Hypertension

ALDOSTERONE-PRODUCING ADENOMA

Subtypes of Histopathologically Classical Aldosterone-Producing Adenomas Yield Various Transcriptomic Signaling and Outcomes

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ABSTRACT: There have been no reports of outcome differences between distinctive subgroups in patients with unilateral primary aldosteronism with histopathologically classical adenomas. The characteristics and incidence of complete clinical success in the patients with unilateral primary aldosteronism could be associated with the concomitant existence of multiple aldosterone-producing micronodules/nodules. Altogether, 74 classical adenomas (mean, 52.9 years and 40 men [54.1%]) were identified among 98 operated patients with unilateral primary aldosteronism. Among them accompanying multiple aldosterone-producing micronodules/nodules (group A) were identified in 38 (51.3%) of the adrenal glands; these patients showed lower likelihood (42.1%) to achieve complete clinical success than that (80.6%) of those with aldosterone-producing adenoma/nodules alone (group B, P=0.001). Additionally, group A adenomas were associated with less complete clinical success (odds ratio, 5.53, P=0.005) in the multivariable regression analysis. Group A patients also had higher baseline aldosterone production (absolute aldosterone ratio) from the contralateral adrenal gland (P=0.039). Based on the patterns of genes with highly differential expressions as uncovered by RNA-seq analysis, Group A adenomas showed distinct transcriptomic profiles in comparison to the gene expressions from Group B adenomas. Pathway enrichment analysis revealed that HTR2B-mediated calcium pathway, in terms of *HTR2B*, and *PLCE1* was prominently downregulated in Group A adenomas. There was 51.3% of the patients with unilateral primary aldosteronism with classical group A adenomas. These patients were more likely to have hypertension-persistence after adrenalectomy. The functional signatures of Group A adenomas showed attenuated HTR2B-mediated PLC/IP3/$Ca2^+$ pathway; this may provide some mechanistic explanation to various clinical outcomes. **(*Hypertension*. 2021;78:1791–1800. DOI: 10.1161/HYPERTENSIONAHA.121.18006.)**

• **Supplemental Material**

Key Words: adrenal gland ■ aldosterone ■ classical adenoma ■ hypertension ■ *HTR2B* ■ multiple aldosterone-producing micronodules

Primary aldosteronism (PA) is characterized by autonomous production of aldosterone in the presence of suppressed renin concentrations. Patients with PA have higher risks for cardiovascular diseases than those of matched essential hypertensives.[1]

Recent findings suggest that some somatic mutations could be the first step in aldosterone-producing adenoma (APA) development from multiple aldosterone-producing micronodules/nodules (mAPM/mAPN). mAPM/mAPN express CYP11B2 but could not be differentiated from surrounding adrenal cortical cells under hematoxylin and eosin (H&E) staining; are located at outer zona glomerulosa in contact with the adrenal capsule and/or inner zona fasciculata; these mAPM/mAPN cells could be associated with autonomous aldosterone production. However, in contrast to *KCNJ5* being the most frequent somatic mutated gene in APAs, only very limited cases of mutated *KCNJ5* have been identified in mAPM/mAPN of the excised adrenal gland specimens among computer tomography–negative unilateral PA (uPA) patients.[2] It is hypothesized that mAPM/mAPN could

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Novelty and Significance

What Is New?

- There was 51.3% of patients with unilateral primary aldosteronism with histopathologically classical adenoma harbored coexistent multiple aldosterone-producing micronodule (mAPM)/multiple aldosterone-producing nodule (mAPN).
- They were less likely to have complete clinical success after adrenalectomy.
- HTR2B- mediated PLC/IP3/$Ca2^{+}$ pathway, involving *HTR2B* and *PLCE1*, were attenuated both in mRNA and protein levels in these aldosterone-producing adenoma (APA) harboring mAPM/mAPN.

What Is Relevant?

- Accompanied by mAPM/mAPN or not has been alleged to form 2 possible origins of tumorigenesis in histopathologic classical APA/APN.
- After adrenalectomy, classical APA patients without mAPM/mAPN have higher clinical complete success than those with concomitant mAPM/mAPN (80.6% versus 42.1%).
- Classical APA patients harboring mAPM/mAPN had higher absolute aldosterone concentrations in the contralateral adrenal gland.

Summary

Comparison of the transcriptomic landscapes between the adenomas of the classical APA/APNs with mAPM/mAPN or not could provide some insights into a possible different origin of tumorigenesis in APA/APN.

Nonstandard Abbreviations and Acronyms

APA	aldosterone-producing adenoma
mAPM	multiple aldosterone-producing micronodule
mAPN	multiple aldosterone-producing nodule
PA	primary aldosteronism
PLC	phospholipase C
TAIPAI	Taiwan Primary Aldosteronism Investigation
uPA	unilateral primary aldosteronism

be potential precursors of APAs, and/or mAPM/mAPN are contributors to wild-type *KCNJ5*-uPA, or they carry APA driver gene mutations.[2] However, in an adrenal gland with a histopathologic classical CYP11B2-positive APA/aldosterone-producing nodule (APN), it could be accompanied with or without concomitant surrounding mAPM/mAPN. Little is known regarding associations of the histopathologic readings, clinical presentations and outcomes, and molecular functional pathway patterns in various combinations. The histopathology of primary aldosteronism consensus based on H&E staining and CYP11B2 immunohistochemistry is helpful to standardize nomenclature and achieve consistency among the diagnosis of uPA.[3] Herein, we explored the associations of the histopathologic/immunohistochemical (IHC) characterizations of the excised adrenal glands in patients with uPA, the functional transcriptome enrichment of various APA/APN, and their clinical outcomes of the classical uPA with or without concomitant subcapsular mAPM/mAPN.

MATERIALS AND METHODS

The authors declare that all supporting data are available within the article and its Supplemental Material.

Study Population

This study was conducted by investigators from the TAIPAI (Taiwan Primary Aldosteronism Investigation) study group via using a prospectively collection of patients with uPA and specimens.[4,5] From July 2017 to January 2019, all uPA patients with an image-identifiable adrenal nodule/adenoma and underwent unilateral adrenalectomy were collected. The diagnosis of PA was made via standardized screening test and stringent confirmatory tests, including the saline infusion test or captopril test; and subsequently rigorous subtype identification tests were performed to assure its functional unilateral lateralization of the PA.[6,7] PA subtype lateralization tests included adrenal venous sampling (supplementary methods) (Figure S1 in the Supplemental Material).

Ethical Statement

The study was conducted in accordance with the requirements of the Human Tissue Act and approved by our institutional ethical committee (200611031R).

Tissue Immunohistochemistry

Standard H&E staining and immunohistochemistry (IHC) were performed. Mouse monoclonal antibody for CYP11B2 and 17α-hydroxylase (CYP17A1), rat monoclonal antibody for CYP11B1 (a kind gift from Professor Celso Gomez-Sanchez) were used for IHC.[8,9] The rabbit polyclonal antibody was used to detect human HTR2B (26408-1-AP, Proteintech, Chicago, IL), PLCD1 (204409-T46, Sino Biological, Beijing, China), PLCE1 (LS C804166, LifeSpan BioSciences, Seattle, WA).

Histopathologic Evaluation of Adrenals

The adrenal specimens were categorized as classical or nonclassical histopathologic findings according to the

histopathology of primary aldosteronism consensus.[3] The classical group comprised adrenals with a solitary APA or a dominant APN.[10] The classical uPA histopathology was further subclassified to group A or B for further evaluation of treatment outcomes and molecular signaling. Group A included those APA/APN with concomitant surrounding mAPM/mAPN, and group B denoted those APA/APN with no concomitant mAPM/mAPN. mAPM/mAPN were defined as cortical micronodules or nodules demonstrating positive CYP11B2 staining (micronodules could not be differentiated from surrounding adrenal cortical cells under H&E staining).[3] The nonclassical histopathology uPA patients with positive mAPM/mAPN but with negative CYP11B2 staining in the solitary APA/APN were not included in our final analysis, because they were not the classical adenoma.

Transcriptome Sequencing

Total RNA was isolated and purified from visible adenomas with positive CYP11B2 staining of the adrenalectomy specimens (from 7 classical APA/APN with positive surrounding, 5 with and 2 without a KCNJ5 mutation; mAPM/mAPN and 3 classical APA/APN without concurrent mAPM/mAPN, all 3 with a KCNJ5 mutation). The quality and concentrations of the RNA samples were respectively measured with Agilent 2100 Bioanalyzer (Agilent) and Nanodrop ND-2000 spectrophotometer (Thermo Scientific). Samples with OD260/280 ratio of ≥1.8, RIN values of ≥8.5 were further selected for library construction. Partek Genomics Suite and statistical package were used to perform statistical analysis, hierarchical clustering, differential expression analysis, and pathway enrichment.

Outcome Definitions

We defined the clinical and biochemical outcomes according to the consensus of PA Surgical Outcome with at least 2 follow-up visits.[11]

Statistical Analyses

Statistical analysis was performed using the R 4.0.1 software (R Foundation for Statistical Computing, Vienna, Austria). In statistical testing, 2-sided $P \leq 0.05$ was considered statistically significant.

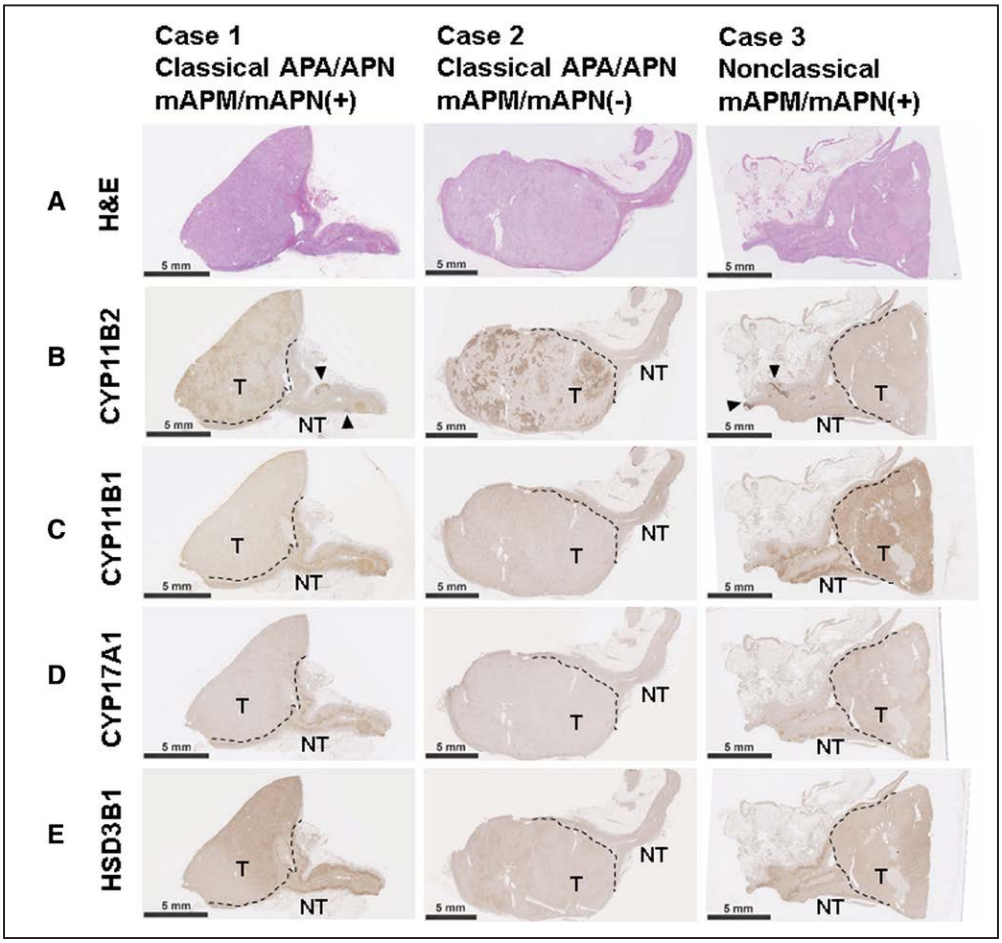

Figure 1. Representative images of histopathologic classical or nonclassical adenoma accompanied by positive or negative multiple aldosterone-producing micronodule (mAPM)/multiple aldosterone-producing nodule (mAPN).
Tumor in case 1, 2 depicted mottled CYP11B2 expression in adenoma harboring KCNJ5 mutation. In case 3, the negative CYP11B2 adenoma stained with mild CYP11B1 expression. The mAPN(M) were expressed at zona glomerulosa area with positive CYP11B2 staining. Representative images of hematoxylin and eosin (H&E), CYP11B1, and CYP11B2 immunohistochemistry are shown. Scale bars in pictures are 5 mm. **A**, Hematoxylin and eosin, (**B**) CYP11B2 IHC, (**C**) CYP11B1 IHC, (**D**) CYP17A1 IHC, and (**E**) HSD3B1 IHC. No. arrowhead, mAPM/mAPN. NT indicates nontumor; and T, tumor.

RESULTS

Clinical Characteristics

A total of 98 patients with uPA (mean 52.7 years and 49 men [50.0%]) were collected in this study (Table 1). Among them, 74 of 98 adenomas showed histopathologic classical CYP11B2 positive staining APA/APN (Figure 1, S2). Among the 74 patients with uPA, the basal characteristics of the patients exhibiting concomitant surrounding mAPM/mAPN (group A; n=38, 51.3%) were similar to that of those without mAPM/mAPN (group B; n=36, 48.7%). The LI and their CLS were similar between the 2 groups. However, the absolute aldosterone concentration in the contralateral adrenal vein relative to that of the peripheral vein (absolute aldosterone ratio, AAR) was increased in group A than that in group B (P=0.039). CYP11B1 staining and the H score of CYP11B1 as well as CYP11B2 were not different between the 2 groups.

Chance for Complete Clinical Success Grouped by the Concomitant Surrounding mAPM/mAPN

After adrenalectomy, the log[ARR] levels and the possibility of biochemistry success were not different between the 2 groups; however, APA/APN patients harboring mAPM/mAPN (group A) had a lower possibility to gain complete clinical success (42.1%) after the adrenalectomy than that of the group B patients (80.6 %, P<0.001).

The logistic regression model, adjusted with covariates, showed that those APA/APN patients with higher preoperative systolic BP (odds ratio, 1.04 [95% CI, 1.01–1.07], P=0.023), higher serum creatinine level (odds ratio, 17.2 [95% CI, 2.03–144.8], P=0.009), and group A patients (odds ratio, 5.53 [95% CI, 1.66–18.4], P=0.005) had a lower possibility to achieve complete clinical success after adrenalectomy (Table 2).

Distinctive Transcriptomic Signatures in Adenoma With or Without mAPM/mAPN

We performed RNA-seq transcripts on CYP11B2-positive classical adenomas obtained from 7 group A adenomas/nodules (5 with and 2 without a KCNJ5 mutation) and compared their results with that from 3 group B adenomas/nodules (all 3 with a KCNJ5 mutation). We identified 515 out of 8441 annotated genes that displayed distinct expression patterns between these 2 groups of adenomas: 124 genes were downregulated, while 150 genes were upregulated in the group A adenomas (fold change ≤–2 or ≥2, P≤0.05), indicative of a generally different transcriptional status between the 2 subtypes of histopathologic classical adenomas (Figure 2A). Furthermore, as shown by unsupervised hierarchical clustering analysis, none of the prominent expressions of the group A adenomas clustered with those of group B samples. All

Table 1. Preoperative Characteristics of Unilateral PA Patients With Classical APA/APN Accompanied by Surrounding mAPM/mAPN or Not*

Histopathologic finding	mAPM/ mAPN(+) (group A)	mAPM/ mAPN(–) (group B)	*P* Value
Number	(n=38; 51.3%)	(n=36; 48.7%)	
Female	19 (50.0%)	15 (41.7%)	0.494
Age, y	52.5±10.6	50.7±11.8	0.495
Family history of HTN	27 (71.1%)	22 (61.1%)	0.463
Duration of HTN, y	0.13±0.34	0.22±0.42	0.312
BMI, kg/m^2	26.2±5.5	24.4±3.8	0.107
sBP, mm Hg	160.2±22.0	151.2±16.8	0.051
DBP, mm Hg	95.0±13.9	91.4±13.1	0.258
DM	6 (15.8%)	3 (8.3%)	0.481
CAD	5 (13.2%)	1 (2.8%)	0.200
Tumor size	1.63±0.69	1.05±0.38	0.334
Adrenal venous sampling profiles†			
LI	3.3±1.6	6.8±2.8	0.257
CL	0.71±1.17	0.54±0.58	0.508
AAR	4.57±2.79	2.41±2.04	0.039
Biochemistry			
PAC, ng/dL	55.7±42.0	62.8±48.1	0.500
PRA, ng/mL per h	0.33±0.37	0.32±0.33	0.896
Log[ARR], ng/dL per ng/mL per h	2.43±0.71	2.44±0.54	0.940
Potassium, mmol/L	3.6±0.71	3.5±0.70	0.710
Creatinine, mg/dL	0.87±0.25	0.85±0.30	0.795
CYP11B1 H score	85.1±44.6	76.6±41.2	0.471
CYP11B2 H score	84.4±33.9	101.8±45.5	0.153
CYP11B1 positive in adenoma	16 (42.1%)	14(38.9)	0.999
Clinical outcome			
Complete success	16 (42.1%)	29 (80.6%)	0.003
Partial success	18 (47.4%)	5 (13.9%)	
Absent success	4 (10.5%)	2 (5.6%)	
Biochemical outcome			
Complete success	34 (89.5%)	34 (94.4%)	0.736
Partial success	2 (5.3%)	1(2.8%)	
Absent success	2 (5.3%)	1(2.8%)	
PAC, ng/dL	33.2±20.4	33.8±27.2	0.921
PRA, ng/mL per h	3.67±4.87	3.51±2.20	0.871
Potassium, mmol/L	4.29±0.46	4.46±0.32	0.098

AAR indicates absolute aldosterone ratio; APA/APN, aldosterone-producing adenoma/aldosterone-producing nodules; mAPM, multiple aldosterone-producing micronodule; ARR, aldosterone-renin ratio; BMI, body mass index; CAD, coronary artery disease; CLS, contralateral suppression ratio; Cre, creatinine; DM, diabetes; dBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; EH, essential hypertension; HTN, hypertension; LI, lateralization index, PAC, plasma aldosterone concentration; PRA, plasma renin activity; and sBP, systolic blood pressure.

*Data are expressed as mean±SD unless otherwise indicated.

†The LI was calculated as the ratio of the aldosterone/cortisol (A/C) concentration quotient on the dominant side over the A/C concentration quotient on the contralateral side.[1] CLS is calculated as nondominant adrenal (A/C ratio)/(IVC A/C ratio). Absolute aldosterone ratio was defined as aldosterone concentration in contralateral adrenal vein to the peripheral vein ratio.[2]

Table 2. Baseline Characteristics Predicting Noncured HTN at 12 mo After Adrenalectomy by Univariate and Multivariate Logistic Regression Analysis*

Methods	Univariate		Multivariate	
Items	Odds ratio (95% CI)	*P* Value	Odds ratio (95% CI)	*P* Value
Sex (female)	0.39 (0.077–1.95)	0.250		
Age, y	1.00 (0.94–1.07)	0.968		
mAPM/mAPN (group A)	6.93 (1.71–28.12)	0.007	5.53 (1.66–18.4)	0.005
BMI, kg/m^2	1.03 (0.87–1.20)	0.758		
CAD (yes)	1.08 (0.10–12.04)	0.952		
DM (yes)	0.87 (0.09–8.13)	0.899		
SBP, mm Hg	1.05 (1.02–1.08)	0.002	1.04 (1.01–1.07)	0.023
dBP, mm Hg	1.04 (1.00–1.08)	0.050		
Cre, mg/dL	17.2 (2.03–144.8)	0.009	10.90 (1.18–100.58)	0.035
K, mEq/L	1.60 (0.72–3.52	0.246		
PAC, ng/dL	1.00 (0.99–1.01)	0.797		
PRA, ng/mL per h	1.79 (0.41–7.89)	0.441		
Log ARR, ng/dL per ng/mL per h	1.48 (0.70–3.16)	0.308		

AAR indicates absolute aldosterone ratio; BMI, body mass index; CAD, coronary artery disease; Cre, creatinine; dBP, diastolic blood pressure; DM, diabetes; HTN, hypertension; mAPM, multiple aldosterone-producing micronodule; PAC, plasma aldosterone concentration; PRA, plasma renin activity; and sBP, systolic blood pressure.

*Data are expressed as mean±SD unless otherwise indicated.

group A samples were coordinately assigned to a single group, suggesting that at the transcriptome level they resembled each other to a greater extent and were different from that of the group B (Figure 2B).

Next, to provide functional interpretation, we generated a list of genes with significant expression changes based on the RNA-seq data and conducted ingenuity pathway analysis of canonical pathway as well as Kyoto Encyclopedia of Genes and Genomes pathway analysis.

Ingenuity pathway analysis of canonical pathway showed Inositol 1,4,5-trisphosphate (IP3) biosynthesis was attenuated (r=−1.71; Figure S3A) in group A. This analysis uncovered the enrichment of the calcium signaling pathway (enrichment score of 7.2, $P \leq 0.001$; Figure 2C) in the transcriptomes from group B adenomas, while principal component analysis of all detected genes clearly separated the genes expressed in group A adenomas from those in group B adenomas (Figure 2D).

Additionally, the pathway enrichment analysis of calcium signaling demonstrated that *HTR2B, PLCD1, PLCE1* gene expression were downregulated in group A adenomas (Figure 3A, Figure S3B). Such findings could also be supported by the lower expressions of *HTR2B, PLCE1* in the group A adenomas from our IHC staining (Figure 3B) and western plots (Figure 3C).

To further validate candidate genes in HTR2B- mediated PLC/IP3/Ca2+ pathway, we used transcriptome datasets available at the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO)[12] (from GEO Query DataSets for GSE60042). The *CYP11B2* is positively correlated with *HTR2B, PLCD1, PLCE1* in adenomas but not in their counterpart adjacent adrenal gland (Figure S4). Thus, we speculated that the enriched calcium signaling pathway in adenomas is correlated with the *CYP11B2* expression.

A recent study of APA with and without *KCNJ5* mutations showed an upregulated *HTR2B* expression in patients with APA with mutation.[13] Therefore, we further did a sensitive analysis of the calcium signaling enrichment in adenomas exhibiting *KCNJ5* mutations (supplementary results, Figure S5). The results of the sensitive genetic mutational analysis coincided the initial analysis, indicating similar distinctive transcriptome expressions between the 5 group A adenoma and 3 group B adenoma with all *KCNJ5* mutations, and minimal effect of having *KCNJ5* mutation on the clinical outcomes when mAPM/mAPN was present.

DISCUSSION

As accompanied by mAPM/mAPN or not has been alleged to form 2 possible origins of tumorigenesis in histopathologic classical APA/APN, we first compared the baseline demography, transcriptome signaling, and outcomes of patients with uPA with classical APA/APN adenomas harboring mAPM/mAPN or not. Group A patients were less likely to achieve complete clinical success after adrenalectomy. Comparison of the transcriptomic landscapes between the adenomas of the 2 groups has provided some insights into a possible different origin of tumorigenesis in APA/APN. We have further showed the expression of *HTR2B*, and *PLCE1,* components in HTR2B-mediated PLC/IP3/Ca2+ pathway, were decreased both in mRNA and protein levels in group A adenomas. It is also remarkable from our data that group A patients with uPA harboring mAPM/mAPN

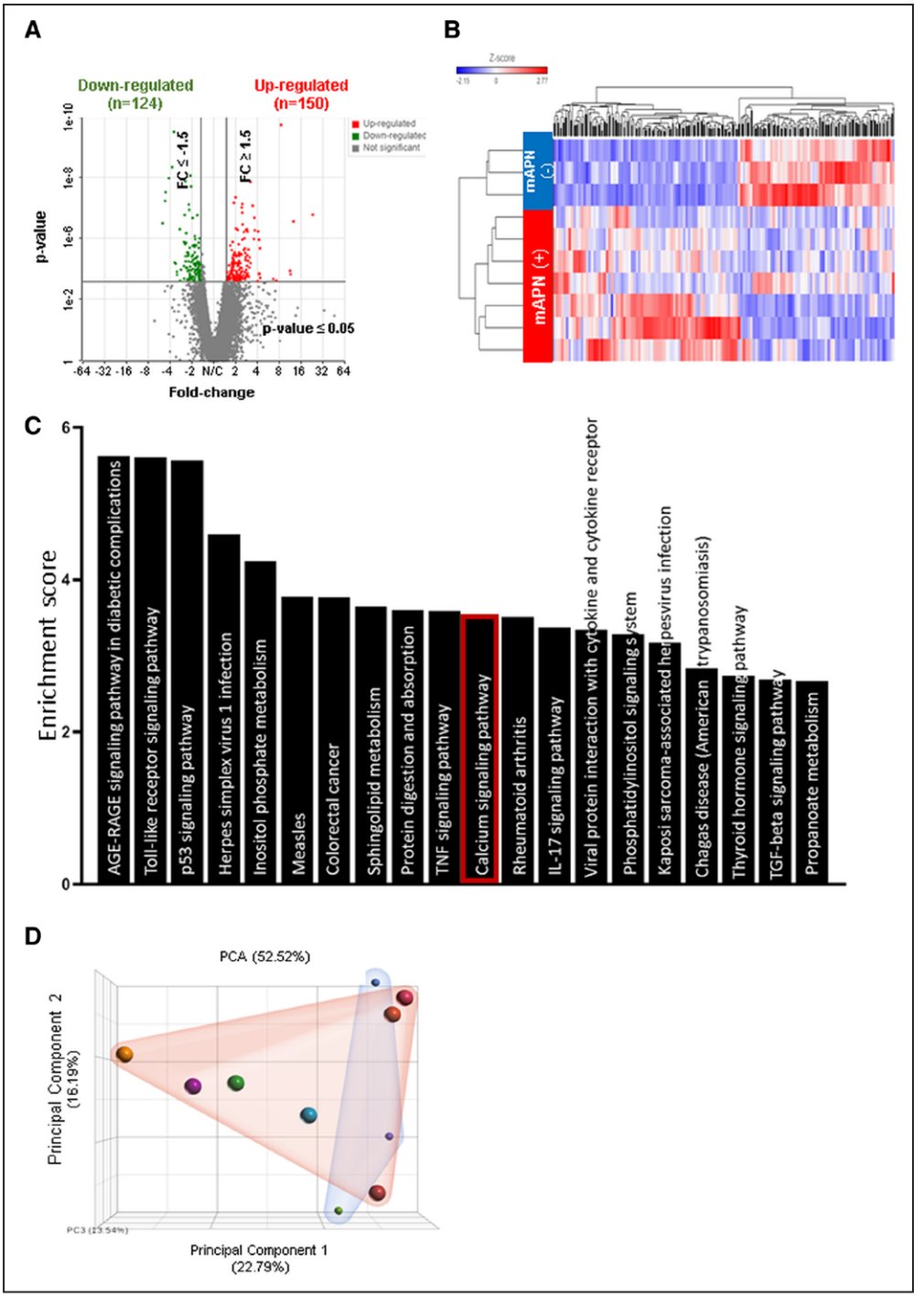

Figure 2. Analysis of the transcriptome distinctions between histopathologic classical CYP11B2 (+) adenoma accompanied by multiple aldosterone-producing micronodule (mAPM)/multiple aldosterone-producing nodule (mAPN) or not.
A, Volcano plot shows the distribution of differentially expressed genes (statistical significance vs magnitude of fold change). Vertical lines denote the fold change cutoff, while the horizontal line denotes the *P* cutoff. Each dot represents one gene. Genes above the thresholds (fold change ≤− 2 or ≥ 2, ANOVA $P \leq 0.05$) are colored, with red and green representing upregulated and downregulated genes, respectively. **B**, The heatmap shows the hierarchical clustering of all differentially expressed protein-coding gene between the 2 groups. The transcriptome similarity among clusters of the adenoma was evaluated by the Euclidean distance and visualized via the heatmap. **C**, Pathway enrichment analysis of the significantly differentially expressed genes shows the top 20 KEGG pathways, based on the enrichment scores. To be note, the calcium signaling pathway is enriched in aldosterone-producing adenoma (APA accompanied by mAPN(M). **D**, Principal component analysis of samples based on the RNA-seq transcriptome data. Red platform is adenoma harboring mAPN(M) (+), while blue platform was adenoma without mAPN(M).

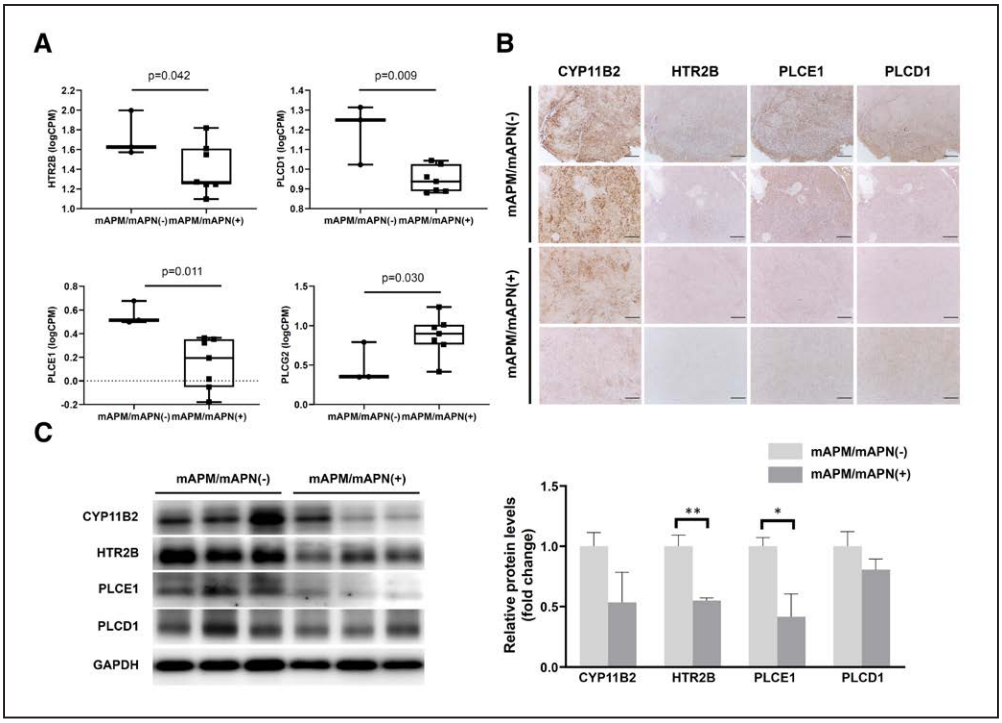

Figure 3. Analysis of the transcriptome distinctions of Ca signaling enrichment between histopathologically classical CYP11B2 (+) adenoma accompanied by multiple aldosterone-producing micronodule (mAPM)/multiple aldosterone-producing nodule (mAPN) or not.

A, The candidate HTR2B, PLCD1, and PLCE1 gene decreased expression in CYP11B2(+) adenoma accomplished by mAPN(M) than no mAPN(M). Enrichment score (*y* axis) and plotted against gene expression (*x* axis) for the candidate genes were detected from the transcriptome. Bar and linear graph: fold change. **B**, IHC showed the expression of candidate protein between mAPM/mAPN (+) and mAPM/mAPN (–) adenoma (C) Western blot showed the different protein expression of classical adenoma between mAPM/mAPN (+) and mAPM/mAPN.

had higher absolute aldosterone concentrations in the contralateral adrenal gland.

Histopathologic Classical APA/APN and mAPM/mAPN

Among our clinically confirmed patients with uPA, 3/4 of them had classical APA/APN defined by immunohistochemistry finding, which was compatible with the percentages previously reported.[10,14] A salient feature of mAPM/mAPN is that their coexistence with concurrent solitary APA/APN was reported only in one article; reporting 57.7%[10] among all classical APA/APN patients—similar to the 51.3% in this cohort.

Previous data supported that the APA/APN could derive from the MAPM/MAPN after the appearance of APA driver mutation, for example, pAATLs or micro-APA-like portion.[15] Our observation raised an interesting debate about the hypothesis of mAPM/mAPN transitioning to CYP11B2 positive adenoma and then uPAs. The histological characteristics of coexistent phenotypes of mAPM/mAPN (at subcapsular region) and classical adenoma (in adrenal outer cortex) could coincide in the same adrenal gland, but usually they do not exist immediately next to each other—instead, they often were separated by a CYP11B2-negative bridging adrenal cortical zone (Case 1). However, our transcriptome profiling from excised adrenal adenomas demonstrated significant differences in transcript types and levels between the classical adenoma with or without mAPM/mAPN. Scrutinizing the microscopic IHC observation of all our specimens, we did not observe any obvious relationship of mAPM/mAPN to APA transitional changes, either. Interestingly, the mAPM/mAPN adjacent to APA tumor mass usually carried different somatic mutations, and mutated *KCNJ5* only counted for very limited cases in mAPM/mAPN in previous reports.[2,16] Therefore, specific perturbations in gene expressions and regulation in adenomas could point toward the existence of significant heterogeneities in group A versus group B classical adenomas. The greater variation could potentially arise from an inherent heterogeneity of the disease caused by its exceptionally high mutational burden. Sun et al[17] investigate the metabolic phenotypes between APA and mAPM/mAPN, they concluded specific subgroups of APM with strikingly divergent distribution patterns of metabolites. Thus, we speculate that some mAPM/mAPN somatic mutations and signaling are different from the ones commonly carried by the solitary APA/APN.[2]

Contralateral mAPM/mAPN

We further showed that group A patients were less likely to achieve complete clinical success after adrenalectomy from the multivariable logistic regression analysis. mAPM/mAPN could be found in adrenal glands with functionally uPAs or even within normal adult adrenal glands in autopsy series[18]; they are composed of zona glomerulosa-like cells. The numbers of mAPM/mAPN seem to increase in an age-dependent manner.[19] Of note, all adrenals of bilateral idiopathic hyperaldosteronism were found to have at least one MAPN/MAPM.[16] The wide variations in histopathologic characteristics of the adenomas and concurrent presence of mAPN/mAPM raise the possibility that most cases of unilateral production of aldosterone might represent bilateral asymmetrical hyperplasia with nodules.[20] Accordingly, it is reasonable to suspect that there could also be mAPM/mAPN located in the contralateral adrenal gland in group A patients.[21] This assumption was supported by our findings of a higher AAR in the contralateral compared with ipsilateral adrenal gland. Therefore, we speculate elevated aldosterone production from the contralateral gland at baseline in group A patients when compared with that in group B patients.[3] Such evidence supports our hypothesis that the presence of mAPM/mAPN could lead to either diagnostic confusion during the lateralization test (like interpreting AVS test results) or subsequent absence of complete success after adrenalectomy.

It has also been found that mAPM/mAPN is the source of aldosterone excess in computer tomography–negative uPA, idiopathic hyperaldosteronism, or adrenals with bilateral aldosterone excess and no abnormalities on computed tomography scan.[16] Nonclassical adrenals composited of mAPM/mAPN only are associated with less biochemical success[3]; yet they underlined the importance of the concomitant associated hyperplasia.[22] Our observation further showed higher risks of persistent hypertension after ipsilateral adrenalectomy in group A patients.

mAPM/mAPN and Calcium Signaling Among Adenomas

Kyoto Encyclopedia of Genes and Genomes pathway analysis further identified the downregulated representation of *HTR2B* and *PLCE1* in the adenoma tissues, in terms of calcium signaling expression, both in mRNA and protein levels, in group A adenomas. Importantly, the calcium signaling pathway is strongly correlated to CYP11B2 expression in our result. This finding is consistent in our external validation using the gene expression profile from APA.[12] Thus, the attenuation of calcium signaling profiles was also uniquely found in group A APA/APN.

Based on our further analysis, in comparison with the calcium signaling profile in classical APA without *mAPM/mAPN*, the attenuation of calcium signaling profile was found in classical APA with adjacent concomitant *mAPM/mAPN*, and this finding was not affected by the presence of *KCNJ5* mutation or not. *HTR2B* is a protein-coding gene. It is well known that activation of the 5-HT2B receptor can increase intracellular calcium levels through activating PLC (phospholipase C) and subsequently triggering inositol 1,4,5-trisphosphate (IP3) production.[23] *PLCE1* gene encodes an isoform of PLCε (phospholipase C epsilon), which is one of the enzymes that activate the phospholipid signaling cascade to generate the second messenger molecule IP3.[24]

Although there is no direct evidence demonstrating the uninterrupted correlation between 5-HT2B receptor/PLCE1/Ca2+ pathway and aldosterone production. There are several studies showing that the serotonin signaling pathway can affect aldosterone synthesis in adrenocortical cells.[25]

Strengths and Limitations

Using a multicenter prospectively collected tissue bank, we confirmed that mAPM/mAPN carriers of the classical APA/APN histopathology, identified according to the newly published histopathology of primary aldosteronism criteria, was associated with higher baseline aldosterone production from the unresected contralateral adrenal glands.[3] In PA, previous studies revealed the potential role of mAPM/mAPN in idiopathic hyperaldosteronism, where there was no lateralization on imaging or adrenal venous sampling for the aldosterone excess.[16] Our current finding is the first one to reveal various outcomes among the histopathologically classical APA/APN patients with or without coexistent mAPM/mAPN and concluded higher risks of residual hypertension after adrenalectomy in group A patients.

Comparison of the transcriptomic landscapes between the group A versus B patients has provided some insights into a possibly different origin of tumorigenesis in APA. mAPM/mAPN adjacent to classical tumor could carry different functional and pathway enrichment, with the adenomas showing decreased expression of *HTR2B*, and *PLCE1,* which are important components in HTR2B-mediated PLC/IP3/$Ca2^+$ pathway, both in mRNA and protein levels.

Third, since only patients with solitary CYP11B2 staining APA/APN who underwent adrenalectomy were recruited to this study, it remains uncertain if the existence of mAPM/mAPN in patients with unilateral or bilateral adrenal hyperplasia (without a prominent APA) is also associated with change of calcium signaling. Fourth, many risk factors will also have impacts on residual hypertension after adrenalectomy, for example, arterial stiffness,[26] vascular changes due to cortisol excess,[9] or kidney function;[27] they need to be put into the big picture during the interpretation.

Perspectives

Given that 40.5% (30/74) of the histopathologically classical APA/APN adrenals were characterized with positive CYP11B1 staining, which is similar to what was reported previously,[28] their interactive pathophysiological function with mAPM/mAPN clearly needs to be further addressed.

Furthermore, it is also reasonable to speculate the existence of mAPM/mAPN in the contralateral adrenal gland in whom with enriching mAPM/mAPN in the adrenalectomized specimens; these patients were originally thought to have uPA according to the current stringent PA diagnosis guidelines. There could be functional differences of bilateral mAPM/mAPN, if they do exist, in expressing the aldosterone excess. mAPM/mAPN may contribute to subclinical PA, and this assumption may help explain findings from the PATHWAY-2 study, showing the mineralocorticoid receptor antagonist is the most effective drug in resistant hypertension.[29]

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Disclosures

None.

REFERENCES

1. Funder JW, Carey RM, Mantero F, Murad MH, Reincke M, Shibata H, Stowasser M, Young WF Jr. The Management of Primary Aldosteronism: case detection, diagnosis, and treatment: an Endocrine Society Clinical Practice Guideline. *J Clin Endocrinol Metab*. 2016;101:1889–1916. doi: 10.1210/jc.2015-4061
2. De Sousa K, Boulkroun S, Baron S, Nanba K, Wack M, Rainey WE, Rocha A, Giscos-Douriez I, Meatchi T, Amar L, et al. Genetic, cellular, and molecular heterogeneity in adrenals with Aldosterone-Producing Adenoma. *Hypertension*. 2020;75:1034–1044. doi: 10.1161/HYPERTENSIONAHA.119.14177
3. Williams TA, Gomez-Sanchez CE, Rainey WE, Giordano TJ, Lam AK, Marker A, Mete O, Yamazaki Y, Zerbini MCN, Beuschlein F, et al. International histopathology consensus for unilateral primary aldosteronism. *J Clin Endocrinol Metab*. 2021;106:42–54. doi: 10.1210/clinem/dgaa484
4. Wu VC, Kuo CC, Chang HW, Tsai CT, Lin CY, Lin LY, Lin YH, Wang SM, Huang KH, Fang CC, et al; TAIPAI study group. Diagnosis of primary aldosteronism: comparison of post-captopril active renin concentration and plasma renin activity. *Clin Chim Acta*. 2010;411:657–663. doi: 10.1016/j.cca.2010.01.027
5. Peng KY, Chang HM, Lin YF, Chan CK, Chang CH, Chueh SJ, Yang SY, Huang KH, Lin YH, Wu VC, et al. miRNA-203 modulates Aldosterone levels and cell proliferation by targeting Wnt5a in Aldosterone-Producing Adenomas. *J Clin Endocrinol Metab*. 2018;103:3737–3747. doi: 10.1210/jc.2018-00746
6. Williams TA, Lenders JWM, Mulatero P, Burrello J, Rottenkolber M, Adolf C, Satoh F, Amar L, Quinkler M, Deinum J, et al; Primary Aldosteronism Surgery Outcome (PASO) investigators. Outcomes after adrenalectomy for unilateral primary aldosteronism: an international consensus on outcome measures and analysis of remission rates in an international cohort. *Lancet Diabetes Endocrinol*. 2017;5:689–699. doi: 10.1016/S2213-8587(17)30135-3
7. Wu VC, Hu YH, Er LK, Yen RF, Chang CH, Chang YL, Lu CC, Chang CC, Lin JH, Lin YH, et al; TAIPAI group. Case detection and diagnosis of primary aldosteronism - The consensus of Taiwan Society of Aldosteronism. *J Formos Med Assoc*. 2017;116:993–1005. doi: 10.1016/j.jfma.2017.06.004
8. Wu VC, Wang SM, Chueh SJ, Yang SY, Huang KH, Lin YH, Wang JJ, Connolly R, Hu YH, Gomez-Sanchez CE, et al. The prevalence of CTNNB1 mutations in primary aldosteronism and consequences for clinical outcomes. *Sci Rep*. 2017;7:39121. doi: 10.1038/srep39121
9. Peng KY, Liao HW, Chan CK, Lin WC, Yang SY, Tsai YC, Huang KH, Lin YH, Chueh JS, Wu VC. Presence of subclinical hypercortisolism in clinical Aldosterone-producing Adenomas predicts lower clinical success. *Hypertension*. 2020;76:1537–1544. doi: 10.1161/HYPERTENSIONAHA.120.15328
10. Meyer LS, Handgriff L, Lim JS, Udager AM, Kinker IS, Ladurner R, Wildgruber M, Knosel T, Bidlingmaier M, Rainey WE, et al. Single-Center Prospective Cohort Study on the Histopathology, Genotype, and Postsurgical Outcomes of Patients With Primary Aldosteronism. *Hypertension*. 2021;78:XX-XX. doi: 10.1161/HYPERTENSIONAHA.121.17348
11. Chan CK, Kim JH, Chueh E, Chang CC, Lin YF, Lai TS, Huang KH, Lin YH, Wu VC; TAIPAI Study Group. Aldosterone level after saline infusion test could predict clinical outcome in primary aldosteronism after adrenalectomy. *Surgery*. 2019;166:362–368. doi: 10.1016/j.surg.2019.05.001
12. Murakami M, Yoshimoto T, Nakabayashi K, Tsuchiya K, Minami I, Bouchi R, Izumiyama H, Fujii Y, Abe K, Tayama C, et al. Integration of transcriptome and methylome analysis of aldosterone-producing adenomas. *Eur J Endocrinol*. 2015;173:185–195. doi: 10.1530/EJE-15-0148
13. Monticone S, Hattangady NG, Nishimoto K, Mantero F, Rubin B, Cicala MV, Pezzani R, Auchus RJ, Ghayee HK, Shibata H, et al. Effect of KCNJ5 mutations on gene expression in aldosterone-producing adenomas and adrenocortical cells. *J Clin Endocrinol Metab*. 2012;97:E1567–E1572. doi: 10.1210/jc.2011-3132
14. Weisbrod AB, Webb RC, Mathur A, Barak S, Abraham SB, Nilubol N, Quezado M, Stratakis CA, Kebebew E. Adrenal histologic findings show no difference in clinical presentation and outcome in primary hyperaldosteronism. *Ann Surg Oncol*. 2013;20:753–758. doi: 10.1245/s10434-012-2670-2
15. Nishimoto K, Seki T, Kurihara I, Yokota K, Omura M, Nishikawa T, Shibata H, Kosaka T, Oya M, Suematsu M, et al. Case report: nodule development from subcapsular Aldosterone-producing cell clusters causes Hyperaldosteronism. *J Clin Endocrinol Metab*. 2016;101:6–9. doi: 10.1210/jc.2015-3285
16. Omata K, Satoh F, Morimoto R, Ito S, Yamazaki Y, Nakamura Y, Anand SK, Guo Z, Stowasser M, Sasano H, et al. Cellular and genetic causes of idiopathic hyperaldosteronism. *Hypertension*. 2018;72:874–880. doi: 10.1161/HYPERTENSIONAHA.118.11086
17. Sun N, Meyer LS, Feuchtinger A, Kunzke T, Knösel T, Reincke M, Walch A, Williams TA. Mass spectrometry imaging establishes 2 distinct metabolic phenotypes of Aldosterone-producing cell clusters in primary Aldosteronism. *Hypertension*. 2020;75:634–644. doi: 10.1161/HYPERTENSIONAHA.119.14041
18. Omata K, Anand SK, Hovelson DH, Liu CJ, Yamazaki Y, Nakamura Y, Ito S, Satoh F, Sasano H, Rainey WE, et al. Aldosterone-producing cell clusters frequently harbor somatic mutations and accumulate with age in normal adrenals. *J Endocr Soc*. 2017;1:787–799. doi: 10.1210/js.2017-00134
19. Zennaro MC, Boulkroun S, Fernandes-Rosa F. Genetic causes of functional Adrenocortical Adenomas. *Endocr Rev*. 2017;38:516–537. doi: 10.1210/er.2017-00189

20. Gomez-Sanchez CE, Kuppusamy M, Reincke M, Williams TA. Disordered CYP11B2 expression in Primary Aldosteronism. *Horm Metab Res*. 2017;49:957–962. doi: 10.1055/s-0043-122238
21. Kometani M, Yoneda T, Aono D, Karashima S, Demura M, Nishimoto K, Yamagishi M, Takeda Y. Impact of aldosterone-producing cell clusters on diagnostic discrepancies in primary aldosteronism. *Oncotarget*. 2018;9:26007–26018. doi: 10.18632/oncotarget.25418
22. Meyer LS, Wang X, Sušnik E, Burrello J, Burrello A, Castellano I, Eisenhofer G, Fallo F, Kline GA, Knösel T, et al. Immunohistopathology and steroid profiles associated with biochemical outcomes after Adrenalectomy for Unilateral Primary Aldosteronism. *Hypertension*. 2018;72:650–657. doi: 10.1161/HYPERTENSIONAHA.118.11465
23. Millan MJ, Marin P, Bockaert J, Mannoury la Cour C. Signaling at G-protein-coupled serotonin receptors: recent advances and future research directions. *Trends Pharmacol Sci*. 2008;29:454–464. doi: 10.1016/j.tips.2008.06.007
24. Smrcka AV, Brown JH, Holz GG. Role of phospholipase Cε in physiological phosphoinositide signaling networks. *Cell Signal*. 2012;24:1333–1343. doi: 10.1016/j.cellsig.2012.01.009
25. Lopez AG, Duparc C, Naccache A, Castanet M, Lefebvre H, Louiset E. Role of mast cells in the control of Aldosterone secretion. *Horm Metab Res*. 2020;52:412–420. doi: 10.1055/a-1119-1063
26. Chan CK, Yang WS, Lin YH, Huang KH, Lu CC, Hu YH, Wu VC, Chueh JS, Chu TS, Chen YM. Arterial stiffness is associated with clinical outcome and cardiorenal injury in lateralized primary aldosteronism. *J Clin Endocrinol Metab*. 2020;105:dgaa566. doi: 10.1210/clinem/dgaa566
27. Wu VC, Chueh SC, Chang HW, Lin LY, Liu KL, Lin YH, Ho YL, Lin WC, Wang SM, Huang KH, et al. Association of kidney function with residual hypertension after treatment of aldosterone-producing adenoma. *Am J Kidney Dis*. 2009;54:665-673. doi: 10.1053/j.ajkd.2009.06.014
28. Nanba K, Omata K, Gomez-Sanchez CE, Stratakis CA, Demidowich AP, Suzuki M, Thompson LDR, Cohen DL, Luther JM, Gellert L, et al. Genetic characteristics of Aldosterone-producing Adenomas in blacks. *Hypertension*. 2019;73:885–892. doi: 10.1161/HYPERTENSIONAHA.118.12070
29. Scholl UI, Stölting G, Nelson-Williams C, Vichot AA, Choi M, Loring E, Prasad ML, Goh G, Carling T, Juhlin CC, et al. Recurrent gain of function mutation in calcium channel CACNA1H causes early-onset hypertension with primary aldosteronism. *Elife*. 2015;4:e06315. doi: 10.7554/eLife.06315