miRNA299 involvement in *CYP11B2* expression in aldosterone-producing adenoma

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Abstract

Objective: The pathophysiology of aldosterone-producing adenomas (APAs) has been intensively investigated using genetic and epigenetic approaches. However, the role of miRNAs in APA is not fully understood. The present study profiled miRNAs in APAs as an exploratory approach to elucidate their pathophysiological roles in APAs.

Design: Tissues of APAs and other adrenocortical adenomas were obtained from patients who underwent adrenalectomy.

Methods: Candidate miRNAs differentially detected from samples were examined by whole miRNA sequencing. The expression of candidate miRNAs in APA tissues were further validated by real-time quantitative polymerase chain reaction (qPCR). Further, differential miRNA expression between APAs with and without *KCNJ5* somatic mutations was examined. Prediction of miRNA target genes was performed by bioinformatics analysis. For specific miRNAs, correlation analysis between the levels of their target genes and *CYP11B2* was analyzed in APA tissues.

Results: Our study determined differential expression of six miRNAs in APA or APA with *KCNJ5* mutations. We further demonstrated that miR299 levels were negatively correlated with mRNA levels of *CACNB2*, which encodes the beta-subunit of the L-type calcium channel. Additionally, we found significant correlations among miR299, *CACNB2*, and *CYP11B2* levels in APA tissues.

Conclusions: Our study suggests the possible pathophysiological involvement of specific miRNAs in calcium signaling and aldosterone hypersecretion in APAs. Further studies, including *in vitro* analyses, are required to clarify these findings.

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Introduction

Primary aldosteronism (PA) is an important cause of secondary hypertension (1, 2) and has been associated with severe cardiovascular and renal complications, as shown by comparison with age-, sex- and blood pressure-matched patients with essential hypertension (3, 4). Among the PA subtypes, aldosterone-producing adenoma

(APA) often causes severe forms of resistant hypertension that are curable by unilateral adrenalectomy, making APAs therapeutically attractive (1, 5).

Recently, the pathogenesis of APA has been clarified at the molecular level using high-throughput technologies, including next-generation sequencing, transcriptomics

and methylomics (6, 7, 8). In particular, a series of exome sequencing studies have uncovered that somatic mutations in genes encoding ion channels or carriers (*KCNJ5*, *ATP1A1*, *ATP2B3* and *CACNA1D*) (9, 10, 11, 12, 13) cause activation of calcium (Ca) signaling. This activation leads to autonomous aldosterone hypersecretion from APA through increased expression of *CYP11B2*, a gene encoding a steroidogenic enzyme responsible for a rate-limiting step of aldosterone synthesis (14). Furthermore, methylome studies have revealed that hypomethylation of the proximal promoter region of *CYP11B2* is involved in its increased expression in APA (15, 16). Thus, the pathophysiology and pathogenesis of APA are currently being clarified in both genomic and epigenomic studies.

miRNA, another important epigenetic factor, is a small non-coding RNA molecule. It is widely accepted that miRNAs bind to their target mRNAs through their complementary sequences (17, 18) and negatively regulate the expression of their target gene through mRNA degradation and post-transcriptional modification (19). A series of studies have shown that miRNAs are crucial regulators of biological processes, including various pathological conditions, such as neoplasms (20, 21). However, to date, data on miRNA expression in APAs are limited.

In the present study, we profiled miRNAs that are differentially detected in tumor tissues from patients with APAs via whole miRNA sequencing, as an exploratory approach to elucidate the pathophysiological significance of miRNAs in APAs. The differential expression of miRNA between APAs with or without *KCNJ5* somatic mutations was also examined.

Subjects and methods

Subjects

The study protocol was approved by the Ethics Committee for Human Research at Tokyo Medical and Dental University (Tokyo, Japan) (nos. 191 and G2016-002). Written informed consent was obtained from all participants. Adrenal tissue was obtained from 62 patients with APA, 12 patients with adrenocortical adenoma (ACA) with subclinical hypercortisolism (SH) and four patients with non-functioning adrenal adenoma (NF) who underwent adrenalectomy at Tokyo Medical and Dental University Hospital as described previously (8). Among them, 12 APAs and 5 SHs were from cases that were used in previous studies (8).

The diagnosis of PA and SH was based on diagnostic criteria as described in the Supplementary materials (see section on [supplementary data](#) given at the end of this article). NF patients did not fulfill diagnosis criteria of either PA or SH. All adrenal tumors were pathologically diagnosed as benign adrenocortical adenoma.

Detection of somatic mutations in *KCNJ5*, *ATP1A1*, *ATP2B3*, *CACNA1D* and *CTNNB1* by PCR and direct sequencing

APA-specific somatic mutations in *KCNJ5*, *ATP1A1*, *ATP2B3*, *CACNA1D* and *CTNNB1* were analyzed by PCR and direct sequencing. The detailed methods have been described previously (22), and the primers used are described in Supplementary Table 1.

Extraction of RNA

Adrenocortical tumor samples were obtained at the time of surgery and stored at -80°C . Detailed protocols of RNA extraction are provided in the Supplementary materials.

Whole miRNA sequencing and differential expression analysis

A total of 14 adrenocortical tumors from patients with APA, 5 patients with SH and three patients with NF were investigated by whole miRNA sequencing as 'profiling samples'. Following RNA extraction, 500–1000 ng of total RNA were used for whole miRNA sequencing and subsequent differential expression analysis. Detailed protocols are described in the Supplementary materials.

Hierarchical cluster analysis using whole miRNA sequence data showed that SHs clustered more closely to NFs than APAs (Supplementary Fig. 1). Due to the similarity of miRNA distributions of SHs and NFs, we set a combination of SHs and NFs as a control group for the subsequent analysis.

To search for miRNAs potentially involved in the pathogenesis of APAs, we defined the following profiling criteria to screen miRNAs by whole miRNA sequencing: Criterion (1) differentially detected miRNAs (adjusted *P* value <0.05) in APA tissues compared to the control (SH and NF tissues) and Criterion (2) differentially detected miRNAs (adjusted *P* value <0.05) between APAs with and without *KCNJ5* somatic mutations (Fig. 1).

Figure 1

Strategy for miRNA profiling by whole miRNA sequencing. A total of 14 APAs (9 with *KCNJ5* mutation and 5 without *KCNJ5* mutation) and 8 non-APAs (5 SHs and 3 NFs) were examined as 'profiling samples'. Using whole miRNA sequencing, 513 miRNAs were detected; of which, 12 miRNAs met criterion 1 and 24 miRNAs met criterion 2. Four miRNAs met both criteria 1 and 2. These 32 miRNAs are listed in Supplementary Table 5. APA, aldosterone-producing adenoma; miRNA, microRNA; n, numbers of detected miRNAs; NF, non-functioning adrenal adenoma; qPCR, real-time quantitative polymerase chain reaction; SH, adrenocortical adenoma with subclinical hypercortisolemia.

Real-time qPCR analyses of miRNAs

To validate results of the whole miRNA sequencing, reverse transcription and qPCR were performed as described in the Supplementary materials. For qPCR analysis, ten patients with APA, seven patients with SH and one patient with NF were added to the 'profiling samples' used for whole miRNA sequencing. Accordingly, 24 patients with APA, 12 patients with SH and four patients with NF were investigated as 'validation samples'.

Prediction of miRNA target genes

Prediction of miRNA target genes was performed by bioinformatics analysis using an online tool (<http://www.mirdb.org/mirDB/>). First, candidate target genes with a target score >75 were extracted from the miRDB database. We postulated that if the miRNA controlled expression of the target gene, the expression level of the miRNA and target gene would be inversely correlated. The candidate target genes were, therefore, narrowed down using the gene expression profiles of our previously published transcriptome database based on RNA sequencing (<https://doi.org/10.1530/ERC-17-0117>) (8). Selected genes

consisted of 34 APAs (29 with and 5 without *KCNJ5* mutations) and 5 SHs, as follows. For miR34a, which corresponds to Criterion 1, we selected genes showing greater than two-fold mRNA expression in APAs than SH in the RNA sequence database. For miR370, which corresponds to Criterion 1, we selected genes showing lesser than two-fold mRNA expression in APAs than SH in the RNA sequence database. For miR24, miR299, miR30e and miR93, which correspond to Criterion 2, we selected genes showing greater than two-fold mRNA expression in APAs with *KCNJ5* mutations than APAs without *KCNJ5* mutations in the RNA sequence database. The correlation of expression levels between these genes predicted by bioinformatics analysis and the corresponding miRNAs were further examined by qPCR as described below.

Reverse transcription and qPCR analyses for gene expression

For mRNA quantification in adrenocortical tumor specimens, reverse transcription and qPCR analysis were performed for the 29 candidate target genes by the method described previously (22). Notably, to examine the relation of *CACNB2* gene expression with miRNA and *CYP11B2*, we analyzed 62 APA samples (43 APA with *KCNJ5* mutations and 19 APA without *KCNJ5* mutations) as 'confirmation samples'. The detailed protocols and primers used for the qPCR assay are described in the Supplementary materials.

Statistical analysis

Statistical analysis was carried out using SPSS (version 21.0; IBM Corp). Data are presented as the median and interquartile range (IQR). Due to skewed distribution, miRNA levels of miR370, miR24 and miR93 and mRNA levels of *NEFM*, *GRIP1*, *FRMD6*, *HSPB7*, *CACNB2*, *CTNND2*, *CHST11*, *HSPA12A*, *TMEM154* and *CYP11B2* were log-transformed for statistical analysis. In the whole miRNA sequence, adjusted *P* values were calculated using the Benjamini and Hochberg false discovery rate correction as a reference. The hierarchical cluster analysis was performed by edgeR (v.3.18.1) (23). Mann-Whitney *U* test was used to compare quantitative variables between two groups. Univariate correlations were evaluated as Pearson's correlation coefficient, since each parameter was normally distributed. Adjusted *P*-value <0.05 in the whole miRNA sequence and *P* values <0.05 in the qPCR were considered to be statistically significant.

Results

Profiling of tissue miRNAs in patients with PA

Clinical characteristics of the APA, SH and NF patients were divided into profiling samples, validation samples, and confirmation samples, as described in Table 1. Profiling revealed *KCNJ5* somatic mutations in 70% of APA specimens. The somatic mutational state of APAs in the present study is described in Supplementary Table 4.

Our strategy for miRNA profiling by whole miRNA sequencing is shown in Fig. 1. In analysis of adrenocortical adenomas, 513 miRNAs were detected. Of which, 12 miRNAs met Criterion 1, and 24 miRNAs met Criterion 2. Four miRNAs (miR1307, miR370, miR532 and miR675) met both Criteria 1 and 2. These 32 miRNAs are listed in Supplementary Table 5.

Validation of miRNAs profiling by qPCR in adrenocortical adenoma samples

To validate the results of whole miRNA sequencing, we analyzed the levels of these 32 miRNAs in adrenocortical adenoma samples by qPCR (Supplementary Table 5). The levels of six miRNAs (miR24, miR299, miR30e, miR34a, miR370 and miR93) showed significant differential expression, which corresponded with the whole miRNA sequencing data (Fig. 2 and Supplementary Table 6). The levels of miR34a and miR370 were significantly lower and higher, respectively, in APA than in the reference control (NFs and SHs), which aligned with the 12 miRNAs of Criterion 1. Further, the levels of miR24, miR299, miR30e and miR93 were significantly lower in APAs with *KCNJ5* mutations than in those without *KCNJ5* mutations, which aligned with the 24 miRNAs of Criterion 2 (Fig. 2).

Table 1 Clinical characteristics of ACA patients. All data are presented as the median and interquartile range. 'Profiling samples', 'validation samples' and 'confirmation samples' indicate samples used for whole miRNA sequencing, qPCR for validation of miRNA expression and validation of the correlation between miRNAs and target genes, respectively. Validation samples contain the all of 'Profiling samples' and confirmation samples contain all APAs of 'Validation samples'. Hypokalemia was defined by serum potassium <3.5 mEq/L or by treatment with any oral potassium supplement.

	Non-APA		APA	
	NF	SH	Non- <i>KCNJ5</i>	<i>KCNJ5</i>
Profiling samples				
Number	3	5	5	9
Age	63 (60–66)	52 (46–54)	62 (57–62)	54 (54–60)
Female (%)	33	80	20	77
Duration of HT (Y)	0 (0–4)	0 (0–13)	16 (7–17)	7 (2–12)
Hypokalemia (%)	0	0	100	88
PRA (ng/dL/h)	0.6 (0.5–2.1)	0.2 (0.2–0.3)	0.2 (0.2–0.7)	0.1 (0.1–0.3)
PAC (pg/mL)	77.0 (59.5–79.5)	76.0 (71.0–84.0)	283 (258–395)	317 (285–457)
Validation samples				
Number	4	12	11	13
Age	60 (57–64)	50 (45–54)	57 (49–62)	54 (50–57)
Female (%)	25	92	37	69
Duration of HT (Y)	0 (0–2)	1 (0–5)	15 (7–19)	9 (3–12)
Hypokalemia (%)	0	25	100	84
PRA (ng/dL/h)	0.5 (0.3–1.3)	0.3 (0.2–1.0)	0.2 (0.1–0.5)	0.2 (0.1–0.3)
PAC (pg/mL)	79.5 (68.2–89.7)	77.0 (68.7–91.0)	241 (198–367)	326 (289–413)
Confirmation samples				
Number	–	–	19	43
Age	–	–	57 (49–62)	51 (42–57)
Female (%)	–	–	27	71
Duration of HT (Y)	–	–	9 (6–16)	9 (4–15)
Hypokalemia (%)	–	–	83	94
PRA (ng/dL/h)	–	–	0.2 (0.2–0.6)	0.2 (0.1–0.3)
PAC (pg/mL)	–	–	270 (198–416)	403 (273–491)

ACA, adrenocortical adenoma; APA, aldosterone-producing adenoma; HT, hypertension; K, serum potassium; NF, non-functioning adrenal adenoma; Non-*KCNJ5*, APA without *KCNJ5* somatic mutations; PAC, plasma aldosterone concentration; PRA, plasma renin activity; SH, ACA with subclinical hypercortisolemia.

Figure 2

Differential expression of miRNAs in APA or APA with *KCNJ5* somatic mutations validated by qPCR. Data were analyzed by qPCR. Relative miRNA levels were calculated and are presented as box plots. Controls consisted of 12 adrenocortical adenomas with subclinical hypercortisolemia and four non-functioning adrenal adenomas. APA consisted of 24 APA specimens regardless of *KCNJ5* somatic mutation status. Non-*KCNJ5* consisted of 11 APAs without *KCNJ5* somatic mutations. *KCNJ5* consisted of 13 APAs with *KCNJ5* somatic mutations. *P* values were calculated using the Mann-Whitney *U* test. * indicates $P < 0.05$ between two groups. APA, aldosterone-producing adenoma; *KCNJ5*, APA with *KCNJ5* somatic mutations; miRNA, microRNA; non-*KCNJ5*, APA without *KCNJ5* somatic mutations; qPCR, real-time quantitative polymerase chain reaction.

The expression of miRNAs profiled by Criterion 1 in the whole miRNA sequence was investigated by qPCR to validate use of the SH and NF combination as a reference control to APA. The qPCR analysis revealed that the distribution of all miRNAs, except miR34, was not significantly different between SHs and NFs (Supplementary Fig. 2).

Nine target genes negatively correlated with levels of the corresponding miRNAs

Integration of bioinformatics analysis using the online tool and our transcriptome database (8) predicted 29 candidate genes that are possibly regulated by the six miRNAs (Supplementary Table 7). We examined mRNA levels of these 29 genes by qPCR using the validation samples and then analyzed the correlation between expression levels of the miRNAs and their target genes (Table 2). The mRNA levels of *CACNB2*, which encodes L-type Ca channel and could potentially affect cellular Ca signaling, showed a significantly negative correlation to miR299 (Table 2). The mRNA levels of *CTNND2*, *ANK2*, *RIMS3*, *MSTN*, *LRRK1*, *MAP3K8*, *GRIP1* and *PPP4R4* also showed significant correlation to the corresponding miRNA levels (Table 2). The mRNA levels of the other genes showed no significant correlation to the corresponding miRNA levels (Table 2).

Expression levels of *CACNB2* positively correlated with *CYP11B2*

CACNB2 encodes the $\beta 2$ subunit of the L-type Ca channel, which interacts with the $\alpha 1$ subunit to modulate function of the Ca channel (24, 25). Dysregulation of Ca signaling in adrenocortical cells is considered as one of the crucial pathogenic factors leading to *CYP11B2* upregulation and subsequent autonomous aldosterone hypersecretion in PA. We therefore further examined the relationship of *CACNB2* mRNA levels with those of *CYP11B2* using the confirmation samples. The mRNA levels of *CACNB2* in the confirmation samples showed significant negative correlations to miR299 levels (Fig. 3). Moreover, *CACNB2* mRNA levels showed a significantly positive correlation with *CYP11B2* (Fig. 3).

We also compared the miR299 levels between the entire APA and the control as a secondary sub-analysis using validation samples, although this comparison did not show significant difference in the miRNA profiling conducted using whole miRNA sequencing using the profiling samples. We found that the levels of miR299 were also significantly lower in the entire APA than in the controls using the validation samples (Supplementary Fig. 3). The mRNA levels of *CACNB2* showed significant negative and positive correlations to miR299 levels and *CYP11B2* mRNA levels, respectively, in the entire ACA that consist of the confirmation samples and controls (Supplementary Fig. 3).

Table 2 Correlations between miRNA and gene expression levels of candidate genes profiled by bioinformatics analysis. Pearson's correlation coefficient was evaluated in each combination of miRNAs and candidate target genes, which were shown by bioinformatics analysis. Only the significant genes are described by their gene description.

miRNA	Gene symbol	<i>r</i>	<i>P</i> value	Gene description
APA compared with other ACA				
hsa-miR-34a-5p	<i>BMP3</i>	−0.350	0.054	
	<i>CTNND2</i>	−0.442	0.013	Catenin (cadherin associated protein) delta 2 (35, 36)
	<i>TPD52</i>	−0.343	0.059	
	<i>RELN</i>	−0.174	0.437	
	<i>ANK2</i>	−0.629	< 0.001	Ankyrin 2 (adaptor protein to membrane) (37)
	<i>RIMS3</i>	−0.467	0.008	Regulating synaptic membrane exocytosis 3 (38)
	<i>MSTN</i>	−0.456	0.011	Myostatin (39)
hsa-miR-370-3p	<i>LRRK1</i>	−0.589	0.010	Leucine rich repeat kinase 1 (40)
	<i>CHST15</i>	−0.387	0.102	
	<i>CHST11</i>	−0.338	0.512	
	<i>HSPA12A</i>	−0.372	0.117	
	<i>PMAIP1</i>	−0.267	0.269	
	<i>TMEM154</i>	−0.332	0.227	
	<i>MAP3K8</i>	−0.417	0.038	MAP kinase kinase kinase 8 (41, 42)
APA with <i>KCNJ5</i> mutations compared with those without mutations				
hsa-miR-24-3p	<i>NEFM</i>	−0.087	0.637	
	<i>PTPRD</i>	−0.084	0.733	
	<i>GRIP1</i>	−0.406	0.021	Glutamate receptor interacting protein 1 (43, 44)
hsa-miR-299-3p	<i>FRMD6</i>	−0.264	0.275	
	<i>PAIP2B</i>	−0.206	0.371	
	<i>HSPB7</i>	−0.158	0.493	
	<i>CACNB2</i>	−0.560	0.002	Voltage-dependent calcium channel, β 2 subunit (24, 25)
hsa-miR-30e-5p	<i>PPP4R4</i>	−0.535	0.018	Serine/threonine protein phosphatase 4, regulatory subunit 4 (45)
	<i>GRM3</i>	−0.279	0.247	
	<i>CACNB2</i>	−0.200	0.273	
	<i>CTNND2</i>	−0.193	0.289	
	<i>ELOVL7</i>	−0.314	0.191	
hsa-miR-93-3p	<i>CACNB2</i>	−0.216	0.236	
	<i>ATP9A</i>	−0.082	0.654	
	<i>DLGAP1</i>	−0.280	0.246	

P values <0.05 are indicated in bold.

ACA, adrenocortical adenoma; APA, aldosterone-producing adenoma; *r*, correlation coefficient.

mRNA levels of *CYP11B2* correlated with miR299

We examined the correlation of *CYP11B2* mRNA levels with levels of miR299 using the confirmation samples. *CYP11B2* mRNA levels showed significant negative correlations with miR299 levels (Fig. 4). *CYP11B2* mRNA levels in the entire ACA that consist of the confirmation samples and controls also showed significant negative correlations with miR299 levels (Supplementary Fig. 3).

Discussion

We identified six miRNAs (24, 299, 30e, 34a, 370 and 93) by a combination of whole miRNA sequencing and qPCR. In addition, we revealed that miR299 levels in APA tissues were inversely correlated with *CACNB2* expression

levels. Furthermore, we revealed that *CYP11B2* expression levels in APA tissues were positively and negatively correlated with *CACNB2* and miR299 expression levels, respectively, suggesting pathophysiological relevance of the relationship among miR299, *CACNB2*, and *CYP11B2*.

The two analytical methods for miRNA, whole miRNA sequencing and qPCR, have several advantages and disadvantages. Whole miRNA sequencing is capable of comprehensively profiling miRNA expression by processing a large number of samples. However, qPCR can accurately quantify individual miRNAs, although it is difficult to profile a large number of miRNAs. Therefore, in the present study, we first screened samples using whole miRNA sequencing and then validated the obtained candidate miRNAs using qPCR. We screened 32 candidate miRNAs by whole miRNA sequencing and validated six

Figure 3

Correlations between *CACNB2* mRNA levels and miR299 or *CYP11B2* mRNA levels. Univariate correlations between *CACNB2* mRNA levels and miR299 expression levels (A), and *CYP11B2* mRNA levels (B) are shown. Pearson's correlation coefficient was used to analyze 62 APAs regardless of *KCNJ5* somatic mutation status. APA, aldosterone-producing adenoma; *r*, correlation coefficient.

miRNAs by qPCR. Similar to the present study, Tiedt *et al.* comprehensively examined circulating miRNAs in patients suffering from acute ischemic stroke using whole miRNA sequencing and qPCR and reported that six miRNAs were validated by qPCR from 22 miRNAs profiled by whole

Figure 4

Correlations between miR299 levels and *CYP11B2* mRNA levels. Univariate correlations between *CYP11B2* mRNA levels and miR299 expression levels are shown. Pearson's correlation coefficient was used to analyze 62 APAs regardless of *KCNJ5* somatic mutation status. APA, aldosterone-producing adenoma; *r*, correlation coefficient.

miRNA sequencing (26). Therefore, consistency between results of whole miRNA sequencing and qPCR does not appear to be high. In a different viewpoint, although our whole miRNA sequence did not show the significant difference in miR299 levels between APAs and controls, the qPCR showed a significant difference between them. It is, therefore, possible to speculate that the miRNA profiling method that we adopted overlooked some other miRNAs that are relevant in the pathophysiology of APA. Further studies are required to develop a more efficient screening method to examine miRNAs relevant to disease models.

Of the six miRNAs validated by qPCR, four miRNAs were differentially expressed in APAs with *KCNJ5* mutations compared with those without *KCNJ5* mutations. We have recently reported that APAs with *KCNJ5* mutations show marked differences in mRNA expression and DNA methylation at the whole genomic level compared to those without *KCNJ5* mutations (8, 27). In accordance with this finding, Monticone *et al.* also showed that expression levels of several genes in APAs with *KCNJ5* mutations were different from those without *KCNJ5* mutations (28). These results strongly support that APAs with *KCNJ5* mutations undergo different epigenetic regulation, in mRNA expression, DNA methylation, and miRNA expression, as compared to those without *KCNJ5* mutations.

Bioinformatics analysis and qPCR revealed five miRNAs that showed negative correlation between their expression levels and the mRNA levels of their respective target gene: miR34a and *CTNND2*, *ANK2*, *RIMS3* or *MSTN*; miR370 and *LRRK1* or *MAP3K8*; miR24 and *GRIP1*; miR299 and *CACNB2*; and miR30e and *PPP4R4*. Although, at present, it is difficult to infer the pathophysiological relevance of *CTNND2*, *ANK2*, *RIMS3*, *MSTN*, *LRRK1*, *MAP3K8*, *GRIP1* and *PPP4R4* in APA (Table 2), they may be new candidate factors for clarifying the unsolved pathogenic mechanism in PA in the future.

CACNB2 encodes the beta-subunit of the L-type Ca channel (24, 25). A previous study showed that *CACNB2* upregulation in cardiomyocytes induced activation of Ca signaling, which causes arrhythmia (29). It has been recognized that membrane depolarization and subsequent activation of Ca signaling play crucial roles in the autonomous aldosterone hypersecretion from APA through increased *CYP11B2* expression, a steroidogenic enzyme that is a rate-limiting step in the aldosterone synthesis pathway (14). Somatic mutations of *CACNA1D*, which encodes the alpha-subunit of L-type Ca channel (11, 12), have been shown to be associated

with APA pathogenesis. Therefore, we further examined the possible relationship between *CACNB2* and *CYP11B2* using 62 APA tissue specimens. The expression levels of *CACNB2* positively correlated to those of *CYP11B2* and negatively correlated with those of miR299 in APA tissues. In addition, miR299 levels were negatively correlated to *CYP11B2* levels, although bioinformatics analysis did not show any possible interaction between *CYP11B2* and miR299. It is, therefore, intriguing to hypothesize that decreased miR299 levels induce *CACNB2* expression in APA tissues, which leads to activation of the L-type Ca channel and subsequent activation of Ca signaling, resulting in increased *CYP11B2* expression via the effect of these miRNAs on *CACNB2* expression. However, further *in vitro* studies are required to confirm this hypothesis.

There have been a few studies examining the miRNA expression profiles in APAs (30, 31, 32). Velázquez-Fernández *et al.* (30) and He *et al.* (31) showed that miR-Let7f, 10b, 139-5p, and miR375 were differentially expressed in APAs compared with adjacent adrenal glands or adrenal tissues from idiopathic hyperaldosteronism. Peng *et al.* reported that miR203 regulated aldosterone production and tumorigenesis in APA, which was determined by comparing APAs with adjacent adrenal gland tissues (32). These discrepancies, including those of the present study, could be explained by the different study design and modality. In particular, our study used ACAs (SHs and NFs) as reference controls, whereas previous studies used adjacent adrenal glands of APAs as reference controls.

In the present study, we set a combination of SH and NF as a reference control of APAs. We originally thought that ACAs, except APA and adrenal Cushing syndrome (CS), should be reference controls to APAs. These ACAs include ACAs with weak cortisol secretion, but not showing CS, so-called SH (33, 34), and NF. In fact, cases of ACA with SH have more opportunities to undergo adrenalectomy than those with NFs. Our hierarchical cluster analysis using the whole miRNA sequence data showed that SHs were more closely clustered to NFs than to APAs. In addition, our qPCR analysis revealed that the distribution of miRNAs, which were profiled by Criterion 1 in the whole miRNA sequencing, were comparable between SHs and NFs. We, therefore, considered that it is reasonable to use the combination of SH and NF as the reference control to APA.

In the present study, although our whole miRNA sequence did not show statistically significant difference in miR299 levels between APAs and the controls, qPCR analysis showed a significant difference between them. In addition, correlations among miR299, *CACNB2*, and

CYP11B2 in APAs were also found in entire ACAs. These results are intriguing and suggest that miR299 may be involved in relationship among *CACNB2* and *CYP11B2*, not only in *KCNJ5* mutation-positive APAs, but also in the entire APA. However, they were obtained from a sub-analysis based on the negative results of the original screening, and therefore, care should be taken to interpret these data.

There are some limitations in this study. First, our study used correlation analyses of the expression levels of miRNAs and their target genes. Since miRNA can regulate their target gene expression through mRNA degradation and interference with mRNA translation (19), it is possible that our experimental design overlooked target genes for which mRNA translation was inhibited by miRNA. However, the small number of available tumor specimens was not sufficient for additional analysis at the protein level. Secondly, some of our results are based on simple correlation analyses that do not directly prove causality and cannot exclude possible confounding factors. Therefore, additional *in vitro* studies using cell culture, such as a luciferase assay to confirm *CACNB2* as a miR299 target, are required.

In conclusion, we revealed six miRNAs specifically expressed in APAs: differential expression of miR34a and miR370 in APAs compared to control ACAs and differential expression of miR24, 299, 30e, and 93 in APAs with *KCNJ5* mutations compared with those without *KCNJ5* mutations. We also found a significant correlation in APA tissues among miR299, *CACNB2* and *CYP11B2* levels, suggesting possible pathophysiological roles of these miRNAs in the dysregulation of Ca signaling and aldosterone hypersecretion in APAs. The present study postulates a novel concept that miRNA, as an epigenetic factor, plays a key role in the pathogenesis and pathophysiology of APAs. Further studies, including *in vitro* analyses, are required to clarify the pathophysiological significance of miRNAs in APAs.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/EJE-18-0882>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this study.

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