Tohoku J. Exp. Med., 2021, **254**, 1-15

Invited Review for the 100th Anniversary of the TJEM

Pathology of Aldosterone Biosynthesis and its Action

Xin Gao,[1] Yuto Yamazaki,[1] Yuta Tezuka,[2,3] Kei Omata,[2,3] Yoshikiyo Ono,[3] Ryo Morimoto,[3] Yasuhiro Nakamura,[4] Takashi Suzuki,[5] Fumitoshi Satoh[2,3] and Hironobu Sasano[1]

[1]Department of Pathology, Tohoku University, Graduate School of Medicine, Sendai, Miyagi, Japan
[2]Division of Clinical Hypertension, Endocrinology and Metabolism, Tohoku University Graduate School of Medicine, Sendai, Miyagi, Japan
[3]Division of Nephrology, Endocrinology, and Vascular Medicine, Tohoku University Hospital, Sendai, Miyagi, Japan
[4]Division of Pathology, Faculty of Medicine, Tohoku Medical and Pharmaceutical University, Sendai, Miyagi, Japan
[5]Department of Pathology and Histotechnology, Tohoku University Graduate School of Medicine, Sendai, Miyagi, Japan

Aldosterone plays pivotal roles in renin-angiotensin-aldosterone system in order to maintain the equilibrium of liquid volume and electrolyte metabolism. Aldosterone action is mediated by both mineralocorticoid receptor and 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2). Its excessive actions directly induced tissue injuries in its target organs such as myocardial and vascular fibrosis in addition to chronic kidney diseases. Excessive aldosterone actions were also reported to be involved in unbalanced electrolyte metabolism in inflammatory bowel disease and development of pulmonary diseases. Hyperaldosteronism is tentatively classified into primary and secondary types. Primary aldosteronism is more frequent and has been well known to result in secondary hypertension with subsequent cardiovascular damages. Primary aldosteronism is also further classified into distinctive subtypes and among those, aldosterone-producing adenoma is the most frequent one accounting for the great majority of unilateral primary aldosteronism cases. In bilateral hyperaldosteronism, aldosterone-producing diffuse hyperplasia and aldosterone-producing micronodules or nodules are the major subtypes. All these aldosterone-producing lesions were reported to harbor somatic mutations including *KCNJ5, CACNA1D, ATP1A1* and *ATP2B3*, which were all related to excessive aldosterone production. Among those mutations above, somatic mutation of *KCNJ5* is the most frequent in aldosterone-producing adenoma and mostly composed of clear cells harboring abundant aldosterone synthase expression. In contrast, *CACNA1D*-mutated aldosterone-producing micronodules or aldosterone-producing nodules were frequently detected not only in primary aldosteronism patients but also in the zona glomerulosa of normal adrenal glands, which could eventually lead to an autonomous aldosterone production resulting in normotensive or overt primary aldosteronism, but their details have remained unknown.

Keywords: aldosterone; 11β-hydroxysteroid dehydrogenase; mineralocorticoid receptor; pathology; primary aldosteronism
Tohoku J. Exp. Med., 2021 May, **254** (1), 1-15.

Introduction

Aldosterone is a pivotal component of the renin-angiotensin-aldosterone system (RAAS), which maintains the homeostasis of liquid volume and electrolyte metabolism (Laragh et al. 1972, Laragh and Sealey 2011, Patel et al. 2017) and is produced in the zona glomerulosa (ZG) of adrenal cortex as a result of the activation of angiotensin II (ANG II). Aldosterone could bind to mineralocorticoid receptor (MR) to regulate the reabsorption of water, sodium

Received March 22, 2021; revised and accepted April 7, 2021. Published online May 18, 2021; doi: 10.1620/tjem.254.1.
Correspondence: Hironobu Sasano, M.D., Ph.D., Department of Pathology, Tohoku University School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980- 8575, Japan.
e-mail: hsasano@patholo2.med.tohoku.ac.jp

and potassium (Booth et al. 2002; Nakamura et al. 2016; Seccia et al. 2018). However, MR could be activated not only by mineralocorticoids such as aldosterone but also by glucocorticoids including cortisol and corticosterone due to their similar binding affinity to MR (Krozowski and Funder 1983; Arriza et al. 1987; Sheppard and Funder 1987). Therefore, the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) plays pivotal roles in conferring mineralocorticoid specificity through *in situ* degradation of cortisol or converting cortisol to cortisone which has little binding affinity to MR and has been reported to be co-localized with MR in almost all human tissues by our group (Edwards et al. 1988; Funder et al. 1988; Hirasawa et al. 1997, 1999, 2000; Stewart et al. 1987; Suzuki et al. 1998; Takahashi et al. 1998). In addition, aldosterone has been also reported to influence functions of many organs other than kidney, including cardiac tissues, vascular smooth muscles, colon, lacrimal glands, sweat glands, bronchial epithelium and others and exert harmful or compensatory effects on these organs above (Brilla et al. 1990; Weber and Brilla 1991; Takahashi et al. 1999; Iwakura et al. 2014).

An autonomous overproduction of aldosterone or primary aldosteronism (PA) is frequently associated with somatic mutations of the genes including potassium inwardly rectifying channel subfamily J member 5 (*KCNJ5*), calcium voltage-gated channel subunit alpha1 D (*CACNA1D*), ATPase Na^+/K^+ transporting subunit alpha 1 (*ATP1A1*), and ATPase plasma membrane Ca^{2+} transporting 3 (*ATP2B3*) (Zennaro et al. 2017). In addition, these somatic mutations were frequently detected in the unilateral or bilateral aldosterone-producing adenoma (APA), aldosterone-producing micronodule (APM) and aldosterone-producing nodule (APN), all of which could lead to normotensive or clinically overt PA (Nanba et al. 2017; Williams et al. 2021), although its details have remained unknown. It is thus pivotal to emphasize the followings at this juncture; 1. Aldosterone works on a wide variety of tissues and plays pivotal roles in their pathology; 2. PA is the common disease and must be detected and treated at earlier clinical stages in order to avoid its direct intractable organ damages. Therefore, in this review, we summarized the pathophysiology of aldosterone biosynthesis and its actions as well as the pathology of PA.

Physiological roles of aldosterone in the renin-angiotensin-aldosterone system (RAAS)

RAAS has been well known to play pivotal roles in the regulation of extracellular volume, sodium and potassium balance and tonus of vascular system within physiological status in human (Laragh et al. 1972; Laragh and Sealey 2011; Patel et al. 2017). The initial step of RAAS is the synthesis of angiotensinogen in the liver. Angiotensinogen is subsequently converted to Angiotensin (Ang) I under the activation of renin, which is regulated by the renal baroreceptor and the delivery of sodium chloride (NaCl) to the macula densa in juxtaglomerular apparatus (Ames et al. 2019, Sparks et al. 2014). Therefore, the changes of blood pressure and electrolytes balance could result in the elevation of renin production, which further catalyzes the conversion of angiotensinogen to Ang I as a rate-limiting step of the system (Ames et al. 2019, Sparks et al. 2014). Ang I is subsequently converted to Ang II by angiotensin converting enzyme (ACE), which is ubiquitously released from endothelial cells or others (Laragh et al. 1972, Laragh and Sealey 2011, Patel et al. 2017). ACE-2 was reported to convert Ang II to another subtype, RAAS peptide Ang-(1-7), which could attenuate the function of Ang II and be regarded as the degradation product of Ang II (Santos et al. 2018). Ang II binds to angiotensin II type 1 receptor (AT1R) in adrenocortical ZG, which subsequently results in promoting aldosterone biosynthesis (Nakamura et al. 2016, Seccia et al. 2018). AT2R has been known as another subtype and to antagonize that of AT1R to reduce the blood pressure (Horiuchi et al. 1999). Therefore, elevated aldosterone stimulated by AT1R is the final lead factor in RAAS and contributes to the systemic regulation of sodium and fluid volume, and the excretion of potassium from the kidney. Aldosterone is also known to bind to mineralocorticoid receptor (MR) to increase the activity of luminal epithelial Na^+ channel (ENaC), luminal K^+ channel, serosal Na^+/K^+ - ATPase and others. (Booth et al. 2002). In addition, water follows the transcellular movements of Na^+, maintaining the equilibrium of fluid volume in human (Booth et al. 2002).

Aldosterone biosynthesis in adrenocortical ZG

The binding of Ang II to AT1R in adrenocortical ZG generates inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) following an activation of phosphoinositide-specific phospholipase C (PLC) (Hattangady et al. 2012). Activated IP_3 could result in transient increase of intracellular calcium levels which subsequently activates calcium/calmodulin-dependent protein kinases (CaMK) and finally promotes the expression of aldosterone biosynthesis in the ZG of normal adrenal by activating cAMP response element binding (CREB) (Barrett et al. 2000, Fern et al. 1995, Hattangady et al. 2012, Spat et al. 2016). Both of those above increased cytosolic Ca and DAG activate protein kinase C (PKC), which could act on protein kinase D (PKD) to activate CREB stimulating steroidogenic acute regulatory protein (StAR) transcription and subsequently increase the levels of CYP11B2 expression (Fig. 1) (Bollag 2014, Hattangady et al. 2012).

Cholesterol is the precursor of aldosterone as in other corticosteroids and subsequently converted to pregnenolone under the activation of cytochrome P450 side-chain cleavage (CYP11A1) (Nakamura et al. 2016, Sasano et al. 1989b, Seccia et al. 2018). Pregnenolone is then converted to progesterone by 3β-hydroxysteroid dehydrogenase (3β-HSD) (Nakamura et al. 2016, Sasano et al. 1990, Seccia et al. 2018). 21-hydroxylase (CYP21A2) then catalyze progesterone into deoxycorticosterone (Sasano et al. 1988) and

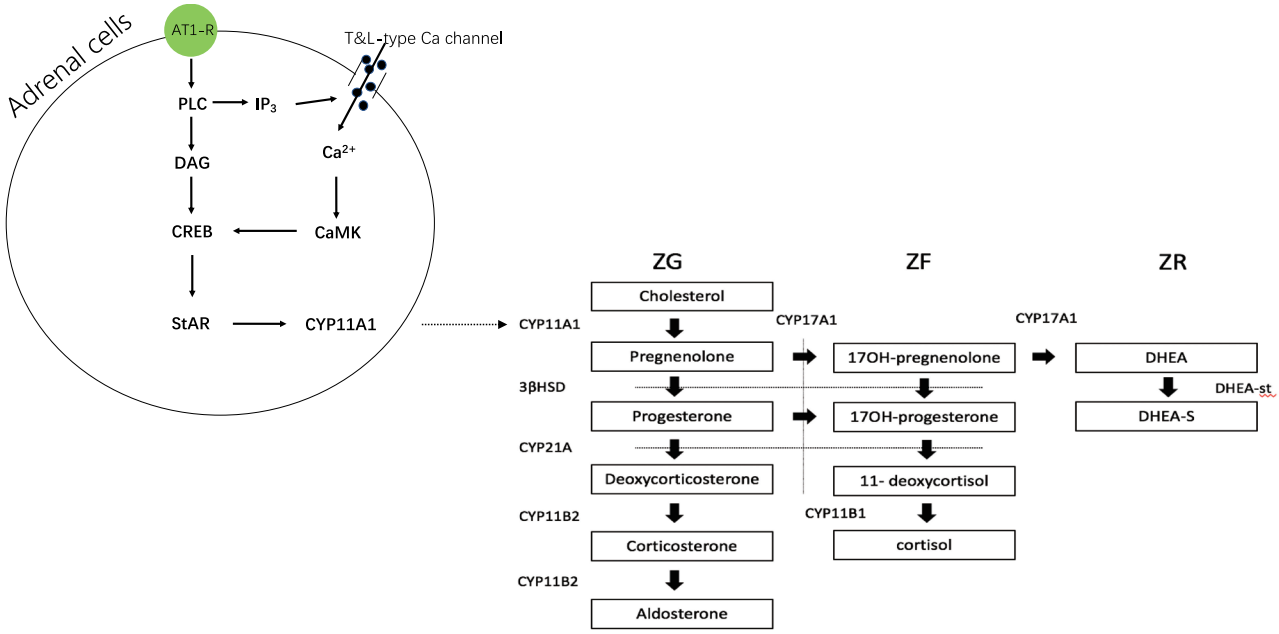

Fig. 1. The pathway of aldosterone biosynthesis.
Angiotensin II (ANG II) binds to its receptor, angiotensin II type 2 receptor (AT1R) in the adrenocortical zona glomerulosa (ZG) cells which initiates the activation of phosphoinositide-specific phospholipase (PLC), inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), subsequently resulting in the transcription of steroidogenic acute regulatory protein (StAR) and aldosterone biosynthesis. Aldosterone is synthesized through the cascades of the following steroidogenic enzymes, CYP11A1, 3β-HSD, CYP21A and CYP11B2. PLC, phosphoinositide-specific phospholipase C; DAG, diacylglycerol; CREB, cAMP response element binding; StAR, steroidogenic acute regulatory protein; CaMK, calcium/calmodulin-dependent protein kinases; CYP11A1, cytochrome P450 side-chain cleavage; 3β-HSD, 3β-hydroxysteroid dehydrogenase; CYP21A, 21-hydroxylase; CYP11B2, aldosterone synthase; CYP17A1, 17-alpha-hydroxylase/17,20 lyase; CYP11B1, 11β-hydroxylase.

Aldosterone Synthase (CYP11B2) finally catalyzed it into aldosterone (Nakamura et al. 2016, Seccia et al. 2018). On the other hand, cortisol is produced in the zona fasciculata (ZF) through the cascades of various steroidogenic enzymes stimulated by adrenocorticotropic hormone (ACTH). The enzymes involved in the conversion of cholesterol to 11-deoxycortisol included CYP11A1, 17-alpha-hydroxylase/17,20 lyase (CYP17A1) and HSD3B (Nakamura et al. 2016, Sasano et al. 1989a, Seccia et al. 2018). Cortisol is finally biosynthesized under the activation of 11β-hydroxylase (CYP11B1) (Fig. 1) (Nakamura et al. 2016, Seccia et al. 2018).

Aldosterone-producing lesions in normal adrenal glands

We have recently demonstrated that the great majority of normal human adrenal glands, aldosterone is not necessarily produced diffusely but focally in the ZG as aldosterone-producing micronodules (APMs) previously termed aldosterone-producing cell clusters (APCCs) (Omata et al. 2017). An APM is defined as CYP11B2-positive ZG cells beneath the adrenal capsule whose maximum diameter is usually less than 10 mm and discernible only by CYP11B2 immunohistochemistry (Nishimoto et al. 2010). Both CYP11B1 and CYP17A1 immunoreactivity were absent in these APMs (Nishimoto et al. 2010; Williams et al. 2021). In contrast to CYP11B2 above, the key steroidogenic enzymes including CYP17A1 and CYP11B1 were diffusely expressed throughout the normal zona fasciculata (ZF) (Gomez-Sanchez et al. 2014; Nakamura et al. 2014). In addition, we recently reported that the number of APMs in normal ZG increased in conjunction with aging (Nanba et al. 2017; Omata et al. 2017). However, with the increased number of APMs, the total CYP11B2 positive areas in APMs were also significantly inversely correlated with aging (Nanba et al. 2017). These results above all indicated that RAAS-independent APMs harboring the features of more autonomous and less physiological aldosterone production were considered to develop in conjunction with aging and those focal or localized nodular aldosterone production in the ZG could be also considered to represent the senescent changes of the adrenal cortex (Nanba et al. 2017). We will discuss those later in this review.

11β-Hydroxysteroid Dehydrogenase Type 2 (11β-HSD2)

11β-HSD mediated aldosterone actions in human tissues

Aldosterone exerts its effects by binding to MR in kidney, colon, salivary glands and others as described above. However, results of early *in vitro* studies did demonstrate that MR also bound with equal affinity to glucocorticoids such as cortisol and corticosterone (Krozowski and Funder 1983; Arriza et al. 1987; Sheppard and Funder 1987).

Therefore, it is easily postulated that under physiological conditions, in which serum cortisol is much higher than aldosterone, MR could be constantly occupied by cortisol and no aldosterone specific actions could be possible in human. However, results of subsequently reported studies revealed that MR was selectively activated by picomolar levels of aldosterone but not by the higher nanomolar levels of cortisol in distal nephron *in vivo* (Moguilewsky and Raynaud 1980; Coirini et al. 1985; Reul and de Kloet 1985). Therefore, the discrepancy of the binding affinity of aldosterone and cortisol to MR between *in vivo* and *in vitro* studies provided interesting but perplexing problems to those studying the mechanisms of aldosterone actions. The number of interesting hypothesis was historically proposed to explain this enormous discrepancy between *in vitro* and *in vivo* findings above. For instance, aldosterone was proposed to cross the cell membrane in more specific manner than cortisol or there could be some unknown mechanisms modifying the intracellular concentrations of these two hormones, aldosterone and cortisol (Krozowski and Funder 1983). This rather mysterious discrepancy eventually came to be solved by detailed clinical studies of rare disorders. The deficiency of the conversion from cortisol to cortisone had been clinically reported in the literature (Ulick et al. 1977). This rare hereditary condition, also termed the syndrome of apparent mineralocorticoid excess, has been reported in children manifesting marked hypertension, sodium retention, potassium loss and suppressed plasma renin activity despite undetectable levels of mineralocorticoids (Ulick et al. 1977). Subsequent analysis did reveal that actually cortisol itself functioned as mineralocorticoid in those patients (Stewart et al. 1988). The suppression of endogenous cortisol by dexamethasone was subsequently reported to reverse various symptoms due to mineralocorticoid excess, which were also resumed after the termination of dexamethasone treatment (Stewart et al. 1988). This rather mysterious mechanism of this rare but unique genetic disease was finally clarified in late 1980s by studying the activities of 11β-hydroxysteroid dehydrogenase (11β-HSD) and the status of this enzyme, not binding affinity of MR in various aldosterone target tissues defined *in vivo* aldosterone specificity (Stewart et al. 1987; Edwards et al. 1988; Funder et al. 1988). In kidney, 11β-HSD catalyzes both cortisol and corticosterone into cortisone and 11β-dehydrocorticosterone, respectively and both of which had no binding capacity to MR. Only aldosterone, which is by no means catalyzed by 11β-HSD could bind to MR, which does eventually confer *in vivo* hormone specificity in target tissues.

The isozyme of 11β-HSD: type 1 and type 2

However, along with the discovery of potential roles of 11β-HSD enzyme in mineralocorticoid actions, some contradicting or inconsistent findings had been also reported. For instance, 11β-HSD was ubiquitously expressed in the liver but the liver itself did not have MR in any of cell types. In addition, 11β-HSD was also not necessarily co-colocalized with MR in the distal nephron (Edwards et al. 1988; Tannin et al. 1991). These findings above all suggested the possible presence of the isozymes of 11β-HSD. Subsequent studies did reveal the presence of the new isozyme or subtype termed 11β-HSD type 2 (11β-HSD2) in addition to its original form, 11β-HSD type 1 (11β-HSD1) (Brown et al. 1996a, b). 11β-HSD2 could metabolize cortisol into cortisone, which could by no means compete the binding to MR with aldosterone and 11β-HSD1 above could convert the inactive cortisone to active cortisol, making it possible to bind to MR or GR. 11β-HSD1 is widely distributed in human tissues such as liver, vasculature, adipose tissue, ovary, testis, brain and others (Tannin et al. 1991; Walker et al. 1991; Bujalska et al. 2002). We subsequently demonstrated that 11β-HSD2 was specifically co-localized with MR in almost all of aldosterone-target tissues including kidney, cardiac tissues, vascular smooth muscles, colon, lacrimal glands, sweat glands, bronchial epithelium and others using the methods of double immunohistochemistry and/or immunohistochemical analysis using the serial tissue sections of mirror images (Hirasawa et al. 1997, 1999, 2000; Suzuki et al. 1998; Kato et al. 1999; Konishi et al. 2003). We subsequently demonstrated that 11β-HSD2 played pivotal roles not only in physiological but also pathophysiological actions of aldosterone in these human organs (Hirasawa et al. 1997, 1999, 2000; Suzuki et al. 1998; Kato et al. 1999; Konishi et al. 2003). We will further discuss the pathophysiological roles of this very interesting local regulation of aldosterone actions in the following chapters.

The Pathology of Aldosterone Actions

Aldosterone actions in inflammatory bowel disease

Aldosterone could regulate Na^+ reabsorption by binding to MR in colonic epithelial cells (Levitan and Ingelfinger 1965). In addition, inflammatory bowel disease (IBD) including ulcerative colitis or Crohn's disease was reported to accompany the impaired epithelial Na^+ absorption (Hawker et al. 1980; Sandle et al. 1990). Ulcerative colitis was characterized as the inflammatory disorders of the superficial mucosa from the rectum to the colon, in a continuous fashion although many exceptions reported. On the other hand, Crohn's disease is defined as transmural inflammation which could influence any parts of the gastrointestinal tract from mouth to anus not necessarily in the continuous fashion (Shapiro et al. 2016). Those findings above suggested the possible involvement of aldosterone actions in IBD, especially with correlation to clinically severe diarrhea, considering the importance of aldosterone actions identified in regulation of gastrointestinal tract function. We demonstrated the expression of 11β-HSD2 in normal colonic mucosa, ulcerative colitis and Crohn's disease (Takahashi et al. 1999). In normal colonic mucosa, a clear gradient of 11β-HSD2 expression was detected in the colonic epithelium along with colonic crypt, i.e., more pro-

nounced toward the surface of the mucosa (Takahashi et al. 1999). However, 11β-HSD2 was decreased or even absent in the surface epithelium around severely ulcered lesions of ulcerative colitis (Takahashi et al. 1999). In addition, the absence or decreased 11β-HSD2 expression was detected at both protein and mRNA levels in ulcerative colitis compared to colonic epithelial cells of adjacent uninflamed mucosa (Takahashi et al. 1999). Of particular importance, there were also no significant differences of 11β-HSD2 expression between corticosteroid treated and untreated patients prior to surgery in the patients with ulcerative colitis (Takahashi et al. 1999). This interesting finding above indicated that the glucocorticoid treatment in ulcerative colitis patients exerted little effects on 11β-HSD2 expression in colonic epithelial cells. This decreased 11β-HSD2 expression in ulcerative colitis was also reported to be mediated by pro-inflammatory cytokines including tumor necrosis factor (TNF)-alpha and interleukin (IL)-1 by both *in vitro* study and mice model (Mercer et al. 1993). Collectively, inflammatory cells in ulcerative colitis could mediate 11β-HSD2 expression through both transcriptional and translational processes resulting in abnormal absorption of water and Na^+ leading to clinically marked diarrhea of the patients. Those findings above shed new lights on pathophysiology of ulcerative colitis patients.

Aldosterone actions in human lung and its disorders

We firstly reported the expression of 11β-HSD2 expression in columnar epithelium of the respiratory bronchiole of human lung and its co-localization with MR, which also indicated that aldosterone could contribute to local or *in situ* regulation of sodium and water in human lung (Suzuki et al. 1998). In rat models, aldosterone was also reported to increase both mRNA and protein levels of Na^+, K^+-ATPase and to subsequently increase lung edema clearance (Olivera et al. 2000). In addition, fetal lung tissue was reported to harbor more increased 11β-HSD2 expression compared to adult lung in order to avoid the potential harmful effects of excessive glucocorticoid and to further induce the retention of liquid within airway to stimulate their growth (Suzuki et al. 1998). Of particular interest, aldosterone was also reported to be involved in tumorigenesis of lung cancer. Glucocorticoid was known to suppress lung tumorigenesis via cyclooxygenase-2 mediated pathway (Chang et al. 2015). Therefore, the inhibition of 11β-HSD2 could execute anti-tumor effects in association with increased tissue active glucocorticoid levels and decreased COX-2 expression in pulmonary tissues (Chang et al. 2015). In addition, the co-treatment of RAAS blockers with systemic therapy of advanced non-small cell lung cancer improved the clinical outcome of the patients (Rachow et al. 2021). Therefore, aldosterone actions as well as 11β-HSD2 may be considered as one of the adverse prognostic factors in lung cancer patients and studies on this interesting area are being proceeded at this juncture.

Aldosterone actions on cardiovascular system and its disorders

Aldosterone was originally considered to regulate the equilibrium of sodium and water in the body mainly through its actions in kidneys. However, results of several studies have demonstrated that aldosterone could directly induce myocardial and vascular fibrosis resulting in increased morbidity and mortality independently from the status of systemic hypertension or electrolytes abnormalities (Brilla et al. 1990; Weber and Brilla 1991). The aldosterone-dependent fibrosis of cardiac tissues was reported to be mediated by an activation of NADPH oxidase, production of reactive oxygen species (ROS) and proinflammatory mediators derived from cardiac tissues as a result of aldosterone actions (Rocha et al. 2002; Sun et al. 2002). In the rat model of myocardial infarction, myocardial infarction resulted in two-fold increased aldosterone synthase (CYP11B2) mRNA levels and 3.7-fold increased plasma aldosterone level (Silvestre et al. 1999). In the patients with congestive heart failure, increased MR mRNA and protein levels were also detected in the left ventricle of the failing heart (Yoshida et al. 2005). An increased MR expression was also reported in the atrial tissue of the patients with atrial fibrillation (Tsai et al. 2010). In addition, 11β-HSD2 overexpression was also reported in the left ventricle of rats with cardiac fibrosis and atrial tissue of the patients with atrial fibrillation (Konishi et al. 2003; Lavall et al. 2014). Of particular interest, mRNA levels of collagen types 1 and 3 were significantly higher in stroke-prone spontaneously hypertensive than control rats (Konishi et al. 2003). In normal heart, cardiomyocytes are constantly occupied by endogenous glucocorticoids due to the relatively low expression levels of 11β-HSD2 (Edwards et al. 1988; Sheppard and Autelitano 2002; Gray et al. 2017). Therefore, MR occupied by endogenous glucocorticoids could provide protective effects compared to aldosterone-mediated activations of MR (Qin et al. 2003), although there have been controversies in this aspect. In addition, the treatment of MR blocker spironolactone was reported to significantly reduce the subsequent risks of death from progressive hear failure and sudden death from cardiac failure and could improve various symptoms of heart failure (Pitt et al. 1999). This was considered primarily due to the spironolactone-mediated prevention of potassium loss and myocardial fibrosis (Pitt et al. 1999).

Aldosterone actions on vascular smooth muscle cells and their significance in vasculopathy

The presence of MR in human vasculature was first reported by Sasano et al., in 1986 (Sasano et al. 1986) and subsequently reported to be present not only in endothelial but also in smooth muscle cells of human vasculature (Koenig and Jaffe 2014; Nakamura et al. 2006; Rizzoni et al. 1996). Vascular structural remodeling in small resistance medium sized arteries was also reported in the patients with primary aldosteronism compared to essential

hypertensive patients, which indicated that MR in smooth muscle cells could directly mediate the remodeling process of the vessels (Rizzoni et al. 1996). We also previously demonstrated that aldosterone could promote the expression of MDM2, a nuclear protein involved in the prevention of p53-mediated cell cycle, which was subsequently attenuated by aldosterone receptor blocker, eplerenone (Nakamura et al. 2006). In addition, results of our *in vivo* study also demonstrated that MDM2 expression was significantly higher in smooth muscle cells of small arteries of APA than that of non-functional adenomas and normal adrenal glands (Nakamura et al. 2006). Aldosterone-mediated vascular remodeling was also reported in smooth muscle cells of MR knockout mice models (Pruthi et al. 2014). In addition, MR mediated pathway was also associated with 11β-HSD2 activities despite its lower expression levels (McCurley et al. 2013). MR was also reported to be involved in SMC proliferation via Rho-kinase signaling, placental growth factor signal, gal-3, int-alpha 5 and other pathways (Koenig and Jaffe 2014). Those findings above indicated that MR antagonists could play pivotal roles in the prevention of vascular remodeling as well as the development of further cardiovascular disease independent of plasma aldosterone levels.

Aldosterone actions on chronic kidney disease

We previously reported that the prevalence of chronic kidney disease (CKD) in PA patients was significantly lower before than after treatment of PA throughout the follow-up period of 12 months (Iwakura et al. 2014). Both aldosterone-producing adenoma and bilateral hyperaldosteronism (BHA) patients harbored an increased prevalence of CKD reaching up to approximately 20% due to decreased estimated glomerular filtration rate (eGFR). Those findings above did also indicate discernible effects of PA on the pathogenesis of CKD. Increased plasma aldosterone levels were also reported as one of the major risk factors of developing human renal injuries and could be attenuated by MR antagonists therapy (Spencer et al. 2020). For instance, APA patients harbored the more marked histologically proven renal damages than those expected from the preoperative renal evaluation (Danforth et al. 1977). MR activation also induced renal endothelial dysfunction characterized by inflammatory activation, impaired vasodilation and fibrosis. (Arima et al. 2004; Oberleithner et al. 2004; Lai et al. 2006; Duprez 2007; Gromotowicz et al. 2011; Bauersachs et al. 2015). In addition, MR-mediated glomerulosclerosis could reduce the ability of capillary oxygen delivery, which could finally result in ischemic renal injuries (Hollenberg 2004; Nangaku 2006; Cowgill et al. 2016; Brown et al. 2019). Spironolactone, one of the MR blockers, was also reported to attenuate the decreased eGFR and severity of histopathological lesions to eventual protection of the patients from potential ischemic renal injuries (Sanchez-Pozos et al. 2012; Barrera-Chimal et al. 2013; Barrera-Chimal et al. 2015). Spironolactone was also reported to process nephroprotective function by reducing proteinuria (Bomback et al. 2008). Eplerenone is another MR antagonist providing its attenuated effects on CKD and was reported to be more selective to MR compared to spironolactone which could also bind with progesterone receptor (Weinberger et al. 2002). Esaxerenone is a newly developed MR antagonist which has much higher selectivity to MR compared with spironolactone and eplerenone and its anti-hypertensive effects were also demonstrated by *in vitro* study (Arai et al. 2016; Barfacker et al. 2012; Li et al. 2019). Results of phase III clinical trials of esaxerenone also demonstrated its therapeutic efficacy not only in hypertensive patients but also in those with type 2 diabetes and microalbuminuria, which could also place esaxerenone as the choice of medical therapy in CKD patients (Itoh et al. 2019; Wan et al. 2021).

The Pathology of Primary Aldosteronism

Primary aldosteronism accounts for 5-10% of all hypertensive patients and is considered one of the most frequent endocrinological disorders at this juncture (Rossi et al. 2006; Williams et al. 2006; Funder 2012). Considering the direct adverse actions of aldosterone on various tissues mentioned above, it is pivotal to detect and treat PA patients earlier than other hypertensive diseases. In addition, clarification of the pathological characteristics of early or prodromal lesions of primary aldosteronism is very much warranted at this juncture in order to establish the appropriate clinical management of PA patients. This will also provide clinical benefits to the patients who did not meet the classical criteria of PA considering systemic adverse effects of aldosterone excess independent of the levels of blood pressure and serum electrolytes and the high frequency of PA among general population. Therefore, we will discuss the recently discovered new development of pathology of primary aldosteronism in the following chapters.

Somatic mutations in PA patients

One of the novel concepts of pathology of PA is that the great majority of the adrenocortical cells involved in autonomous aldosterone production and secretion harbors the somatic mutations of ion channels or pumps including potassium inwardly rectifying channel subfamily J member 5 (*KCNJ5*), calcium voltage-gated channel subunit alpha1 D (*CACNA1D*), ATPase Na^+/K^+ transporting subunit alpha 1 (*ATP1A1*), and ATPase plasma membrane Ca^{2+} transporting 3 (*ATP2B3*) as previously described. These findings above also indicated the significance or importance of those somatic mutations in the pathology of PA. In addition, the prevalence of somatic mutations among PA patients was reported to be dependent on ethnicity or race of the patients and the clinicopathological and histopathological characteristics varied among different somatic mutations (Gao et al. 2021a, b). For instance, the mutation of *KCNJ5* was the most frequent one among all somatic mutations reported in PA patients but this is also more frequent in East Asian APA

patients whose incidence reached up to almost 70% of all PA patients compared to Caucasian patients who had 38% incidence (Fernandes-Rosa et al. 2014; Kitamoto et al. 2015; Gao et al. 2021a). On the other hand, the mutations of *ATP1A1* and *CACNA1D* were more frequent in Caucasian than in East Asian PA patients (Fernandes-Rosa et al. 2014; Kitamoto et al. 2016).

Mutations of *KCNJ5* encoding inward rectifier K^+ channel 4, resulted in increased Na^+ permeability, which subsequently lead to continuous depolarization of the cell membrane of aldosterone-producing cells (Choi et al. 2011). This depolarized cell membrane induced an influx of cytosolic Ca^{2+} as the second messenger, which finally initiated aldosterone biosynthesis (Choi et al. 2011). Mutations of *CACNA1D* encode for L-type calcium channel α-subunit $Ca_V1.3$, which regulated intracellular calcium homeostasis (Fernandes-Rosa et al. 2014). Mutations of *ATP1A1*, the gene of α1 subunit of the Na^+/K^+-ATPase, resulted in an influx of calcium by altering sodium and potassium homeostasis (Azizan et al. 2013). Mutations of *ATP2B3* directly influenced the status of calcium pump plasma membrane Ca^{2+} transportation, which subsequently increased cytosolic calcium levels (Di Leva et al. 2008). All of those above did change intracellular Ca levels and finally resulted in aldosterone overproduction of those cells harboring mutations.

Somatic mutations in normal adrenal glands

Somatic mutations were identified not only in APAs but also in APMs present in normal adrenocortical ZG (Nanba et al. 2013; Nishimoto et al. 2015; Omata et al. 2017). We previously reported that 21/61 APMs (34%) of normal adrenocortical ZG cells harbored somatic mutations including fourteen *CACNA1D* mutations; three *ATP2B3* mutations, two *ATP1A1* mutations, and two with simultaneous *CACNA1D* and *ATP2B3* mutations (Omata et al. 2017). Somatic mutations were also reported to be detected in 8/23 APMs (35%) with six *CACAN1D* and two *ATP1A1* mutations in normal adrenocortical ZG cells (Nishimoto et al. 2015). In addition, somatic mutation of *KCNJ5* was detected in five APMs from adjacent non-pathological adrenocortical ZG of APAs (De Sousa et al. 2020). On the other hand, aging was reported one of the potential factors in the development of APMs due to the fact that the number of APMs in the normal ZG increased throughout aging (Nanba et al. 2017; Omata et al. 2017). This also indicated an association between aging and somatic mutations of the relevant genes in the development of APMs in human adrenal glands, but further investigations are required for clarification.

Terminology of PA based on international histopathology consensus

PA is characterized by autonomous aldosterone overproduction and clinically classified into unilateral hyperaldosteronism (UHA) and bilateral hyperaldosteronism (BHA) based on the results of adrenal vein sampling (Young et al. 2004; Toniato et al. 2006). In addition, the main causes of PA are APAs and bilateral adrenal cortical hyperplasia based on the results of CYP11B2 immunohistochemistry. Along with the development of CYP11B2 antibody, many studies have demonstrated variable histopathological features of aldosterone-producing lesions. Therefore, in order to avoid the confusing nomenclatures of aldosterone-producing lesions and to standardize the terminology, those nomenclatures have been also recently unified by international histopathology consensus as summarized in Table 1 (Williams et al. 2021).

(1) Aldosterone-producing adenoma (APA)

APA is defined as the neoplasm whose diameter measures usually larger than 10 mm and is composed of ZG-like/compact cells and ZF-like/clear cells. APA frequently harbored somatic mutation including *KCNJ5, CACNA1D, ATP1A1* and *ATP2B3* resulting in clinical aldosterone overproduction. The diagnosis of an APA should therefore depend on both clinical endocrinological and his-

Table 1. Histopathological classification of aldosterone-producing lesions.

	Aldosterone producing lesions	Diameter	Visible by H&E	Genotype	CYP11B2 immunoreactivity	Clinical status
Non-neoplastic	Aldosterone-producing micronodule (APM)	< 10mm	×	*KCNJ5 CACNA1D ATP2B3 ATP1A1*	A polarized expression pattern	Normal/ Normotensive PA/ Overt PA
	Aldosterone-producing nodule (APN)	< 10mm	✓			Normal/ Normotensive PA/ Overt PA
	Aldosterone-producing diffuse hyperplasia (APDH)	-	△	×	A broad and uninterrupted strip	Overt PA
Neoplastic	Aldosterone-producing microadenoma (micro APA)	< 10mm	△	*KCNJ5 CACNA1D ATP2B3 ATP1A1*	A ubiquitous expression pattern	Overt PA
	Aldosterone-producing adenoma (APA)	> 10mm	✓			Overt PA

PA, primary aldosteronism; H&E, hematoxylin and eosin staining; △, sometimes visible; ×, undetectable; ✓, detectable.

topathological findings including immunohistochemistry using a validated CYP11B2 antibody (Fig. 2). Therefore, it is pivotal to note that pathologists involved in correct diagnosis of aldosterone producing adrenocortical lesions must have full knowledge of endocrine abnormalities in order to reach accurate histopathological diagnosis of the disorders.

(2) Aldosterone-producing nodule/micronodule (APN/APM)

Aldosterone-producing nodule (APN) is tentatively defined as a CYP11B2-positive lesion usually measuring less than 10 mm in greatest diameter and is also composed of compact or clear cortical cells or their mixture. The characters of an aldosterone-producing micronodule (APM), previously termed APCC or aldosterone producing cell cluster, were also reported to be similar to those of APN, which was usually lower than 10 mm in its maximum diameter and demonstrated CYP11B2 expression. APM is currently considered to be derived from ZG cells beneath the adrenal capsular. Both APM and APN were detected in the adrenal glands with or without PA status and also sometimes harbored somatic mutations, especially of *CACNA1D*, which made it extremely difficult to differentiate between these two disease entities. The only plausible method to achieve histopathological differentiation was based on the findings based on morphological features obtained by classical hematoxylin and eosin (H&E) stained tissue sections, i.e., APN could be visible on routinely processed H&E sections compared to APM, some of which could be discernible only by immunohistochemistry of CYP11B2. However, further investigations are required for clear differentiation between APM and APN (Fig. 3).

(3) Aldosterone-producing diffuse hyperplasia (APDH)

APDH is a broad and uninterrupted strip of hyperplastic ZG cells harboring CYP11B2-positive immunoreactivity in more than 50% of cells (Williams et al. 2021). APDH was reported as one of the main causes of clinically diagnosed idiopathic hyperaldosteronism (IHA) and to also harbor no discernible somatic mutations in their aldosterone producing adrenocortical cells (Fig. 4) (Omata et al. 2018; Yamazaki et al. 2017).

The differentiation among APM, APN and micro APA

Increasing number of PA cases have been clinically identified by development of adrenal venous sampling but most of those lesions not necessarily discernible by routine CT imaging examination (Yamazaki et al. 2017). These so called "CT negative lesions" include APDH, APN, APM and aldosterone-producing microadenoma (micro APA), which must be confirmed by detailed histopathological examination. APN and APM could be histopathologically differentiated by H&E stained tissue sections as mentioned above. However, micro APAs also demonstrated histological characteristics similar to APN or APM, which was reported to be smaller than 10 mm in its greatest dimension

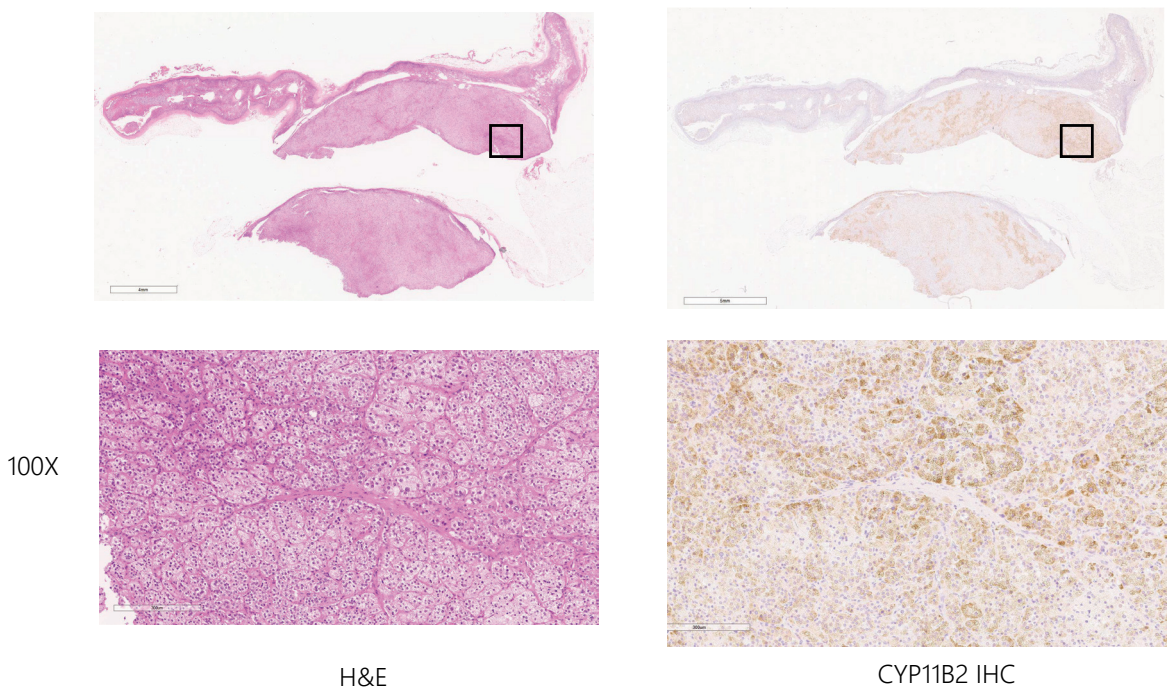

Fig. 2. Representative microscopic images of aldosterone-producing adenoma (APA).
An APA is a neoplastic lesion whose diameter is usually larger than 10 mm in its greatest dimension. An APA is composed of compact and clear tumor cells and frequently harbors somatic mutations including *KCNJ5, ATP1A1, ATP2B3* and *CACNA1D*. These molecular features above could lead to an aldosterone overproduction in the patients. In addition, APA harbors non-polarized or unidirectional expression patterns of CYP11B2, which contributes to its differential diagnosis from other lesions. Therefore, the diagnostic criteria of APA must be based upon its clinical and histopathological features of the patients.

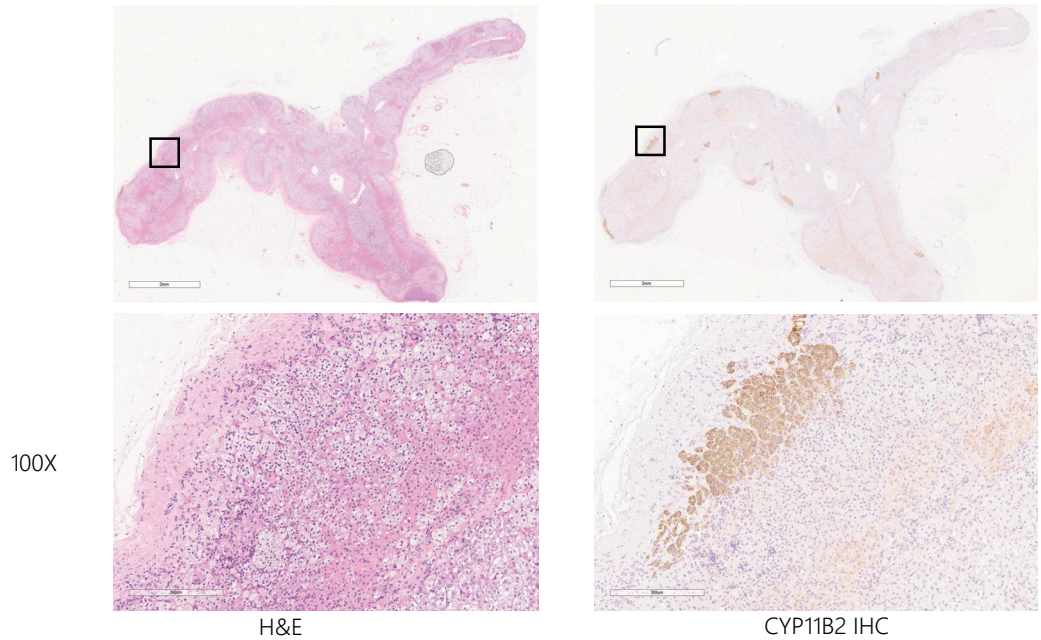

Fig. 3. Representative microscopic images of aldosterone-producing micronodule (APM).
An APM, previously termed aldosterone-producing cell clusters (APCC), originates from zona glomerulosa (ZG) of normal adrenal cortex which plays pivotal roles in the physiological aldosterone production. In principle, APM is smaller than 10 mm in its greatest dimension and expresses CYP11B2 but not CYP11B1 nor CYP17A1. APM cannot be differentiated from surrounding ZG cells only by hematoxylin and eosin (H&E) stained tissue sections. APM is detected not only in the normal adrenal gland but also in non-pathological adrenal glands of PA patients and sometimes harbors somatic mutations including those of *CACNA1D*. Therefore, the valid CYP11B2 immunohistochemistry is required for definitive histopathological diagnosis.

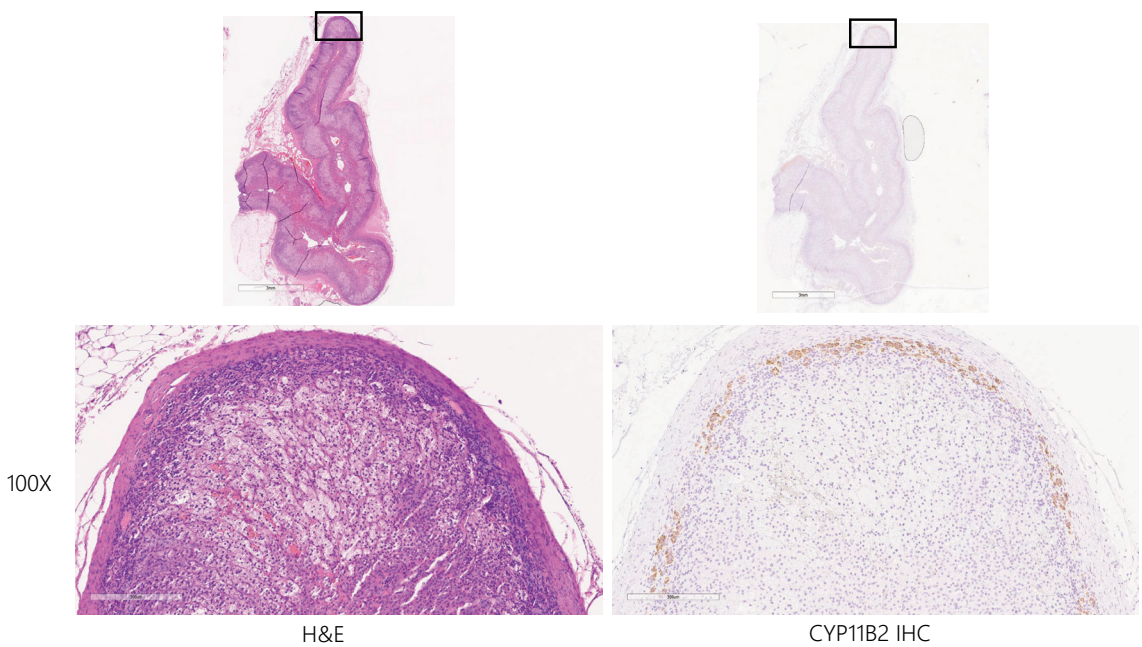

Fig. 4. Representative microscopic images of aldosterone-producing diffuse hyperplasia (APDH).
Aldosterone-producing diffuse hyperplasia (APDH) demonstrates uninterrupted of continuous (more than 50% of the ZG) CYP11B2 expression in histologically hyperplastic ZG. APDH is frequently detected in PA patients harboring no somatic mutations and is another histopathological subtype of clinically diagnosed idiopathic hyperaldosteronism (IHA). Therefore, both clinical or endocrinological information and histopathological findings based on H&E stained tissue sections as well as CYP11B2 immunohistochemistry are definitively required for the final diagnosis of APDH.

and only sometimes visible by examination on routine H&E stained tissue sections (Williams et al. 2021). Therefore, the definitive differentiation between APN/APM and micro APA should be based on detailed histopathological analysis including valid CYP11B2 immunohistochemistry. A gradient of the patterns of CYP11B2 immunoreactivity from the outer to inner part could be detected in non-neoplastic APNs/APMs and this distinctive polarity of CYP11B2 expression patterns are by no means detected in neoplastic micro APAs.

Potential associations among APM, APN and APA

Along with the detection of APM, the associations of these aldosterone-producing lesions among each subtype have been recently reported. It is true that there have been no established transitional pathways from one lesion to others reported at this juncture. However, results of our previously reported studies did reveal that both APN and APM had similar spectrum of somatic mutations, i.e., *CACNA1D* mutations were predominantly detected, although it is also true that APM and APN could not be well differentiated at the time of analysis (Nishimoto et al. 2015; Omata et al. 2017; Yamazaki et al. 2017; Omata et al. 2018). These findings above also indicated that there could be non-neoplastic transitional pathways present between APN and APM. However, whether a non-neoplastic APN could be transformed into neoplastic APA has remained virtually unknown because of the predominant *KCNJ5* somatic mutation patterns of APAs compared to the predominant *CACNA1D* somatic mutations of APNs.

Morphology of aldosterone-producing adenoma

Normal adrenal cortex was composed of ZG, ZF and zona reticularis (ZR) from outer to inner part but when normal adrenocortical cells differentiate or develop into nodules or tumors including aldosterone-producing lesions, the cells of these lesion were usually rather complex mixture of those simulating normal cortical cells, especially of ZF and ZR cells (Tsuchiyama et al. 1980; Yamazaki et al. 2018; Gao et al. 2020). We recently termed these cells simulating ZF and ZG/ZR cells as clear and compact cells instead of ZG or ZF-like cells in order to avoid potential confusions, possibly alluding the functional features of those designated terms above (Williams et al. 2021). Compact cells harbor relatively high ratio of nuclear to cytoplasm with eosinophilic lipid-poor cytoplasm. Whereas clear cells harbor a relatively low ratio of nuclear to cytoplasm with a lipid-rich cytoplasm. In APAs, we previously demonstrated that *KCNJ5* mutated APAs were predominantly composed of clear cells compared with *KCNJ5* wild type APAs (Yamazaki et al. 2018). In addition, the relative intensity of CYP11B2 immunoreactivity was significantly positively correlated with the number of clear cells, especially in *KCNJ5* mutated APAs (Yamazaki et al. 2018). On the other hand, we also demonstrated that both *ATP1A1* and *CACNA1D* mutated APAs were composed of more compact cells than clear cells (Ono et al. 2019). In contrast, *ATP2B3* mutated APAs were predominantly composed of clear tumor cells, in the same extent as *KCNJ5* mutated APAs (Ono et al. 2019). In APMs or APNs, 13 of 32 nodules or micronodules were predominantly composed of compact cells and 18 of 32 nodules of clear cells (Yamazaki et al. 2017). The lower ratio of clear to compact cells was due to predominant *CACNA1D* mutated genotype compared with that in predominant *KCNJ5* mutated APA. In APDH, aldosterone-producing lesions were composed of morphologically normal ZG cells rather than compact nor clear cells.

Clinicopathological correlations of aldosterone-producing lesions

APA and BHA account for the great majority of clinically overt PA cases, which usually do not pose diagnostic difficulties in most clinicians managing PA patients. However, some patients harboring suppressed plasma renin activity, but normal or slightly elevated plasma aldosterone levels were reported to have normal blood pressure (Brown et al. 2017). APMs were usually detected in normal adrenocortical ZG resulting in the physiological aldosterone production. However, APMs were also reported to be identified in adjacent normal ZG of APA lesions, which indicated autonomously renin-independent APMs (Nishimoto et al. 2015). In addition, APMs frequently harbored somatic mutations and their numbers increased with aging as mentioned above. These findings above indicated that physiologically renin-dependent APMs could be potentially developed to pathologically renin-independent APMs possibly due to aging or genetic disorders, which could also account for normotensive PA (Nanba et al. 2017; Gao et al. 2021b). In addition, the lager areas of APMs or APNs were detected in IHA patients than those in normal patients, which suggested that pathological APMs could be also related to the changes of normotensive PA to overt PA, but it awaits further investigations for clarification.

Summary and Future Perspectives

Aldosterone is biosynthesized in ZG cells of adrenal cortex under the stimulation of Ang II and acts not only on kidney but also on other organs such as cardiac tissues, vascular smooth muscles, colon, lacrimal glands, sweat glands, bronchial epithelium and others. In particular, direct actions of aldosterone, not necessarily via high blood pressure and electrolytes imbalance, could induce myocardial and vascular fibrosis resulting in higher morbidity and mortality of the patients with cardiovascular diseases. Aldosterone has been well known to maintain water and electrolytes balance through its actions on kidneys but also to increase the incidence of CKD at its excessive levels through its direct actions. In colon, aldosterone regulates the balance of sodium and water through colonic epithelial cells and its *in situ* decreased activities turned out to result in intractable diarrhea in the patients with ulcerative colitis. In lung, aldosterone plays pivotal roles not only in main-

taining water and electrolyte balance but also in the pathway of its tumor development.

Primary aldosteronism is the most frequent cause of hyperaldosteronism and accounts for 5 to 10% of all hypertensive patients, which could make it the most frequent endocrine disorders. Among those with primary aldosteronism, an APA is the neoplastic disorder accounting for the most frequent subtype of unilateral PA. On the other hand, an APM or APN is the non-neoplastic lesion usually present in both normal adrenal glands and clinically defined IHA. Unilateral or bilateral APDH have been also reported although rare. All of those aldosterone-producing lesions above with an exception of APDH frequently harbored somatic mutations of *KCNJ5, CACNA1D, ATP1A1* and *ATP2B3*. The number of APMs in normal adrenal glands increased with aging and APMs could become renin-independent, suggesting that physiological renin-dependent APMs in normal adrenal glands could potentially become pathological renin-independent APMs resulting in potentially normotensive PA. These renin-independent APMs could continue to develop due to the genetic disorders or aging leading to the changes of normotensive to overt PA. This is the major reason of the high incidence of PA among hypertensive subjects at large. Therefore, the classical concepts, "aldosterone works only on kidneys" and "PA is rare disease" must be changed into the new concepts; "aldosterone works on a wide variety of tissues and plays pivotal roles in their pathology" and "PA is the common disease and must be diagnosed and treated at its earliest clinical stages in order to avoid its intractable organ damages".

Acknowledgments

Fumitoshi Satoh and Hironobu Sasano were supported by grants from Ministry of Health, Labour and Welfare in Japan (H 29 Nanji-Japan 046).

Conflict of Interest

The authors declare no conflict of interest.

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