Identification of crucial miRNAs and the targets in renal cortex of hypertensive patients by expression profiles

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ABSTRACT

Backgrounds: Defect in kidney is one major reason of hypertension. The study aimed to uncovering the regulatory mechanisms of miRNAs and the targets in hypertensive kidney.

Methods: Gene expression profile of GSE28345 and miRNA expression profile of GSE28283 were downloaded from GEO database. After data preprocessing, differently expressed genes (DEGs) and miRNAs (DE-miRs) were identified using limma package. Then targets of miRNAs were predicted according to information in relevant databases. Function and pathway enrichment analyses were performed for DEGs using DAVID software. Furthermore, protein–protein interaction (PPI) networks were constructed for up- and down-regulated genes, respectively, using the Cytoscape. Additionally, for down-regulated DEGs, the integrated regulatory network was established combining PPI network with the miRNA–mRNA interactions.

Results: As a result, 285 DEGs were identified, including 177 up-regulated and 108 down-regulated genes. Combined with the predicted targets of miRNAs, 22 up-regulated DE-miRs were identified. In the integrated network for down-regulated DEGs, three crucial nodes were identified as ASPN, COL12A1, and SCN2A. ASPN was predicted as target of miR-21 and miR-374b, and COL12A1 was the target of miR-30e, miR-21, and miR-195, while SCN2A was the target of miR-30e, miR-374b, and miR-195. Notably, COL12A1 and ASPN were linked with each other in the network.

Conclusion: Three crucial genes were identified in hypertensive kidney, such as COL12A1, ASPN, and SCN2A. ASPN might co-function with COL12A1, and they both might be the targets of miR-21. SCN2A might be a novel target of miR-30e and miR-374b. However, more experiments are needed to validate these results.

Introduction

Hypertension is the major risk factor that leads to cardiovascular disease with high mortality and morbidity. It is often accompanied with numerous complications such as stroke, heart failure, and kidney diseases. In the United States, although the management and control of hypertension have been dramatically improved from 1999 to 2010, the prevalence remains as stable as 30% among these years.

The endothelial dysfunction characterized by decreased synthesis of nitric oxide (NO) is a hallmark of hypertension. As the main extracellular cation, sodium retention in the extracellular could decrease the synthesis of NO. On the contrary, high plasma potassium, the main intracellular cation, has a beneficial effect on endothelial cells’ softness and could activate NO release. Therefore, it is recommended in the prevention of hypertension to keep the low-level of plasma sodium and high-level of potassium. It is theorized that hypertension is caused by a primary defect in the kidney. Kidney has a potent effect on the control of sodium excretion via the renin–angiotensin system (RAS), which was supposed to be the critical mechanism for blood pressure regulation. Therefore, it is urgent to investigate alterations in hypertensive kidneys.

Currently, various approaches including gene candidate analysis and genome wide search have been introduced into the identification of genetic components of hypertension, and several candidate genes were identified, such as SPON1, GDF-15, and STK39. However, gene alterations related to hypertensive kidney are rarely reported.

MicroRNAs (miRNAs) are small non-coding RNAs that could bind to complementary sequences of its targets...
and thereby result in the mRNAs’ degradation and the translation inhibition. They are key regulators for gene expressions. Reportedly, hcmv-miR-UL112 is associated with the increased risk of hypertension. Increased circulating miRNA-34a is detected in hypertensive patients with different liver diseases. Despite these profound findings, the molecular mechanisms of hypertension, especially kidney-related hypertension, remain obscure.

A recent study using microarray technology identifies differentially expressed genes (DEGs) and miRNA (DE-miRs) between kidneys of hypertensive patients and normotensive controls. As a result, the study finds 46 DEGs and 13 DE-miRs in the renal cortex between the two kinds of samples, and gene expressions, such as APOE, SLC13A1, and CD36; and miRNA expressions, such as hsa-miR-21, hsa-miR-181a, and hsa-miR-663 are validated. Nevertheless, the criteria for DEGs selection are not rigorous because the detailed fold change is not provided. In addition, interactions among the DEGs from protein level are not considered. Therefore, we reanalyzed their data by combining the gene expression profile GSE28345 with the miRNA expression profile GSE28283, to take full advantage of this microarray data and thereby uncover the potential gene interactions at protein level and the miRNA–mRNA regulations, thus to provide novel insights into the pathology of hypertension and potential biomarkers for the prognosis.

Methods

Microarray data

The gene expression profile GSE28345 and the miRNA expression profile GSE28283 were all obtained from the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo, Bethesda, MD) database. Samples of the two profiles were the same, and they were from the renal cortex tissues. There were a total of eight samples: five were from hypertensive male patients (hypertensive samples) and three from normotensive male patients (control samples). The profile of GSE28345 was based on the platform of GPL6244 [HuGene-1.0-st] Affymetrix Human Gene 1.0 ST Array, and GSE28283 based on the GPL10850 Agilent-021827 Human miRNA Microarray (V3) (miRBase release 12.0 miRNA ID version) (Agilent Technologies, Palo Alto, CA).

Preprocessing of the two profiles and selection of DEG or DE-miRs

Raw data of the gene expression profile was normalized by Robust Multichip Average (RAM) algorithm, and the samples were divided into hypertensive group and control group. Then, Linear Models for Microarray Analysis (limma, http://www.bioconductor.org/packages/release/bioc/html/limma.html) of Bioconductor R was recruited to identify the differently expressed genes (DEGs). The thresholds were |log 2 fold change (FC)| > 0.58 and p values <.05.

For miRNA profile, the raw data was interpreted by limma package to obtain signal intensity, and then were undergone the normexp background correction and quartile normalization. Thereafter, t-test in limma package was utilized to identify DE-miRs between two groups. The cutoff values were |log 2 FC| ≥ 1 and p values <.05.

The prediction of target genes of miRNA

Databases including miRanda (http://mirdb.org/miRDB), PicTar (http://pictar.mdc-berlin.de), PITA, TargetScan (www.targetscan.org) and MirTarget2 were searched to identify target genes of miRNAs. The interplayed miRNA–mRNAs that were recorded in at least three of the above databases were selected. Considering that miRNAs always regulate mRNA’s degradation and translation inhibition by binding to 3’ UTR of the mRNA, we extracted the interaction information between the corresponding transcripts (RefseqID) and miRNAs to reveal the interactions of miRNA–target.

The correlation analysis between differently expressed mRNAs and miRNAs

In the identified transcript–miRNAs, the interactions with reverse expressions were screened out. Subsequently, the transcript RefseqID was converted into gene symbols.

Functional enrichment analyses of DEGs

The Gene ontology (GO, http://www.geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/pathway.html) pathway enrichment analyses were performed utilizing the Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.abcc.Ncifcrf.gov/) online software, to recognize potential biological processes and pathways that DEGs were involved in. The cutoff values for the significant function and pathways were p values <.05 and the count (gene number that enriched in a specific function or pathway term) ≥ 2.

Construction of protein–protein interaction (PPI) network

By mapping the DEGs into Search Tool for the Retrieval of Interacting Genes (String, http://string-db.org/)
database, the interaction relationships among DEGs at protein level were screened. Then, the protein–protein interaction (PPI) networks for up- and down-regulated DEGs were constructed under the criterion of combine score >0.4, respectively. The Cytoscape software was used to visualize the networks. We focused the down-regulated DEGs that might be down-regulated by the miRNAs, and the integrated regulatory network was constructed combining the PPI network of down-regulated genes with the miRNA–mRNA interaction. The connectivity degrees were calculated through network statistical methods.

Results
Selection of DEGs and DE-miRs
According to the screening criteria, a total of 731 differently expressed transcripts were identified in the renal cortex tissues of hypertensive patients. Among them, 459 were up-regulated, corresponding to 177 DEGs; while 272 were down-regulated, corresponding to 108 DEGs.

Through the comparison of 859 known miRNAs between two groups, 22 up-regulated miRNAs in hypertensive group were identified. No down-regulated miRNAs were detected.

The predicted target genes of miRNAs
Based on the interactions between miRNAs and the target transcripts, and the differently expressed miRNAs, the reversely expressed miRNA–transcript interactions were recognized. For all the identified miRNAs were up-regulated, only the down-regulated mRNAs were chosen to predict the miRNA–mRNA interaction. As a result, 23 transcripts were selected and converted into corresponding genes symbols, and the relevant miRNA–mRNA interactions were identified (Table 1).

The function and pathways of the DEGs
For the up-regulated DEGs, KEGG pathway analyses indicated that they were significantly enriched in four pathways such as arachidonic acid metabolism (e.g., CYP2J2, CYP2B6, and GPX3), metabolism of xenobiotics by cytochrome P450 (MOXBCP) (e.g., UGT1A7, UGT1A10, and UGT1A6), maturity onset diabetes of the young (e.g., HNF1A, SLC2A2, and PKLR) and Drug metabolism (DGM) pathways (e.g., UGT1A7, UGT1A10, and UGT1A6) (Table 2). Notably, genes enriched in MOXBCP and DGM pathways were identical. Meanwhile, GO analysis indicated that these up-regulated DEGs were mainly involved in transmembrane transport process and diverse metabolic processes of cofactor, triglyceride, acylglycerol, neutral lipid, and glycerol ether (Table 3).

On the other hand, the down-regulated DEGs were primarily enriched in MOXBCP and DGM pathways, in which three genes of GSTM1, GSTM3, and ADH1B were all enriched (Table 2). Additionally, function of the down-regulated DEGs were significantly associated with regulation of tube size, regulation of blood vessel size, vascular process in circulatory system, chemical homeostasis, and inorganic anion transport (Table 3).

| microRNA    | RefseqID         | Gene symbol |
|-------------|------------------|-------------|
| hsa-mir-128 | NM_001029854     | PDE8B       |
| hsa-mir-128, hsa-mir-132, hsa-mir-195, hsa-mir-30e | NM_153619 | SEMA6D     |
| hsa-mir-128 | NM_153617        | SEMA6D      |
| hsa-mir-128, hsa-mir-132, hsa-mir-195, hsa-mir-30e | NM_153618 | SEMA6D      |
| hsa-mir-128, hsa-mir-342-3p | NM_172069 | PLEXH12  |
| hsa-mir-132, hsa-mir-223 | NM_022842 | CDCP1      |
| hsa-mir-132, hsa-mir-194, hsa-mir-223, hsa-mir-30e, hsa-mir-374b | NM_052832 | SLC26A7   |
| hsa-mir-132, hsa-mir-215, hsa-mir-30e | NM_015236 | LPHN3      |
| hsa-mir-152 | NM_000222        | KIT         |
| hsa-mir-152 | NM_023037        | FRY         |
| hsa-mir-195, hsa-mir-21, hsa-mir-30e | NM_004370 | COL12A1    |
| hsa-mir-195 | NM_014900        | COBL1       |
| hsa-mir-21, hsa-mir-374b | NM_017680 | ASPN        |
| hsa-mir-223 | NM_144710        | Sep10       |
| hsa-mir-28-5p | NM_032034 | SLC4A11    |
| hsa-mir-30e, hsa-mir-378 | NM_020927 | VAT1L      |
| hsa-mir-30e, hsa-mir-374b | NM_021998 | ZNF711     |
| hsa-mir-342-3p | NM_030925 | CEBPRL     |
| hsa-mir-374b | NM_005398 | PPP1R3C    |
| hsa-mir-374b | NM_002667 | PLN         |
| hsa-mir-374b | NM_006882 | FGL2        |
| hsa-mir-425 | NM_002160        | TNC         |
Moreover, combined with the interactions of miRNA–mRNA, function of miRNAs was inferred according to that of mRNAs. As a result, the functions of miRNAs of seven targets were enriched. PDE8B (targeted by hsa-miR-128) was significantly enriched in cyclic nucleotide catabolic process and nucleoside monophosphate catabolic process; SLC26A7 (targeted by hsa-miR-132 and hsa-miR-223), SCN2A (targeted by hsa-miR-132, hsa-miR-194, hsa-miR-195, hsa-miR-223, hsa-miR-30e, and hsa-miR-374b), and SLC4A11 (targeted by hsa-miR-374b and hsa-miR-21, and COL12A1, predicted as targets of multiple miRNAs. As revealed in the integrated network (Figure 2), ASPN was the target of miRNA-374b and miRNA-21, and COL12A1 was the target of miRNA-30e, miRNA-21, and miRNA-195, while SCN2A was the target of miRNA-30e, miRNA-374b, and miRNA-195. Notably, ASPN and COL12A1 were linked with each other in the integrated network.

**Discussion**

Although hypertension is a chronic disease, it is an important factor for cardiovascular disease and a major cause for mortality. In the present study, based on the analyses of microarray data, three down-regulated DEGs, ASPN, COL12A1, and SCN2A, were identified and predicted as targets of multiple miRNAs. As revealed in the integrated network (Figure 1), the hub nodes (degrees >5) were CYP2B6 (15), CYP2J2 (12), CES2 (10), ABO (9), APOE (7), and APOH (6). For down-regulated DEGs, the integrated network of miRNAs and DEGs was established, combining with the interactions of miRNA-mRNA. In this integrated network, there were 49 nodes and 54 interactions, and ASPN, COL12A1 and SCN2A were three hub nodes. In addition, key miRNAs like hsa-miR-30e, hsa-miR-374b, hsa-miR-128, hsa-miR-195, hsa-miR-132, and hsa-miR-223 were identified.
be highly expressed in subtype three tumors.\textsuperscript{26} 
\textit{COL12A1} is identified as the only collagen gene that up-regulated almost two fold in the \textit{db/db} diabetic nephropathy mouse model, compared with normal mice.\textsuperscript{27} Moreover, a recent study reveals that \textit{COL12A1} is up-regulated in salt-induced hypertensive rat.\textsuperscript{28} As a class II secreted leucine-rich proteoglycans (SLRP), \textit{ASPN} encodes a cartilage extracellular protein that could bind collagen and calcium.\textsuperscript{29} In response to venous hypertension, \textit{ASPN} is found to be significantly downregulated in varicose saphenous veins, compared with normal saphenous veins (NSV) (expression value: 40.0 versus 207.1).\textsuperscript{30} However, most studies demonstrate \textit{ASPN} is associated with osteoarthritis.\textsuperscript{31,32} \textit{ASPN} was firstly implicated in renal cortex of hypertensive patients based on our study, suggesting it might be a novel gene marker for hypertensive kidney prognosis. Both of \textit{ASPN} and \textit{COL12A1} are extracellular matrix (ECM) genes. Although there are no reports about their correlations, they are simultaneously down-regulated in mouse models and patient cells.\textsuperscript{33} In addition, \textit{COL6A3}, one family member of \textit{COL12A1}, is also linked to \textit{ASPN} in the sub-networks in early phase of Duchenne muscular dystrophy, based on the expression profile of GSE6011.\textsuperscript{34} These collectively provide potent evidence that \textit{ASPN} might function synergistically with \textit{COL12A1}, as predicted in our integrated network (Figure 2).

Regarding to the targeting interactions with miRNA, \textit{COL12A1} is reported as the target of miR-300-5p in adult rat cardiac fibroblasts\textsuperscript{35} and the target of hsa-miR-16 in human obesity.\textsuperscript{36} In the present study, \textit{COL12A1} was predicted as the target of hsa-miR-21, hsa-miR-30e, and hsa-miR-195. Reportedly, miR-21 is one of the miRNAs that induced by Angiotensin II. It is enriched in cardiac fibroblasts and could promote cardiac fibrosis via increasing expression of MMP2 in fibroblast.\textsuperscript{35} Commonly, aberration of miR-21 links to development of various cancer types, and involves in numerous cellular process, such as proliferation, apoptosis and metastasis.\textsuperscript{37,38} In addition, \textit{COL12A1} is identified as one novel target of miR-26b in carcinoma-associated fibroblasts of breast cancer.\textsuperscript{39} However, no targeting relationships between \textit{COL12A1} and miR-21, and miR-195 are reported yet. Interestingly, in renal mesangial cells,
**COL12A1** is suggested as a potential target of miR-30e further supporting our prediction of the targeting of hsa-miR-30e and COL12A1. For the targeting relationships between **ASPN** and miRNAs, **ASPN** is predicted as the target of miR-21 in hippocampus after traumatic brain injury. Considering that **COL12A1** was also predicted as the target of miR-21 but no validation and other prediction have been reported, it might be inferred the targeting of miR-21 on **COL12A1** might be via the mediation of **ASPN**. However, more validation experiments are required.

Deletion of angiotensin II type 1 receptors could result in the disruption of epithelial sodium transport in proximal tubule, and then cause blood pressure alteration. The sodium channels, voltage-gated alpha subunit gene **SCN2A** is considered as one candidate for hypertension detection. However, based on current studies, this gene is mostly related to neurological disorders. Reportedly, mutations in **SCN2A** could cause seizures and epilepsy. With regard to the targeting miRNA and this gene, it is predicted as the target of miR-132, one of the neuronal activity-dependent miRNAs. Unfortunately, there are few studies reported potential targeting of miR-30e or miR-374b and this gene, as predicted in our study. This suggests that **SCN2A** might be a novel target of these miRNAs in renal cortex of hypertensive patients. Likewise, we need extensive experiments to validate the findings in our study.

Although we provided more regulatory information than the study of Marques, there remained a limitation that all the predictive results need to be validated via substantial experiments. Nevertheless, our study is still of great value and provide novel insight into regulatory mechanisms on hypertensive kidney.

In conclusion, our results identified three crucial genes in hypertensive kidney, such as **COL12A1**, **ASPN**, and **SCN2A**. Among them, **ASPN** was linked to **COL12A1** and they were both targets of miR-21. **SCN2A** might be the target of miR-30e and miR-374b. However, these regulatory relationships need to be further validated.

**Disclosure statement**

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.
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