteronism is the most common form of secondary hypertension with a prevalence of 5–10% in hypertensive patients. Aldosterone-producing adenoma (APA) is a subtype of primary aldosteronism, and somatic mutations in KCNJ5, ATP1A1, ATP2B3, CACNA1D, CLCN2, or CTNNB1 were identified and recognized to drive aldosterone production and/or contribute to tumorigenesis in APA. Mutations of KCNJ5, ATP1A1, ATP2B3, CACNA1D, and CLCN2 are known to activate calcium signaling, and its activation potentiate CYP11B2 (aldosterone synthesis) transcription in adrenal cells. Transcriptome analyses combined with bioinformatics using APA samples were conductive for each gene mutation mediated pivotal pathway, gene ontology, and clustering. Several important intracellular molecules in increase aldosterone production were detected by transcriptome analysis, and additional functional analyses demonstrated intracellular molecular mechanisms of aldosterone production which focused on calcium signal, CYP11B2 transcription and translation. Furthermore, DNA methylation analysis revealed that promoter region of CYP11B2 was entirely hypomethylated, but that of other steroidogenic enzymes were not in APA. Integration of transcriptome and DNA methylome analysis clarified some DNA methylation associated gene expression, and the transcripts have a role for aldosterone production. In this article, we reviewed the intracellular molecular mechanisms of aldosterone production in APA, and discussed future challenges for basic studies leading to clinical practice.

Key words: Aldosterone-producing adenoma, CYP11B2, Calcium signaling, DNA methylation, Molecular mechanism

Introduction

Primary aldosteronism is the most common form of secondary hypertension with a prevalence of 5–10% in hypertensive patients. The most common subtypes of primary aldosteronism are aldosterone-producing adenoma (APA) and bilateral idiopathic hyperaldosteronism [1]. Whole exome sequencing has revealed that somatic mutations in the ion channel genes: KCNJ5, CACNA1D, CLCN2 and pumps ATP1A1 and ATP2B3, or in CTNNB1 gene were identified in APAs [2-6]. Recently, somatic mutations identified by next generation sequencing in CYP11B2 expressing APA covering all coding exons and intron-exon junctions of these genes were present in more than 93% of APAs [7]. The development of advanced technology such as next generation sequencing, RNA sequencing, and DNA methylation have helped clarification of aldosterone production machinery in APA. Omics analysis including genome, methylome, proteome and transcriptome have provided important and meaningful pathway, gene ontology, clustering and others information in APA.

The mutations of genes, which code for membrane channel or pumps, commonly lead to activating intracellular calcium signaling, ultimately resulting in autonomous over-production of aldosterone [2-4]. Transcriptome and DNA methylation analysis led to the identification of multiple important molecules for calcium signaling in APA. Activation of calcium signaling stimulates transcription of rate-limiting enzymes, CYP11B2 (aldosterone synthase), in the biosynthesis of aldosterone in APA. The CYP11B2 protein is detected in APA by immunohistochemistry analysis using specific CYP11B2 monoclonal antibodies [8].

The molecular mechanisms of aldosterone production in APA have expanded greatly in recent years. In this review article, we provide an overview of the molecular
mechanisms of aldosterone production in APA. Some familial forms of PA, familial hyperaldosteronism (FHA), have been recognized, and germline mutations of responsible gene such as KCNJ5 (FHA3), CLCN2 (FHA2) and CACNA1H (FHA4) have been reported [2, 9-11]. In this review, we focus on the reports related with isolated APA, but not FHA or bilateral idiopathic hyperaldosteronism.

**Steroidogenic Enzymes in APA**

The precursor of steroid hormones is cholesterol, which is utilized from low-density lipoprotein and high-density lipoprotein. Cholesterol is transported into the mitochondria via steroidogenic acute regulating (StAR) protein, and converted to pregnenolone by the CYP11A1. Pregnenolone is then acted sequentially by several enzymes including the HSD3B2 and CYP21A2 in the endoplasmic reticulum (ER) to generate deoxycorticosterone which then moves into the mitochondria where it is acted upon the CYP11B2 to generate aldosterone (Fig. 1).

CYP11B2 is the rate-limiting enzyme to produce aldosterone. In fact, only CYP11B2 was elevated among steroidogenic enzymes when we transduced a KCNJ5 mutant in HAC15 cells [12]. CYP11B2 mRNA is highly expressed in APA compared with normal adrenal tissue by microarray analysis [13, 14]. Proteome analysis revealed that APA had marked increase of CYP11B2 protein levels compared with adjacent adrenal cortex [15]. CYP11B2 protein detection is useful for the pathological diagnosis of APA, as nonfunctioning adrenocortical adenomas (NFAs) are common in adrenals, but are not the source of aldosterone production as they do not express the CYP11B2 [8]. APA with any of the mutations have CYP11B2 expression, although there are some differences of staining intensity among the mutations [16, 17]. Collectively, it is suggested that CYP11B2 immunostaining should be included in diagnostic criteria of APA [18].

The amino acid sequences of CYP11B2 are 93% homologous with 11 β-hydroxylase (CYP11B1), catalyzing the final step of cortisol synthesis. The development of monoclonal antibodies for CYP11B2 enabled CYP11B2

![Fig. 1 Proposed molecular mechanisms focused on calcium signaling for aldosterone production in aldosterone-producing adenoma (APA). The somatic mutations of genes such as KCNJ5, ATP1A1, ATP2B3, CACNA1D, and CLCN2 lead to membrane depolarization and/or open of voltage gated Ca^{2+} channels, thereby increasing intracellular Ca^{2+} in cytosol. The intracellular molecules such as VSNL1, PCP4, and GSTA1 serve as transmitters of calcium signaling, and they stimulate CYP11B2 transcription. Cytosol Ca^{2+} levels are also regulated via inositol 1,4,5-trisphosphate receptor (IP3R) mediated Ca^{2+} release from endoplasmic reticulum (ER), and the Ca^{2+} storage in ER is regulated by sarcoendoplasmic reticulum calcium transport ATPase (SERCA) in collaboration with CALN1. Some transcription factors due to calcium signal bind to CYP11B2 promoter regions which are already hypo-methylated, resulted in acceleration of CYP11B2 transcription. Subsequently, CYP11B2 translation is initiated, and it may be mediated partly by CLGN. CYP11B2 protein which located in ER, and increased CYP11B2 stimulated aldosterone production and secretion.](image-url)
expression in APA, and separate from CYP11B1 expression [8]. The antibody is also useful for western blotting, and thus it is utilized in basic studies focused on CYP11B2 expression using cell culture [19].

The expressions of steroidogenic enzymes except for CYP11B2 were influenced by the detection method and adrenal reference tissue, and the difference appeared to be slight if any. APAs had lower expression of CYP17A1 and CYP11B1 compared to adjacent adrenal cortex [20-22]. In comparison with NFA using microarray data, we showed that APA had lower CYP11B1 expression and comparable levels of CYP17A1 [23, 24]. A recent proteome analysis revealed that HSD3B2 and CYP21A2 protein levels were slightly elevated in APA, whereas CYP17A1 and CYP11B1 protein levels were not different compared with adjacent adrenal cortex [15]. Some differences in the expression of steroidogenic enzymes are likely among APA genotypes. Transcriptome analysis demonstrated that CYP17A1 expression in KCNJ5 mutated APA was more abundant than in APA without other or no mutations [22]. APA expressing CYP11B1 by immunohistochemistry analysis were more frequent with KCNJ5 mutation [17]. CYP17A1 immunoreactivity was higher in KCNJ5 or CACNA1D-mutated APAs compared with ATP2B3-mutated APAs [16].

Gene Expression Profiles by Transcriptome Analysis in APA

Transcriptome analysis has enabled to know of all the gene expression changes including coding and non-coding genes in tissues in a single study. Data obtained from the transcriptome lead to the comparison of the difference of gene expression profile among two or more tissues, and bioinformatics help to identify potential associations of gene sets with pathway or gene ontology terms.

After discovery of somatic gene mutations in APA, gene expression profiles were compared among somatic mutations or between with and without a somatic mutation. Initial analysis of the transcriptome using hierarchical clustering between APA with and without KCNJ5 mutations showed no differences of gene expression profile [25]. Clustering analysis using differentially expressed genes between them could separate KCNJ5 mutated APA from APA without KCNJ5 mutation [25]. Subsequent reports also indicated that clustering analyses using differentially expressed genes between APA with and without KCNJ5 mutation or among some mutations including KCNJ5, ATP1A1 and ATP2B3 showed gene expression profiles were different among mutations [26, 27].

The clustering analysis revealed that APAs without any mutation had heterogenous characteristics of gene expression [27], however the study analyzed only hot spot of each genes. Recent genetic analysis using next generation sequencing covering all coding exons and intron-exon junctions may clearly differentiate APA without mutations from APA with the mutations. The APAs without known mutations may have somatic mutations in undiscovered region or genetic abnormalities except for a somatic mutation.

Taken together, the results of hierarchical clustering analysis were influenced by the data analysis method and sample selection. Although hierarchical clustering shows rough difference of gene expression among mutations, the important point is that functional analyses are needed to investigate the effect of each mutation on aldosterone production or tumorigenesis.

Functional Analysis Based on Transcriptome Analysis in APA

The KCNJ5 mutation around the pore region of the channel cause loss in K⁺ selectivity and an increase Na⁺ influx into cytoplasm, resulting in the depolarization of the plasma membrane. Subsequently, activation of the voltage-gated Ca²⁺ channel, followed by accumulation of intracellular Ca²⁺ and CYP11B2 transcription [2, 12]. Activation of intracellular calcium signaling is a common pathway in ATP1A1, ATP2B3, CACNA1D and CLCN2 mutations for autonomous aldosterone overproduction [3, 4]. Even before the elucidation of the somatic mutations of channels and pumps, calcium signaling has been known to be one of the main intracellular regulators for CYP11B2 transcription [28, 29]. We and other researchers have focused on the key factors in calcium signaling mediated aldosterone production in APA.

VSNL1 is neuronal calcium signal protein and act as modulator of intracellular targets is reported to be highly expressed in APAs [24, 30]. VSNL1 over-expression induced CYP11B2 transcription and silencing VSNL1 inhibited angiotensin II mediated CYP11B2 transcription in adrenal cells, and suggesting to have a role in aldosterone production [30]. Additionally, it has antiapoptotic function in the inhibition of calcium-induced apoptosis especially in APAs harboring KCNJ5 mutations [30].

On the basis of transcriptome analysis using APA samples, CALN1, encoding calneuron 1, was one of the most upregulated genes, and the most highly correlated with CYP11B2 among 155 genes encoding calcium binding protein with EF-hand, a motif of Ca²⁺ binding [24]. CALN1 which colocalizes with ER increased Ca²⁺ storage in ER to collaborate with sarcoendoplasmic reticulum calcium transport ATPase (SERCA) [24]. The stored Ca²⁺ were released by inositol 1,4,5-trisphosphate (IP3)
stimulation or extruded by leakage via IP3 receptor, and potentiated CYP11B2 transcription and aldosterone production in adrenal cells [24, 31].

GSTA1, encoding Glutathione-S-transferase A1, is enzyme for intracellular detoxification, and GSTA1 inhibition and downregulation up-regulated intracellular Ca^{2+} levels and stimulated aldosterone production in human adrenal cells [32]. In KCNJ5 mutated APA with high aldosterone levels compared with those with low aldosterone levels, GSTA1 was the lowest expressed in transcriptome analyses [32, 33]. Furthermore, oxidative stress involved with GSTA1 seem to regulate aldosterone production in adrenal cells [32, 33].

Wnt/β-catenin signal is also activated in 70% of APA [34], whereas somatic mutation of CTNNB1, encoding β-catenin, is detected only in small group of APA [5]. SFRP2, an inhibitor of Wnt signal, has lower expression in APA, and play a role for APA development and indirect activation of CYP11B2 promoter [34]. NPNT, nephronectin, has the highest expression in zona glomerulosa type cells, compact cells, like APA with CTNNB1 mutation. NPNT is a secreted matrix protein and regulated by canonical Wnt pathway [35]. In adrenal cells, overexpression or silencing of NPNT increased or decreased aldosterone production, respectively [36].

CLGN, calmin, is the highest expressed genes among ER related genes in APA in comparison to NFA by transcriptome analysis [19]. CLGN over-expressing adrenal cells increased CYP11B2 protein levels and aldosterone production, but not CYP11B2 transcription [19]. The RNA sequencing analysis using CLGN over-expressing adrenal cells revealed that tRNA metabolic process and aminoacyl-tRNA biosynthesis were the most enriched pathways, indicating translation activation [19].

We summarized these molecules mediating the aldosterone production machinery in the Fig. 1. The molecules related with intracellular calcium signaling involve the activation, Ca^{2+} storage and antiapoptotic effects, and the activated calcium signal potentiation of CYP11B2 transcription.

**DNA Methylation Profile in APA**

DNA demethylation at the cytosine of 5'-cytosine-guanine-3' dinucleotides of the gene promoter region typically potentiate gene transcription [37]. Gene transcription due to the alterations of DNA methylation implicate hormone production as well as tumor progression, cell survival, and DNA damage repair [37, 38].

Recent advanced technology using Infinium Human-Methylation450 BeadChip kit (Illumina, San Diego, CA, USA) enable to determine >485,000 individual methylation site in a single analysis [39]. According to our previous analysis, the number of differentially methylated CpG sites was ~56,000 in comparing between APA and NFA [23]. The results described below are still only a small fraction of DNA methylation mediated aldosterone production at present, researchers will have to spend more time to clarify the entire landscape of gene transcriptional regulation via DNA methylation in APA.

The promoter region of the CYP11B2 gene in APA is extensively hypo-methylated compared with NFA among the genes of steroidogenic enzymes [23]. Hypomethylation of CYP11B2 in APA compared with adjacent adrenal gland was also observed [40, 41]. These methylation levels were not different among APAs with or without any gene mutations, and a KCNJ5 mutation did not alter the methylation levels in human adrenal cells [23].

We summarized the reported genes which might be regulated by DNA methylation and regulate aldosterone production or tumorigenesis in APA (Table 1). These genes were extracted by integrated analysis of transcriptome and methylome. MC2R, HPX, HTR4, PTGER1 and PCP4 genes showed hypomethylation and mRNA up-regulation in APA, and suggested to have a role in aldosterone production [40, 42, 43]. PRRX1, RAB38, FAP, GCNT2 and ASB4 related with tumorigenesis were up-regulated and hypomethylated in APA as compared to adjacent adrenal gland [40].

The expression of PCP4 which is a calmodulin accessory protein were increased in APA, and PCP4 suppression decreased CYP11B2 transcription in adrenal cells [44]. PCP4 was one of the most hypomethylated genes among calcium signal related genes in APA, and the methylation levels was inversely correlated with PCP4 expression levels in APA [43]. The study also demonstrated that CEBPA binds to the hypomethylation site by chromatin immunoprecipitation assay, thereby indicating that PCP4 expression may be associated with DNA methylation in conjunction with the transcriptional factors CEBPA [43].

Not only CYP11B2 is hypomethylated in APA, but also several molecules involved in CYP11B2 transcription are hypo- or hyper-methylated (Table 1). The collaboration of intracellular signaling and DNA methylation may regulate several gene expression for aldosterone over-production in APA. Analyses using chromatin immunoprecipitation assays are laborious and involve an immense amount of time and effort to clarify the entire regulation by which DNA methylation mediated gene transcription occurs. In addition, there are no methods to modulate methylation levels of specific and interesting regions of DNA, and thus gene transcription via direct effects of DNA methylation cannot be assessed at the present moment.
Conclusions and Perspectives

The clarifications of molecular mechanisms for aldosterone production and the association between molecular mechanisms and clinical findings has potentials to lead to pharmacological therapeutic targets, the development of new drugs may produce improvements in the methods for screening, confirmation tests, and medical therapy of APA. As we summarized, some molecules related with calcium signaling have important roles for suppression of aldosterone production in APA. Entire intracellular mechanisms of aldosterone production are still complex. In addition, recent basic studies suggested that mRNA translation and translation via miRNA also participate aldosterone regulation in APA [19, 45-47], however these have not been fully elucidated. Significant more work including basic studies on the regulation of aldosterone biosynthesis will be necessary for the development of medical therapies for primary aldosteronism and aldosterone-producing adenomas. Current molecular technologies and bioinformatics will be crucial in obtaining the information to solve this complicated issue.

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Table 1 Summary of genes associated with DNA methylation in APA

| Gene name | Description | Methylation level in APA | Reference tissue | Reference |
|-----------|-------------|--------------------------|------------------|-----------|
| AVPR1A    | arginine vasopressin receptor 1A | Hypermethylated | normal adrenal cortex and NFA | Howard, et al. [41]. |
| PRKCA     | protein kinase C alpha | Hypermethylated | normal adrenal cortex and NFA | Howard, et al. [41]. |
| MC2R      | melanocortin 2 receptor | Hypomethylated | adjacent adrenal gland | Murakami, et al. [40]. |
| HPX       | hemopexin | Hypomethylated | adjacent adrenal gland | Murakami, et al. [40]. |
| PRRX1     | paired related homeobox 1 | Hypomethylated | adjacent adrenal gland | Murakami, et al. [40]. |
| RAB38     | member RAS oncogene family | Hypomethylated | adjacent adrenal gland | Murakami, et al. [40]. |
| FAP       | fibroblast activation protein alpha | Hypomethylated | adjacent adrenal gland | Murakami, et al. [40]. |
| GCNT2     | glucosaminyl (N-acetyl) transferase 2 | Hypomethylated | adjacent adrenal gland | Murakami, et al. [40]. |
| ASB4      | ankyrin repeat and SOCS box containing 4 | Hypomethylated | adjacent adrenal gland | Murakami, et al. [40]. |
| HTR4      | 5-hydroxytryptamine receptor 4 | Hypomethylated | NFA | Itcho, et al. [42]. |
| PTGER1    | prostaglandin E receptor 1 | Hypomethylated | NFA | Itcho, et al. [42]. |
| PCP4      | Purkinje cell protein 4 | Hypomethylated | NFA | Kobuke, et al. [43]. |

APA, aldosterone-producing adenoma; NFA, nonfunctioning adrenocortical adenoma.

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