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Integrated analysis of the lncRNA-associated competing endogenous RNA network in salt sensitivity of blood pressure

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ABSTRACT

Accumulating evidence showed that competing endogenous RNA (ceRNA) mechanism plays a pivotal role in salt sensitivity of blood pressure (SSBP). We constructed a ceRNA network based on SSBP-related differently expressed lncRNAs (2), mRNAs (73) and miRNAs (18). Bioinformatic analyses were utilized to analyze network and found network genes participate in biological pathways related to SSBP pathogenesis such as regulation of nitric oxide biosynthetic process (GO:0045,428) and cellular response to cytokine stimulus (GO:0071,345). Fourteen candidate ceRNA pathways were selected from network to perform *q*RT-PCR validation and found nine RNAs (KCNQ1OT1, SLC8A1-AS1, *IL1B*, *BCL2L11*, *KCNJ15*, *CX3CR1*, *KLF2*, hsa-miR-362–5p and hsa-miR-423–5p) differently expressed between salt-sensitive (SS) and salt-resistant (SR) groups ($P < 0.05$). Four ceRNA pathways were further validated by luciferase reporter assay and found KCNQ1OT1→hsa-miR-362–5p/hsa-miR-423–5p→*IL1B* pathways may influence the pathogenic mechanism of SS. Our findings suggested the ceRNA pathway and network may affect SS occurrence mainly through endothelial dysfunction and inflammatory activation.

1. Introduction

Large epidemiology studies had demonstrated the adverse effects of high salt intake on elevated blood pressure (BP), such as Intersalt study [1] and PURE study [2]. Some populations show heterogeneous BP elevations in response to high salt intake, this phenomenon is a physiological trait generally referred as salt sensitivity of blood pressure (SSBP) [3,4]. Individuals could be classified into salt-sensitive (SS) and salt-resistant (SR), SS display BP elevations due to high salt intake, while SR not. A longitudinally follow-up

Abbreviations: BP, blood pressure; SSBP, Salt sensitivity of blood pressure; SS, salt-sensitive; SR, salt-resistant; CVDs, cardiovascular diseases; lncRNA, long non-coding RNA; *q*RT-PCR, quantitative real-time PCR; miRNA, microRNA; ceRNA, competing endogenous RNA; EpiSS, systemic epidemiology of salt sensitivity; MSAOSL-DST, modified Sullivan's acute oral saline load and diuresis shrinkage test; MAP, mean arterial pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; WHR, waist-to-hip ratio; FBG, fasting blood glucose; TC, total cholesterol; TG, triglycerides; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol.

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study found individuals with high salt sensitivity could increase risk of developing hypertension [5], when compared with moderate sodium sensitivity. SS could elevate the morbidity and mortality of cardiovascular diseases (CVDs) independent of BP, and the associations between CVDs with SS is as strong as BP [6,7]. Therefore, it is of great public health significance to explore the molecular mechanism of SS.

Long non-coding RNAs (lncRNAs) are crucial part of transcriptome that are longer than 200 nucleotides [8]. Animal studies found that lncRNAs participate in SS development, such as lncRNA-NONRATG012674.2 [9] and lncRNA-sONE [10]. In human, we identified five *SS*-related lncRNAs (lnc-IGSF3-1:1, SCOC-AS1, SLC8A1-AS1, KCNQ1OT1 and lnc-GNG10–3:1) by microarray and quantitative real-time PCR (*q*RT-PCR) methods previously [11]. MicroRNAs (miRNAs) are endogenous non-coding RNAs that typically measure 22 nucleotides in length [12]. Zhu et al. demonstrated that inhibiting miRNA-429 expression in renal medulla could increase SSBP in Sprague Dawley rats [13], and later, reported that the overexpression of miRNA-429 transgene into renal medulla attenuated salt-sensitive hypertension in Dahl S Rats [14]. In human, researchers found 45 urinary exosome miRNAs were associated with salt sensitivity [15]. We previously found 36 differently expressed miRNAs between SS and SR hypertensives by high-throughput sequencing and further identified hsa-miR-361–5p and hsa-miR-362–5p are down-regulated in SS hypertensives [16].

In molecular biology, RNA transcripts, such as lncRNA and circular RNA, contain an abundance of miRNA bindings sites, and these competing endogenous RNAs (ceRNA) could regulate the expression of downstream target genes of miRNA by sponge adsorption miRNA [17,18]. This regulatory mechanism underlying the crosstalk among lncRNAs, miRNAs and mRNAs plays a pivotal role in the pathophysiological processes of CVDs [19,20], including hypertension [21,22]. However, few studies have reported on the ceRNA mechanism of SS in human. Therefore, this study aimed to uncover the ceRNA interaction underlying SSBP through methods of statistical analysis, bioinformatics analysis and experimental verification.

2. Methods

2.1. Study subjects

Participants were selected from the System Epidemiology Study on Salt Sensitivity of Blood Pressure (EpiSS) cohort study, which aimed to uncover both genetic and environmental factors of salt sensitivity of BP. The EpiSS study was registered in the Chinese Clinical Trial Registry (http://www.chictr.org.cn/index.aspx, NO: ChiCTR-EOC-16009980). The protocol for selection of the subjects, sample collection and measurement methods have been described in detail previously [23]. In brief, individuals with Han nationality and aged 35–70 years old were recruited from community health centers in Beijing and Liaoning Province during July 2014 and July 2016. Patients with secondary stage and above hypertension, cardiovascular disease, kidney disease, liver disease, malignant tumor or pregnancy or who had used antihypertensive drugs in the past month were excluded. In the current study, 20 subjects (10 S S and 10 SR) were involved in conducting the microarray, 200 subjects (100 S S and 100 SR) were conducted in *q*RT-PCR. This study was approved by the Ethics Committee of Capital Medical University (NO: 2019SY016). Written informed consent was obtained from all subjects.

2.2. Saline loading and diuresis shrinkage protocol

In EpiSS study, the modified Sullivan's acute oral saline load and diuresis shrinkage test (MSAOSL-DST) [24–26]. As descripted in the previous study [23], MSAOSL-DST mainly contains the following steps: (i) the 1st BP was measured after taking a rest at least 15 min; (ii) subjects were asked to take 1 L of saline solution orally within half an hour, and the 2nd BP was measured 2 h after taking saline; (iii) the subjects were given 40 mg furosemides immediately after the 2nd BP measurements, and the 3rd BP was measured 2 h after taking furosemide. Mean arterial pressure (MAP) was defined as the one-third of systolic BP (SBP) plus two-third of diastolic BP (DBP). The ΔBP_1 was calculated as the 2nd BP minus the 1st BP. The ΔBP_2 was defined as the 3rd BP minus the 2nd BP. Individuals with $\Delta MAP_1 \geq 5$ mmHg or $\Delta MAP_2 \leq -10$ mmHg were identified as SS, and the others were SR [27].

2.3. Data collection and variables

To obtain demographic information, behavior habits and medical history, questionnaires were completed through face-to-face interviews. The physical measurements included seated BP, height, weight, waist and hip circumference. BP were measured after at least 15-min rest with an automatic sphygmomanometer (Omron HEM-7118, Japan) [28]. BP measurement was carried out twice with a 1 min interval, the average of the two readings was used in data analysis. Body mass index (BMI) was calculated by dividing the weight in kilogram over the squared height in meters, and waist-to-hip ratio (WHR) by dividing waist circumference (cm) by hip circumference (cm). Fasting venous blood samples were collected before the test. Serum biochemical were examined, including fasting blood glucose (FBG), total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C).

2.4. LncRNA-miRNA-mRNA ceRNA network

The "lncRNA-miRNA-mRNA" ceRNA network was constructed based on the five *SS*-related lncRNAs (lnc-IGSF3-1:1, SCOC-AS1, SLC8A1-AS1, KCNQ1OT1 and lnc-GNG10–3:1) [11]. Firstly, we performed correlation analysis and screened lncRNA-mRNA coexpression pairs. The mRNAs were chosen from the microarray and meet the following criteria: (i) *P*-value$<$0.05; (ii) average expression

level>10; (iii) foldchange>1.5; (iv) significant correlated with lncRNAs (Pearson's correlation coefficient>0.65). Then, the ceRNA network was built using the following steps: (i) predicting the targeted miRNAs of these five lncRNAs by starBase 3.0 (https://starbase.sysu.edu.cn/) and miRDB (http://mirdb.org/miRDB/); (ii) retaining the same targeted miRNAs as in the 36 S S-associated miRNAs [16] and forming the crucial lncRNA-miRNA pairs; (iii) predicting the target mRNAs of miRNAs by starBase 3.0 and miRWalk 3.0 (http://mirwalk.umm.uni-heidelberg.de/); (iv) Combining these lncRNA-mRNA, lncRNA-miRNA and miRNA-mRNA interactions together for ceRNA network construction using Cytoscape software.

2.5. Network analysis

CytoNCA [29] (http://apps.cytoscape.org/apps/cytonca) was applied to identify the essential miRNAs. The CytoNCA is a Cytoscape plugin for centrality analysis and supports eight different centrality measures, including betweenness centrality (BC), closeness centrality (CC), degree centrality (DC), eigenvector centrality (EC), local average connectivity-based method (LAC), network centrality (NC), subgraph centrality (SC) and information centrality (NC). Metascape [30] (http://metascape.org/) was utilized to explore biological functions of the ceRNA network by enrichment analysis. DAVID [31] (https://david.ncifcrf.gov/) was performed to identify genes in the network associated with SSBP by gene annotation.

2.6. Microarray and quantitative real-time PCR test

Whole blood samples from SS and SR subjects were collected for the extraction of total RNA for microarray using standard procedure and *q*RT-PCR using SYBR Green method. In microarray test, the SBC human (4 × 180 K) ceRNA array v1.0 was used, which contains 68,423 lncRNAs and 18,853 mRNAs. The microarray data are available from the Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/) database under the accession number GSE135111. After ceRNA network construction and network analysis, we selected twelve RNAs (two lncRNAs, two miRNAs and eight mRNAs) from the network to detected their expression levels in 200 subjects (100 S S and 100 SR) by *q*RT-PCR test. The GAPDH and U6 were used as internal controls. The relative expression level was determined with the $2^{-\Delta\Delta ct}$ method [32]. Each sample was performed in triplicate. The primer sequences for *q*RT-PCR are summarized in Table S1.

2.7. Prediction of binding sites between miRNAs and target genes

RNAhybrid (https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid) and LncBase v.3 (http://www.microrna.gr/LncBase) softwires were conducted to predict the binding sequence between miRNAs and 3′UTR region of target genes (hsa-miR-362–5p/KCNQ1OT1, hsa-miR-362–5p/*IL1B*, hsa-miR-362–5p/SLC8A1-AS1, hsa-miR-362–5p/*KCNJ15*, hsa-miR-423–5p/KCNQ1OT1 and hsa-miR-423–5p/*IL1B*). And the wild-type and mutant reporter vector of target genes were synthesized according to the sequence information.

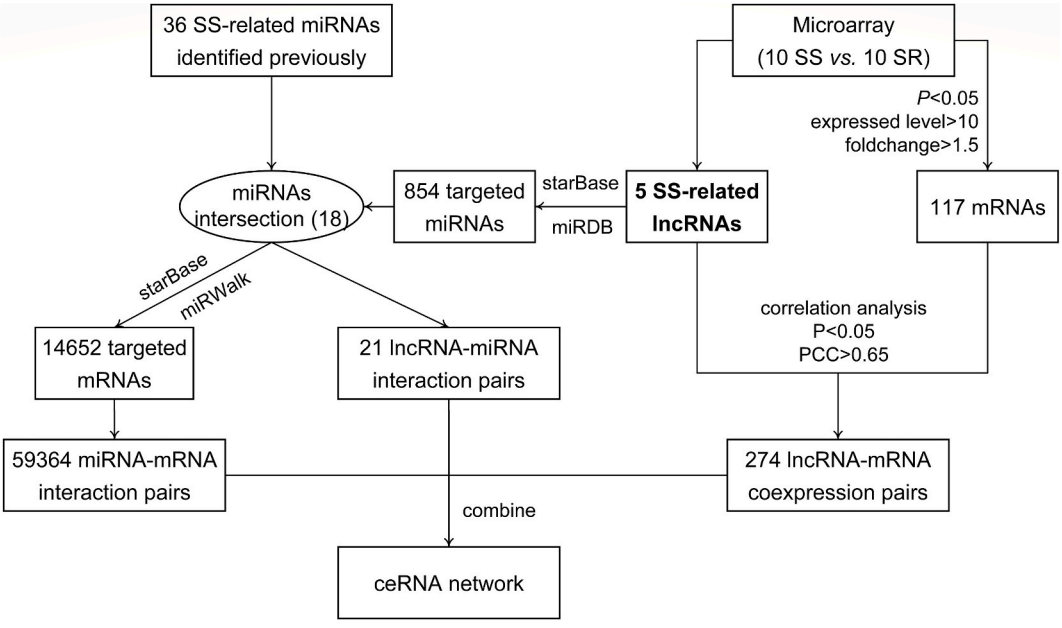

Fig. 1. Study approach of constructing the ceRNA coregulatory network. CeRNA, competing endogenous RNA; SS, salt-sensitive; SR, salt-resistant; lncRNA, long non-coding RNA; miRNA, microRNA; PCC, Pearson's correlation coefficient.

2.8. Dual luciferase reporter assay

HEK 293 T cells were seeded into 24-well plates and co-transfected with GV272-KCNQ1OT1-WT, GV272-KCNQ1OT1-Mut, GV272-SLC8A1-AS1-WT, GV272-SLC8A1-AS1-Mut, GV272-KCNJ15-WT, GV272-KCNJ15-Mut, GV272-IL1B-WT or GV272-IL1B-Mut, along with the GV272 control vector used as internal control and miRNA mimics (hsa-miR-362–5p or hsa-miR-423–5p) or mimic control. Luciferase activity was measured using the Dual-Luciferase® Reporter Assay System (cat. No. E1910, Promega, Madison, WI, USA) after 48 h of incubation according to the manufacturer's instructions. Independent experiments were performed in triplicate, and the average value was calculated. Relative luciferase activity was normalized to the Renilla luciferase internal control.

2.9. Statistical analysis

The SPSS 24.0 (SPSS, Inc, Chicago, IL) and R 3.4.4 software were used for statistical analysis and $P < 0.05$ was considered significant. In the microarray data, the comparison of mRNAs between SS and SR groups was performed by limma package and correlation analysis between lncRNAs and mRNAs was conducted by cor. test () function. In the *q*RT-PCR data, differential expression levels of RNAs between groups were analyzed using independent *t*-test. The Spearman correlation analysis was performed to analyze the correlation between RNAs in one ceRNA pathway. The associations between RNAs with SS prevalence, ΔBP_1 and ΔBP_2 were evaluated using multivariate logistic regression models (odds ratio [*OR*] and 95 % confidence interval [*95%CI*]) or multivariate linear regression models (beta coefficient [β] and *95%CI*), adjusted for covariables including age, sex, current smoking, TG, TC, LDL-C and HDL-C. Log_2 transformations were used to transform skewed RNA expressional data in the multivariate analyses.

3. Results

3.1. LncRNA-miRNA-mRNA ceRNA coregulatory network

The study approach of constructing the ceRNA coregulatory network (Fig. 1). 117 mRNAs were screened out from microarray to perform correlation analysis with lncRNAs and 274 lncRNA-mRNA coexpression pairs were acquired (Table S2). We obtained 854

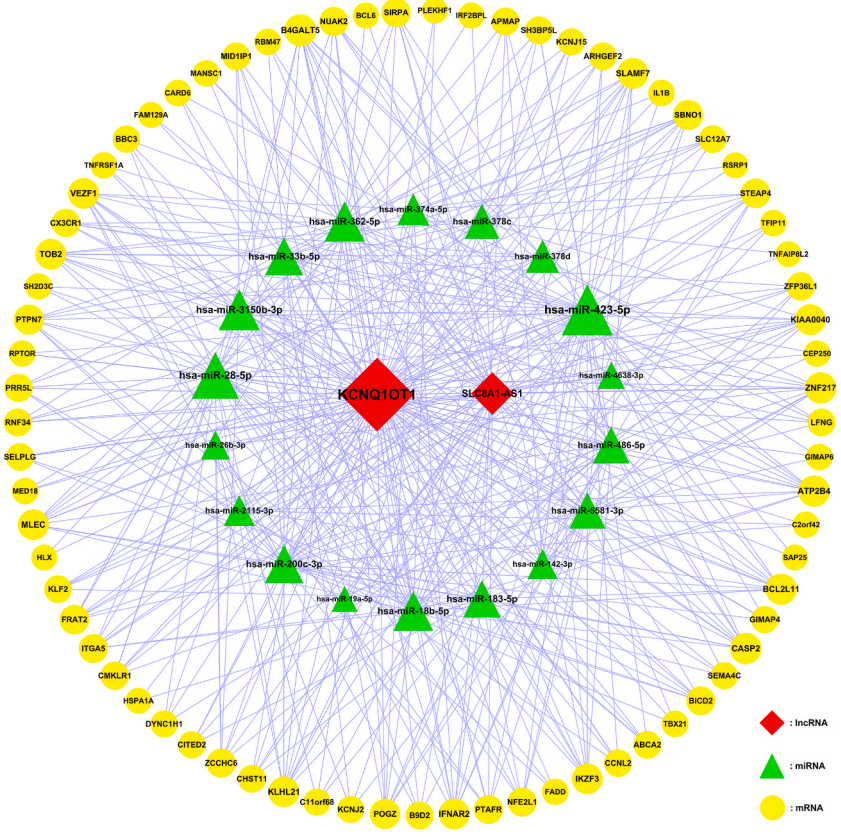

Fig. 2. LncRNA-miRNA-mRNA ceRNA network. In this network, the triangle, diamond and circular nodes represent miRNAs, lncRNAs and mRNAs, respectively. Edges between lncRNAs with mRNAs indicate the coexpression relationship. Edges between miRNAs and lncRNAs/mRNAs represent lncRNA-miRNA and miRNA-mRNA associations predicted by bioinformatic software respectively.

targeted miRNAs of lncRNAs and 21 lncRNA-miRNA interaction pairs (Table S3). A total of 14,652 targeted mRNAs of miRNAs and 59,364 miRNA-mRNA interaction pairs were predicted by online tools. After combining RNA-RNA pairs, we constructed the ceRNA coregulatory network with 339 "lncRNA-miRNA-mRNA" ceRNA loops (Fig. 2). The network was consisted of 93 nodes (two lncRNAs, 18 miRNAs and 73 genes) and 430 edges (91 lncRNA-mRNA pairs, 318 miRNA-mRNA pairs and 21 lncRNA-miRNA pairs).

3.2. Network analysis and selection of ceRNA pathways for validation

We calculated centrality indexes of miRNAs in the network by CytoNCA (Table 1), hsa-miR-423–5p and hsa-miR-28–5p were among the top three in seven centrality indicators (BC, CC, EC, IC, SC, NA and DC) and hsa-miR-362–5p was among the top three in five indicators (BC, CC, LAC NC and DC). Then, hsa-miR-423–5p, hsa-miR-28–5p and hsa-miR-362–5p were considered as hub miRNAs of the network.

We summarized the results of enrichment analysis for 73 genes in the network (Fig. 3, Table 2). Network genes participate in the regulation of nitric oxide biosynthetic process (GO:0045,428), cellular response to cytokine stimulus (GO:0071,345) and negative regulation of cytokine production (GO:0001818), as well as biological pathways related to SS pathogenic mechanisms such as cytokine-cytokine receptor interaction (hsa04060).

In the network, thirteen genes (*BCL2L11*, *CX3CR1*, *STEAP4*, *FAM129A*, *HSPA1A*, *PTAFR*, *KCNJ15*, *RPTOR*, *VEZF1*, *ATP2B4*, *KLF2*, *IL1B* and *KCNJ2*) were found to be associated with potential SSBP-related biological function (Table 3). For example, *IL1B* participated with regulation of vascular endothelial growth factor receptor signaling pathway, *CX3CR1* was related to positive regulation of angiogenesis and *KCNJ15* participated the potassium ion transport.

We reviewed these thirteen genes to find evidence that they may be involved in the mechanism of SSBP. However, *FAM129A*, *HSPA1A*, *PTAFR*, *RPTOR* and *KCNJ2* had no relevant supporting evidence reports. The increase in *FAM129A* (aliases: *NIBAN1*) expression has been related to cellular mechanisms that minimize the damage caused to cellular homeostasis [33]. *HSPA1A* is overexpressed in different tumor types and reported as a theranostic target for cancer therapy [34]. *PTAFR* is found to be an anti-psychotic target receptor potentially associated with pneumonia [35]. *RPTOR* plays an important role during mitosis [36]. The mutations in *KCNJ2* cause genetic sudden cardiac death syndromes [37]. The remained eight mRNAs (*BCL2L11*, *CX3CR1*, *STEAP4*, *KCNJ15*, *VEZF1*, *ATP2B4*, *IL1B* and *KCNJ2*) formed fourteen ceRNA pathways with lncRNAs (KCNQ1OT1 and SLC8A1-S1) and miRNAs (hsa-miR-423–5p and hsa-miR-362–5p), and were selected for further validation.

3.3. Characteristics of enrolled participants

The 20 participants in the microarray test included 10 hypertensive patients and 10 normotensives, with the average age of 64.55 ± 2.21 years old. There was no significant difference in gender, age, SBP, DBP, BMI, heart rate, TC, HDL, LDL or FBG between groups ($P > 0.05$), except TG ($P = 0.018$) (Table S4).

Twelve RNAs (KCNQ1OT1, SLC8A1-S1, hsa-miR-423–5p, hsa-miR-362–5p, *BCL2L11*, *CX3CR1*, *STEAP4*, *KLF2*, *KCNJ15*, *VEZF1*, *IL1B* and *ATP2B4*) were selected from the coregulatory network to perform *q*RT-PCR validation. We summarized the characteristics of study subjects and the comparison between SS and SR groups (Table 4). A total of 200 (100 S S *vs.* 100 SR) participants were enrolled in the study, with an average age of 62.68 years old, and 117 (57.5 %) of hypertensive patients. There was no significant difference in gender, age, BP, BMI, WHR, TC, TG, HDL-C, LDL-C, chronic diseases, current smoking or drinking between two groups, while the mean FBG was little lower in SS compared to SR (5.81 ± 1.50 *vs.* 5.78 ± 1.75, $P = 0.005$).

Table 1
The centrality indexes of miRNAs in the network.

MiRNAs	BC	CC	EC	IC	LAC	SC	NC	DC
hsa-miR-423–5p	**661.21**	**0.63**	**0.25**	**5.51**	1.95	**678207.30**	**12.04**	**86**
hsa-miR-362–5p	**505.39**	**0.58**	0.16	5.10	**2.46**	275982.62	**9.23**	**64**
hsa-miR-28–5p	**425.14**	**0.60**	**0.23**	**5.39**	1.95	**534729.06**	**9.48**	**72**
hsa-miR-33 b-5p	379.23	0.57	0.14	4.94	**2.45**	212734.39	7.00	54
hsa-miR-3150 b-3p	211.10	0.56	**0.17**	**5.14**	1.93	**307360.75**	5.95	52
hsa-miR-5581–3p	177.85	0.56	0.13	4.79	**2.42**	185890.25	5.25	46
hsa-miR-200c-3p	170.06	0.55	0.14	4.99	1.91	211862.72	5.92	44
hsa-miR-18 b-5p	158.78	0.55	0.17	5.07	1.92	291154.80	6.28	48
hsa-miR-183–5p	115.45	0.54	0.14	4.90	1.90	206059.31	5.41	40
hsa-miR-486–5p	97.27	0.53	0.14	4.85	1.90	198801.20	4.54	38
hsa-miR-378c	69.21	0.53	0.12	4.67	1.88	161744.00	4.13	32
hsa-miR-378 d	36.93	0.52	0.11	4.45	1.86	117913.87	2.98	26
hsa-miR-37s4a-5p	30.61	0.51	0.09	4.26	1.83	91510.63	3.26	22
hsa-miR-2115–3p	23.72	0.51	0.08	4.02	1.80	58939.81	3.13	18
hsa-miR-142–3p	16.06	0.50	0.07	3.88	1.78	54847.42	2.38	16
hsa-miR-26 b-3p	10.29	0.41	0.04	3.55	1.71	14275.35	2.25	12
hsa-miR-4638–3p	1.50	0.39	0.02	2.81	1.50	5144.07	2.00	6
hsa-miR-19a-5p	0	0.39	0.01	2.06	1.00	2334.13	2.00	2

BC, betweenness centrality; CC, closeness centrality; DC, degree centrality; EC, eigenvector centrality; LAC, local average connectivity-based method; NC, network centrality; SC, subgraph centrality; IC, information centrality.

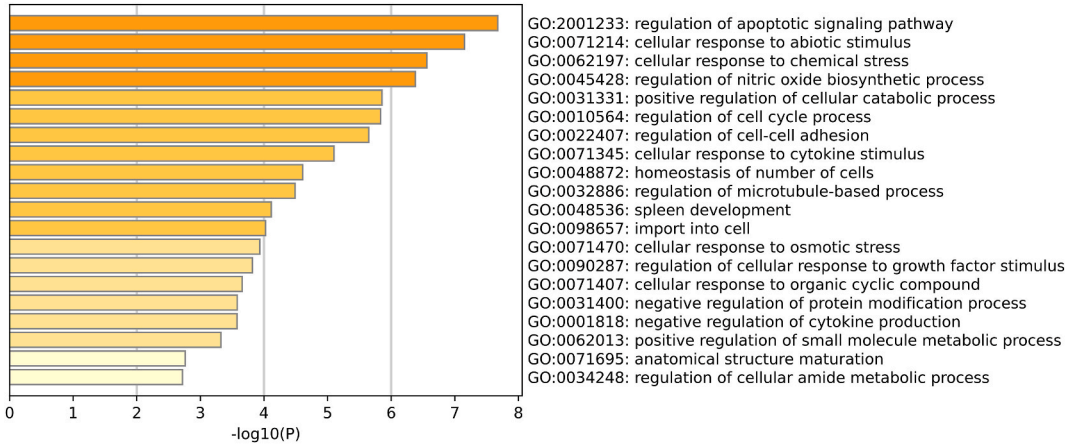

Fig. 3. The GO Term enrichment analysis of network genes by Metascape. GO, Gene Ontology.

Table 2
Results of KEGG pathways enrichment analysis.

NO.	KEGG	Description	log 10(*P*)	Genes
1	hsa 04010	MAPK signaling pathway	−2.24724	*HSPA1A*, *IL1B*, *PTPN7*, *TNFRSF1A*
2	hsa 04621	NOD-like receptor signaling pathway	−2.9804	*IFNAR2*, *IL1B*, *FADD*, *CARD6*
3	hsa 04380	Osteoclast differentiation	−3.57297	*IFNAR2*, *IL1B*, *TNFRSF1A*, *SIRPA*
4	hsa 04668	TNF signaling pathway	−2.59217	*IL1B*, *TNFRSF1A*, *FADD*
5	hsa 04620	Toll-like receptor signaling pathway	−2.68364	*IFNAR2*, *IL1B*, *FADD*
6	hsa04060	Cytokine-cytokine receptor interaction	−2.2421	*CX3CR1*, *IFNAR2*, *IL1B*, *TNFRSF1A*
7	hsa 05418	Fluid shear stress and atherosclerosis	−3.43679	*IL1B*, *TNFRSF1A*, *ARHGEF2*, *KLF2*
8	hsa 04150	mTOR signaling pathway	−2.18997	*TNFRSF1A*, *RPTOR*, *PRR5L*
9	hsa04068	FoxO signaling pathway	−2.4005	*BCL6*, *BCL2L11*, *KLF2*

KEGG, Kyoto Encyclopedia of Genes and Genomes.

Table 3
The potential SSBP-related biological function of genes in the network.

Genes	Related Disease	Biological process GO term	KEGG pathway
BCL2L11	Neovascularization		hsa04068: FoxO signaling pathway
CX3CR1	Atherosclerosis, Coronary vascular endothelial dysfunction, Diabetes	GO:0016,525~negative regulation of angiogenesis, GO:0045,766~positive regulation of angiogenesis	
STEAP4	Insulin resistance		
FAM129A	Insulin resistance		
HSPA1A	Diabetes, Insulin resistance, Stroke		
PTAFR	Atherosclerosis, Diabetes	GO:0045,776~negative regulation of blood pressure, GO:0045,907~positive regulation of vasoconstriction, GO:0045,909~positive regulation of vasodilation	
KCNJ15	Diabetes	GO:0006813~potassium ion transport, GO:0010,107~potassium ion import	
RPTOR		GO:0001938~positive regulation of endothelial cell proliferation	hsa 04910: Insulin signaling pathway
VEZF1		GO:0001525~angiogenesis, GO:0001885~endothelial cell development	
ATP2B4	Hypertension	GO:0010,751~negative regulation of nitric oxide mediated signal transduction, GO:0045,019~negative regulation of nitric oxide biosynthetic process, GO:0051,001~negative regulation of nitric-oxide synthase activity, GO:0071,872~cellular response to epinephrine stimulus, GO:1,900,082~negative regulation of arginine catabolic process	
KLF2	Diabetes	GO:0045,429~positive regulation of nitric oxide biosynthetic process	hsa04068: FoxO signaling pathway
IL1B	Acute coronary syndrome, Stroke, Arteriosclerosis, Hypertension, Diabetes	GO:0030,949~positive regulation of vascular endothelial growth factor receptor signaling pathway, GO:0045,766~positive regulation of angiogenesis, GO:0046,627~negative regulation of insulin receptor signaling pathway	hsa 04940: Type I diabetes mellitus
KCNJ2	Stroke, Diabetes	GO:0006813~potassium ion transport, GO:0010,107~potassium ion import	hsa 04924: Renin secretion

SSBP, salt sensitivity of blood pressure; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

3.4. Expression levels of RNAs between groups

The expression levels of KCNQ1OT1, SLC8A1-AS1, *KCNJ15*, *CX3CR1*, *BCL2L11* and *IL1B* were higher in SS than those in SR, while *KLF2*, hsa-miR-362–5p and has-miR-423–5p were lower (Fig. 4). After acute sodium-volume repletion by oral saline, KCNQ1OT1, SLC8A1-AS1, *KCNJ15*, *CX3CR1, BCL2L11*, *IL1B* and *ATP2B4* had higher expression levels in higher ΔMAP_1 group than those in lower ΔMAP_1 group, while *KLF2* appeared opposite results. After acute sodium-volume depletion by taking furosemides, the expressions of RNAs had no significant difference between higher and lower ΔMAP_2 groups, except for *STEAP4*.

3.5. Associations between RNAs and salt sensitivity of blood pressure

The associations between RNAs with SS or ΔBP, and there were nine RNAs associated with SSBP in total (Table 5). In multiple logistic regression analysis, we found that for every unit increment in expressions of KCNQ1OT1, SLC8A1-AS1, *IL1B*, *BCL2L11*, *KCNJ15* and *CX3CR1*, SS prevalence significantly increased, with *OR* (*95%CI*) of 6.156 (3.395, 12.038), 4.450 (2.616, 8.025), 3.394 (2.190, 5.553), 2.494 (1.257, 5.131), 4.030 (2.403, 7.142) and 2.480 (1.682, 3.843), respectively; for every unit increase in expressions of *KLF2*, hsa-miR-362–5p and hsa-miR-423–5p, the prevalence of SS significantly decreased, with *OR* (*95%CI*) of 0.141 (0.067, 0.274), 0.577 (0.409, 0.796) and 0.662 (0.479, 0.898), respectively.

In multiple linear regression analysis, we found that ΔMAP_1 was positively associated with the expressions of KCNQ1OT1, SLC8A1-AS1, *IL1B*, *KCNJ15* and *CX3CR1*, with β (*95%CI*) of 4.093 (2.602, 5.583), 3.827 (2.419, 5.234), 1.845 (0.673, 3.017), 1.907 (0.551, 3.262) and 1.648 (0.560, 2.737), respectively; while ΔMAP_1 was negatively correlated with *KLF2* (β=-1.717, *95%CI*: −3.314, −0.120). In addition, ΔMAP_2 was significantly associated with KCNQ1OT1 ($\beta = -1.701$, *95%CI*: −3.387, −0.015) and *KLF2* ($\beta = 2.824$, *95%CI*: 1.089, 4.559).

3.6. Correlations between RNAs in ceRNA pathways associated with SSBP

These nine SSBP-associated RNAs (KCNQ1OT1, SLC8A1-AS1, *IL 1B*, *BCL2L11*, *KCNJ15*, *CX3CR1*, *KLF2*, hsa-miR-362–5p and hsa-miR-423–5p) made up nine ceRNA loops. The correlations between RNAs in ceRNA pathways were presented in Table 6. According the ceRNA theory, in one ceRNA pathway, miRNA should be negatively correlated with lncRNA and mRNA, while lncRNA be positively

Table 4
Characteristics of the participants classified by salt-sensitive and salt-resistant.

Variables	Total ($N = 200$)	SS ($N = 100$)	SR ($N = 100$)	*P*
Baseline characteristics				
Age (years)[b]	62.68 ± 6.98	61.84 ± 7.01	63.51 ± 6.87	0.070
Gender (male, n, %)[c]	69 (34.5)	33 (33.0)	36 (36.0)	0.655
SBP (mmHg)[b]	137.81 ± 19.39	135.71 ± 20.09	139.91 ± 18.53	0.175
DBP (mmHg)[b]	80.01 ± 10.97	79.54 ± 11.47	80.48 ± 10.47	0.284
MAP (mmHg)[a]	99.27 ± 12.31	98.26 ± 13.01	100.29 ± 11.54	0.244
BMI (kg/m^2)[a]	26.04 ± 3.51	26.09 ± 3.54	25.99 ± 3.50	0.835
WHR[b]	0.91 ± 0.06	0.91 ± 0.06	0.90 ± 0.07	0.748
TC (mmol/L)[b]	5.23 ± 0.98	5.19 ± 0.97	5.28 ± 0.99	0.458
TG (mmol/L)[b]	1.93 ± 1.15	1.96 ± 1.32	1.90 ± 0.95	0.538
LDL-C (mmol/L)[a]	2.74 ± 0.86	2.63 ± 0.92	2.86 ± 0.79	0.063
HDL-C (mmol/L)[b]	1.56 ± 0.54	1.65 ± 0.65	1.48 ± 0.39	0.304
FBG (mmol/L)[b]	5.81 ± 1.50	5.78 ± 1.75	5.85 ± 1.20	0.005
Hypertension (n, %)[c]	115 (57.5)	54 (54.0)	61 (61.0)	0.317
Diabetes (n, %)[c]	33 (16.5)	18 (18.0)	15 (15.0)	0.568
Coronary artery disease (n, %)[c]	27 (13.5)	12 (12.0)	15 (15.0)	0.535
Stroke (n, %)[c]	32 (16.0)	19 (19.0)	13 (13.0)	0.247
Current smoking (yes, n, %)[c]	81 (40.5)	35 (35.0)	46 (46.0)	0.113
Current drinking (yes, n, %)[c]	105 (52.5)	51 (51.0)	54 (54.0)	0.671
Changes of blood pressure during saline loading and diuresis shrinkage stages				
ΔSBP_1 (mmHg)[b]	11.42 ± 11.74	18.02 ± 10.57	5.68 ± 9.51	<0.001
ΔDBP_1 (mmHg)[b]	−1.34 ± 7.03	3.64 ± 5.55	−5.67 ± 5.03	<0.001
ΔMAP_1 (mmHg)[b]	2.87 ± 7.27	7.62 ± 5.77	−1.88 ± 5.22	<0.001
ΔSBP_2 (mmHg)[a]	−5.35 ± 11.44	−8.78 ± 12.44	−2.35 ± 9.58	<0.001
ΔDBP_2 (mmHg)[a]	1.12 ± 7.10	−1.35 ± 7.86	3.27 ± 5.57	<0.001
ΔMAP_2 (mmHg)[b]	−1.12 ± 7.68	−3.64 ± 8.33	1.39 ± 6.03	<0.001

SS, salt-sensitive; SR, salt-resistant; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; BMI, body mass index; WHR, waist-to-hip ratio; TC, total cholesterol; TG, triglycerides; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; FBG, fasting blood glucose; ΔBP_1, changes of blood pressure during saline loading stage; ΔBP_2, changes of blood pressure during diuresis shrinkage stage.

[a] , Statistical testing by Student's t-test.

[b] , Statistical testing by Mann-Whitney *U* test.

[c] , Statistical testing by $\chi 2$ test.

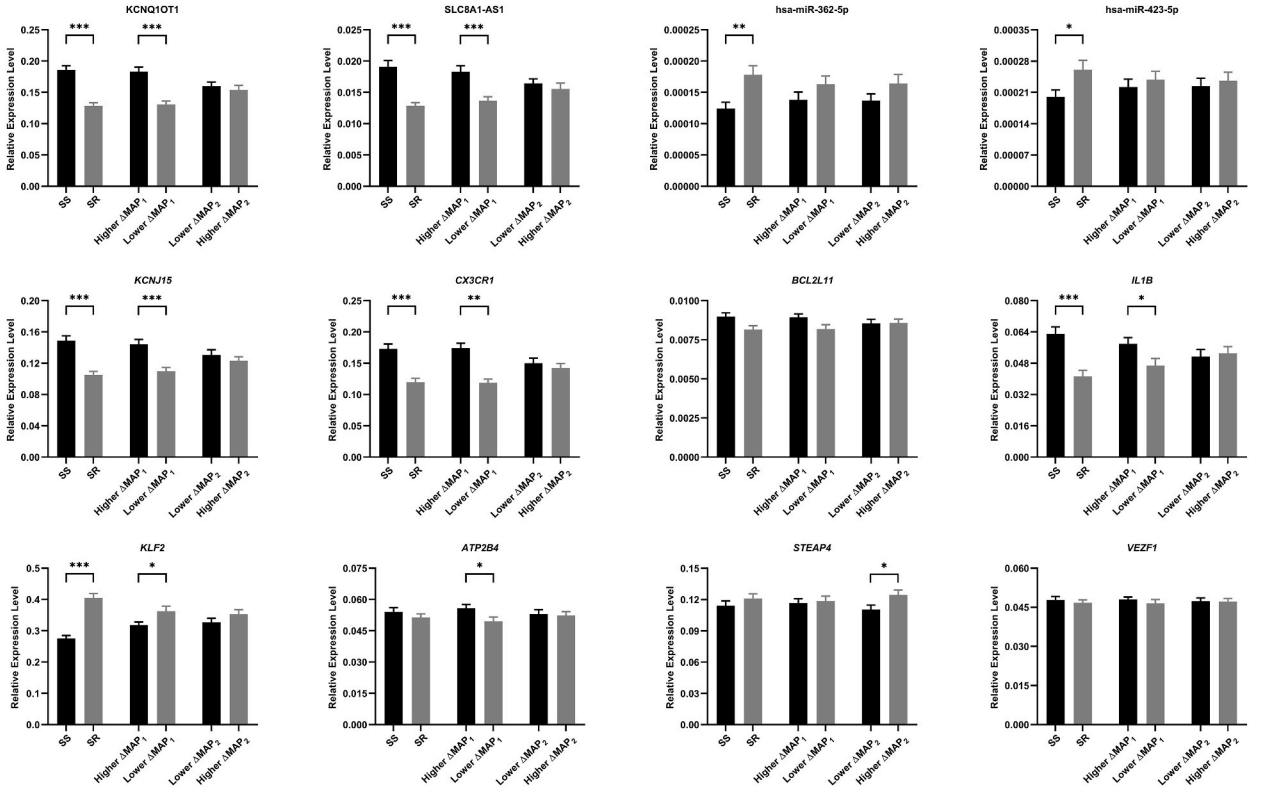

Fig. 4. The expression levels of RNAs between groups. SS, salt-sensitive; SR, salt-resistant; ΔMAP_1, change of mean blood pressure during acute sodium-volume repletion period; ΔMAP_2, change of mean blood pressure during acute sodium-volume depletion period; Higher $\Delta MAP_1/\Delta MAP_2$, individuals with $\Delta MAP_1/\Delta MAP_2$ larger than the median value; Lower $\Delta MAP_1/\Delta MAP_2$, individuals with $\Delta MAP_1/\Delta MAP_2$ lower than the median value. Error bars indicate the mean ± SD. Statistical testing by Student's *t*-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Table 5
Associations of RNAs with salt sensitivity of blood pressure.

Variables	SS[a]	ΔMAP_1[b] (mmHg)	ΔMAP_2[c] (mmHg)
	OR (95%CI)	β (95%CI)	β (95%CI)
KCNQ1OT1	6.156 (3.395, 12.038) ***	4.093 (2.602, 5.583) ***	−1.701 (−3.387, −0.015) *
SLC8A1-AS1	4.450 (2.616, 8.025) ***	3.827 (2.419, 5.234) ***	−1.204 (−2.782, 0.374)
hsa-miR-362–5p	0.577 (0.409, 0.796) **	−0.155 (−1.200, 0.900)	0.225 (−0.911, 1.360)
hsa-miR-423–5p	0.662 (0.479, 0.898) **	−0.096 (−1.117, 0.924)	−0.178 (−1.291, 0.934)
IL1B	3.394 (2.190, 5.553) ***	1.845 (0.673, 3.017) **	−0.446 (−1.702, 0.810)
BCL2L11	2.494 (1.257, 5.131) *	1.744 (−0.580, 4.068)	0.015 (−2.499, 2.528)
KCNJ15	4.030 (2.403, 7.142) ***	1.907 (0.551, 3.262) **	−0.468 (−1.939, 1.002)
CX3CR1	2.480 (1.682, 3.843) ***	1.648 (0.560, 2.737) **	−0.119 (−1.292, 1.054)
KLF2	0.141 (0.067, 0.274) ***	−1.717 (−3.314, −0.120) *	2.824 (1.089, 4.559) **
VEZF1	1.403 (0.629, 3.176)	0.595 (−2.217, 3.407)	0.605 (−2.440, 3.651)
STEAP4	0.826 (0.507, 1.320)	−0.521 (−2.169, 1.126)	1.602 (−0.128, 3.332)
ATP2B4	1.192 (0.754, 1.895)	1.301 (−0.323, 2.925)	0.689 (−1.081, 2.458)

SS, salt-sensitive; SR, salt-resistant; ΔMAP_1, change of mean blood pressure during acute sodium-volume repletion period; ΔMAP_2, change of mean blood pressure during acute sodium-volume depletion period. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

[a] , Statistical testing by multiple logistic regression analysis; adjusted for age, sex, high-density lipoprotein cholesterol and current smoking.

[b] , Statistical testing by multiple linear regression analysis; adjusted for age, sex, high-density lipoprotein cholesterol and triglycerides.

[c] , Statistical testing by multiple linear regression analysis; adjusted for age, sex, low-density lipoprotein cholesterol and total cholesterol.

correlated with mRNA. There were four ceRNA pathways (KCNQ1OT1/hsa-miR-362–5p/*IL1B*, SLC8A1-AS1/hsa-miR-362–5p/*KCNJ15*, KCNQ1OT1/hsa-miR-362–5p/KCNJ15 and KCNQ1OT1/hsa-miR-423–5p/IL1B) satisfied ceRNA theory and further validated with Dual-luciferase reporter assay.

KCNQ1OT1 might regulate the expression of IL1B by competitively sponging hsa-miR-362–5p and hsa-miR-423–5p.

Sequence information of predicted binding sites between miRNAs and 3′UTR region of target genes in wild-type and mutant

reporter vector were summarized in Table S5. Compared with the control group, co-transfection with GV272-KCNQ1OT1-WT vector and hsa-miR-362–5p or hsa-miR-423–5p mimics reduced luciferase reporter activity significantly (Fig. 5A and C). Similarly, the luciferase activity of GV272-*IL1B*-WT was reduced after hsa-miR-362–5p or hsa-miR-423–5p mimics treatment (Fig. 5B and D). These results indicated that KCNQ1OT1 might regulate the expression of *IL1B* by competitively sponging hsa-miR-362–5p and hsa-miR-423–5p. However, hsa-miR-362–5p mimics reduced the luciferase activity of GV272-SLC8A1-AS1-WT reporter but not GV272-*KCNJ15*-WT (Fig. 5E and F).

4. Discussion

This study constructed a SSBP-related ceRNA regulatory network containing 339 "lncRNA-miRNA-mRNA" pathways. The network might be involved in the pathogenesis of SS through endothelial dysfunction and inflammatory activation, and thirteen genes (including *IL1B* and *KCNJ15*, *etc*.) might play an important role. We identified nine SSBP-associated RNAs and formed nine ceRNA pathways, and four of them satisfied ceRNA theory. Further experimental verification of these four ceRNA pathways revealed that KCNQ1OT1 might regulate the expression of *IL1B* through sponge adsorption of hsa-miR-423–5p and hsa-miR-362–5p.

Up to now, the mechanism of ceRNA network related to hypertension has been reported [21,22,38]. There might be a large number of RNA crosstalk and ceRNA regulation processes in the potential SSBP pathogenesis. We previously constructed a *SS*-related ceRNA network containing six lncRNAs, six miRNAs and sixteen mRNAs in essential hypertensive patients, which contained 23 "lncRNA--miRNA-mRNA" ceRNA pathways [39]; However, the results are only suitable for hypertensive population, and focused on finding hub biomarkers from the network for salt-sensitive hypertension. In the current research, we further collected both hypertensive and normotensive participants and constructed a ceRNA network. The network contained two lncRNAs, 18 miRNAs and 73 mRNAs and was centered on KCNQ1OT1 and SLC8A1-AS1, involving 339 "lncRNA-miRNA-mRNA" pathways. In addition, to further study the ceRNA mechanism, we further performed the network analyses through bioinformatics and validation of ceRNA pathways by dual-luciferase reporter assay.

Previous studies reported that SS was resulted from several biological pathways [40]. The present study found that the constructed network participated in the regulation of nitric oxide biosynthetic process, cellular response to cytokine stimulus, negative regulation of cytokine production and cytokine-cytokine receptor interaction. Genes in the network involved with regulation of vascular endothelial growth factor receptor signaling pathway, potassium ion transport and so on. These results indicated that the network might participate in the pathogenesis of SS through multiple systems such as Endothelial system, Natriuretic peptide system, Ion and water channels, transporters, and exchangers.

We identified nine SSBP-associated RNAs (KCNQ1OT1, SLC8A1-AS1, *IL1B*, *BCL2L11*, *KCNJ15*, *CX3CR1*, *KLF2*, hsa-miR-362–5p and hsa-miR-423–5p) and they formed nine ceRNA pathways in total. Endothelial dysfunction plays a crucial role in SS pathogenesis [41], and KCNQ1OT1 is one of the important mediators in endothelial cell physiologic processes. KCNQ1OT1 was associated with the inflammation [42], proliferation and apoptosis [43] of vascular smooth muscle cells. SLC8A1-AS1 regulates *SLC8A1* expression [44], and SLC8A1 protein is also known as Na^+/Ca^{2+} exchanger protein 1 (NCX1). NCX1 is highly expressed in renal vascular smooth muscle, which may regulate arterial Ca^{2+} and vascular smooth muscle contraction through the mechanism of local regulation of Na^+ gradient, and participate in blood pressure regulation under sodium load [45]. Iwamoto et al. claimed that salt-sensitive hypertension is caused by Ca^{2+} entry through NCX1 in arterial smooth muscle, indicating that NCX1 could be a trigger of salt-sensitive hypertension [46].

IL1B is an important mediator of inflammatory response. Single nucleotide polymorphisms (SNPs) of *IL1B* have been reported to be related with hypertension [47,48]. For example, SNP (C-31 T) could increase risk of hypertension in populations of Japanese [49] and Polish [50]. *BCL2L11* participated in regulating the pulmonary microvascular endothelial cells apoptosis [51] and hypoxia/reoxygenation-induced renal tubular epithelial cell apoptosis and injury [52], suggesting that *BCL2L1*1 might affect SSBP through endothelial dysfunction. The protein encoded by *KCNJ15* is an integral membrane protein and inward-rectifier type potassium channel, which mainly enables potassium ions to enter cells. Increased plasma glucose induced *KCNJ15* expression, and *KCNJ15* overexpression influenced insulin secretion [53]. CX3CR1 is the homologous receptor of CX3CL1 chemokine. The mechanisms involving chemokines and their receptors in the pathogenesis of hypertension include the impact of the migration of macrophages and monocytes to the vascular wall and endothelial dysfunction [54]. CX3CR1 deficiency protects against hypoxia-induced pulmonary hypertension by modulating monocyte recruitment, macrophage polarization, and pulmonary artery smooth muscle cell proliferation [55]. KLF2 plays a role in many SSBP-related pathophysiological processes, including blood pressure regulation [56,57] endothelial function [58,59], inflammatory activation [60,61] and nitric oxide production [62,63].

Hsa-miR-423–5p and hsa-miR-362–5p were considered as hub miRNAs of the network. Hsa-miR-423–5p was associated with several CVDs, such as hypertension [64], myocardial infarction [65] and acute coronary syndrome [66]. MicroRNA-423–5p participated in glucolipid metabolism, hepatic miR-423–5p inhibition suppressed gluconeogenesis and improved insulin resistance, hyperglycemia, and fatty liver in obese diabetic mice [67]. Clinical studies also showed that the expression levels of circulating hsa-miR-423–5p altered in patients with metabolic syndrome [68] and severe obesity [69]. Studies on hsa-miR-362–5p were mostly focused on tumors, but we previously found that the expression of circulating hsa-miR-362–5p was down-regulated in salt-sensitive hypertensives compared with salt-resistant hypertensives [16], which was consistent with the result of this study.

According to the ceRNA theory [17,18], if lncRNA expression was up-regulated in SS, miRNA expression would be down-regulated and mRNA up-regulated. Both lncRNA and mRNA were negatively correlated with the target miRNA, and the expression levels of lncRNA and mRNA are positively correlated [70–72]. In the current study, four ceRNA pathways (KCNQ1OT1/hsa-miR-362–5p/IL1B, SLC8A1-AS1/hsa-miR-362–5p/KCNJ15, KCNQ1OT1/hsa-miR-362–5p/KCNJ15 and KCNQ1OT1/hsa-miR-423–5p/IL1B) satisfied this

Table 6
Correlations between RNAs in ceRNA pathways.

No.	lncRNA-miRNA-mRNA ceRNA pathway	miRNA-lncRNA	miRNA-mRNA	lncRNA-mRNA
1	SLC8A1-AS1/hsa-miR-362–5p/*BCL2L11*	−0.215*	−0.001	0.088
2	SLC8A1-AS1/hsa-miR-362–5p/*KCNJ15*	−0.215*	−0.221*	0.209**
3	KCNQ1OT1/hsa-miR-362–5p/*BCL2L11*	−0.218*	−0.001	0.199*
4	KCNQ1OT1/hsa-miR-423–5p/*BCL2L11*	−0.259*	−0.077	0.199*
5	KCNQ1OT1/hsa-miR-423–5p/*CX3CR1*	−0.259*	−0.072	−0.024
6	KCNQ1OT1/hsa-miR-362–5p/*IL1B*	−0.218*	−0.259*	0.218**
7	KCNQ1OT1/hsa-miR-423–5p/*IL1B*	−0.259*	−0.258*	0.218**
8	KCNQ1OT1/hsa-miR-362–5p/*KCNJ15*	−0.218*	−0.221*	0.234**
9	KCNQ1OT1/hsa-miR-423–5p/*KLF2*	−0.259*	0.173*	−0.269***

Statistical testing by Spearman correlation analysis. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

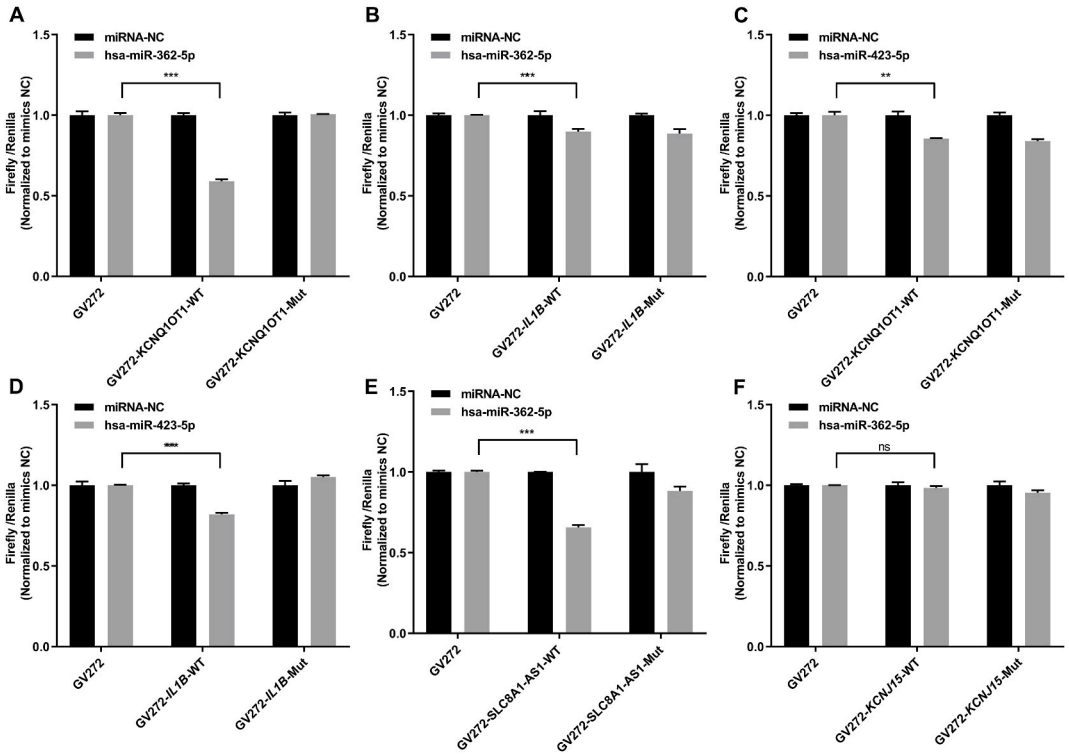

Fig. 5. Results of Dual Luciferase reporter assay. Luciferase activity was measured in 293 T cells co-transfected with GV272-KCNQ1OT1-WT or GV272-KCNQ1OT1-Mut and hsa-miR-362–5p mimic (A); GV272-*IL1B*-WT or GV272-*IL1B*-Mut and hsa-miR-362–5p mimic (B); GV272-KCNQ1OT1-WT or GV272-KCNQ1OT1-Mut and hsa-miR-423–5p mimic (C); GV272-*IL1B*-WT or GV272-*IL1B*-Mut and hsa-miR-423–5p mimic (D); GV272-SLC8A1-AS1-WT or GV272- SLC8A1-AS1-Mut and hsa-miR-362–5p mimic (E); GV272-*KCNJ15*-WT or GV272- *KCNJ15*-Mut and hsa-miR-362–5p mimic (F). WT, wild-type sequence; MUT, mutated sequence. Error bars indicate the mean ± SD. The results represent three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

theory. In addition, we further verified that KCNQ1OT1 serves as a miRNA sponge for hsa-miR-362–5p or hsa-miR-423–5p and regulates the expression of *IL1B* by dual luciferase reporter assay.

Some strengths and limitations of the current study should be acknowledged. First, all RNAs in the constructed ceRNA network were derived from experimental results of SS and SR populations. Second, we utilized two bioinformatic softwires for prediction during network construction, so that the prediction results are complementary and sufficient. Third, we performed network analysis, and found the potential biological functions of the network and the main mechanism affecting SSBP. Forth, the selected ceRNA pathways were validated experimentally at the population and cellular levels. The limitations are as follows, although the methods for determining SS are not uniform yet, the dietary intervention protocol is more accurate than the acute saline load test protocol; during the construction of ceRNA network, many RNAs were gradually removed from the construction of ceRNA network, so the SSBP-related RNA crosstalk may not be comprehensively analyzed; only a few "lncRNA-miRNA-mRNA" pathways were selected for verification; only dual luciferase reporter assay was adopted for verification, further gain and loss of function experiments are needed.

5. Conclusions

In conclusion, our study constructed a SSBP-related ceRNA network with lncRNAs as the core, which contains 339 "lncRNA-miRNA-mRNA" pathways. The network may affect the occurrence of SS through endothelial dysfunction and inflammatory activation and other pathogenic mechanisms. Furthermore, thirteen genes, including *IL1B*, *KCNJ15* and *KLF2*, may play important roles in SS pathogenesis. This study found nine ceRNA pathways were associated with SSBP and experimental verification showed that KCNQ1OT1 might sponge hsa-miR-362–5p and hsa-miR-423–5p, and affect the inhibition of miRNAs on *IL1B*, thus regulating the expression of *IL1B*.

Human subjects/informed consent statement

This study was complied with the ethical standards of the institutional research committee, the Helsinki Declaration of 1964 and its subsequent modifications, or comparable ethical standards. The study protocol was analyzed and approved by the Ethics Committee of the Capital Medical University (number: 2019SY016). All subjects in the study signed an informed consent.

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Availability of data and materials

The microarray data are available from the Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/) database under the accession number GSE135111. The other experimental data used and/or analyzed during the current study are available from the corresponding author on reasonable request.

CRediT authorship contribution statement

Wenjuan Peng: Data curation, Methodology, Writing - original draft, Investigation. **Yunyi Xie:** Investigation. **Juan Xia:** Investigation. **Han Qi:** Investigation. **Kuo Liu:** Investigation. **Bingxiao Li:** Investigation. **Fengxu Zhang:** Investigation. **Fuyuan Wen:** Investigation. **Ling Zhang:** Conceptualization, Investigation, Writing - review & editing.

Declaration of competing interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e22466.

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