Scientific Research Publishing

Health, 2024, 16, 173-186
https://www.scirp.org/journal/health
ISSN Online: 1949-5005
ISSN Print: 1949-4998

Identification and Validation of Novel Biomarkers Related to the Calcium Metabolism Pathway in Hypertension Patients Based on Comprehensive Bioinformatics Methods

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How to cite this paper: Chang, X.G., Guo, L., Zou, L.Y., Ma, Y.Z. and Feng, J.L. (2024) Identification and Validation of Novel Biomarkers Related to the Calcium Metabolism Pathway in Hypertension Patients Based on Comprehensive Bioinformatics Methods. *Health*, **16**, 173-186.
https://doi.org/10.4236/health.2024.163015

Received: February 6, 2024
Accepted: March 15, 2024
Published: March 18, 2024

Abstract

Background: Hypertension is a universal risk factor for cardiovascular diseases and is thus the leading cause of death worldwide. The identification of novel prognostic and pathogenesis biomarkers plays a key role in disease management. **Methods:** The GSE145854 and GSE164494 datasets were downloaded from the Gene Expression Omnibus (GEO) database and used for screening and validating hypertension signature genes, respectively. Gene Ontology (GO) enrichment analysis was performed on the differentially expressed genes (DEGs) related to calcium ion metabolism in patients with hypertension. The core genes related to immune infiltration were analyzed and screened, and the activity of the signature genes and related pathways was quantified using gene set enrichment analysis (GSEA). The infiltration of immune cells in the blood samples was analyzed, and the DEGs that were abnormally expressed in the clinical blood samples of patients with hypertension were verified via RT-qPCR. **Results:** A total of 176 DEGs were screened. GO showed that DEGs was involved in the regulation of calcium ion metabolism in biological processes (BP), actin mediated cell contraction, negative regulation of cell movement, and calcium ion transmembrane transport, and in the regulation of protease activity in molecular functions (MF). KEGG analysis revealed that the DEGs were involved mainly in the cGMP-PKG signaling pathway, ubiquitin-protein transferase, tight junction-associated proteins,

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and the regulation of myocardial cells. MF analysis revealed the immune infiltration function of the cells. RT-qPCR revealed that the expression of Cacna1d, Serpine1, Slc8a3, and Trpc4 was up regulated in hypertension, the expression of Myoz2 and Slc25a23 was down regulated. **Conclusion:** Cacna1d, Serpine1, Slc8a3, Trpc4, Myoz2 and Slc25a23 may be involved in the regulation of calcium metabolism pathways and play key roles in hypertension. These differentially expressed calcium metabolism-related genes may serve as prognostic markers of hypertension.

Keywords

Hypertension, Biomarkers, Differentially Expressed Genes, Ca^{2+} Metabolism, Bioinformatics Analysis

1. Introduction

Hypertension is a worldwide health problem and one of the common causes of death. High blood pressure is often an underlying and progressive process, so it is often difficult to diagnose in a timely manner [1]. Its pathogenesis is complex and includes genetic susceptibility and the influence of environmental factors [2]. In addition, studies have shown that hypertension is associated with the development of cardiovascular diseases, which increase the risk of cardiovascular diseases and their complications and may also lead to cognitive and functional decline in elderly individuals [3]. However, by lowering blood pressure to a target level, or "controlling" high blood pressure with lifestyle changes and medication, the risk of adverse outcomes from high blood pressure can be greatly reduced [4]. Although drug treatment has been used for a long time, in many cases, patients' blood pressure still cannot reach the ideal range [5].

In recent years, the rapid development of bioinformatics technology has provided important support for the identification of biomarkers and the identification of hub genes in the module of clinical significance [6]. Through comprehensive analysis of high-throughput data, bioinformatics and machine learning analyses can aid in understanding the molecular mechanisms of this disease and screening key genes [7]. These methods have played key roles in transcriptomic, metabolomic and proteomic studies, facilitating the search for disease driver genes, discovery of drug targets, classification of tumor subtypes, discovery of molecular markers, drug sensitivity screening, etc., and querying and predicting protein and RNA interactions [8] [9] [10].

This study aimed to provide basic insights into the etiology and related molecular basis of hypertension by analyzing the differentially expressed genes (DEGs) through comprehensive bioinformatics. Our study aimed to elucidate the role of calcium ion metabolism-related genes in the pathogenesis of hypertension and to create a strategy for potential drug prediction.

2. Materials and Methodology

2.1. Sample Collection

We collected blood samples from 51 non-hypertensive people (Age: 9 - 99; Male: n = 25; Female: n = 26) and 51 hypertensive patients (Age: 43 - 88; Male: n = 27; Female: n = 24) in Yangbi Yi Autonomous County People's Hospital. The study was conducted with the informed consent of the patients and with the approval of the Ethics Committee of Yangbi Yi Autonomous County People's Hospital.

2.2. RT-qPCR

According to the manufacturer's instructions, we used a magnetic bead method and total RNA extraction kit (G3611-50T; Servicebio, China) to extract total RNA from blood and a SweScript RT I first-strand cDNA synthesis kit (G3331-50; Servicebio, China) for cDNA synthesis. In subsequent experiments, we used SYBR Green qPCR Master Mix (G3320-01; Servicebio, China) for RT-qPCR. The RT-qPCR amplification reaction program was as follows. First, the amplification reaction was performed at 95˚C for 10 min, followed by 40 cycles of amplification at 95˚C for 15 s and amplification at 60˚C. The amplification reaction was performed at 60˚C for 30 s. For the quantitative analysis of gene expression levels, we used GAPDH as an internal reference gene and the $2^{-\Delta\Delta Ct}$ method to calculate the relative expression level. The sequences of primers used were as follows: (Table 1).

Table 1. Primer sequences.

Genes	Sequence (F: Forward primer, R: Reversed primer)
Cacna1d	F: 5'-AAATCCAAACTCAGCCGAC-3' R: 5'-AAACGTGACAGACTTCACG-3'
Cbarp	F: 5'-GCCCCAUTTGTCACCAT-3' R: 5'-CTGATCTCTGCGAAGTCAGTG-3'
Ccl19	F: 5'-CCTGCTGTAGTGTTCACCA-3' R: 5'-TCTGGATGATGCGTTCTACC-3'
Serpine 1	F: 5'-TTCAAGTTGATGACAGGGC-3' R: 5'-CTCATCCTTGTTCCATGGC-3'
Slc8a3	F: 5'-GATGGGAAAGCCAGTATTGG-3' R: 5'-TCCACCGTAGTCTTGAACTC-3'
Trpc4	F: 5'-CATTTGTAAGTACAGTGCCC-3' R: 5'-CAAATAAAGCCTCTGCCAC-3'
Gt2i	F: 5'-TGCAAAGGAAAGGATTCGT-3' R: 5'-CTTCCTTTACTCCTGAAGCTG-3'
Myoz2	F: 5'-TGCTCCAGGATATTCTGGAC-3' R: 5'-GATTGATAGTACTTAGGGACAGC-3'
Nos1ap	F: 5'-GGATACGGTATGAGTTAUGC-3' R: 5'-TCACTTTCACTCCATCCAC-3'
Slc25a23	F: 5'-TCTACGAGACTCTGAAGUACTG-3' R: 5'-CTGGATATGGTACCGCAGG-3'
Spink 1	F: 5'-TTTCTTCTCAGTGCCTTGG-3' R: 5'-CATTGTAACATTTGGCCTCTC-3'
GAPDH	F: 5'-TCAAGATCATCAGCAATGCC-3' R: 5'-CGATACCAUAGTTGTCATGGA-3'

2.3. Determination of DEGs

When using R software for statistical analysis and visualization, we used the GEOquery package to download the hypertension-related datasets GSE145854 and GSE164494 from the GEO database (https://www.ncbi.nlm.nih.gov/geo/). GSE145854 using the GPL17117 platform, we describe the transcriptome sequencing results of Wistar Kyoto (WKY) Rats and Spontaneously Hypertensive Rats (SHR) thoracic aorta, where we used 3 SHR samples as hypertensive samples and 3 WKY samples as normal controls. GSE164494 using the GPL21273 platform and the Ang II-induced hypertensive mouse model, we used 5 wild-type mice treated with 500 ng/kg/min AngII infusion as hypertensive samples and 5 wild-type mice treated with saline infusion as normal controls. During data processing, we excluded the case in which one probe corresponded to multiple molecules and when multiple probes corresponding to the same molecule were encountered, we retained only the probe with the maximum signal value. Next, we analysed the differences between the two samples using the limma package and normalised them using the DESeq2 package. The threshold for identifying DEGs was set at a $P < 0.05$. We created volcano plots and heatmaps to visualize the expression patterns of the DEGs. To further understand the relationships between different gene sets, we performed overlap analysis using the Venny 2.1 tool (https://bioinfogp.cnb.csic.es/tools/venny/index.html). This approach helped to reveal common and unique gene sets that were biologically important in our study.

2.4. Gene Ontology (GO) Analysis

We used R software for GO enrichment analysis, and the cluster Profiler package was used for GO annotation analysis of the DEGs, which included Molecular Function (MF), Cellular Component (CC), and Biological Process (BP) terms. In the KEGG pathway enrichment analysis, we also used the cluster Profiler package, and a significance level of $P < 0.05$ was used as the standard for statistical significance.

2.5. Gene Set Enrichment Analysis (GSEA)

Genome enrichment analysis (GSEA) was performed using R software to identify biological pathways and functions associated with phenotype groupings. Specifically, the gene expression profile and phenotype grouping data were organized into an appropriate format, the risk characteristic-related biological pathway and functional analysis of the DEGs were performed using the cluster Profiler package. To investigate the association of differential DEGs with relevant signalling pathways and biological processes in hypertension, the gene set database was selected as "M2: curated gene sets (CP: Canonical pathways)". The minimum gene set was 5, the maximum gene set was 5000, the resampling was 1,000 times, the significance thresholds were set to $P < 0.05$ and FDR < 0.25, and the enrichplot package was used to visualize the GSEA results.

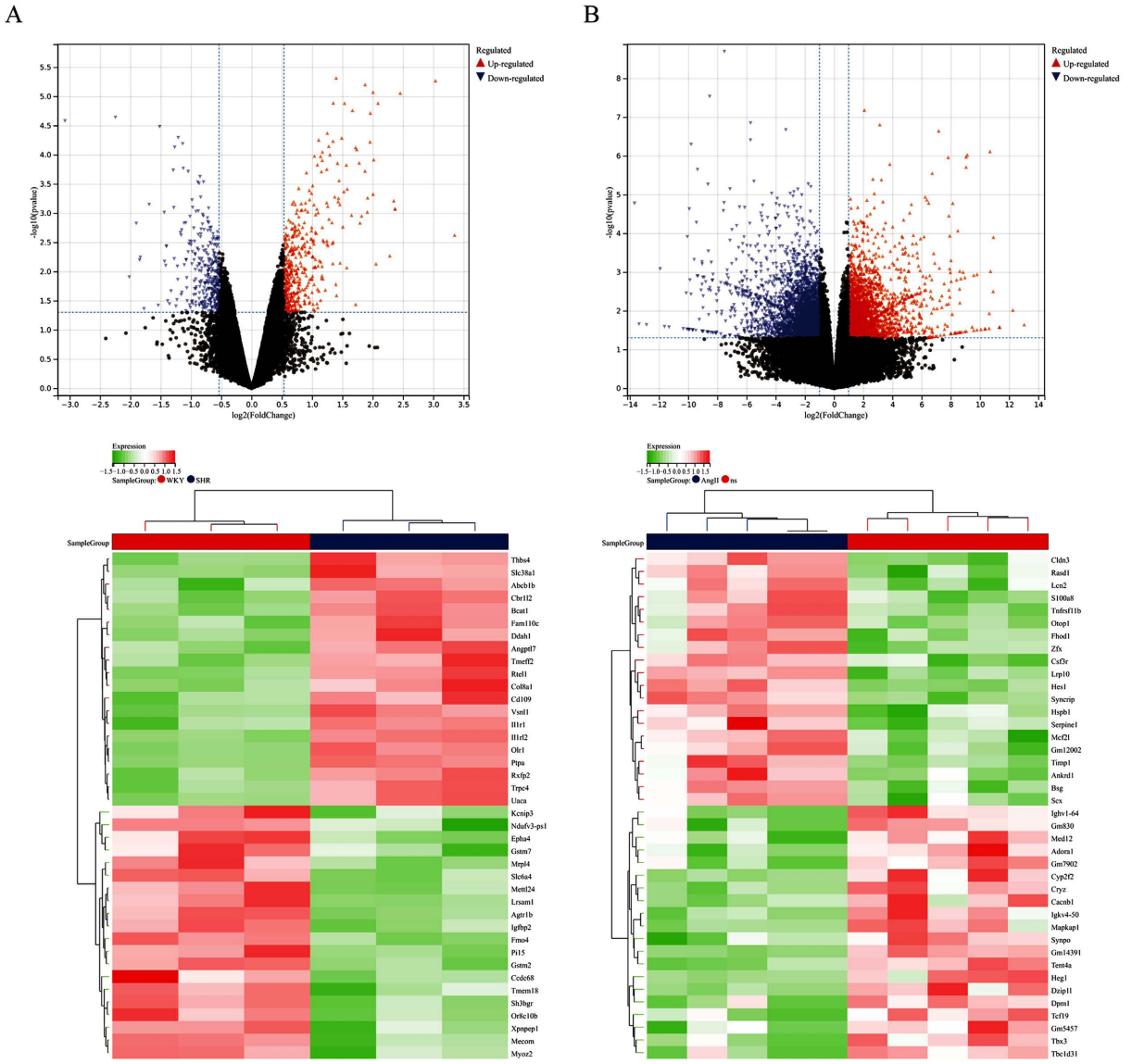
2.6. Immune Infiltration Assay

Immune infiltration analysis was performed using R software, and the gene expression data of each sample were compared with those of immune cells through the mMCP-counter package to assess the infiltration level of immune cells.

3. Results

3.1. DEGs that were Significantly Differentially Expressed

According to the search terms and retrieval conditions, the GSE145854 and GSE164494 datasets were downloaded from the GEO database. Among them, GSE145854 had 570 upregulated DEGs and 425 downregulated DEGs (**Figure 1(A)**), and GSE164494 had 2278 upregulated DEGs and 2301 downregulated DEGs (**Figure 1(B)**). In addition, 176 overlapping DEGs were identified in the 2 datasets using Venny 2.1 (**Figure 1(C)**).



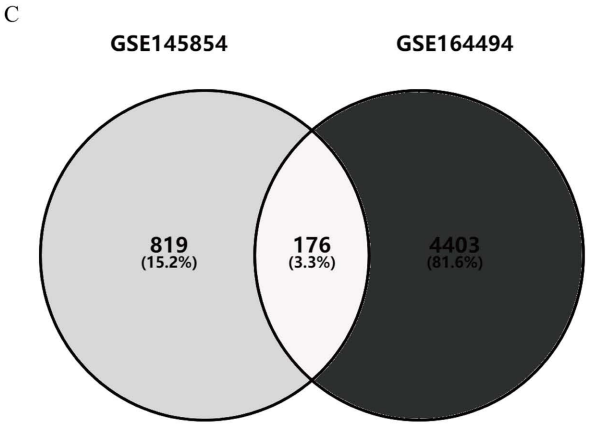
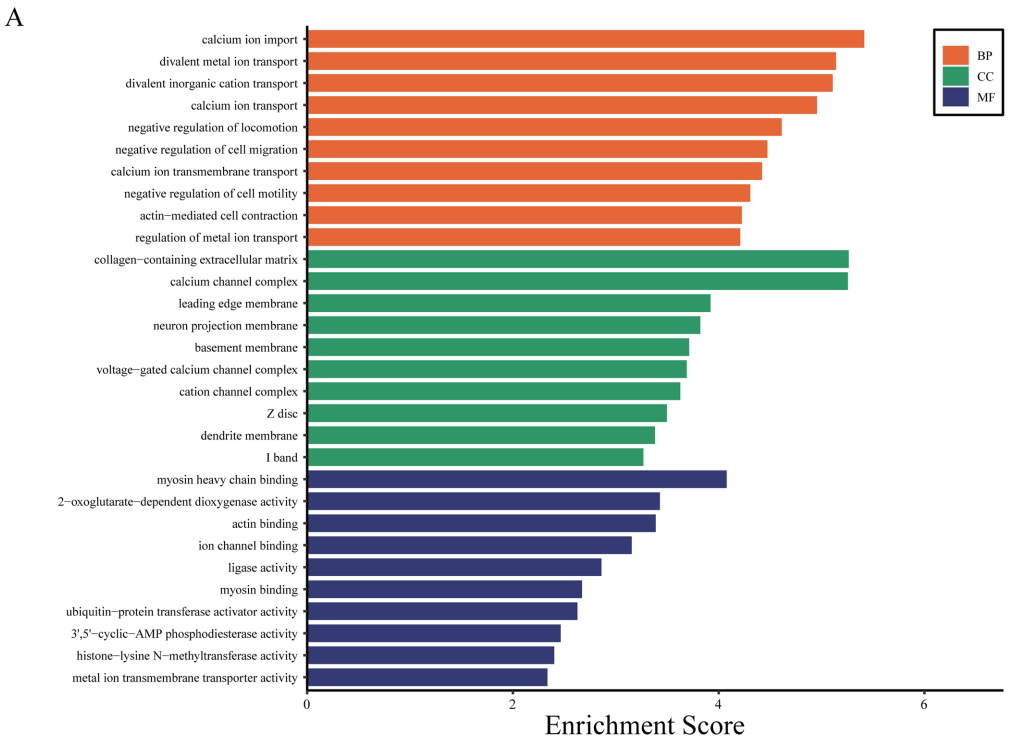
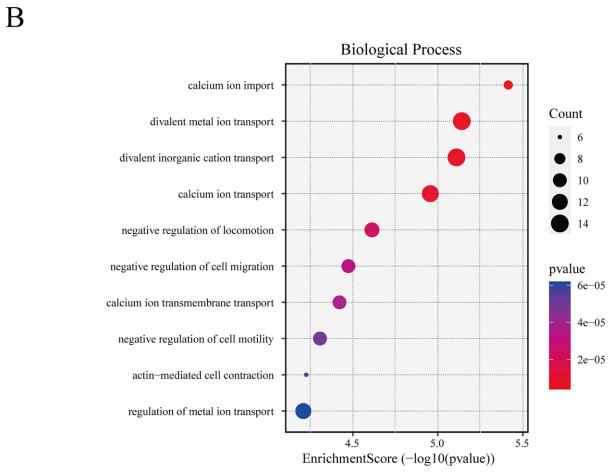
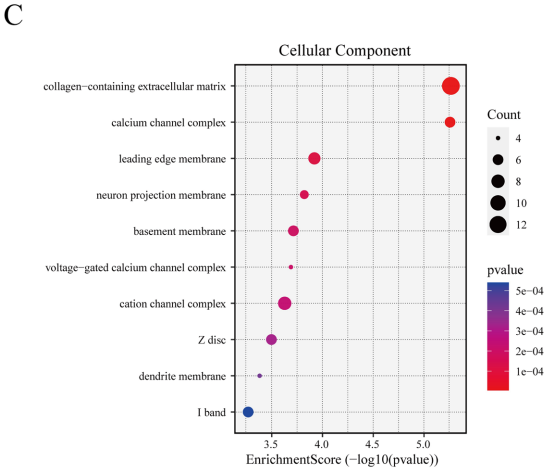


Figure 1. Significantly differentially expressed DEGs. (A) Volcano map; (B) Heatmap; (C) Dataset intersection DEGs.







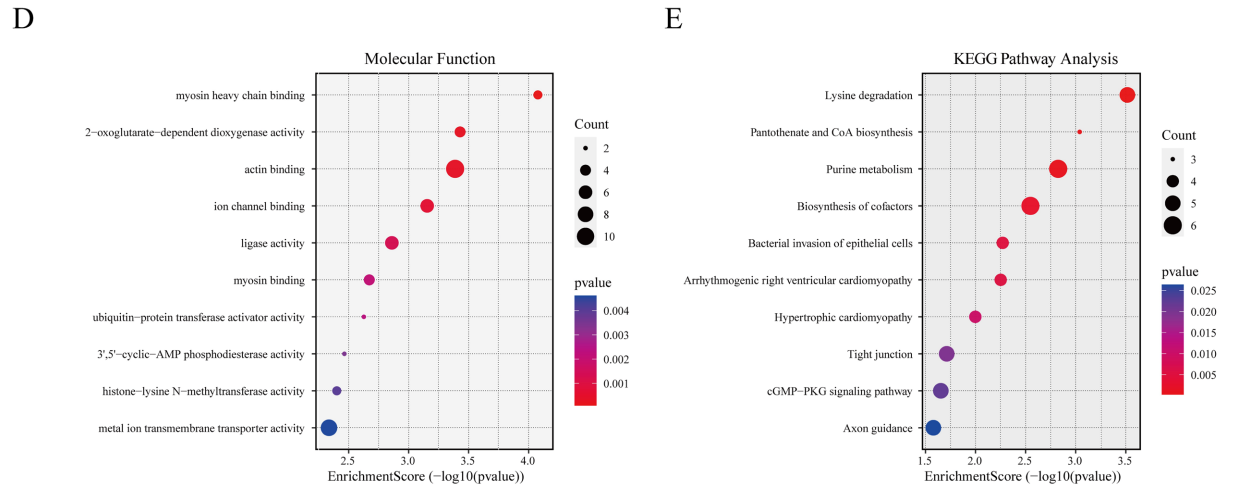


Figure 2. Enrichment analysis. (A) GO enrichment analysis; (B) Biological Process; (C) Cellular Component; (D) Molecular Function; (E) KEGG pathway.

Table 2. Key genes involved in calcium ion metabolism.

name	logFC	
	GSE164494	GSE145854
Cacna1d	2.767629029	1.067715
Cbarp	1.038753192	0.396428
Ccl19	3.583316657	0.475507333
Gt2i	−1.855870609	−0.307862667
Myoz2	−1.654615041	−0.877018667
Nos1ap	−1.097176652	−0.553486333
Serpine 1	1.432787915	0.951215
Slc25a23	−2.905241285	−0.714465
Slc8a3	2.344115167	0.775163333
Spink 1	−2.262386725	−0.563688333
Trpv6	−1.099406098	0.303186

3.2. Enrichment Analysis

To demonstrate the hypertension-related functional annotation and pathway enrichment, GO function and KEGG pathway analyses were performed on the DEGs. The GO annotation of DEGs consisted of three parts: biological process (BP), cellular component (CC) and molecular function (MF). The functional enrichment of the DEGs was analyzed. The DEGs were involved mainly in the regulation of calcium ion transmembrane transport, the immune response, cell physiological functions, cell migration, and cytokine production (**Figures 2(A)-(D)**). The results of KEGG analysis showed that these genes were mainly associated with the interactions of the cGMP-PKG signaling pathway, ubiquitin-protein transferase, tight junction-related proteins and the regulation of myocardial cells (**Figure 2(E)**). The intersection DEGs were significantly associated with calcium ion metabolism and were simultaneously enriched in other metabolic pathways. The related key genes are listed in the table below (**Table 2**).

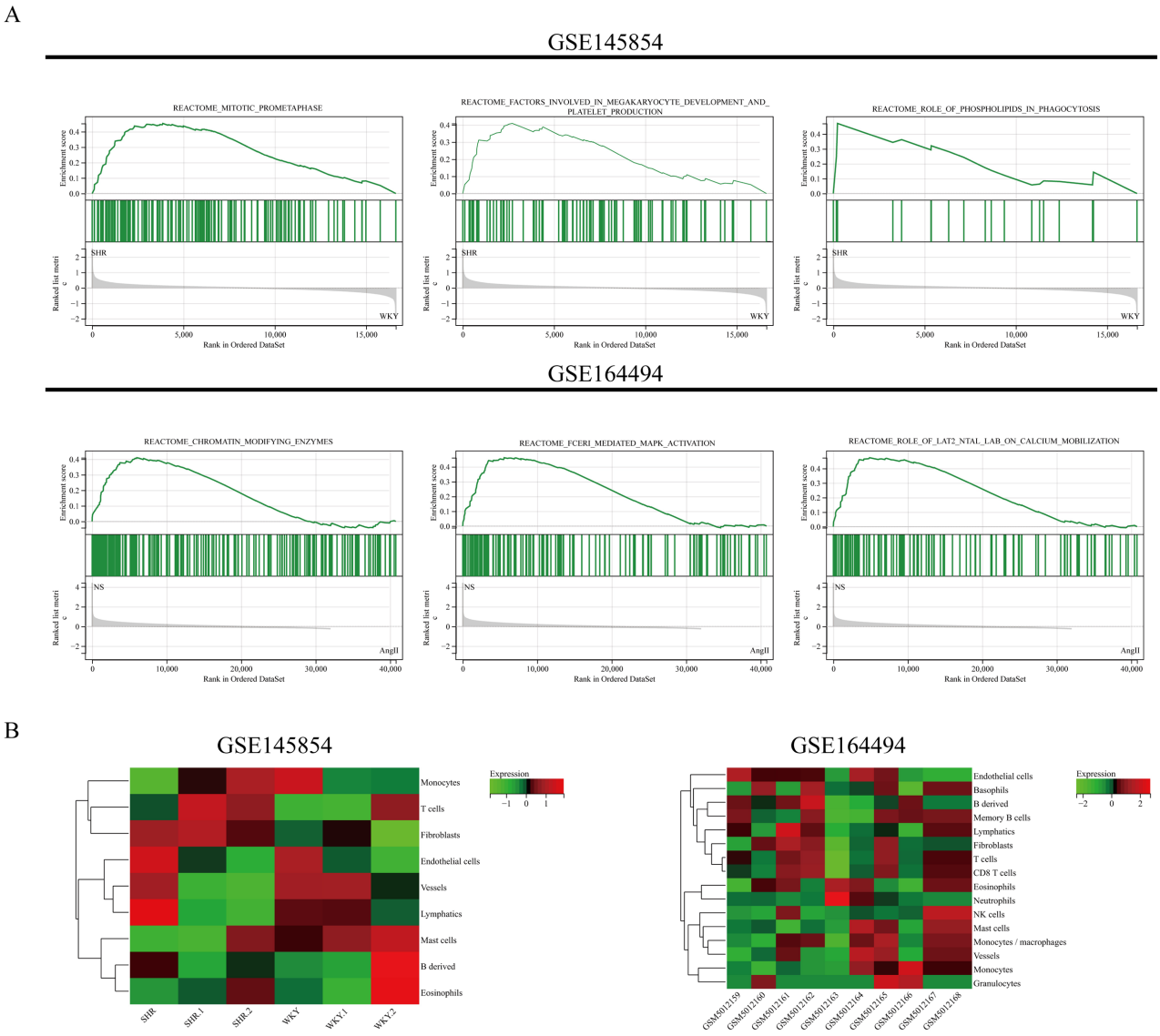


Figure 3. Immune infiltration assay. (A) GSEA analysis; (B) Immune infiltration analysis.

3.3. Immune Infiltration Assay

The GSEA technique was used to investigate the changes and possible underlying mechanisms in hypertensive patients and healthy volunteers. In hypertensive patients, Role of Phospholipids in Phagocytosis, Mitotic Prometaphase, Factors Involved in Megakaryocyte Development and Platelet Production, Role of Lat2 Ntal Lab on Calcium Mobilization, Fceri Mediated Mapk Activation, Chromatin Modifying Enzymes pathway was significantly enriched, but the enrichment patterns were different between the two groups (**Figure 3(A)**). To determine whether hypertensive patients have different immune infiltrates, we compared immune infiltration among hypertensive patients. Immune infiltration assay is mainly based on analysis of tissue sample transcriptome sequencing data or gene chip data. Different cells have some signature markers, as do immune cells, so immune cell infiltration can be assessed and quantified based

on the amount of expression of these marker genes in tissues. The results of our analysis show that the expression of monocytes, T cells, mast cells, B cells, endothelial cells, basophils, memory B cells, lymphatic vessels, fibroblasts, cd8 T cells, eosinophils, neutrophils, NK cells, macrophages, and vessels were significantly different between the control group and the hypertensive patients (**Figure 3(B)**). These results suggest that this feature model has a potential role in predicting the immune response to immunotherapy in hypertensive patients.

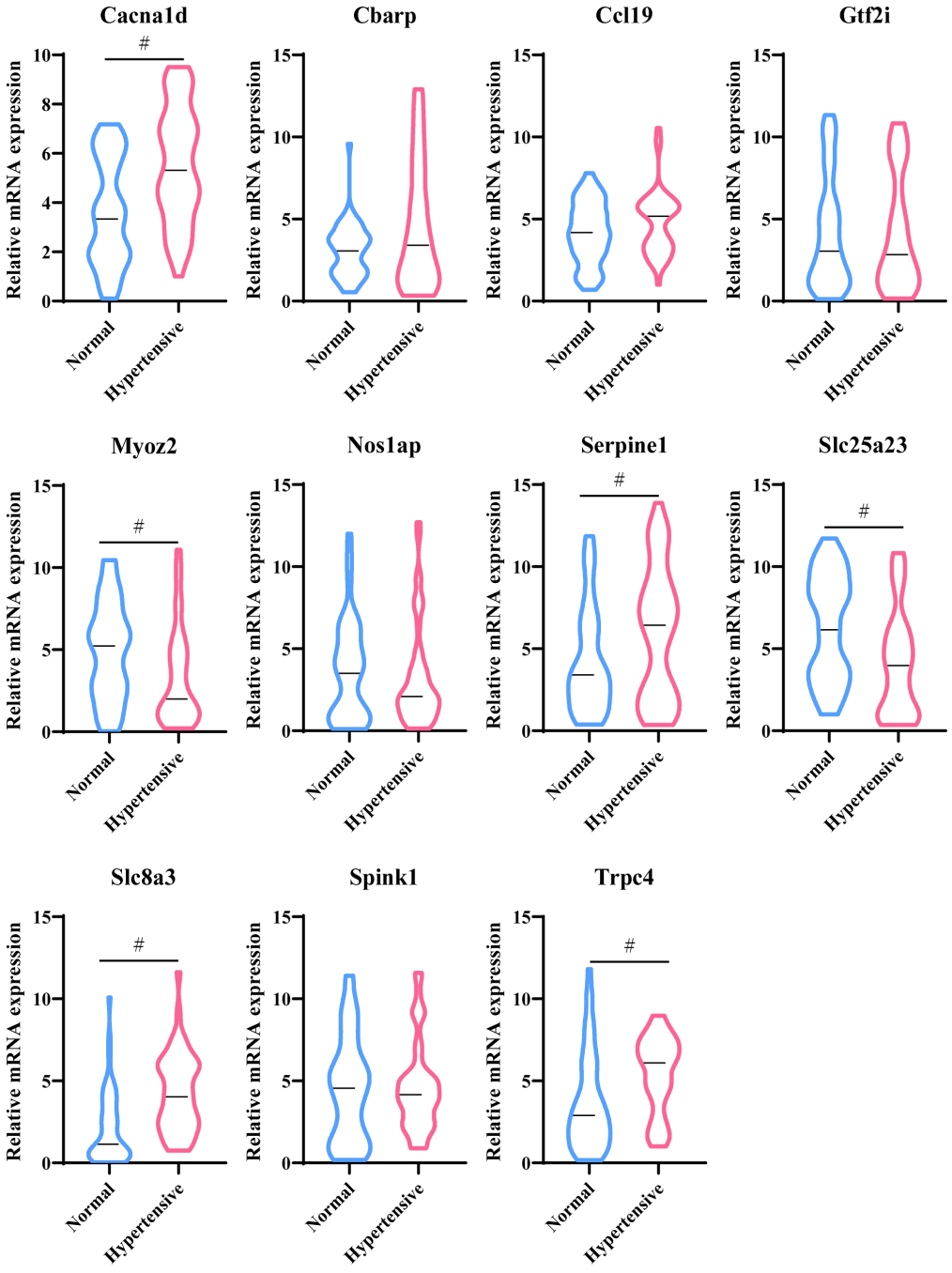


Figure 4. RT-qPCR validation of 11 DEGs in the blood of patients with clinical hypertension.

3.4. RT-qPCR validation of 11 DEGs in the Blood of Patients with Clinical Hypertension

To further verify the expression levels of 11 novel DEGs in the blood of hypertensive patients. RT-qPCR analysis was performed on 51 blood samples from hypertensive patients and non-hypertensive patients. Cacna1d, Cbarp, Ccl19, Serpine1, Slc8a3, and Trpc4 tended to be upregulated, while Cacna1d ($P < 0.05$), Serpine1 ($P < 0.05$), Slc8a3 ($P < 0.05$), and Trpc4 ($P < 0.05$) were significantly upregulated. Gtf2i, Myoz2, Nos1ap, Slc25a23, and Spink1 tended to be downregulated, and Myoz2 ($P < 0.05$) and Slc25a23 ($P < 0.05$) had significant trends (**Figure 4**).

4. Discussion

According to the Global Health Observatory, 1.13 billion people worldwide have hypertension, which increases the incidence of diseases such as left ventricular hypertrophy, coronary heart disease, heart failure, atrial fibrillation and peripheral arterial disease [11] [12]. Many pathophysiological factors are known to be associated with the pathogenesis of hypertension, including structural and functional abnormalities as well as molecular and cellular mechanisms of cardiovascular changes [13] [14]. In addition, impaired vasodilation [15], impaired Ca^{2+} signaling [16], oxidative stress [17], proinflammatory cytokines [18] and fibrotic growth factor [19] are considered to play important roles in this process. Ca^{2+} serve as universal secondary messengers and participate in various biological processes, such as proliferation, cell death, migration and immune response [20]. There is now growing evidence that calcium ion homeostasis is an important driving factor in the occurrence and development of cancers and affects the treatment response of cancer patients [21]. With the understanding of cellular calcium homeostasis mechanisms and specific calcium signaling-targeted therapies (e.g., cardiovascular disease [22] and neuronal diseases [23]), Ca^{2+} signaling has become a very attractive target for the development of novel disease treatment drugs [24]. The researchers used bioinformatics technology to analyze the molecules that regulate calcium metabolism mediating hypertension, and preliminary screening analysis found that core targets such as Cacna1d, Cbarp, Ccl19, Serpine1, Slc8a3, Trpc4, Gtf2i, Myoz2, Nos1ap, Slc25a23, and Spink1 may regulate calcium metabolism and hypertension.

Bioinformatics methods have been used to analyze gene sequencing data of various diseases to identify DEGs and perform various analyses. An increasing number of powerful databases and powerful online tools have been established to help us reposition known complex disease mechanisms [25]. In a growing body of studies, microarray technology is being used to search for DEGs and their molecular functions (MFs), biological processes (BPs) or cellular components (CCs), and related regulatory pathways in specific disease states [26]. For example, Habib Rahman and his colleagues used bioinformatics and machine learning methods to identify novel factors that improve the identification and

characterization of glioblastoma tumors and their progression [27]. With neighborhood-based benchmarking and multilayer network topology techniques, they also identified novel putative biomarkers showing how T2D interacts [28]. In our study, key genes were identified via comprehensive bioinformatics analysis techniques, such as GO and KEGG enrichment analysis and MF analysis, in the GEO datasets GSE145854 and GSE164494. Relying on the GEO database, the specific infiltration of immune cells in the blood of hypertensive patients and healthy patients was compared to elucidate the pathogenesis and potential therapeutic targets of hypertension.

The results of this study revealed 11 common DEGs between the GSE145854 dataset and the GSE164494 dataset, including 6 upregulated DEGs, *i.e.*, Cacna1d, Cbarp, Ccl19, Serpine1, Slc8a3, and Trpc4, and 5 downregulated DEGs, *i.e.*, Gtf2i, Myoz2, Nos1ap, Slc25a23 and Spink1. A recent genome-wide association study (GWAS) of Nos1ap, Slc25a23, and Spink1 revealed that the rs9810888 polymorphism of the calcium voltage-gated channel subunit α1 D gene (Cacna1d) was associated with blood pressure in Chinese adults [29]. Stanton AM et al. also showed that the Cacna1d allele is associated with elevated blood pressure and blood pressure salt sensitivity and that obstruction of Cacna1d expression can relieve the upregulation of blood pressure [30]. Cacna1d is an important gene in the calcium channel pathway. It plays an important role in the regulation of cellular calcium and iron levels and is a key signal for the contraction and dilation of vascular smooth muscle [31]. Our results also indicated that Cacna1d was involved in the regulation of calcium ion metabolism and the process of hypertension.

In summary, this study conducted bioinformatics analysis based on GEO data set, and we found that genes Cacna1d, Serpine1, Slc8a3, Trpc4, Myoz2 and Slc25a23 were the most significant markers of calcium ion metabolism and hypertension. In addition, down-regulating the expression of Cacna1d, Serpine1, Slc8a3, Trpc4, or promoting the expression of Myoz2 and Slc25a23 are molecules that regulate calcium ion metabolism and predict the occurrence of hypertension, and can also be considered as potential targets to prevent the concentration of calcium ions in the blood vessel wall and calcium ion metabolism, so as to prevent the occurrence of vascular calcification and vascular diseases. Although it is currently unclear how the immune system affects vascular remodeling and calcium ion metabolism, it can be speculated that the immune system has a long way to go in this pathological process. The study has the problems of insufficient data and result bias, so further experiments are needed to verify and improve the study results. Further experiments on immune cells can help to identify the specific targets of the immune system involved in vascular remodeling and calcium ion metabolism, facilitating the development of immunomodulatory therapy for patients with hypertension. Moreover, it is necessary to explore the specific underlying mechanism through animal and cell experiments.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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