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micro-RNA screening and prediction model construction for diagnosis of salt-sensitive essential hypertension

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Abstract

Commonly used tests for diagnosis of salt-sensitive hypertension (SSH) are complex and time-consuming, so new methods are required. Many studies have demonstrated roles for miRNAs in hypertension; however, the diagnostic value of miRNAs has yet to be determined for human SSH. In this study, we examined miRNA expression profiles by initial high-throughput miRNA sequencing of samples from patients with salt-sensitive and salt-resistant hypertension (SSH and SRH, respectively; n = 6, both groups), followed by validation by quantitative real-time polymerase chain reaction (qRT-PCR) in a larger cohort (n = 91). We also evaluated differences in baseline characteristics (e.g., age, sex, body mass index, consumption of specific foods) between the SSH and SRH groups. Of 36 miRNAs identified as differentially expressed between SSH and SRH groups by RNA-Seq, 8 were analyzed by qRT-PCR. There were significant differences in the expression levels of hsa-miR-361-5p and hsa-miR-362-5p between the 2 groups ($P = .023$ and.049, respectively). In addition, there were significant differences in sauce and poultry consumption between the 2 groups ($P = .004$ and.001, respectively). The areas under the curve (AUC) determined by receptor operating characteristic (ROC) analysis for hsa-miR-361–5p and all 8 miRNAs were 0.793 (95% CI, 0.698–0.888; sensitivity = 73.9%, specificity = 74.4%; $P < .001$) and 0.836 (95% CI, 0.749–0.922; sensitivity = 80.4%, specificity = 81.4%; $P < .001$), respectively, when sauce and poultry consumption were included in the models. Assay feasibility and economic considerations make hsa-miR-361-5p combined with the dietary factors the preferred markers for diagnosis of SSH.

Abbreviations: Ang II = angiotensin II, AUC = area under the curve, BMI = body mass index, DBP = diastolic blood pressure, GIR = glucose infusion rate, GLU = glucose, GO = gene ontology, HDL = high-density lipoprotein, KEGG = kyoto encyclopedia of genes and genomes, LDL = low-density lipoprotein, MAP = mean arterial pressure, MMPs = metalloproteinases, NS = normal saline, OR = odds ratio, qRT-PCR = quantitative real-time polymerase chain reaction, RAAS or RAS = renin–angiotensin–aldosterone system, RNA-seq = RNA sequencing technology, ROC = receptor operating characteristic curve, SBP = systolic blood pressure, SNP = single nucleotide polymorphism, SRH = salt-resistant hypertension, SSH = salt-sensitive hypertension, TC = total cholesterol, TG = triglyceride, WHR = waist–hip ratio.

Keywords: dietary factors, miRNAs, ROC curve, salt-sensitive hypertension

1. Introduction

Salt-sensitive hypertension (SSH), an independent risk factor for cardiovascular disease,[1] has received much attention since the positive association between salt and blood pressure was proved by the famous Intersalt study.[2] The definition of SSH, an increase in blood pressure in response to high dietary salt intake, was first proposed by Kawasaki et al[3] in 1978. Then, Sullivan summarized the different criteria for salt sensitivity and gave a unified method to distinguish salt-sensitive from salt-resistant individuals.[4] The incidence of salt sensitivity is approximately 15% to 42% and 28% to 74% among normotensive and hypertensive individuals, respectively.[5] The development of epigenetics and precision medicine provides great opportunities to study the underlying genetic mechanisms of SSH. A small number of allelic variants of genes are associated with SSH and these may have important roles in the heterogeneity of blood pressure responses to salt.[6] As SSH is regarded as an intermediate inheritance phenotype of essential hypertension, understanding the pathogenesis of SSH can contribute to clarification of the pathogenic mechanisms underlying essential hypertension. Acute or chronic oral normal saline (NS) loading, diet NS-loading, and Sullivan tests are commonly used to diagnose SSH.[7] However, all of these tests involve complex procedures, which can be time-consuming; therefore, it is necessary to develop a flexible and noninvasive method for the diagnosis of SSH.

MicroRNAs (miRNAs) are endogenous noncoding RNAs of 16 to 22 nucleotides, belonging to the family of small RNAs.[8] MiRNAs regulate gene expression at the posttranscriptional level through their interactions with the 3'untranslated regions of

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target mRNAs.[9] The known functions of miRNAs include control of the developmental timing of cells,[10] regulation of signal transduction pathways,[11] effecting apoptosis and metabolism,[12] and playing essential roles in embryonic development.[13] Cardiovascular diseases, such as hypertension and coronary disease, are areas of intense research. The miRNA, rno-miR-132, may play a role in cardiac fibrosis and hypertension in response to treatment with angiotensin II (Ang II) for 24 hours. Metalloproteinases (MMP) 9 is a target gene for rno-miR-132; therefore, this miRNA may participate in cardiac fibrosis and hypertension through targeting matrix *MMPs* and *SPRY1*.[14] Similarly, hsa-miR-145 has significantly higher expression levels in atherosclerotic plaques from hypertensive patients, compared with controls.[15] Hsa-miR-155[16] as well as hsa-miR-122[17,18] also associate with the development and pathologic progress of hypertension. In addition, hsa-miR-21 was proved to be sufficient to reduce blood pressure and suggested a theoretical ground for the miRNA-based therapeutics against hypertension.[19] Although the function of miRNAs in the development of hypertension has been validated by many researches, the role of miRNAs in SSH has never been reported before, which restricted by the methodology of the diagnosis of SSH and the examination of miRNAs. RNA Sequencing technology (RNA-Seq) achieves base-pair-level resolution, has a much higher dynamic range of expression levels than other methods of transcriptome analysis, and is capable of de novo annotation; this technique has, therefore, been widely used to reveal the complex landscape of the transcriptome such as miRNAs.[20] The RNA-seq gives us an opportunity to study the miRNA expression in SSH.

Environment factors are also the important risk factors for SSH, except the genetic factors. As the fact that high salt consumption widely exists in China, the influence of SSH is more severe than other countries. In China, especially the north China, people have the habit to consume the pickled and salted food such as sauce, a kind of flavoring that contains high salt. The habit of having salt is related to the fluctuation of blood pressure, thus we considered the frequent of relevant food consumption could be a significant environmental factor for the incidence of SSH.

In this study, we applied high-throughput miRNA sequencing technology to determine candidate differentially expressed miRNAs with the aim of identifying biomarkers useful for the diagnosis of SSH. First, we used RNA-Seq to investigate miRNA expression levels in salt-sensitive (SS) and salt-resistant (SR) individuals. Next, we selected differently expressing miRNAs for further investigation. Finally, we tested the model that constructed with the miRNAs expression level and the dietary factors using a larger sample size to assess the accuracy of the identified target miRNAs as biomarkers for SSH by qRT-PCR and drew receptor operating characteristic (ROC) curves to evaluate the accuracy of the logistic model constructed using miRNA expression levels and specific environmental factors.

2. Methods

2.1. Participants

This study was an extension of a previous study of SSH[21] as well as a part of EpiSS (System Epidemiology Study on Salt Sensitivity of Blood Pressure) study, which has registered in WHO International Clinical Trials Registry Platform (No: ChiCTR-EOC-16009980). The total sample size of EpiSS is 1806 so far. The present study recruited 6 paired hypertensive patients to present the RNA-Seq, and 50 paired to do the qRT-PCR. Participant was recruited from 3 communities of Beijing from April 2014 to February 2015 (Jin Zhan and Guan Zhuang communities in Chao Yang district and Lu Gu community in Shi Jingshan district) through the assistance of the Community health service centers. All the participants joined the study voluntarily and willingly. Hypertensive patients of Han race and both genders, living in Beijing for more than 5 years, 35 to 70 years of age, with stage 1 essential hypertension were recruited to participate in this study at 3 community sites. Patients were excluded from participation if they had normal blood pressure or stage 2 or 3 hypertension, older than 70 years of age, pregnant women, took antihypertensive drugs in the morning of the saline test and the patients who had severe coronary heart disease, heart failure, stroke, peripheral arterial disease, myocardiopathy, valvular heart disease, congenital heart disease, acute myocardial infarction, severe liver and kidney disease, and cancer. The SSH and salt-resistant hypertensive (SRH) patients were further paired according to their age, gender, and the diagnosis results of saline-loading test. Data collection was planned before the index test.

Twenty-four-hour urine and time-point urine electrolyte concentrations were used to test sodium excretion. Demographic data, the frequency of food consumption, and physical and biochemical measurements were collected by standard questionnaire (combined the questionnaire of China chronic disease risk factors monitoring in 2013 and China food frequency questionnaire) and physical and blood biochemistry examinations, respectively. Blood samples were used to measure total cholesterol (TC), triglyceride (TG), high-density lipoprotein (HDL), low-density lipoprotein (LDL), plasma glucose (GLU), and the miRNAs expression level by qRT-PCR. Weight, height, waist circumference, and hip circumference were used to calculate body mass index (BMI) and waist–hip ratio (WHR). The collection of blood was conducted by professional nurses from the community health centers on the third day after the saline-loading test. This study was approved by the Ethical Committee of Capital Medical University, in compliance with the Declaration of Helsinki. All participants have signed informed consent before the study began.

2.2. Measurement of blood pressure

We used the definition of hypertension from the 2010 Chinese guidelines for management of hypertension.[22] Blood pressure (BP) was measured in the same arm using a mercury sphygmomanometer with an 22 cm length and 12 cm width cuff by trained persons. Before being measured, patients should rest in the seated position for 5 minutes. Blood pressure was measured twice, with an interval of at least 1 to 2 minutes between each measurement. If the results of twice measurement differed greatly, the third measurement was needed. Pulse rate was measured simultaneously.

2.3. Diagnosis of salt-sensitive hypertension

A modified Sullivan acute NS-loading test was used to distinguish SRH and SSH patients, and mean arterial pressure (MAP) was calculated with the systolic blood pressure (SBP) and diastolic blood pressure (DBP) for each time point.[23] Blood pressure was measured on 3 time points: baseline BP, 2 hours after sodium-loading, and 2 hours after diuresis reduction (Fig. 1). Three MAP values (MAP_1, MAP_2, and MAP_3) were calculated from the corresponding 3 BP values. MAP was considered to be the DBP

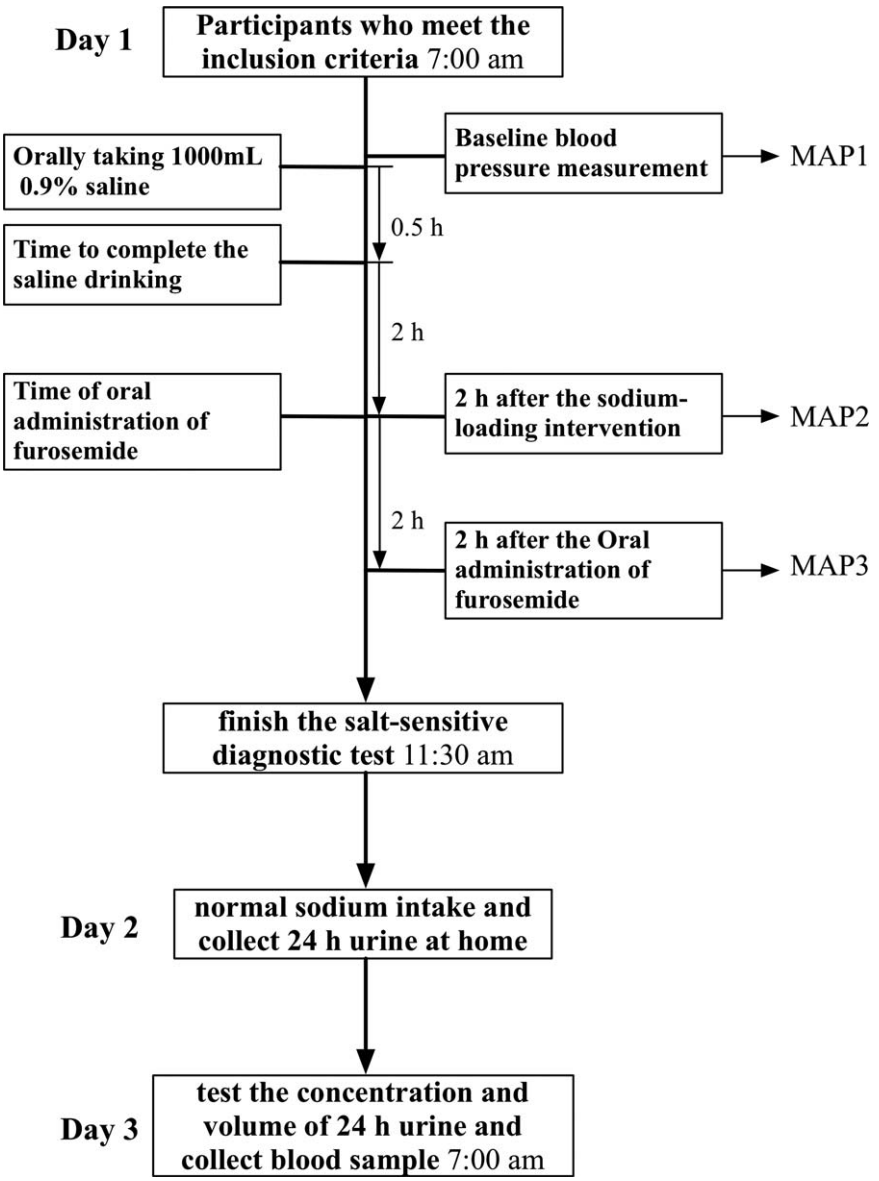

Figure 1. The process of the modified Sullivan acute NS-loading test and the collection of urine and blood sample. MAP_1, MAP_2, MAP_3 were calculated from the baseline blood pressure, the blood pressure of 2 h after the sodium-loading intervention and 2 h after the oral administration of furosemide, respectively. MAP = mean arterial pressure, NS = normal saline.

plus one-third of pulse pressure. Patients with $MAP_2 - MAP_1 \geq 5$ mm Hg or $MAP_3 - MAP_2 \leq -10$ mm Hg were diagnosed with SSH, and others were diagnosed with SRH. This diagnosis method was widely used to distinguish the SSH from SRH and was validated to have the same accuracy with the long period sodium-loading test.[24]

2.4. Total RNA extraction

Whole blood samples containing ethylenediaminetetraacetic acid (300 μL) in 1.5 mL Eppendorf tubes were thoroughly mixed with 900 μL TRI Reagent (Invitrogen, Carlsbad, CA) and incubated for 5 minutes at room temperature. Next, they were thoroughly mixed with 0.2 mL chloroform, using a QT-1 Whirlpool Mixer (Shqite, Shanghai) and stood at room temperature for 2 minutes. Mixtures were subsequently centrifuged at 12,000 *g* for 15 minutes at 4°C. The aqueous phase was carefully transferred into a 1.5 mL Eppendorf tube, mixed with isopropanol and left to stand for 10 minutes at room temperature, followed by centrifugation at 12,000 *g* for 10 minutes at 4°C, and discarding of the aqueous phase. Next, 75% ethanol was added to the sample, the centrifugation step repeated and the aqueous phase discarded, followed by the addition of 15 μL DEPC water and incubation at 65°C for 3 to 5 minutes to dissolve the RNA pellet. Finally, RNA samples were stored at −80°C for further processing.

2.5. MiRNA sequencing

Total RNA samples were reverse transcribed into cDNA and a Truseq DNA Sample Preparation Kit (Illumina, San Diego, CA) was used to construct a cDNA library for next-generation sequencing. The specific steps of library construction included shearing of the cDNA fragments, repair of paired-ends, addition of an "A-tail" to the 3'end of the cDNA fragments, ligation of

Table 1

Differential read counts between samples from SRH and SSH patients for eight miRNAs.

miRNAs	Read counts		*P* value	
	SRH (n = 6)	SSH (n = 6)	DESeq2	EdgeR
hsa-miR-423-5p	10113.33	6880.28	.031	.040
hsa-miR-15b-5p	3502.00	5059.35	.032	.040
hsa-miR-210-3p	666.00	450.49	.003	.015
hsa-miR-362-5p	269.50	424.23	.020	.023
hsa-miR-19a-3p	258.50	155.42	.041	.038
hsa-miR-26b-3p	154.33	108.85	.025	.037
hsa-miR-361-5p	97.00	173.26	.006	.006
hsa-miR-382-5p	12.67	7.96	.039	.028

R packages, DESeq2, and edgeR were used to analyze the differences between miRNA read counts in the 2 groups. $P < .05$ was considered statistically significant.
SRH = salt-resistant hypertension, SSH = salt-sensitive hypertension.

adaptors, and size selection. The resulting cDNA fragments were cleaned using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). The library was then amplified by PCR. An Agilent Custom SureSelect Enrichment Kit (Agilent Inc) was used to enrich exome target regions by hybridization of the library followed by hybrid capture. The samples were then sequenced using an Illumina Hiseq 2000 system (Illumina, San Diego, CA). Original image data were converted into sequence data by base calling with FastQC (version 0.11.3) to evaluate the quality of reads. The high-throughput data of this study was submitted to Sequence Reads Archive of NCBI. The accession number of our experiment is SRR3739704.

2.6. Fluorescent qRT-PCR

For the validation of candidate miRNAs identified by RNA-Seq, qRT-PCR was performed using the SYBR Green PCR method (Geneskies, Shanghai, China). A multi-RT stem-loop technology invented by Shanghai Geneskies corporation was chosen to carry out reverse transcription with a miR-Real Quant Kit (Geneskies). SnU6 was used as a stable endogenous control. Threshold cycle values (Ct values) were determined from amplification curves. The $2^{-\Delta Ct}$ method was used to calculate relative quantitative expression ($\Delta Ct = Ct_{miRNA} - Ct_{U6}$). Eight candidate miRNAs selected from RNA-Seq were analyzed by qRT-PCR: hsa-miR-15b-5p, hsa-miR-19a-3p, hsa-miR-382-5p, hsa-miR-26b-3p, hsa-miR-362-5p, hsa-miR-423-5p, hsa-miR-210-3p, and hsa-miR-361-5p. The readers of the qRT-PCR were not blind to the results of the other test, such as the biochemical test and physical examination.

2.7. Statistical analyses

Relative qualitative datasets are presented as means ± standard deviation (SD), and differential expression levels between the SSH and SRH groups were analyzed using an independent 2-sample *t* test with SPSS 19.0 software. Non-normally distributed data were analyzed using a Wilcoxon rank sum test. Qualitative variables were compared using Pearson χ^2 or Fisher exact tests. An unconditional logistic regression model was used to select diagnostic miRNA biomarkers, adjusted for age, gender, BMI, and 2 diet factors. Odds ratios (OR) represent the risk of developing SSH. The discriminant analysis included Mahal distant and Jackknife distant analyses using MATLAB software. The R packages, DEseq2 and EdgeR, were used to identify the different expressed miRNAs between the SSH and SRH groups ($P < .05$). GraphPad Prism 6 (GraphPad software, San Diego, CA) was used to draw charts. We applied Omicsbean (http://www.omicsbean.com) to perform target gene prediction and pathway analysis.

3. Results

3.1. High-throughput miRNA sequence analysis

RNA-Seq analysis of 12 essential hypertension patients (n = 6 each, SSH and SRH groups) identified a total of 1250 known and 570 novel miRNAs. Analysis with DEseq2 and EdgeR identified 36 miRNAs with significantly different expression levels. These 36 miRNAs were then screened with the following 4 criteria: differential expression ratio > 2 ($\log_2 FC > 1$) or < -2 ($\log_2 FC > -1$); expression levels in SSH or SRH of more than 5 copies; miRNAs related to pathways involved in hypertension pathology, as determined by literature analysis; and the miRNA that had single nucleotide polymorphism (SNP) in the seed region of it. Eight miRNAs that met these 4 criteria were finally selected for expression verification in a larger sample by qRT-PCR. These 8 miRNAs were hsa-miR-15b-5p, hsa-miR-19a-3p, hsa-miR-210-3p, hsa-miR-26b-3p, hsa-miR-361-5p, hsa-miR-362-5p, hsa-miR-382-5p, and hsa-miR-423-5p (Table 1).

Hsa-miR-15b-5p, hsa-miR-362-5p, and hsa-miR-361-5p were found to be upregulated in SSH compared with SRH patients, while the other 5 miRNAs were downregulated.

3.2. Baseline characteristics of the patients included in the validation stage

The original sample size was 100; after performing outlier detection, including discriminant analysis and principal component analysis, 9 abnormal samples were excluded, and the final sample size was 91. There were 46 and 45 patients in the SSH and SRH groups, respectively. Table 2 shows the baseline characteristics of the study participants. There were no adverse events happened in the process of saline-loading test and the collection of biological fluid sample. The average concentration of Na^+ at 24 hours was 100.13 mmol/L in SRH and 91.67 mmol/L in SSH. The average concentration of K^+ at 24 hours was 27.94 mmol/L in SRH and 26.75 mmol/L in SSH. In addition, the average urinary volume at 24 hours was 2057.14 mL for the 2 groups. There was no statistically significant difference in gender, age, BMI, blood biomedical indices, family history of hypertension, or urinary results between the SSH and SRH groups ($P > .05$). However, the groups did demonstrate significant differences in the reported frequencies of consumption of sauce (e.g., preserved bean curd, yellow soybean paste) and poultry ($P = .004$ and.001, respectively).

Table 2

Baseline characteristics of study participants in the salt-resistant and salt-sensitive groups.

Variables	Total	SRH	SSH	*P* value
Number, %	91 (100.0)	45 (49.5)	46 (50.5)	–
Gender, male, %	45 (49.5)	23 (51.1)	22 (48.9)	.835‡
Age, y	60.90 ± 5.59	60.78 ± 5.76	60.95 ± 5.53	.981†
BMI, kg/m^2	27.82 ± 3.44	28.03 ± 3.80	27.62 ± 3.08	.570*
WHR	0.91 ± 0.06	0.91 ± 0.06	0.91 ± 0.06	.847*
TC, mmol/L	4.81 ± 1.09	4.84 ± 0.94	4.78 ± 1.23	.829‡
TG, mmol/L	1.67 ± 1.38	1.77 ± 1.42	1.58 ± 1.36	.590†
HDL, mmol/L	1.34 ± 0.28	1.37 ± 0.32	1.31 ± 0.24	.263*
LDL, mmol/L	2.94 ± 0.89	2.99 ± 0.82	2.89 ± 0.96	.600*
GLU, mmol/L	6.23 ± 1.76	6.33 ± 1.98	6.12 ± 1.53	.821†
Family history of hypertension, no., %				.319‡
Yes	69 (75.8)	37 (82.2)	32 (71.1)	
No	21 (23.1)	8 (17.8)	13 (28.9)	
Sauce, no., %				.004*
<125 g/mo	57 (62.6)	35 (77.8)	22 (47.8)	
125–250 g/mo	21 (23.1)	5 (11.1)	16 (34.8)	
>250 g/mo	12 (13.2)	4 (8.9)	8 (17.4)	
Poultry, no., %				.001‡
Every day	13 (14.3)	2 (4.4)	11 (23.9)	
4–6 times/wk	6 (6.6)	2 (4.4)	4 (8.7)	
1–3 times/wk	45 (49.5)	22 (48.9)	23 (50.0)	
1–3 times/mo	20 (22.0)	14 (31.1)	6 (13.0)	
Never	7 (7.7)	5 (11.1)	2 (4.3)	
24 h UCNa, mmol/L	95.86 ± 49.13	100.13 ± 54.92	91.67 ± 42.92	.533†
24 h UCK, mmol/L	27.34 ± 11.90	27.94 ± 11.56	26.75 ± 12.32	.471*
24 h urine volume, mL	2057.14 ± 876.37	2133.33 ± 915.77	1982.61 ± 839.39	.441†

24 h UCK = the concentration of 24 h urinary K^+, 24 h UCNa = the concentration of 24 h urinary Na^+, BMI = body mass index, GLU = glucose, HC = hip circumference, HDL = high-density lipoprotein, LDL = low-density lipoprotein, SRH = salt-resistant hypertension, SSH = salt-sensitive hypertension, TC = total cholesterol, TG = triglyceride, WC = waist circumference, WHR = waist-hip ratio.

* Statistical testing by independent-samples *t* test.

† Wilcoxon rank sum test.

‡ χ^2 test; $P < .05$ was considered statistically significant.

3.3. Hsa-miR-361-5p and hsa-miR-362-5p were differently expressed between salt-sensitive and salt-resistant patients

We performed qRT-PCR to test the relative expression levels of the 8 candidate miRNAs identified by RNA-Seq. Among the 8 miRNAs, hsa-miR-19a-3p, hsa-miR-210a-3p, and hsa-miR-382–5p were upregulated with fold changes of 1.10, 1.14, and 1.14, respectively. The other 5 miRNAs were downregulated in SSH compared with SRH patients. Furthermore, the only statistically significant differences identified at the validation stage among the 8 miRNAs were in expression of hsa-miR-361-5p and hsa-miR-362-5p; these miRNAs were downregulated by approximately both 0.86 ($P = .023$, $P = .049$, respectively). Figure 2 illustrates the relative expression levels of hsa-miR-361-5p and hsa-miR-362-5p in the SSH and SRH groups.

3.4. Establishing the diagnostic miRNAs model using logistic regression

The associations of the relative expression levels of the 8 candidate miRNAs with SSH were assessed using stepwise logistic regression adjusted for age, sex, BMI, baseline MAP, and eating sauce and poultry. Ultimately, hsa-miR-361-5p was the only miRNA that significantly associated with the risk of SSH: OR (95% CI), 0.091 (0.014–0.595); $P = .012$. In addition, dietary factors, including sauce and poultry consumption, were also included in the final model. The OR for sauce consumption

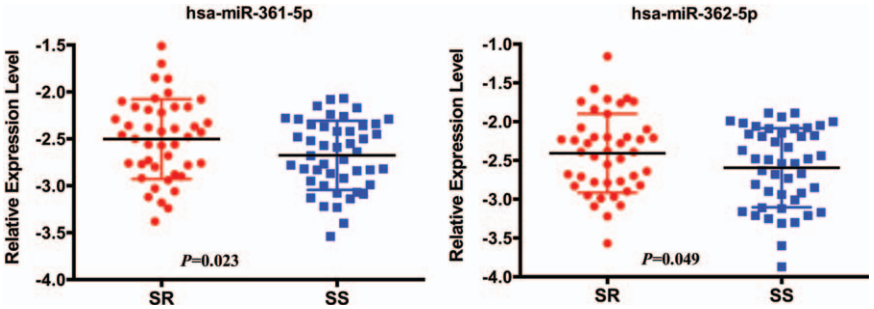

Figure 2. Relative expression levels of hsa-miR-361-5p and hsa-miR-362-5p. Red, salt-resistant; blue, salt-sensitive. *P* values were .023 and .049, respectively.

Table 3

Stepwise regression analysis between miRNAs and salt-sensitive hypertension.

Variables	β	Wald	*P* value	OR	95% CI	
					Lower	Upper
Constant	1.670	2.739	.098	5.314	–	–
Hsa-miR-361-5p	−2.395	6.265	.012	0.091	0.014	0.595
Frequent of sauce consumption	0.948	5.963	.015	2.580	1.206	5.522
Frequent of poultry consumption	−0.707	7.496	.006	0.493	0.297	0.818

Statistical testing by unconditional logistic regression, $P < .05$ was considered statistically significant.

was 2.580 (95% CI, 1.206–5.522; $P = .015$) and the OR for poultry consumption was 0.493 (95% CI, 0.297–0.818; $P = .006$). The final diagnostic model was logit (P = SSH) = 1.67 to $2.395 \times$ miR-361-5p + $0.948 \times$ sauce $-0.707 \times$ poultry (Table 3).

3.5. The diagnostic performance for the establishing logistic model evaluated by ROC analysis

Predicted probabilities were used to construct ROC curves. The AUC of hsa-miR-361-5p was 0.793, when dietary factors were included in the model (95% CI, 0.698–0.888; sensitivity = 73.9%; specificity = 74.4%; $P < .001$). The AUC of all 8 miRNAs alone was 0.690 (95% CI, 0.578–0.795; sensitivity = 78.3%; specificity = 52.3%; $P < .001$); however, this increased to 0.836 when the frequency of sauce and poultry consumption was included in the model (95% CI, 0.749–0.922; sensitivity = 80.4%; specificity = 81.4%; $P < .001$) (Fig. 3). Forty-five patients were diagnosed with SSH using logistic model that includes the 8 miRNAs expression level and the dietary factors. Thirty-seven patients were consistent with the diagnostic results of saline-loading test. Forty-four patients were diagnosed with SRH using the model above and 35 patients were consistent with the diagnostic results of the saline-loading test.

3.6. Target gene prediction and pathway analysis

OmicsBean (http://www.omicsbean.com) analyses were performed to predict the target genes of the 8 miRNAs differentially expressed between the SSH and SRH groups. The output combined the results from 5 databases; TargetScan, PicTar, RNA22, PITA, and miRanda. We identified 1775 target genes for the 8 miRNAs. Next, we used OmicsBean to undertake gene ontology (GO) and pathway analysis of the 1775 target genes, which were found to mainly be involved in biological processes and molecular functions, including homologous to the E6-AP Carboxyl Terminus domain binding and protein binding. To evaluate the possible biological impact of the differentially expressed miRNAs, we preformed Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of the target genes that were found to be mainly involved in the WNT signaling pathway, renin-angiotensin-aldosterone system (RAS) signaling pathway, the dopaminergic synapse, adrenergic signaling in cardiomyocytes, and pathways in cancer. The interaction of miRNAs, target genes, and pathways is illustrated in Fig. 4.

4. Discussion

Our study is the first to identify miRNAs involved in pathogenic pathways of SSH in humans. In the current study, we used RNA-Seq to screen miRNAs differentially identified in whole blood from patients with essential hypertension, with or without salt sensitivity. Among 8 identified differentially expressed miRNAs, only hsa-miR-361-5p and hsa-miR-362-5p retained statistical significance after qRT-PCR analysis. Both hsa-miR-361-5p and hsa-miR-362-5p were downregulated in SSH compared with SSR. The AUC for hsa-miR-361-5p, taking into account dietary factors, was 0.793, which was smaller than that for all 8 miRNAs (0.836). The functional properties of the 8 miRNAs were predicted using KEGG analysis. The target genes of the 8 miRNAs were mainly involved in the WNT signaling pathway, adrenergic signaling in cardiomyocytes, the dopaminergic synapse, the mTOR signaling pathway, and the RAS signaling pathway, among others.

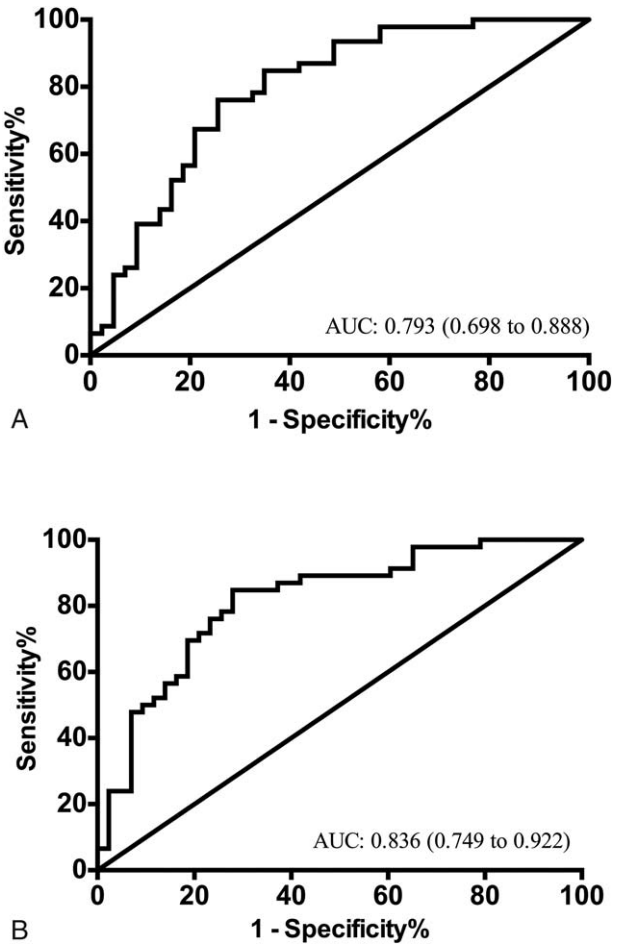

Figure 3. ROC analysis of 8 differentially expressed miRNAs with dietary factors included in the model. A, The AUC of the logistic model was 0.793 for a model including hsa-miR-361-5p and dietary factors. B, A model including all 8 miRNAs and the dietary factors generated an AUC of 0.836. AUC = area under the curve, ROC = receptor operating characteristic curve.

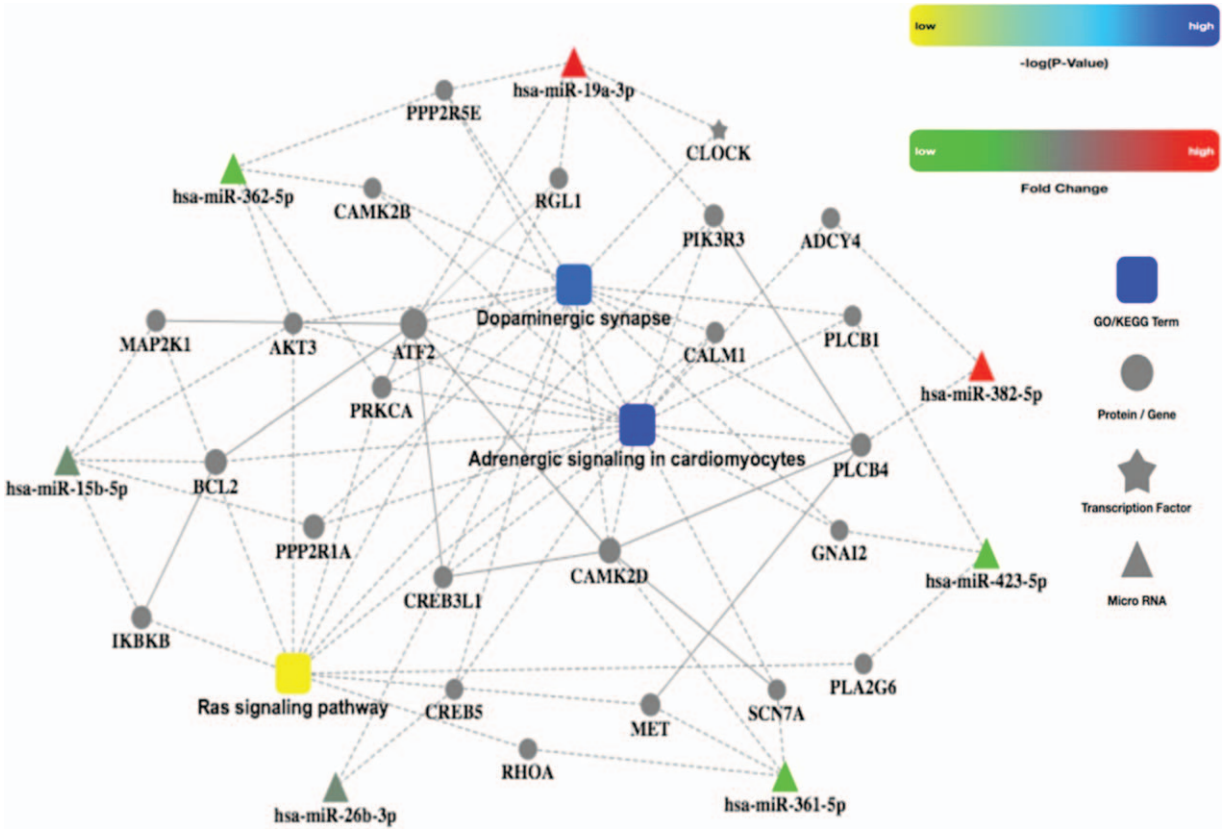

Figure 4. Protein–protein interaction analysis. The network model was generated using the OmicsBean and illustrates the results of KEGG pathway and biological process enrichment analysis. KEGG = kyoto encyclopedia of genes and genomes.

Currently, the diagnosis of SSH is complex and difficult to conduct; therefore, the identification of noninvasive and convenient biomarkers to predict SSH is necessary. Dahl salt-sensitive rats are the most popular animal model for investigations of the role of miRNAs in SSH[25]; however, the results of these investigations may not be applicable to humans, due to species-specific differences. The miRNA panel that we constructed was able to distinguish SSH from SRH groups precisely and accurately. Through testing levels of these circulating miRNAs and determining the frequency of sauce and poultry consumption, it is possible to rapidly determine whether a hypertensive patient is likely to be SSH or SRH. With this information clinicians, or preferably the patients themselves, can guide their daily salt intake.

Although many investigators have reported functional roles for miRNAs in hypertension, the association between miRNAs and SSH remains unclear. In addition, the involvement of hsa-miR-361-5p in the development of SSH has not previously been published and was discovered for the first time in this study. The main biological pathways involved in SSH are reported to be the renin–angiotensin–aldosterone system (RAAS),[26] the endothelial system,[27] ion and water channels, intracellular messengers, the sympathetic nervous system,[28] the Apelin-APJ system, and the natriuretic peptide system.[29] The results of our KEGG pathway analysis similarly indicated that hsa-miR-361-5p is involved in the RAS signaling pathway, through binding to target genes including *PIK3CA, RGL1, CALM1, RHOA, AKT3, MET, MAP2K1, PRKCA, IKBKB*, and *PIK3R3*. Moreover, a genetic association analysis has identified SNPs in *PRKCA* as strongly associated with BP response to hydrochlorothiazide[30]; suggesting that *PRKCA* could be a target of hsa-miR-361-5p affecting the development of SSH. Other pathways, including the cGMP-PKG signaling pathway, the TGF-beta signaling pathway, the dopaminergic synapse, and adrenergic signaling in cardiomyocytes, were also identified as enriched by the analysis of the 8 differentially expressed miRNAs. These pathways are generally consistent with known mechanisms involved in SSH (discussed above); hence, our study confirmed the relationship between miRNAs and SSH, in addition to providing insight into the miRNA-dependent mechanisms involved in the pathology of SSH.

Hsa-miR-361-5p also acts as a tumor suppressor related to some cancers.[31,32] Sun et al[32] reported that hsa-miR-361-5p may act as the putative markers in the diagnosis of different subtypes of breast cancer. We have done target mRNA prediction and KEGG pathway analysis, and discovered that hsa-miR-361-5p played a role in the pathogenic mechanism of glioma. It is interesting that the salt sensitivity of blood pressure was one of the risk factors for cancer.[33] Hsa-miR-361-5p may involve in the pathogenesis of SSH then contribute to cancer onset. Further study to follow up the occurrence of cancer in SSH may help us explain the role of hsa-miR-361-5p against cancer preferably.

In addition, other factors, including the diet[34] and insulin sensitivity,[35] may also influence the salt sensitivity of blood pressure. Yatabe et al[35] showed that the glucose infusion rate (GIR) has an inverse relationship with salt sensitivity. This could be due to the role of hyperinsulinemia, sympathetic overactivation, and reduced suppression of the RAS pathway.

Through KEGG analysis, we discovered that the target genes of the 8 miRNAs, as well as hsa-miR-361-5p alone, were chiefly involved in the WNT signaling pathway, whose functions include roles in embryonic development and insulin sensitivity. Considering the association between insulin sensitivity and salt sensitivity, we hypothesize that hsa-miR-361-5p is likely to play an important role in the pathogenesis of SSH by regulating insulin sensitivity. *PRICKLE2* was a target gene of hsa-miR-361-5p involved in the WNT signaling pathway. The specific function of hsa-miR-361-5p in the progress of SSH requires confirmation by experiments in animal models.

Interestingly, we also identified specific dietary factors, including the frequency of consumption of sauce and poultry, that were significantly different between the SSH and SRH groups. This may be because traditional Chinese cooking methods involve the addition of salt and soy sauce, resulting in diets with high salt contents. In the north of China in particular, the consumption of various sauce, including dark soy sauce, preserved bean curd, and yellow soybean paste, is common and likely to result in increased invisible salt intake, which may influence salt sensitivity over the long term. In Tieling, a city in the northeast of China, our research group found that SSH patients had higher salt intakes than those with SRH after analysis, demonstrating that dietary habits, particularly salt intake, could have a significant influence on the incidence of SSH in this local area. In addition, we found that poultry was a protective factor, which decreased the risk of SSH. Poultry contains lower levels of saturated fat and cholesterol than red and processed meats, the consumption of which is associated with the risk of coronary diseases.[36] In addition, poultry, also known as "white meat," is not significantly associated with cardiovascular death.[37] Thus, the risks of both cardiovascular death and SSH can be reduced through the moderate consumption of poultry, instead of red and processed meats.

In the present study, we describe the first development of a miRNA panel as a biomarker for SSH. The stepwise logistic regression panel ultimately included 3 variables: hsa-miR-361-5p levels and the frequency of sauce and poultry consumption. The logistic model was: logit (P = SSH) = 1.67 to 2.395 × miR-361–5p + 0.948 × sauce − 0.707 × poultry. The OR for hsa-miR-361-5p was 0.091, indicating that it was a protective factor associated with a reduced risk of SSH.

Although our study developed an improved method for the diagnosis of SSH and discovered that hsa-miR-361-5p is a potential biomarker for SSH, it also had some limitations. First, the study was limited to Beijing; therefore, the results may not be applicable more widely. In addition, we did not perform functional experiments; therefore, the specific roles of these miRNAs in SSH require further investigation. Finally, as the levels of miRNAs in the circulation varied over the course of SSH, further experiments should be performed to detect the expression levels of miRNAs at different time points. This study showed a marginal difference in the expression levels of hsa-miR-361-5p and hsa-miR-362-5p between the 2 groups. The result is preliminary at this stage, so further validation analyses are required to confirm the marginal differences.

5. Conclusion

The diagnostic panel integrates genetic and environmental risk factors, indicating that both of them are involved in the etiology and development of SSH. As the diagnostic assay developed in this study provides a noninvasive and diagnostically sensitive indicator of SSH, we believe that it will be widely applicable for the discrimination of SSH from SRH. In particular, hsa-miR-361-5p could be a highly valuable biomarker associated with SSH. Although the prediction performance of hsa-miR-361-5p was inferior to that of all 8 identified miRNAs, it is preferable for the prediction of SSH as, compared with the 8 miRNA panel, screening with hsa-miR-361-5p alone has the advantages of lower cost and ease of implementation, in addition to good diagnostic performance.

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