

The significance of high expression of circulating miR-222-3p in patients with unilateral primary aldosteronism.

Kentaro Okamoto¹, Masakatsu Sone^{1, 2}, Daisuke Taura¹, Youichi Ohno¹, Ichiro Yamauchi¹, Yohei Ueda¹, Takuro Hakata¹, Mika Tsuiki³, Masanori Murakami⁴, Daisuke Yabe¹, Nobuya Inagaki^{1, 5}, Mitsuhide Naruse⁶

¹Department of Diabetes, Endocrinology and Nutrition, Kyoto University Graduate School of Medicine, Kyoto, Japan

²Department of Metabolism and Endocrinology, St. Marianna University School of Medicine, Kawasaki, Japan

³Department of Endocrinology and Metabolism, National Hospital Organization Kyoto Medical Center, Kyoto, Japan

⁴Department of Molecular Endocrinology and Metabolism, Graduate School of Medical and Dental Sciences, Institute of Science Tokyo, Tokyo, Japan

⁵Medical Research Institute KITANO HOSPITAL, PIIF Tazuke-kofukai, Osaka, Japan

⁶Endocrine Center and Clinical Research Center, Ijinkai Takeda General Hospital, Kyoto, Japan

Financial support: This study was supported by research grants from the Japan Agency for Medical Research and Development (AMED) under grant numbers JP17ek0109122 (JPAS) and JP20ek0109352 (JRAS), and the National Center for Global Health and Medicine, Japan (27-1402, 30-1008). This study was also supported by grants from the Japan Smoking Foundation and the Japan Foundation for Applied Enzymology.

Disclosure statement: NI received joint research grants from Terumo, and received speaker honoraria from Nippon Boehringer Ingelheim, Kyowa Kirin, Sanofi, Sumitomo Pharma, and Mitsubishi Tanabe Pharma, and received scholarship grants from Daiichi-Sankyo, Mitsubishi Tanabe, Takeda, Kyowa Kirin, Sumitomo Pharma, Nippon Boehringer Ingelheim, and Kowa. MS and NI were members of the Board of Directors of the Japan Endocrine Society. Other authors have nothing to disclose.

Correspondence and Reprint Requests: Masakatsu Sone, MD, PhD, Department of Metabolism and Endocrinology, St. Marianna University School of Medicine, 2-16-1, Sugao, Miyamae-ku, Kawasaki, Japan. E-mail: sonemasa@marianna-u.ac.jp ORCID ID: 0000-0002-0757-7529

Keywords: Primary aldosteronism, circulating miRNA

Abstract

Context. The significance of circulating miRNAs in primary aldosteronism (PA) is still largely unknown.

Objective. We compared the profiles of circulating miRNAs between unilateral primary aldosteronism (UPA) and bilateral hyperaldosteronism (BHA) and investigated the potential role of a newly identified UPA-related miRNA in the pathogenesis of PA.

Methods. We initially conducted a comprehensive expression analysis of circulating miRNAs using a qPCR panel in a small number of cases matched for background factors, followed by validation analysis with a larger sample size for the candidate miRNAs.

Results. The expression level of one single miRNA, miR-222-3p, was significantly higher in UPA than in BHA, both in the adrenal veins and the inferior vena cava. Moreover, it was significantly higher in the adrenal vein on the tumor side compared to the non-tumor side within the same UPA patients. In primary cultured adrenal cells, miR-222-3p mimics appeared to increase *CYP11B2* expression, although the effect was not statistically significant. However, transfection with miR-222-3p mimics significantly increased cell proliferation, while

transfection with miR-222-3p inhibitors decreased it. *CDKN1B* was identified as a predicted target gene of miR-222-3p. Additionally, the expression level of miR-222-3p exhibited a significant positive correlation with tumor diameter and plasma aldosterone concentration after saline infusion test.

Conclusions. The present results demonstrated higher expression of circulating miR-222-3p in UPA than in BHA, providing a biochemical marker for subtype diagnosis of PA. Moreover, the correlation of miR-222-3p with adrenal cell proliferation and aldosterone secretion indicated its significant involvement in the pathogenesis of PA.

Introduction

Primary aldosteronism (PA) is one of the most common causes of secondary hypertension, although the mechanisms underlying the development and progression of PA remain largely unknown. Since excess aldosterone causes various cardiovascular and renal diseases as well as hypertension, early and exact diagnosis and treatment are indispensable (1-2). There are two major subtypes of PA: unilateral forms of primary aldosteronism (UPA), which can be pathologically classified as aldosterone-producing adenoma (APA), aldosterone-producing nodule or micronodule, aldosterone-producing diffuse hyperplasia, or aldosterone-producing adrenocortical carcinoma, and bilateral hyperaldosteronism (BHA). Surgery is the first-line treatment for UPA, while pharmacotherapy with mineralocorticoid receptor antagonist is the first-line treatment for BHA (3-4). Subtype diagnosis is essential in the clinical management of PA. Although adrenal venous sampling (AVS) is the gold standard for subtype diagnosis, it is invasive, expensive and technically difficult (5-8). Therefore, a biomarker that can differentiate between UPA and BHA before surgery would be highly valuable.

miRNAs are small, non-coding RNAs that can suppress the expression of protein-coding genes by targeting specific mRNAs (9-10). They are thought to play an important role in the pathogenesis of various diseases (11-13). Additionally, miRNAs are known to be released from cells into circulation (14), and circulating miRNAs are considered potential diagnostic

biomarkers (15).

In PA, several miRNAs have previously been reported to be differentially expressed in APA tissue compared to normal adrenal tissue, unilateral adrenal hyperplasia, or nonfunctioning adrenal adenomas, and to affect aldosterone production or cell proliferation *in vitro* (16-20). Regarding circulating miRNAs, several were reported to be differentially expressed in UPA compared with BHA (21-22). Although the sensitivity and specificity of individual miRNAs were insufficient, it has been reported that combining multiple miRNAs with machine learning algorithms can improve diagnostic performance (22). However, the significance of circulating miRNAs and their pathophysiological role in PA are not fully clarified.

In this study, we compared the profiles of circulating miRNAs between UPA and BHA using plasma samples from the adrenal veins obtained through AVS. We also explored novel miRNAs that might serve as biomarker candidates and play a role in PA pathogenesis.

Materials and Methods

Human subjects

All patients were diagnosed with primary aldosteronism with one or more positive functional confirmation tests and were diagnosed as unilateral or bilateral by AVS with ACTH stimulation.

The lateralized diagnosis was based on the AVS criteria in Japan (23). In brief, a selectivity index ≥ 5 was used as the criterion for successful catheter insertion into the right or left adrenal vein and the criteria for lateralization were as follows: UPA was diagnosed in cases meeting the criteria of "lateralized ratio (LR) > 4 " or "LR > 2 and contralateral ratio (CR) < 1 ," while BHA was diagnosed in cases with "LR < 2 ." Fourteen patients were diagnosed with UPA, and fifty-one patients were diagnosed with BHA. Among these patients, we matched age, sex, and peripheral blood aldosterone concentration to create a "discovery cohort" for comprehensive analysis and a "validation cohort" for confirmatory analysis. Adrenal tissues from patients who underwent adrenalectomy were used for primary culture experiments. The clinical characteristics of the patients used in the primary culture experiment are shown in Supplemental Table 1 (24).

Sample Collection and RNA isolation

All plasma samples were obtained during AVS, immediately frozen in liquid nitrogen, and stored at -80°C until analysis. MiRNA isolation from plasma was carried out with a miRNeasy Mini Kit (QIAGEN) following the manufacturer's instructions. The samples were thawed and centrifuged at 3000 xg for 5 min, and 200 μL of supernatant was collected. The supernatant was lysed in 750 μL of QIAzol (QIAGEN) detergent, which includes 1 μg MS2 RNA (Roche). RNA was extracted from this lysate following the manufacturer's instructions included in the

miRNeasy Mini Kit (QIAGEN). Finally, RNA was eluted in 30 µL of nuclease-free water.

miRNA profiling by quantitative PCR panel

Isolated RNA from plasma samples was used for cDNA synthesis using a miRCURY LNA RT Kit (QIAGEN). The synthesized cDNA was diluted 50-fold and utilized for qPCR panel analysis using the Human Serum/Plasma Focus miRCURY LNA miRNA Focus PCR Panels (QIAGEN), which contains 179 different miRNAs commonly found in human serum and plasma. Inter-sample normalization was performed by global normalization.

Quantitative real-time PCR validation of each miRNA expression

Reverse transcription was performed in 10 µL volume using the miRCURY LNATM RT Kit (QIAGEN). One-thirtieth of the obtained cDNA was used for individual qPCR assays using a miRCURY LNATM SYBR Green PCR kit (QIAGEN), miRCURY LNATM miRNA PCR Assays (QIAGEN) of each miRNA and Step One Plus system (Applied Biosystem). We used the following PCR assays: hsa-let-7a-5p (GeneGlobe ID: YP00203906), hsa-let-7f-5p (GeneGlobe ID: YP00204359), hsa-miR-125a-5p (GeneGlobe ID: YP00204339), hsa-miR-136-5p (GeneGlobe ID: YP00204779), hsa-miR-222-3p (GeneGlobe ID: YP00204551), hsa-miR-324-3p (GeneGlobe ID: YP00204303), hsa-miR-2110 (GeneGlobe ID: YP00204328) and hsa-miR-191-5p (GeneGlobe ID: YP00204306). We used miR-191-5p as an endogenous control to

normalize miRNA expression levels, as it was identified as having the lowest standard deviation and the most stable expression in the discovery cohort analysis by NormFinder algorithm (25).

Quantitative real-time PCR of mRNA expression

For quantitative real-time PCR (qPCR) analysis of *CYP11B2*, *CDKN1B* and *ACTB* transcripts, 500 µg of total RNA was reverse transcribed into cDNA using PrimeScript™ RT reagent Kit (TaKaRa, Kyoto, Japan) and performed on a Step One Plus system (Applied Biosystems, Foster City, CA) using Fast SYBR® Green Master Mix (Thermo Fisher Scientific, Vilnius, Lithuania). The sequences of each primer are shown in Supplemental Table 2 (26).

Cell seeding and miRNA inhibitors and miRNA mimics transfection

For the in vitro study, we used primary culture of APA tumoral cells and surrounding “normal adrenal” cells. The primary APA and “normal adrenal” cells were prepared from surgical adrenal tissues immediately after adrenalectomy. From the resected adrenal tissue, we collected APA cells from visibly tumorous areas and surrounding normal adrenal cells from regions grossly adjacent to the tumor. *CYP11B2* mRNA expression was significantly higher in tumorous cells than in normal adrenal cells (Supplemental Figure 1 (27)). They were cultured in Gibco™ DMEM/F12, Glutamax™ supplement (Fisher Scientific), with 10% fetal bovine

serum, ITS Liquid Media Supplement (Sigma-Aldrich), and 0.1 ng/ml basic fibroblast growth factor (Biovender Laboratory System).

The cells were seeded into culture plates 24 hours before transfection. Following the manufacturing protocol, transfection experiments were performed using mirVana™ (Thermo Fisher) miRNA inhibitors and miRNA mimics with Lipofectamine RNAiMAX (Invitrogen). We used mimics at a concentration of 30 nM and inhibitors at 100 nM.

Cell proliferation assay

We evaluated cell proliferation 48 and 72 hours after transfection of miRNA mimics or inhibitors using Cell Count Reagent SF (Nacalai tesque Inc, Kyoto, Japan), in accordance with the manufacturing protocol.

Statistical analysis

R 4.2.2 software was used for statistical analyses. All data are expressed as the mean for parametric variables and as the median for nonparametric variables. Student's t-test was used to compare quantitative variables. The Mann-Whitney U test was used to compare nonparametric variables. Values of $P < 0.05$ were considered to indicate significant differences. In analyzing the accuracy of unilateral PA diagnosis, the optimal receiver operating characteristic (ROC) curve cutoff point for each parameter was determined using the maximum

Youden index. The Youden index was calculated by subtracting 1 from the sum of the sensitivity and specificity. Univariate correlations were evaluated as Pearson's correlation coefficient, since each parameter was normally distributed.

Ethical statement

The study was conducted in accordance with the Declaration of Helsinki Guidelines and the guidelines for clinical studies published by the Ministry of Health and Labor, Japan. It was approved by the ethics committees of the National Hospital Organization Kyoto Medical Center and Kyoto University Graduate School and Faculty of Medicine, Ethics Committee. All participants in this study provided written informed consent for specimen collection.

Results

Comparison of circulating miRNA between UPA and BHA using adrenal vein samples

Initially, we compared 5 UPA cases with 5 BHA cases. These cases, matched based on basic clinical characteristics, comprised the "discovery cohort". The clinical characteristics of the "discovery cohort" are shown in Table 1 and Supplemental Table 3 (28). Patients were matched for age, gender, and peripheral aldosterone levels, and we restricted the "discovery cohort" to male patients only, as even menstrual cycles can alter the overall circulating miRNA profile (29). We compared adrenal venous samples from sites closest to each lesion: the tumor side in

UPA and the aldosterone-higher side in BHA. As a result, we identified 7 miRNAs that were significantly differentially expressed between the UPA tumor side (UPA-T) and the BHA aldosterone-higher side (Supplemental Figure 2 (30) and Excel data (31)). In this analysis, a p-value of <0.05 in the corresponding t-test, without correction for multiple comparisons, was considered statistically significant.

Next, we analyzed the expression of these 7 miRNAs in a “validation cohort” consisting of 9 matched cases. The clinical characteristics of the “validation cohort” are shown in Table 2 and Supplemental Table 3 (28).

Among the 7 miRNAs, miR-222-3p was also significantly elevated on the UPA-tumor side (UPA-T) and miR-136-5p showed a similar pattern as observed in the discovery cohort (Figure 1).

Evaluation of the candidate circulating miRNA using IVC samples

Then, we analyzed these two miRNAs using IVC samples from the same patients. Only miR-222-3p was significantly elevated in the UPA group compared to the BHA group (Figure 2). However, ROC analysis of miR-222-3p for differentiating UPA from BHA indicated that its sensitivity and specificity were insufficient for use as a biomarker, with the sensitivity and specificity values of 64.3 % and 78.6 %, respectively (AUC 0.735[95%CI 0.546-0.923]).

Comparison of circulating miR-222-3p between UPA-tumor side and non-tumor side

On the other hand, when we analyzed all 14 UPA cases, the expression of miR-222-3p was significantly higher on the tumor side (UPA-T) compared to the non-tumor side (UPA-nT) within the same cases (Figure 3).

Transfection of miR-222-3p mimic and miR-222-3p inhibitor to adrenal primary cultured cells

It has been reported that miR-222-3p promotes cell proliferation and inhibits apoptosis in several cell types (32-34), but its significance in adrenal cells has not been clarified yet.

We therefore analyzed the effects of miR-222-3p on primary cultured cells from human APA and adjacent non-tumor adrenocortical cells (surrounding “normal adrenal” tissue). Using miRNA mimics and inhibitors, we evaluated whether miR-222-3p affects cell proliferation or aldosterone production in adrenal primary cultured cells.

Transfection with miR-222-3p mimic resulted in a significant increase in the miRNA expression, approximately one thousand-fold, compared to the negative control in both APA cells and surrounding “normal adrenal” cells. In contrast, transfection with miRNA inhibitor reduced the miRNA expression to approximately 30–40% of the levels observed in the negative control (Figure 4).

Evaluation of the effect of miR-222-3p on *CYP11B2* expression

First, we evaluated the effect of miR-222-3p on *CYP11B2* expression. There appeared to be a tendency for miR-222-3p mimic to increase *CYP11B2* expression in both APA cells and surrounding “normal adrenal” cells, although the effect was not statistically significant. The inhibitor did not affect the expression of *CYP11B2* either in APA cells or in “normal adrenal” cells after 48-hours of transfection (Figure 5 and Supplemental Figure 3 (35)).

Evaluation of the effect of miR-222-3p on adrenal cell proliferation

Next, we evaluated cell proliferation in APA cells and surrounding “normal adrenal” cells using the WST-8 assay. In APA cells, cell proliferation significantly increased after 72 hours of transfection with miR-222-3p mimic, and decreased after 48 and 72 hours of transfection with inhibitors (Figure 6A and Supplemental Figure 4A (36)). Similarly, in surrounding “normal adrenal” cells, proliferation significantly increased after 72 hours of transfection with the mimic, and decreased after 48 and 72 hours of transfection with inhibitor (Figure 6B and Supplemental Figure 4B (36)).

Evaluation for the effect of miR-222-3p on *CDKN1B* expression

Regarding miR-222-3p's effects on cell proliferation, it has been reported that it would target *CDKN1B*, a gene which controls the cell cycle, in several types of cells (37-38). Several

different target prediction tools identify *CDKN1B* as one of the potential target mRNAs of miR-222-3p (39-40).

We then evaluated *CDKN1B* mRNA expression following transfection with miR-222-3p mimic or inhibitor in adrenal primary cultured cells. *CDKN1B* mRNA expression tended to decrease with the mimics and significantly increased with the inhibitors, in both APA cells and surrounding “normal adrenal “cells (Figure 7 and Supplemental Figure 5 (41)).

Correlation between miR-222-3p expression level and clinical parameters

To further investigate the significance of miR-222-3p in the pathogenesis of PA, we examined its correlation with clinical parameters in UPA cases. The expression level of miR-222-3p in the adrenal vein on the tumor side of UPA cases showed a significant positive correlation with tumor diameter (Figure 8A). Additionally, while miR-222-3p expression in the tumor-side adrenal vein did not correlate with baseline plasma aldosterone concentration (PAC) or PAC in adrenal venous blood (data not shown), it showed a significant positive correlation with PAC after saline infusion test (Figure 8B).

On the other hand, when we compared miR-222-3p expression levels between BHA patients with tumors (6 cases) and those without tumors (8 cases), no significant difference in miR-222-3p expression levels was observed between the two groups (Supplemental Figure 6 (42)). Based on these findings, we think that miR-222-3p may not be merely a marker of adrenal

tumor.

Discussion

In the present study, we found that circulating miR-222-3p is more highly expressed in UPA patients than in BHA patients and that this miRNA has a cell-promoting effect on adrenal primary cultured cells.

Previous studies on miRNAs in APA tissue have shown that the expression of miR-10b, miR-24, miR-203, and miR-193a-3p are significantly decreased in APA tissue compared to adjacent adrenal tissues (16, 18, 19). Similarly, the expression of miR-375 has been significantly decreased in APA tissue compared to unilateral hyperplasia (17), and the expression of miR-34a and miR-370 has been significantly decreased in APA tissue compared to nonfunctioning adrenal adenomas (20). Regarding circulating miRNAs, miR-30e-5p, miR-30d-5p, and miR-7-5p were significantly lower in APA compared to bilateral hyperplasia (21). Additionally, miR-24-3p, miR-99b-5p, miR-130b-3p, miR-146a-5p, miR-151a-3p and miR-199a-3p were significantly lower in UPA compared to bilateral hyperplasia (22).

In the current study, miR-99b-5p and miR-199a-3p showed a trend toward lower expression in UPA compared to BHA, consistent with a previous report using adrenal vein samples (Since the difference was not statistically significant in the comprehensive analysis, we did not

proceed to validation for these miRNAs). On the other hand, no obvious differences were observed for miR-7-5p, miR-10b, miR-24, miR-30e-5p, miR-30d-5p, miR-34a, miR-130b-3p, miR-146a-5p, miR-151a-3p, miR-193a-3p and miR-375 in the comprehensive analysis. miR-203 and miR-370 were not examined in the current study, as they are not commonly found in human serum or plasma and were not included in the qPCR panel used for the initial comprehensive analysis.

There are several possible reasons for these differences. First, differences in the presence or absence of somatic gene mutations in APA may have affected miRNA expression. Nakano et al. reported that miR-299 levels were lower in APA with *KCNJ5* mutation than in APA without it (20). However, we were unable to examine APA somatic mutations in our study because surgical specimens from UPA cases used in the discovery and validation cohorts were not archived. Second, patient background factors such as gender and race may have differed between the present and previous studies, influencing miRNA expression (43-44). In this study, the discovery cohort consisted entirely of males, whereas in the previous study, half of the discovery cohort was female. Additionally, this study exclusively involves Japanese participants, while the previous study was conducted in Europe, where the proportion of Asians is considered relatively small. Third, differences in the platforms used to comprehensively miRNA analysis (qPCR, microarray, or NGS) may also account for the variation in results (45).

One of the strengths of this study is the finding that miR-222-3p expression was higher in the adrenal vein on the tumor side compared to the non-tumor side within the same UPA cases. This observation suggests that miR-222-3p may be associated with cell proliferation in the pathogenesis of PA. Several studies have shown that miR-222-3p can promote cell proliferation in various cell types (32-34). However, to our knowledge, no studies have investigated the significance of miR-222-3p in PA or other adrenal diseases.

Increased aldosterone production and dysregulated adrenocortical cell growth are thought to play a role in the etiology of PA. Although pathogenic germline and somatic variants leading to excess aldosterone production have been well identified (46), the mechanisms driving cell proliferation and tumor formation remain unclear (47). Several miRNAs previously implicated in PA have been suggested to contribute to pathogenesis by affecting aldosterone synthase expression and/or cell proliferation. For example, miR-24 has been reported to reduce *CYP11B2* expression and aldosterone secretion (16), while miR-375 has been shown to inhibit cell proliferation (17). Additionally, miR-203 and miR-193a-3p have been reported to decrease cell proliferation and aldosterone hypersecretion (18-19).

In the current study, miR-222-3p tended to increase *CYP11B2* expression in adrenal primary cultured cells and significantly promoted cell proliferation in these cells. Additionally, the expression level of miR-222-3p showed a significant positive correlation with tumor diameter

and with aldosterone concentration after saline infusion test, which is considered a good indicator of autonomous aldosterone secretion. These results suggest that miR-222-3p may contribute to the pathogenesis of PA primarily by promoting cell proliferation and increasing the number of pathological cells, and consequently enhancing autonomous aldosterone secretion. This finding may provide insights into some of the mechanisms underlying PA development.

miRNAs are thought to be involved in the pathogenesis of many diseases by binding to mRNAs of target genes based on sequence complementarity and suppressing their expression (48). Multiple prediction tools have identified *CDKN1B* as a likely target gene for miR-222-3p (39-40). *CDKN1B* regulates the G1 phase of the cell cycle, slowing or arresting cell division (49), and is known to be the causative gene for a subtype of multiple endocrine neoplasia that can also produce adrenal tumors (50-51). Several studies have reported that miR-222-3p may promote cell proliferation by downregulating *CDKN1B* expression in various cell types (37-38). In the current study, we found that *CDKN1B* expression may also be downregulated by miR-222-3p in adrenal cells. However, this finding is based solely on mRNA analysis, which is insufficient to confirm the effect. Further studies, including reporter assays and protein-level analyses, are needed to validate this phenomenon.

miRNAs are thought to be released from various cell types into the extracellular space, where they can be taken up by neighboring or distant cells and subsequently modulate recipient cell function (52). Some circulating miRNAs have been reported to play important roles in cancer progression and cardiovascular diseases (53-54). There are several possible explanations for the finding that miR-222-3p is more upregulated in the adrenal venous blood of UPA patients. One possibility is that miR-222-3p produced in other tissues is transported to the adrenal gland, where it promotes adrenal cell proliferation. Alternatively, tumorigenic adrenal cells or surrounding adrenal tissue may secrete miR-222-3p. To gain further insight, we need to investigate the absolute expression level and specific localization of miR-222-3p in APA adrenal tissue using *in-situ* hybridization or other techniques.

In this study, miR-222-3p expression was significantly higher in the UPA group than in the BHA group, even at IVC levels (Figure 2), but the sensitivity and specificity were insufficient for it to serve as a stand-alone biomarker for differentiating UPA from BHA. However, it may be possible to use miR-222-3p for the differential diagnosis when combined with other clinical indicators, such as serum potassium levels, adrenal imaging findings, or patient gender. Further analysis is needed.

Our study has several limitations. First, the possibility of micro-APA in cases with BHA cannot

be ruled out, which may have resulted in false negatives when comparing miRNA expression between the UPA and BHA groups, potentially obscuring the detection of meaningful miRNAs. Second, the number of cases in this study was relatively small. Due to the limited sample size in the discovery cohort, some differentially expressed miRNAs between the two groups may not have been detected as candidate miRNAs. Moreover, the small sample size in the validation cohort may have prevented the full exclusion of confounding factors, such as comorbidities. Further studies are needed to verify whether similar results can be obtained in a larger, more general population. Third, the miRNA qPCR panel used in this study contained only 179 miRNAs commonly found in human serum and plasma. Therefore, we did not analyze all currently known miRNAs. Fourth, surgical specimens from UPA cases in the discovery and validation cohorts were not archived, so we were unable to examine the presence of known somatic gene mutations (e.g., *KCNJ5*) in UPA cases. Fifth, although it is necessary to analyze whether miR-222-3p acts via *CDKN1B* in adrenal cells at the protein level, the available quantity of primary cultured cells was insufficient for this analysis.

Conclusions

Circulating miR-222-3p was more highly expressed in UPA patients than in BHA patients in the adrenal and peripheral veins. Furthermore, we demonstrated a significant correlation between miR-222-3p levels and adrenal cell proliferation as well as autonomous aldosterone

secretion, suggesting its substantial involvement in the pathogenesis of PA.

Acknowledgments

We thank Maki Kashiwabara, Keiko Umegaki and Yoshie Fukuchi for their technical and secretarial assistance.

Financial support: This study was supported by research grants from the Japan Agency for Medical Research and Development (AMED) under grant numbers JP17ek0109122 (JPAS) and JP20ek0109352 (JRAS), and the National Center for Global Health and Medicine, Japan (27-1402, 30-1008). This study was also supported by grants from the Japan Smoking Foundation and the Japan Foundation for Applied Enzymology.

Author Contributions: KO, MS, NI, and MN were involved in study concept and design. KO, DT and YO were involved in data interpretation and analysis. KO, MS, DT, IY, YU, TH, NI and MN discussed the result. MT and MM provided technical and material support. DY, NI and MN supervised the findings of this work. All authors reviewed the report, commented on drafts of the manuscript, and approved the final report.

Correspondence and Reprint Requests: Masakatsu Sone, MD, PhD, Department of Metabolism and Endocrinology, St. Marianna University School of Medicine, 2-16-1, Sugao, Miyamae-ku, Kawasaki, Japan. E-mail: sonemasa@marianna-u.ac.jp

Disclosure statement: NI received joint research grants from Terumo, and received speaker

honoraria from Nippon Boehringer Ingelheim, Kyowa Kirin, Sanofi, Sumitomo Pharma, and Mitsubishi Tanabe Pharma, and received scholarship grants from Daiichi-Sankyo, Mitsubishi Tanabe, Takeda, Kyowa Kirin, Sumitomo Pharma, Nippon Boehringer Ingelheim, and Kowa. MS and NI were members of the Board of Directors of the Japan Endocrine Society. Other authors have nothing to disclose.

Data availability statement: Some or all datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

References

1. Ohno Y, Sone M, Inagaki N, Yamasaki T, Ogawa O, Takeda Y, Kurihara I, Itoh H, Umakoshi H, Tsuiki M, Ichijo T, Katabami T, Tanaka Y, Wada N, Shibayama Y, Yoshimoto T, Ogawa Y, Kawashima J, Takahashi K, Fujita M, Watanabe M, Matsuda Y, Kobayashi H, Shibata H, Kamemura K, Otsuki M, Fujii Y, Yamamoto K, Ogo A, Okamura S, Miyauchi S, Fukuoka T, Izawa S, Yoneda T, Hashimoto S, Yanase T, Suzuki T, Kawamura T, Tabara Y, Matsuda F, Naruse M. Prevalence of Cardiovascular Disease and Its Risk Factors in Primary Aldosteronism: A Multicenter Study in Japan. *Hypertension*. 2018;**71**(3):530-537.
2. Kawashima A, Sone M, Inagaki N, Takeda Y, Itoh H, Kurihara I, Umakoshi H, Ichijo T,

430 Katabami T, Wada N, Ogawa Y, Kawashima J, Fujita M, Miyauchi S, Okamura S, Fukuoka
 431 T, Yanase T, Izawa S, Yoshikawa Y, Hashimoto S, Yamada M, Kai T, Suzuki T, Naruse M.
 432 Renal impairment is closely associated with plasma aldosterone concentration in patients
 433 with primary aldosteronism. *Eur J Endocrinol.* 2019;**181**(3):339-350.

434 3. Hundemer GL, Curhan GC, Yozamp N, Wang M, Vaidya A. Incidence of Atrial Fibrillation
 435 and Mineralocorticoid Receptor Activity in Patients With Medically and Surgically
 436 Treated Primary Aldosteronism. *JAMA Cardiol.* 2018;**3**(8):768-774.

437 4. Chen YY, Lin YH, Huang WC, Chueh E, Chen L, Yang SY, Lin PC, Lin LY, Lin YH, Wu
 438 VC, Chu TS, Wu KD. Adrenalectomy Improves the Long-Term Risk of End-Stage Renal
 439 Disease and Mortality of Primary Aldosteronism. *J Endocr Soc.* 2019;**3**(6):1110-1126.

440 5. Rossi GP, Barisa M, Allolio B, Auchus RJ, Amar L, Cohen D, Degenhart C, Deinum J,
 441 Fischer E, Gordon R, Kickuth R, Kline G, Lacroix A, Magill S, Miotto D, Naruse M,
 442 Nishikawa T, Omura M, Pimenta E, Plouin PF, Quinkler M, Reincke M, Rossi E, Rump
 443 LC, Satoh F, Schultze Kool L, Seccia TM, Stowasser M, Tanabe A, Trerotola S, Vonend
 444 O, Widimsky J, Jr., Wu KD, Wu VC, Pessina AC. The Adrenal Vein Sampling International
 445 Study (AVIS) for identifying the major subtypes of primary aldosteronism. *J Clin*
 446 *Endocrinol Metab.* 2012;**97**(5):1606-1614.

447 6. Lubitz CC, Economopoulos KP, Sy S, Johanson C, Kunzel HE, Reincke M, Gazelle GS,
 448 Weinstein MC, Gaziano TA. Cost-Effectiveness of Screening for Primary Aldosteronism

- 449 and Subtype Diagnosis in the Resistant Hypertensive Patients. *Circ Cardiovasc Qual*
450 *Outcomes*. 2015;**8**(6):621-630.
- 451 7. Monticone S, Satoh F, Dietz AS, Goupil R, Lang K, Pizzolo F, Gordon RD, Morimoto R,
452 Reincke M, Stowasser M, Mulatero P. Clinical Management and Outcomes of Adrenal
453 Hemorrhage Following Adrenal Vein Sampling in Primary Aldosteronism. *Hypertension*.
454 2016;**67**(1):146-152.
- 455 8. Fuss CT, Treitl M, Rayes N, Podrabsky P, Fenske WK, Heinrich DA, Reincke M, Petersen
456 TO, Fassnach M, Quinkler M, Kickuth R, Hahner S. Radiation exposure of adrenal vein
457 sampling: a German Multicenter Study. *Eur J Endocrinol*. 2018;**179**(4):261-267.
- 458 9. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*.
459 2004;**116**(2):281-297.
- 460 10. Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell*.
461 2009;**136**(2):215-233.
- 462 11. Croce CM. Causes and consequences of microRNA dysregulation in cancer. *Nat Rev*
463 *Genet*. 2009;**10**(10):704-714.
- 464 12. Morozova N, Zinovyev A, Nonne N, Pritchard LL, Gorban AN, Harel-Bellan A. Kinetic
465 signatures of microRNA modes of action. *Rna*. 2012;**18**(9):1635-1655.
- 466 13. Callegari E, Gramantieri L, Domenicali M, D'Abundo L, Sabbioni S, Negrini M.
467 MicroRNAs in liver cancer: a model for investigating pathogenesis and novel therapeutic

- 468 approaches. *Cell Death Differ*. 2015;**22**(1):46-57.
- 469 14. Turchinovich A, Weiz L, Burwinkel B. Extracellular miRNAs: the mystery of their origin
470 and function. *Trends Biochem Sci*. 2012;**37**(11):460-465.
- 471 15. Ho PTB, Clark IM, Le LTT. MicroRNA-Based Diagnosis and Therapy. *Int J Mol Sci*.
472 2022;**23**(13).
- 473 16. Robertson S, MacKenzie SM, Alvarez-Madrazo S, Diver LA, Lin J, Stewart PM, Fraser
474 R, Connell JM, Davies E. MicroRNA-24 is a novel regulator of aldosterone and cortisol
475 production in the human adrenal cortex. *Hypertension*. 2013;**62**(3):572-578.
- 476 17. He J, Cao Y, Su T, Jiang Y, Jiang L, Zhou W, Zhang C, Wang W, Ning G. Downregulation
477 of miR-375 in aldosterone-producing adenomas promotes tumour cell growth via MTDH.
478 *Clin Endocrinol (Oxf)*. 2015;**83**(4):581-589.
- 479 18. Peng KY, Chang HM, Lin YF, Chan CK, Chang CH, Chueh SJ, Yang SY, Huang KH, Lin
480 YH, Wu VC, Wu KD. miRNA-203 Modulates Aldosterone Levels and Cell Proliferation
481 by Targeting Wnt5a in Aldosterone-Producing Adenomas. *J Clin Endocrinol Metab*.
482 2018;**103**(10):3737-3747.
- 483 19. Zhang G, Zou X, Liu Q, Xie T, Huang R, Kang H, Lai C, Zhu J. MiR-193a-3p functions
484 as a tumour suppressor in human aldosterone-producing adrenocortical adenoma by down-
485 regulating CYP11B2. *Int J Exp Pathol*. 2018;**99**(2):77-86.
- 486 20. Nakano Y, Yoshimoto T, Watanabe R, Murakami M, Fukuda T, Saito K, Fujii Y, Akashi T,

- 487 Tanaka T, Yamada T, Naruse M, Ogawa Y. miRNA299 involvement in CYP11B2
488 expression in aldosterone-producing adenoma. *Eur J Endocrinol.* 2019;**181**(1):69-78.
- 489 21. Decmann A, Nyíró G, Darvasi O, Turai P, Bancos I, Kaur RJ, Pezzani R, Iacobone M,
490 Kraljevic I, Kastelan D, Parasiliti-Caprino M, Maccario M, Nirschl N, Heinrich D,
491 Reincke M, Patócs A, Igaz P. Circulating miRNA Expression Profiling in Primary
492 Aldosteronism. *Front Endocrinol (Lausanne).* 2019;**10**:739.
- 493 22. Vékony B, Nyirő G, Herold Z, Fekete J, Ceccato F, Gruber S, Kürzinger L, Parasiliti-
494 Caprino M, Bioletto F, Szücs N, Doros A, Szeredás BK, Syed Mohammed Nazri SK, Fell
495 V, Bassiony M, Dank M, Azizan EA, Bancos I, Beuschlein F, Igaz P. Circulating miRNAs
496 and Machine Learning for Lateralizing Primary Aldosteronism. *Hypertension.*
497 2024;124.23418
- 498 23. Naruse M, Katabami T, Shibata H, Sone M, Takahashi K, Tanabe A, Izawa S, Ichijo T,
499 Otsuki M, Omura M, Ogawa Y, Oki Y, Kurihara I, Kobayashi H, Sakamoto R, Satoh F,
500 Takeda Y, Tanaka T, Tamura K, Tsuiki M, Hashimoto S, Hasegawa T, Yoshimoto T,
501 Yoneda T, Yamamoto K, Rakugi H, Wada N, Saiki A, Ohno Y, Haze T. Japan Endocrine
502 Society clinical practice guideline for the diagnosis and management of primary
503 aldosteronism 2021. *Endocr J.* 2022;**69**(4):327-359.
- 504 24. Okamoto K. Data from: Kyoto University Research Information Repository. Deposited
505 26 March 2025. <https://doi.org/10.57723/kds587687>

- 506 25. Andersen CL, Jensen JL, Ørntoft TF. Normalization of real-time quantitative reverse
507 transcription-PCR data: a model-based variance estimation approach to identify genes
508 suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res.*
509 2004;64(15):5245-5250.
- 510 26. Okamoto K. Data from: Kyoto University Research Information Repository. Deposited
511 26 March 2025. <https://doi.org/10.57723/kds587688>
- 512 27. Okamoto K. Data from: Kyoto University Research Information Repository. Deposited
513 26 March 2025. <https://doi.org/10.57723/kds587681>
- 514 28. Okamoto K. Data from: Kyoto University Research Information Repository. Deposited
515 26 March 2025. <https://doi.org/10.57723/kds587689>
- 516 29. Rekker K, Saare M, Roost AM, Salumets A, Peters M. Circulating microRNA Profile
517 throughout the menstrual cycle. *PLoS One*. 2013;**8**(11):e81166.
- 518 30. Okamoto K. Data from: Kyoto University Research Information Repository. Deposited
519 26 March 2025. <https://doi.org/10.57723/kds587682>
- 520 31. Okamoto K. Data from: Kyoto University Research Information Repository. Deposited
521 26 March 2025. <https://doi.org/10.57723/kds587690>
- 522 32. Liu B, Che Q, Qiu H, Bao W, Chen X, Lu W, Li B, Wan X. Elevated MiR-222-3p
523 promotes proliferation and invasion of endometrial carcinoma via targeting ER α . *PLoS*
524 *One*. 2014;**9**(1):e87563.

- 525 33. Zhang C, Chang Q, Hu Y, Chang W, Guo X, Fu L, Tang G, Chen C. MiR-222-3p
526 promotes the proliferation, migration and invasion of papillary thyroid carcinoma cells
527 through targeting SLC4A4. *Histol Histopathol.* 2021;**36**(11):1199-1207.
- 528 34. Wang W, Kong P, Feng K, Liu C, Gong X, Sun T, Duan X, Sang Y, Jiang Y, Li X, Zhang
529 L, Tao Z, Liu W. Exosomal miR-222-3p contributes to castration-resistant prostate
530 cancer by activating mTOR signaling. *Cancer Sci.* 2023;**114**(11):4252-4269.
- 531 35. Okamoto K. Data from: Kyoto University Research Information Repository. Deposited
532 26 March 2025. <https://doi.org/10.57723/kds587683>
- 533 36. Okamoto K. Data from: Kyoto University Research Information Repository. Deposited
534 26 March 2025. <https://doi.org/10.57723/kds587684>
- 535 37. Yang K, Chen Y, Cui Z, Chen H, Yang L, Zhao J, Luo F, Man S, Zhao J, Jin L.
536 MicroRNA-222-3p participates in the development of oral squamous cell carcinoma by
537 targeting CDKN1B. *J Oral Pathol Med.* 2020;**49**(7):621-629.
- 538 38. Wang Z, Zhu H, Shi H, Zhao H, Gao R, Weng X, Liu R, Li X, Zou Y, Hu K, Sun A, Ge J.
539 Exosomes derived from M1 macrophages aggravate neointimal hyperplasia following
540 carotid artery injuries in mice through miR-222/CDKN1B/CDKN1C pathway. *Cell*
541 *Death Dis.* 2019;**10**(6):422.
- 542 39. TargetScanHuman 8.0 (<https://www.targetscan.org>), McGeary, S. E., et al. (2019). "The
543 biochemical basis of microRNA targeting efficacy." *Science* 366(6472).

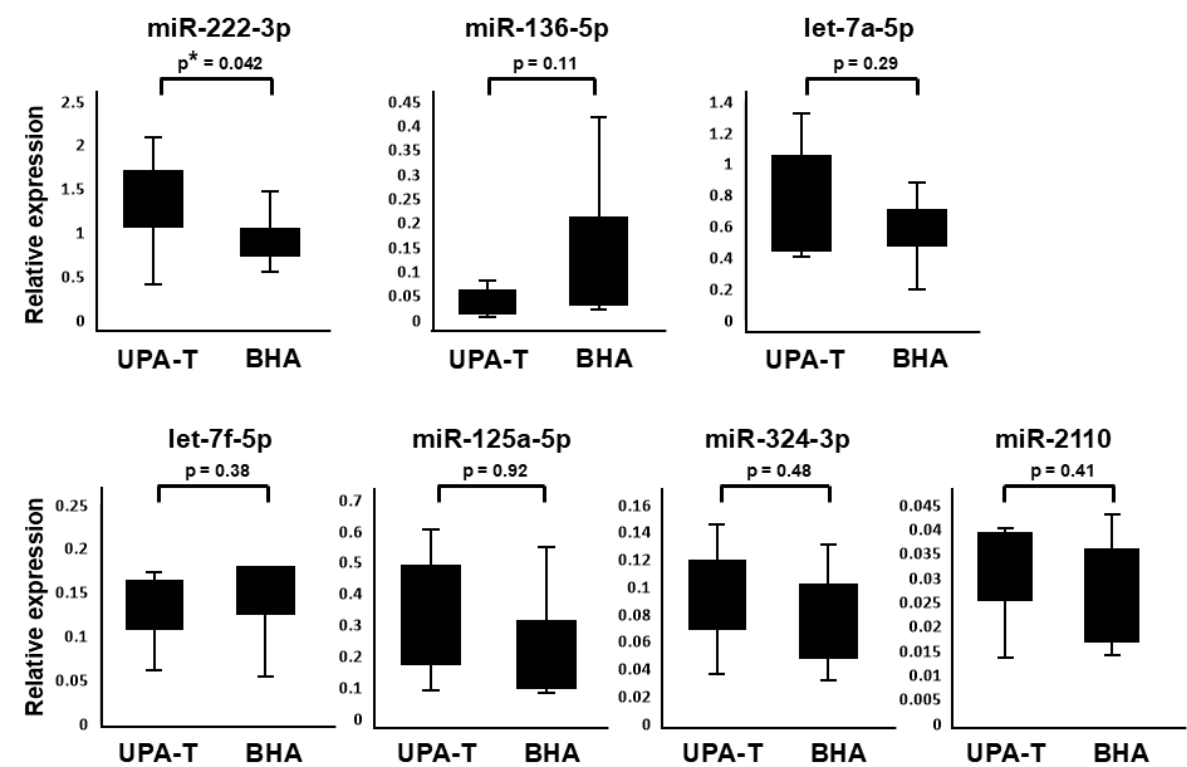
- 544 40. miRDB (<https://mirdb.org>), Chen, Y. and X. Wang (2020). "miRDB: an online database
545 for prediction of functional microRNA targets." *Nucleic Acids Res* 48(D1): D127-d131.
- 546 41. Okamoto K. Data from: Kyoto University Research Information Repository. Deposited
547 26 March 2025. <https://doi.org/10.57723/kds587685>
- 548 42. Okamoto K. Data from: Kyoto University Research Information Repository. Deposited
549 26 March 2025. <https://doi.org/10.57723/kds587686>
- 550 43. Noren Hooten N, Fitzpatrick M, Wood WH, 3rd, De S, Ejiogu N, Zhang Y, Mattison JA,
551 Becker KG, Zonderman AB, Evans MK. Age-related changes in microRNA levels in
552 serum. *Aging (Albany NY)*. 2013;**5**(10):725-740.
- 553 44. Alimena S, Stephenson BJK, Webber JW, Wollborn L, Sussman CB, Packard DG,
554 Williams M, Comrie CE, Wang JY, Markert T, Spiegel J, Rodriguez CB, Lightfoot M,
555 Graye A, O'Connor S, Elias KM. Differences in Serum miRNA Profiles by Race,
556 Ethnicity, and Socioeconomic Status: Implications for Developing an Equitable Ovarian
557 Cancer Screening Test. *Cancer Prev Res (Phila)*. 2024;**17**(4):177-185.
- 558 45. Git A, Dvinge H, Salmon-Divon M, Osborne M, Kutter C, Hadfield J, Bertone P, Caldas
559 C. Systematic comparison of microarray profiling, real-time PCR, and next-generation
560 sequencing technologies for measuring differential microRNA expression. *Rna*.
561 2010;**16**(5):991-1006.
- 562 46. Scholl UI. Genetics of Primary Aldosteronism. *Hypertension*. 2022;**79**(5):887-897.

- 563 47. Tetti M, Gong S, Veglio F, Reincke M, Williams TA. Primary aldosteronism:
564 Pathophysiological mechanisms of cell death and proliferation. *Front Endocrinol*
565 *(Lausanne)*. 2022;**13**:934326.
- 566 48. Valinezhad Orang A, Safaralizadeh R, Kazemzadeh-Bavili M. Mechanisms of miRNA-
567 Mediated Gene Regulation from Common Downregulation to mRNA-Specific
568 Upregulation. *Int J Genomics*. 2014;**2014**:970607.
- 569 49. Hengst L, Dulic V, Slingerland JM, Lees E, Reed SI. A cell cycle-regulated inhibitor of
570 cyclin-dependent kinases. *Proc Natl Acad Sci U S A*. 1994;**91**(12):5291-5295.
- 571 50. Agarwal SK, Mateo CM, Marx SJ. Rare germline mutations in cyclin-dependent kinase
572 inhibitor genes in multiple endocrine neoplasia type 1 and related states. *J Clin*
573 *Endocrinol Metab*. 2009;**94**(5):1826-1834.
- 574 51. Chevalier B, Coppin L, Romanet P, Cuny T, Maïza JC, Abeillon J, Forestier J, Walter T,
575 Gilly O, Le Bras M, Smati S, Nunes ML, Geslot A, Grunenwald S, Mouly C, Arnault G,
576 Wagner K, Koumakis E, Cortet-Rudelli C, Merlen É, Jannin A, Espiard S, Morange I,
577 Baudin É, Cavaille M, Tauveron I, Teissier MP, Borson-Chazot F, Mirebeau-Prunier D,
578 Savagner F, Pasmant É, Giraud S, Vantyghem MC, Goudet P, Barlier A, Cardot-Bauters
579 C, Odou MF. Beyond MEN1, When to Think About MEN4? Retrospective Study on
580 5600 Patients in the French Population and Literature Review. *J Clin Endocrinol Metab*.
581 2024;**109**(7):e1482-e1493.

52. Zhang J, Li S, Li L, Li M, Guo C, Yao J, Mi S. Exosome and exosomal microRNA: trafficking, sorting, and function. *Genomics Proteomics Bioinformatics*. 2015;**13**(1):17-24.
53. Sun Z, Shi K, Yang S, Liu J, Zhou Q, Wang G, Song J, Li Z, Zhang Z, Yuan W. Effect of exosomal miRNA on cancer biology and clinical applications. *Mol Cancer*. 2018;**17**(1):147.
54. Aghabozorgi AS, Ahangari N, Eftekhaari TE, Torbati PN, Bahiraei A, Ebrahimi R, Pashar A. Circulating exosomal miRNAs in cardiovascular disease pathogenesis: New emerging hopes. *J Cell Physiol*. 2019;**234**(12):21796-21809.

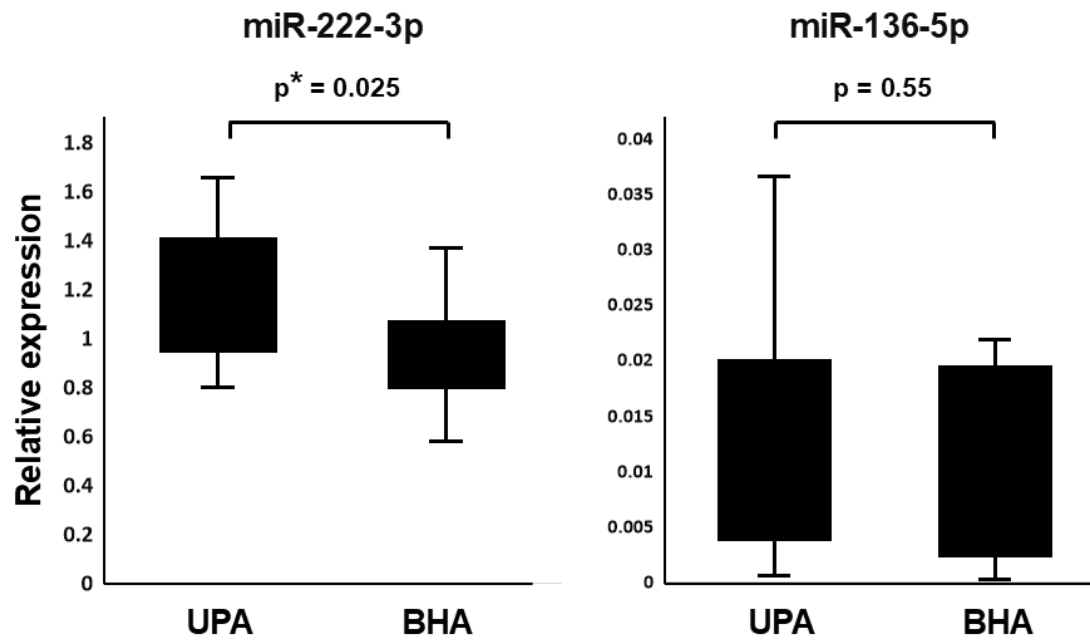
Figure Titles and Figure Legends

Figure 1. Relative expression levels of the 7 miRNAs that were significantly differentially expressed in the discovery cohort were examined in the validation cohort.



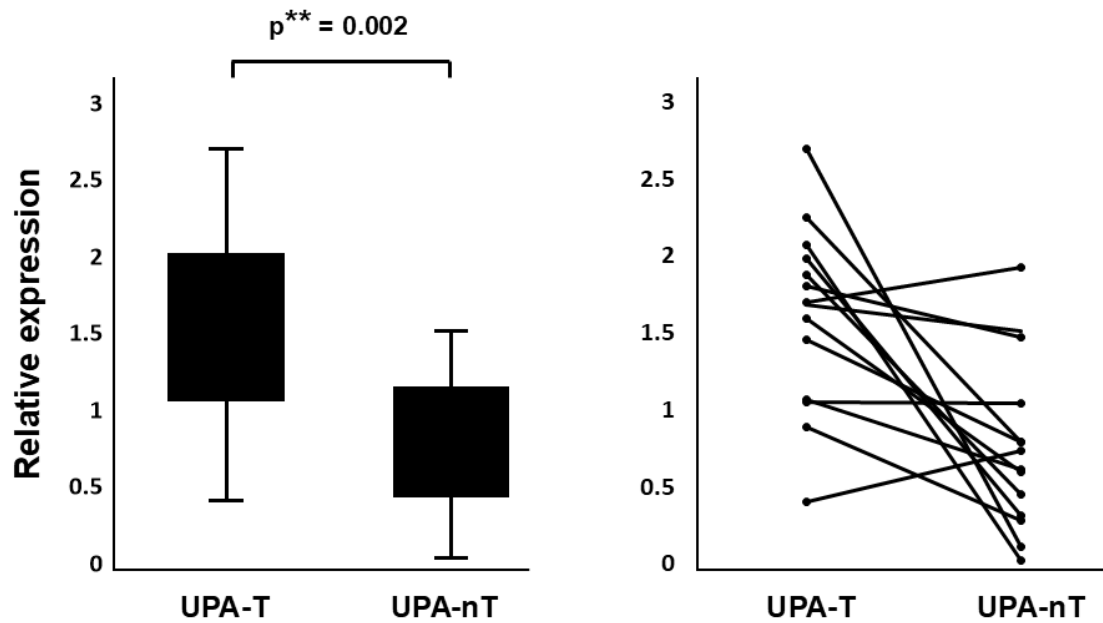
Relative expression levels of each miRNA were quantified by qPCR. miR-191-5p was used as internal control. Data are presented as the mean $\pm$ SEM from nine patients. Asterisks (*) indicate significant differences ($p < 0.05$).

Figure 2. Relative expression levels of miR-222-3p and miR-136-5p in IVC samples.



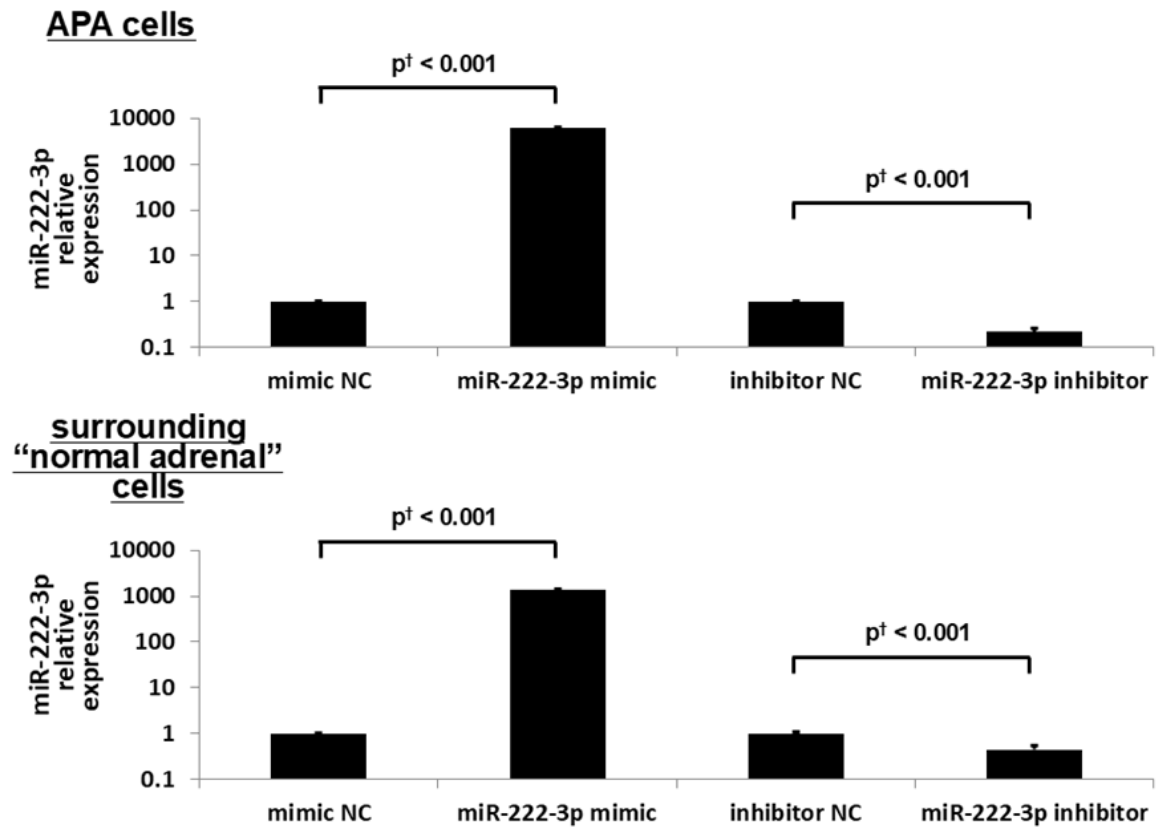
Relative expression levels of each miRNA were quantified by qPCR. miR-191-5p was used as internal control. Data are presented as the mean $\pm$ SEM from fourteen patients. Asterisks (*) indicate significant differences ($p < 0.05$).

Figure 3. The comparison of the miR-222-3p expression in UPA tumor side and in non-tumor side within the same patient.



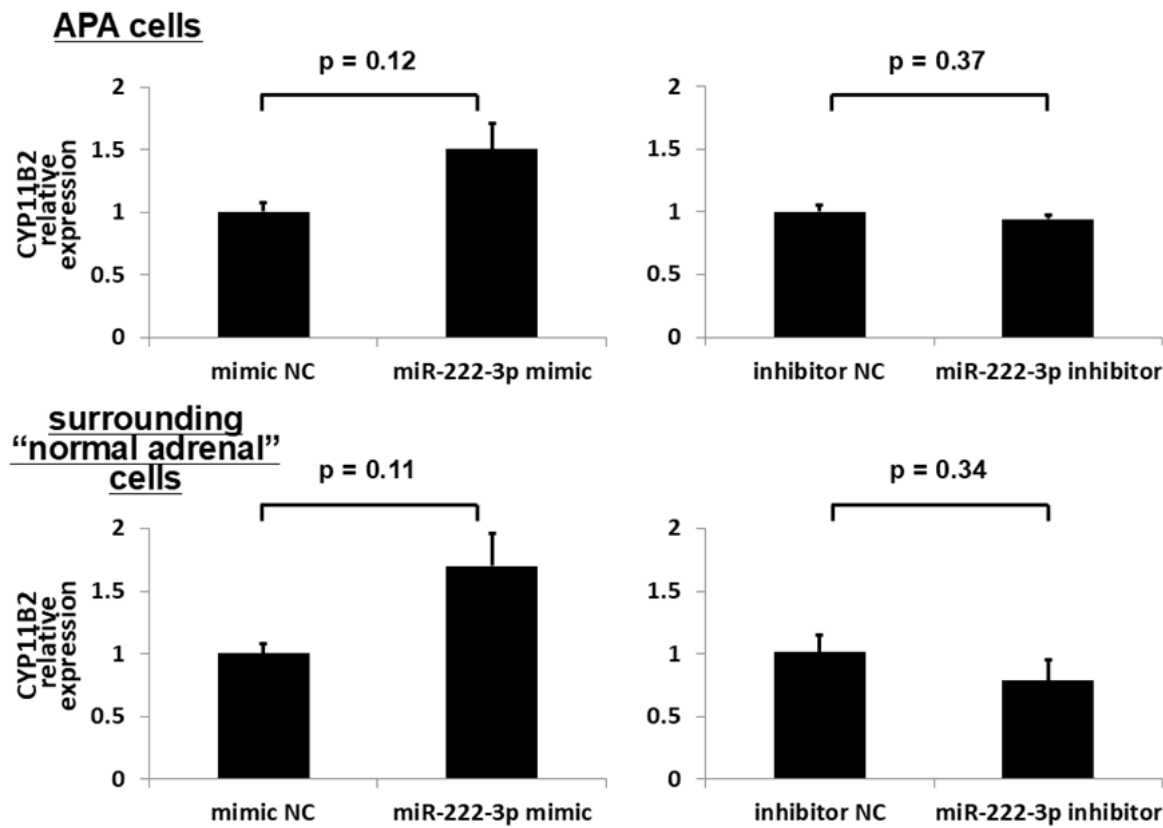
Relative expression level of miR-222-3p was quantified by qPCR. miR-191-5p was used as internal control. Data are presented as the mean $\pm$ SEM from fourteen patients. Double asterisks (**) indicate significant differences ($p < 0.01$).

Figure 4. The changes of miR-222-3p expression by miRNA mimic or inhibitor.



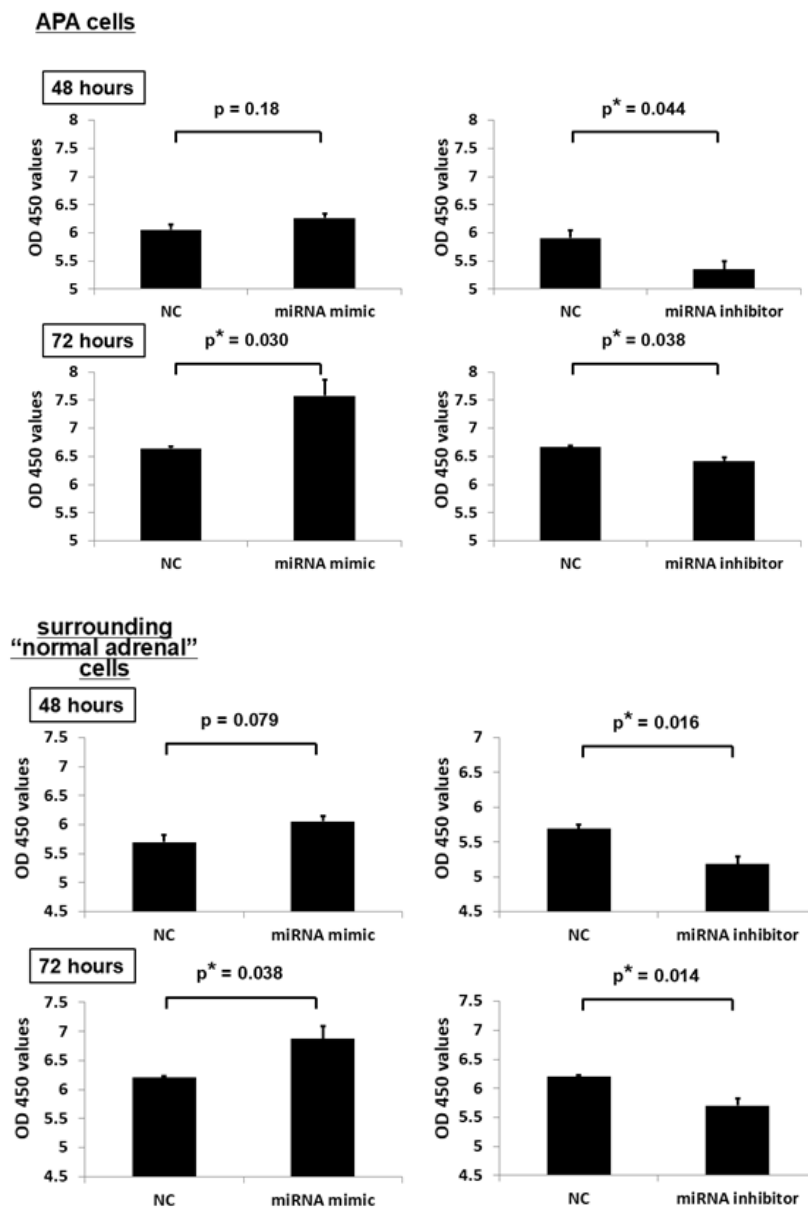
Relative expression level of miR-222-3p mRNA was quantified by qPCR. Data are presented as the mean $\pm$ SEM from three wells. Daggers ($\dagger$) indicate significant differences ($p < 0.001$ vs each NC).

Figure 5. The changes in *CYP11B2* expression induced by miR-222-3p mimic or inhibitor in Patient T1.



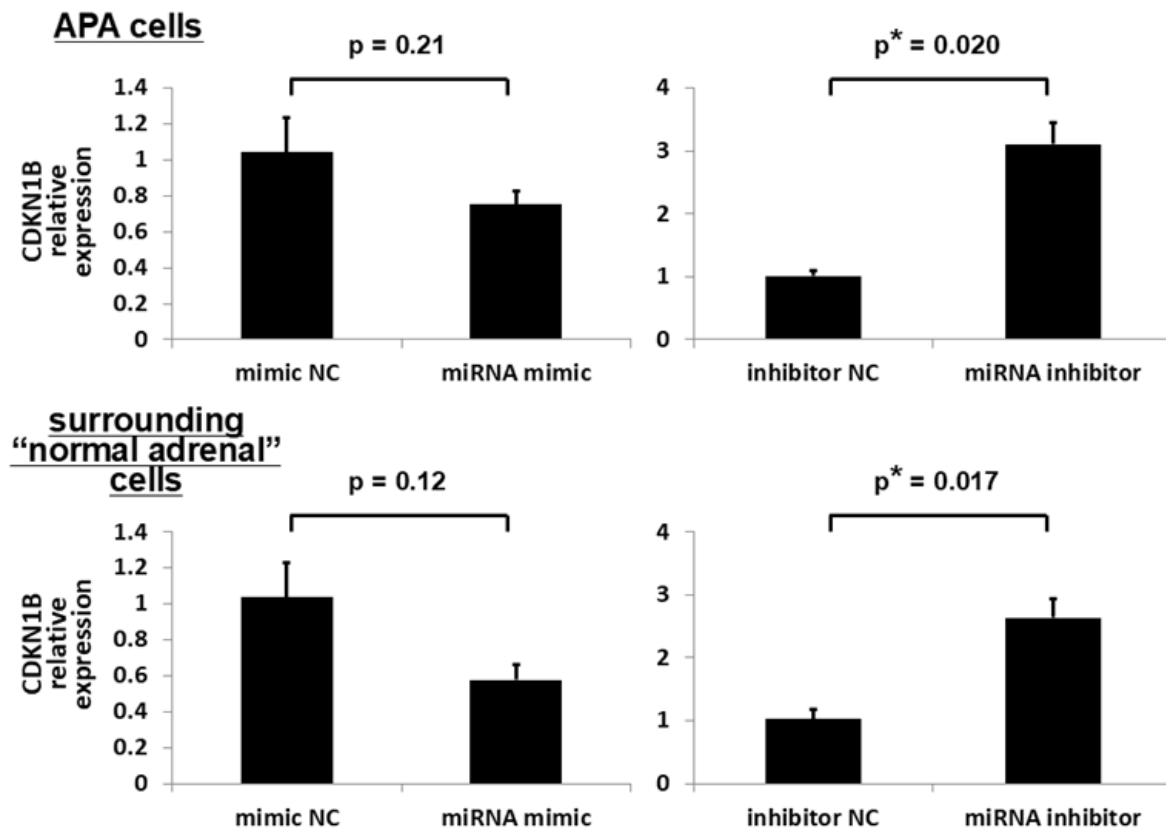
Relative expression level of *CYP11B2* mRNA was quantified by qPCR. *ACTB* mRNA was used as internal control. The transfection experiment was performed once per patient and qPCR analysis was conducted three times. Data are presented as the mean $\pm$ SEM from three wells.

Figure 6. The changes in cell viability induced by miR-222-3p mimic or inhibitor in Patient T1. (A) APA cells. (B) surrounding “normal adrenal” cells.



OD = optical densities at 450nm were measured. The transfection experiment was performed once per patient and cell viability analysis was conducted three times. Data are presented as the mean $\pm$ SEM from three wells. Asterisks (*) indicate significant differences ($p < 0.05$ vs each NC).

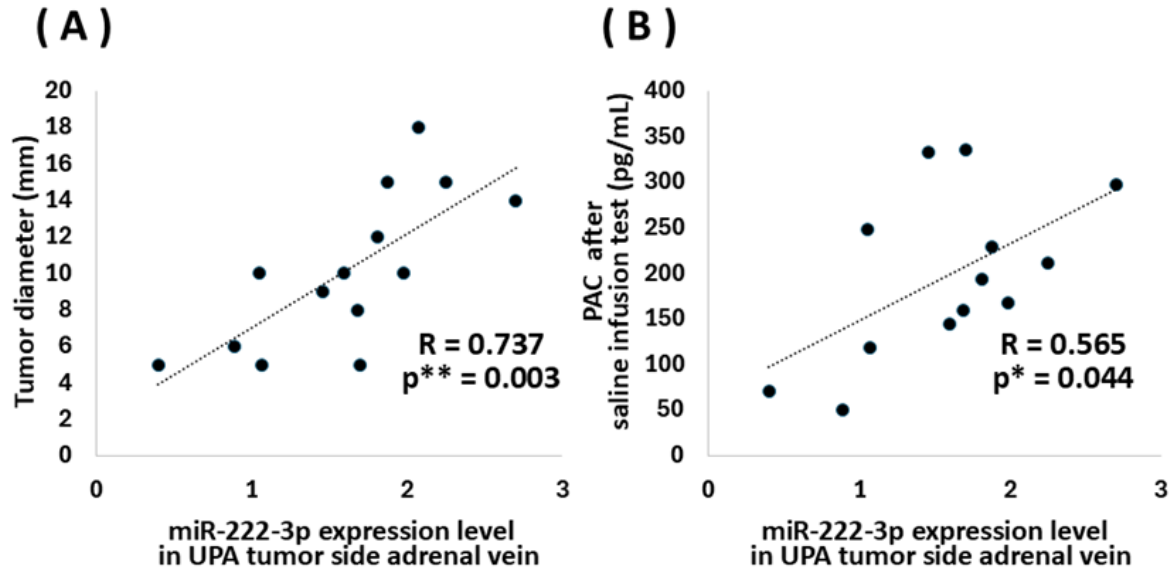
Figure 7. The changes in *CDKN1B* expression induced by miR-222-3p mimic or inhibitor in Patient T1.



Relative expression level of *CDKN1B* mRNA was quantified by qPCR. *ACTB* mRNA was used as internal control. The transfection experiment was performed once per patient and qPCR analysis was conducted three times. Data are presented as the mean $\pm$ SEM from three wells. Asterisks (*) indicate significant differences ($p < 0.05$ vs NC).

Figure 8. (A) Correlation between miR-222-3p expression and tumor diameter (B)

Correlation between miR-222-3p expression and PAC after saline infusion test.



Univariate correlations were evaluated as Pearson's correlation coefficient. R indicates the correlation coefficient. Asterisks (*) and double asterisks (**) indicate significant differences ($p < 0.05$ and $p < 0.01$, respectively).

690 **Table 1.** The clinical characteristics of the “discovery cohort”.

	Group	age (years)	gender	BMI (kg/m ²)	Serum K ⁺ (mmol/L)	PRA (µg/L/h)	PAC (pmol/L)
D1	UPA	48	male	21.3	4.1	0.4	488.224
D2	UPA	65	male	21.6	3.6	0.2	662.986
D3	UPA	44	male	31.0	4.6	0.3	632.472
D4	UPA	60	male	22.7	3.3	0.8	1037.476
D5	UPA	63	male	24.2	3.6	0.1	826.652
mean		56.0		24.2	3.8	0.4	729.562
D6	BHA	53	male	27.5	4.1	0.5	804.46
D7	BHA	48	male	23.3	4.5	0.2	460.484
D8	BHA	66	male	28.5	3.1	0.9	1004.188
D9	BHA	58	male	26.8	3.9	0.6	693.5
D10	BHA	38	male	28.4	3.9	0.2	435.518
mean		52.6		26.8	3.9	0.5	679.63
p value		0.605	1.00	0.223	0.857	0.521	0.734

691 Student’s t test was used to compare parametric variables. Pearson’s χ^2 test was used for

692 categorical parameters.

693 BMI, body mass index; PRA, plasma renin activity and PAC, plasma aldosterone

694 concentration.

695

696 **Table 2.** The clinical characteristics of the “validation cohort”.

	Group	age (years)	gender	BMI (kg/m ²)	Serum K+ (mmol/L)	PRA (µg/L/h)	PAC (pmol/L)
V1	UPA	64	female	17.9	3.6	0.2	685.178
V2	UPA	56	male	25.6	3.1	0.2	405.004
V3	UPA	64	female	30.3	3.0	0.3	851.618
V4	UPA	39	male	31.3	3.4	0.1	490.998
V5	UPA	56	female	26.9	3.9	0.5	438.292
V6	UPA	54	female	30.4	3.6	0.1	527.06
V7	UPA	63	male	26.3	3.9	0.9	868.262
V8	UPA	60	male	23.2	3.0	0.8	1215.012
V9	UPA	44	female	20.3	3.4	0.3	621.376
mean or median		55.6		25.8	3.4	0.3	678.089
V10	BHA	53	female	20.1	3.9	0.1	416.1
V11	BHA	68	female	23.3	4.1	0.2	729.562
V12	BHA	58	male	24.7	3.7	0.1	443.84
V13	BHA	56	female	27.1	3.7	0.8	549.252
V14	BHA	57	female	21.3	4.0	0.3	429.97
V15	BHA	42	male	28.3	3.1	0.8	463.258
V16	BHA	62	female	20.9	5.3	0.1	496.546
V17	BHA	52	male	26.2	4.0	0.6	374.49
V18	BHA	51	male	25.8	4.1	0.4	291.27
mean or median		55.4		24.2	4.0	0.3	466.032
p value		0.977	1.00	0.392	0.011*	0.928	0.063

697 Student’s t test was used to compare parametric variables. The Mann-Whitney U test was
698 used to compare nonparametric variables. Pearson’s χ^2 test was used for categorical
699 parameters. Asterisks (*) indicate significant differences (p <0.05).

700

701