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Comparative analysis of relative gene expression data reveals novel microRNAs in the artery of rats with renovascular hypertension

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Abstract: Hypertension is associated with impaired vascular endothelial function. However, the regulatory mechanisms of vascular dysfunction in rats with renovascular hypertension (RVH) remain poorly understood. In this study, the 2-kidney-1-clip (2K1C) hypertensive rat model was utilized. Next-generation sequencing was then used to detect microRNA (miRNA) expression profiling in the arteries of 2K1C rats. We identified 17 miRNAs that were differentially expressed in the 2K1C group compared with the sham group, of which 9 were downregulated and 8 were upregulated. These differentially expressed miRNAs were found to be associated with immune/inflammatory and metabolic pathways, which are involved in vascular dysfunction. Treatment with losartan maintained the expression of the differentially expressed miRNAs, miR-31a-5p and miR-142-3p, and the levels of TNF-α, IL-1β, IL-6 and MCP-1, indicating that the differentially expressed miRNAs and their associated immune/inflammatory pathways play a pivotal role in the modulation of the vascular dysfunction in 2K1C rats. Our study provides valuable information about miRNA expression in the arteries of 2K1C rats, expanding our understanding of the complex molecular mechanisms underlying the vascular dysfunction in rats with RVH.

Keywords: renovascular hypertension; 2-kidney-1-clip; vascular endothelial function; microRNA expression profiling; losartan

INTRODUCTION

Hypertension, a major risk factor of cardiovascular diseases, remains one of the most important global health problems worldwide [1]. Renovascular hypertension (RVH) is considered a secondary form of hypertension. It is well documented that the activation of the renin-angiotensin-aldosterone system (RAAS) plays a critical role in the pathophysiology of RVH [2]. Studies over several decades have identified multiple actions of angiotensin II (Ang II), including vasoconstriction, the release of aldosterone, retention of sodium and sympathetic adrenergic stimulation [3]. Ang II also plays a key role in the immune response and the propagation of inflammation and subsequently contributes to the progression of arterial hypertension and vascular remodeling [4]. The 2-kidney-1-clip (2K1C) model has been proposed as an experimental model of RVH, and is characterized by elevated arterial pressure and plasma renin activity heavily dependent upon Ang II-mediated pressor effects [5].

RVH induced an alteration of vascular function, including endothelial dysfunction, vascular inflammation, arterial remodeling and atherosclerosis. Although the putative mechanisms of hypertension-mediated vascular disease are complex, important causes involve RAAS activation, oxidative stress, inflammatory responses and immune activation [6]. Moreover, it is well accepted that microRNAs (miRNAs) are participants in a key mechanism in the development of vascular complications of hypertension [7]. Several studies have established that the cardiovascular complications of hypertension are related to specific miRNAs, including miR-21, miR-181a, miR-221, miR-222, miR-143 and miR-145 [8-10]. However, knowledge about the miRNA expression profile in the artery of 2K1C rats is sparse, and the mechanism whereby miRNAs and their associated pathways affect vascular function in rats with RVH is unknown.

In this study, we performed miRNA sequencing to investigate the gene regulatory pathways associated with

vascular dysfunction in 2K1C rats. Our results provide valuable information on the mechanisms of miRNA-mediated vascular function regulation in 2K1C rats.

MATERIALS AND METHODS

Animal experiments

Male Wistar rats aged 7 weeks were acquired from the Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All rats were housed in a temperature-controlled room (21±1°C) under a 12 h light-dark cycle. Animal care and experimental procedures were approved by the Local Animal Care and Ethics Committee at Baotou Medical College. Animals were divided randomly into three groups (n=6/group) as follows: sham group, 2K1C group, losartan group. The renal artery clipping procedure was described in detail previously [11]. In brief, rats were anesthetized with pentobarbital sodium (50 mg/kg body weight by intraperitoneal injection). The left renal artery was isolated and clipped with a silver clip (0.2 mm internal gap). Sham animals underwent identical manipulations except that the renal artery was not clipped. The losartan group received oral losartan at 10 mg/kg per day. The sham and 2K1C groups received normal saline as the vehicle. The treatments were initiated on day 3 after the 2K1C operation. Bodyweight and blood pressure were assessed as described previously [11]. At week 8 after the operation, rats were killed for specimen collection under deep anesthesia induced by pentobarbital sodium.

RNA isolation, small RNA sequencing and analysis

Total RNAs were isolated from thoracic aorta using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's RNA isolation procedure. The quality and concentration of total RNA were determined with a NanoDrop2000 (Thermo Scientific, USA). Each RNA library was prepared from about 1 μg of total RNA using the TruSeq Small RNA Sample Prep Kit (Illumina, San Diego, CA, USA). In brief, the isolated small RNAs (sRNAs) were ligated to 3'- and 5'-adapters using T4 RNA ligase (Epicenter, San Diego, CA, USA). The adapter-ligated sRNAs were converted to cDNA and amplified by PCR. PCR-amplified fragments were extracted and purified by polyacrylamide-gel electrophoresis for the construction of sequencing libraries. Finally, sRNA sequencing (single-end, 50 bp) was performed on the HiSeq 3000 platform (Illumina, San Diego, California) according to the manufacturer's instructions. After generating the sequence reads, the raw reads were preprocessed to remove adapters and junk sequences. Only reads with a length of 15 to 40 nucleotides (nt) were considered. Subsequently, the sequences matching miRNA, rRNA, tRNA, small nuclear RNA (snRNA), small nucleolar RNA (snoRNA) and repeat sequences were classified by alignment to NCBI databases, Rfam (http://rfam.janelia.org) and Repbase (http://www.girinst.org/repbase) using Bowtie software (LC Sciences, Houston, TX, USA). Known miRNAs were identified by performing a BLASTN search against the miRBase (http://www.mirbase.org). The unannotated clean reads were aligned to the reference genome for novel miRNAs prediction using miRDeep2.

Different expression and function enrichment analysis

To understand the differential expression of known and novel miRNAs, the frequency of miRNA read counts were normalized to transcripts per million (TPM) as follows: TPM = actual miRNA count/total count of clean reads × 1000000. The expression level of transcripts was estimated by calculating fragments per kilobase of exon per million fragments mapped (FPKM) using Cufflinks software. Genes with a P-value of <0.05 and $\log_2$-fold change (FC) ≥1 were considered significant. Gene Ontology (GO) annotation and the Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were performed with the genes. The false discovery rate (FDR) was calculated to adjust the P value, which when below 0.05 was considered as statistically significant.

Quantitative real-time RT-PCR

The expression of miRNAs was determined by quantitative real-time RT-PCR (qRT-PCR) analysis, which was performed on the ABI 7500 PCR System using the SYBR assay according to the manufacturer's instructions (Applied Biosystems, Waltham, MA, USA). Primers for qRT-PCR were obtained from Applied Biosystems. The relative expression of miRNAs was calculated by the $2^{-\Delta\Delta Ct}$ method after normalization with housekeeping gene U6 small nuclear RNA (snRNA) [12].

Measurements of cytokines in plasma

Mononuclear chemotactic protein-1 (MCP-1), interleukin (IL)-1β, IL-6 and tumor necrosis factor-α (TNF-α) were measured using ELISA (Thermo Scientific, USA), according to the manufacturer's instructions. The results are determined based on the standard curve derived from cytokine standards.

Statistical analysis

Statistical analysis was performed with SPSS software (ver. 20.0). Data comparisons were analyzed using the independent t-test or paired t-test. Data were expressed as the mean ± standard error of the mean (SEM). The differences were considered statistically significant when the P-value was below 0.05.

RESULTS

Establishment of the 2K1C hypertensive model

The 2K1C hypertensive rat experimental model was produced by clipping the left renal artery. Compared to the sham group, there was a significant increase in systolic blood pressure (SBP) in 2K1C rats after eight weeks of surgery (P<0.05). The SBP was significantly increased after 8 weeks compared to the initial value (0 weeks) in the 2K1C group (P<0.05) (Fig. 1). There was no significant difference in body weight between the three groups (P>0.05) (Supplementary Table S1).

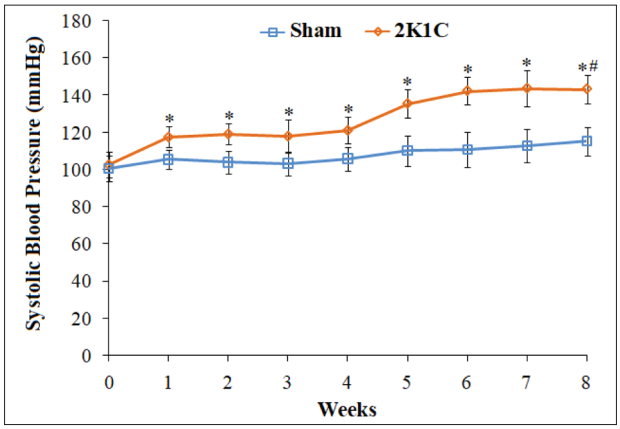

Fig. 1. Development of SBP in the 2K1C group and sham group. *P<0.05, vs sham groups. #P<0.05, vs 0 weeks.

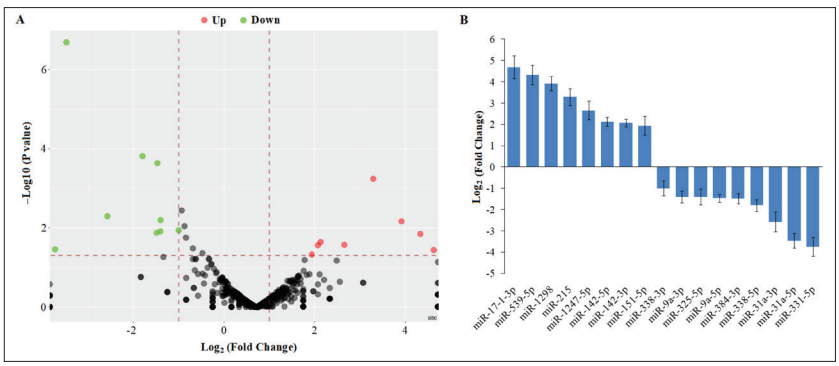

Fig. 2. Differential expressions of miRNAs. **A** – Distribution of differentially expressed genes based on log2 fold-change values. **B** – Differential expression levels of 17 miRNAs.

Identification of differentially expressed miRNAs

Sequencing analysis of sRNA yielded 12854259 raw reads from sham samples and 11562065 raw reads from 2K1C samples, respectively. After filtering the low-quality reads, adaptors and sequence reads (length < 15 nt or > 40 nt), a total of 12290413 and 9954919 clean reads with lengths ranging from 18 to 40 nt were obtained, which represent 444233 and 948333 unique small RNAs in sham samples and 2K1C samples, respectively. All unique sRNA clean reads were compared with the mature miRNAs in the miRbase. The results showed that a total of 296 types of mature miRNAs were present in both sham and 2K1C samples. We further identified 99 novel miRNAs from the remaining sequences with miRDeep2 software. We compared the expression values of miRNAs between sham and 2K1C samples (Fig. 2). The results showed that 17 known miRNAs were significantly differentially expressed (P<0.05), of which 8 were significantly upregulated and 9 were significantly downregulated (Fig. 2).

Target prediction and functional analysis of differentially expressed miRNAs

A total of 35 and 34 transcripts were predicted to be targets of downregulated and upregulated miRNAs, respectively (Supplementary Fig. S1). Our results further showed the top 10 significantly enriched GO terms related to biological process, molecular function and

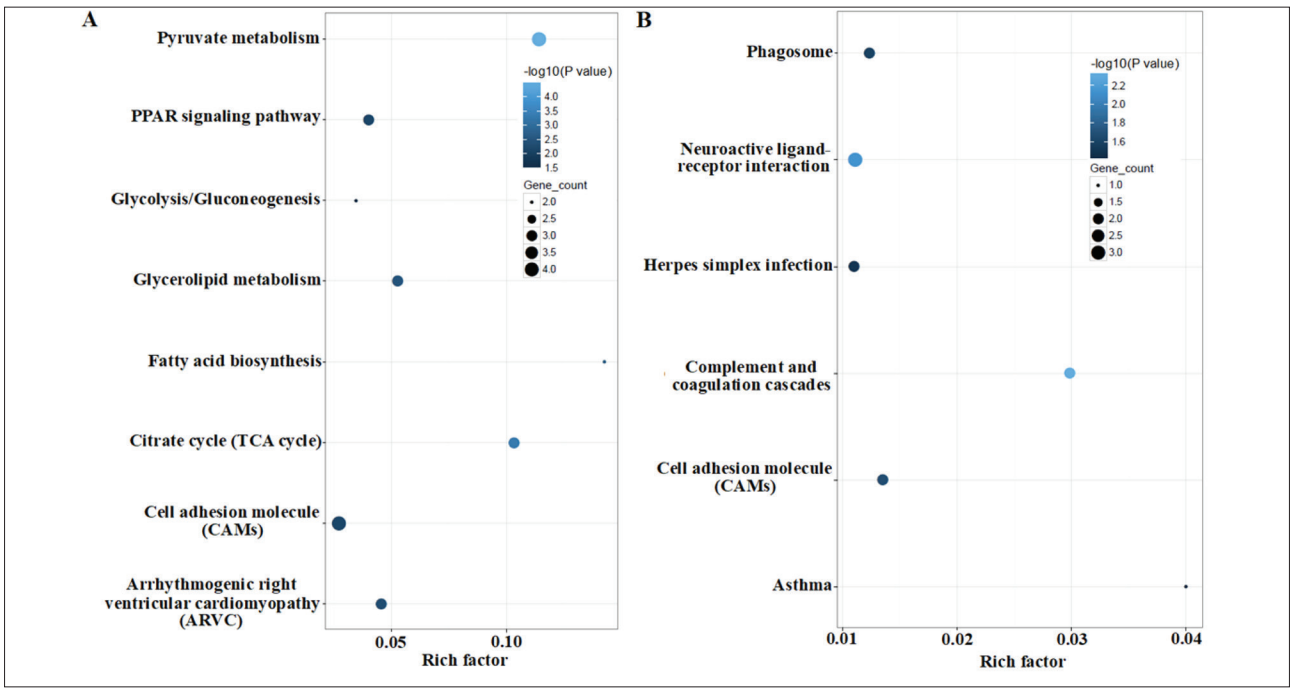

Fig. 3. KEGG pathway of enrichment analysis of miRNA-target genes. **A** – Upregulated miRNAs. **B** – Downregulated miRNAs.

cellular component, respectively, which were associated with the target genes of differentially expressed miRNAs in 2K1C and sham groups (Supplementary Fig. S2). In 2K1C rats, functional categories linked to various immune processes (immune system process, negative regulation of alpha-beta T cell proliferation, negative regulation of leukocyte activation, leukocyte activation) were highly enriched compared to sham rats. To further understand their biological functions, they were mapped to the KEGG pathways. In 2K1C rats, pathways involved in different metabolic pathways (pyruvate metabolism, TCA cycle, glycerolipid metabolism, glycolysis/gluconeogenesis, peroxisome proliferator-activated receptor (PPAR) signaling pathway), biosynthesis and immune-inflammatory responses (complement and coagulation cascades, cell adhesion molecules, and phagosome) were significantly enriched (Fig. 3).

Verification of sequencing data by qRT-PCR

Two miRNAs, including one downregulated miRNA (miR-31a-5p) and one upregulated miRNA (miR-142-3p) were selected for qRT-PCR analysis (Fig. 4A). These results were consistent with those of the analysis of sequencing data.

Treatment with losartan affects miRNA levels

The expression of miR-31a-5p was decreased significantly in the 2K1C group when compared with the sham group ($P<0.05$) (Fig. 4B), while treatment with the Ang II receptor antagonist losartan abolished the hypertension-mediated decrease in aortic miR-31a-5p expression. Similarly, losartan maintained the expression of aortic miR-142-3p ($P<0.05$).

Treatment with losartan affects cytokine concentrations in plasma

Higher levels of MCP-1, IL-1β, IL-6 and TNF-α were observed in 2K1C animals when compared to *sham rats* ($P<0.05$) (Fig. 5), however, in the losartan-treated group, cytokines and chemokine levels were similar to those observed in the sham group ($P>0.05$) (Fig. 5).

DISCUSSION

*H*igh blood pressure due to renal artery disease (RVH) can lead to vessel injury. Identification of miRNAs and their potential targets in the vessels of hypertensive rats will lay the foundation for the elucidation of the

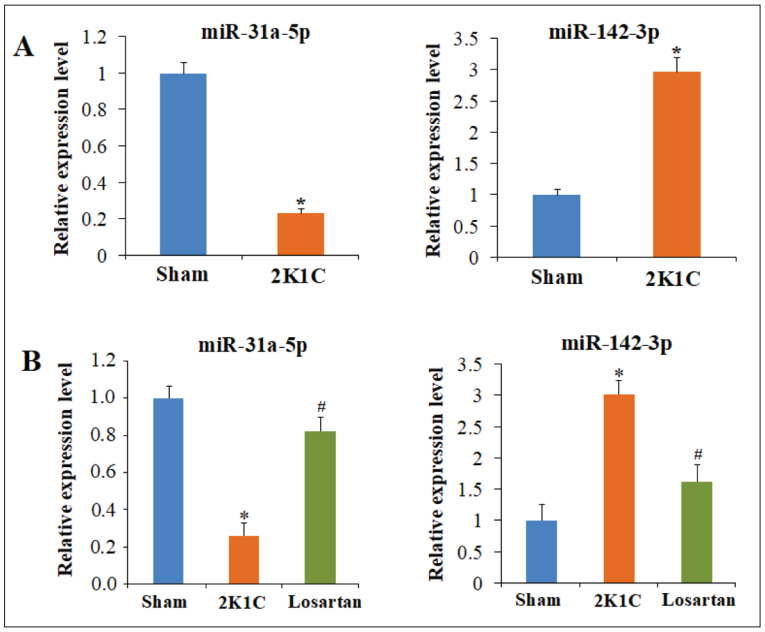

Fig. 4. Expression of miR-31a-5p and miR-142-3p. **A** – qRT-PCR validation. **B** – Effects of losartan on the expression of miR-31a-5p and miR-142-3p. *P<0.05, vs sham group. #P<0.05 vs the 2K1C group.

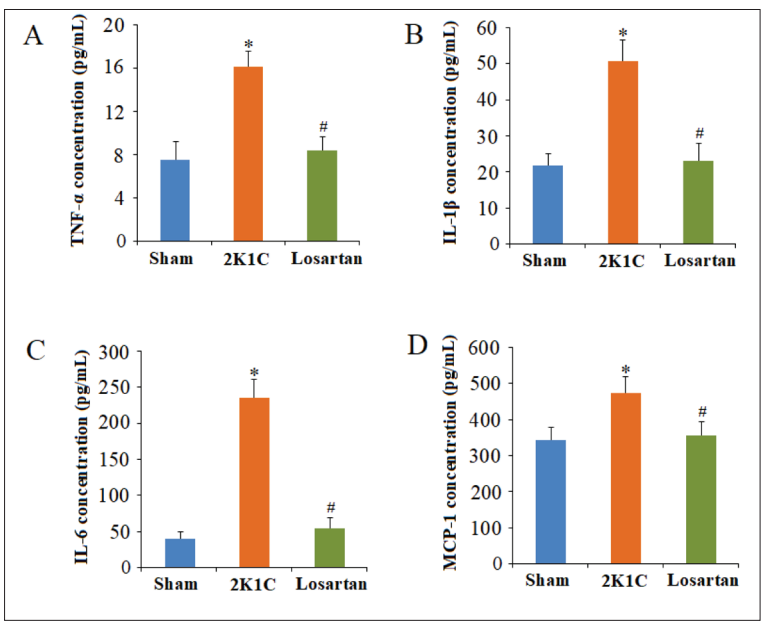

Fig. 5. Effects of losartan on cytokine levels in plasma. **A** – TNF-α; **B** – IL-1β; **C** – IL-6; **D** – MCP-1. *P<0.05, vs sham group. #P<0.05 vs the 2K1C group.

biological roles of miRNAs. Here, we investigated for the first time the expression of miRNAs in the arteries of 2K1C rats, and provide insight into the regulatory mechanisms of miRNAs on vascular function.

Previous studies demonstrated that vascular function is impaired in 2K1C rats [13]. To determine the involvement of regulatory miRNAs in the vascular function of rats with *RVH*, we constructed and sequenced sRNA libraries. Data analysis revealed that the sRNA length distribution was between 14 and 41 nt with a peak at 22 nt. It is well known that miRNAs are approximately 22 nt in length and bind directly to the complementary target in mRNA transcripts to regulate gene expression. Therefore, miRNAs might play a certain role in influencing the vascular function of 2K1C rats. In total, 296 known miRNAs and 99 putative novel miRNAs were detected in both groups, and 17 miRNAs were differentially expressed. We found that 9 miRNAs were significantly downregulated, including miR-9a-5p, miR-9a-3p, miR-31a-5p, miR-31a-3p, miR-331-5p, mir-338-5p, mir-338-3p, miR-325-5p, and miR-384-3p. It has been reported that miR-31a mediates inflammation [14], and miR-331-5p and mir-338 might be crucial regulators in the pathogenesis of artery disease [15,16]. Also, it has been reported that miRNA-384-3p participated in the process of neovascularization [17]. Therefore, these miRNAs might be important in regulating gene expression associated with the mediation of vessel function, consistent with previous observations. However, miR-325-5p was downregulated in our study, whereas it was observed to be upregulated in a rat model with diabetic atherosclerosis [18]. This may be because of the different modulation mechanisms of vascular injury between hypertensive rats and diabetic rats. Moreover, we further identified 8 miRNAs that were upregulated, including miR-17-1-3p, miR-142-5p, miR-142-3p, miR-151-5p, miR-215, miR-539-5p, miR-1247-5p and miR-1298. These findings were consistent with those reported in previous studies [19-24], which revealed that these miRNAs play important roles in vascular function. GO and KEGG-pathway enrichment analysis of predicted

target genes further confirmed that these differentially expressed miRNAs are involved in the immunological inflammatory reactions and metabolisms.

Evidence showed that the gene transcripts, such as Brms1 and Itga8, play an important role in vascular biology, in cell growth, proliferation and inflammation [25,26]. They are target genes of miR-31a-5p and miR-142-3p, respectively. In the present study, losartan, an Ang II receptor blocker, maintained the expression of miR-31a-5p and miR-142-3p *in vivo*, suggesting the involvement of both miRNAs in the modulation of vascular function.

We also validated the inhibitory effects of losartan on the concentrations of cytokines in plasma, including TNF-α, IL-1β, IL-6 and MCP-1. It is well known that these inflammatory markers are increased in 2K1C hypertensive animals and may contribute to vascular inflammation [27]. These findings further confirmed the important role of immunological inflammatory signaling in the pathogenesis of the vascular dysfunction in 2K1C rats, which is consistent with the data of comparative analysis of relative miRNAs expression described above.

CONCLUSIONS

The analysis of sRNA sequencing of arteries isolated from 2K1C rats revealed novel miRNAs. We identified 17 differentially expressed miRNAs. Comparative analysis of target genes of differentially expressed miRNAs revealed that alterations in the pathways associated with immune-inflammatory responses and metabolisms might be involved in vascular dysfunction observed in 2K1C rats. The altered miRNA expression and immunological inflammatory signaling in the aorta can be reversed, at least in part, by treating rats with losartan. Our findings reveal the potential mechanism of miRNA-mediated vascular function regulation in rats with RVH. Further studies will be needed to elucidate the cellular origins of the miRNAs.

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Author contributions: HY conducted the study, collected the data, performed the analysis of the data, and prepared the manuscript; HH conducted the study, collected the data, and performed the analysis of the data; ZW designed and supported the study and edited the manuscript. All authors read and approved the final version of the manuscript.

Conflicts of interest disclosure: The authors declare that they have no competing interests.

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Supplementary Material

The Supplementary Material is available at: http://serbiosoc.org.rs/NewUploads/Uploads/Yu%20et%20al_5814_Supplementary%20Material.pdf