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Research

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Immunological Analysis of Peripheral Artery Disease

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Abstract

Purpose: To explore the pathogenesis of Peripheral arterial disease (PAD) and provide bioinformatics basis for the prevention and treatment of PAD. **Methods:** R software is used to analyze differentially expressed genes (DEGs) in PAD patient and control blood samples in GSE27034 and screen for immune differential genes, and then perform GO and KEGG pathway enrichment analysis for immune differential genes. The protein-protein interaction (PPI) network was constructed by using STRING database, and functional modules were analyzed using Cytoscape software. Coexpedia database was used to analyze the gene co-expression network of immune differential genes. Finally, combined with CIBERSORT database, immune cells were obtained by R software. **Results:** The 21 immune differential genes screened in PAD were mainly involved in TNF signaling pathway, IL-17 signaling pathway, cytokine-cytokine receptor interaction, viral protein interaction with cytokines and cytokine receptor signaling pathways, and rheumatoid arthritis. Compared with the normal group, neutrophils were higher in number in the PAD group, while macrophages M0 were significantly lower ($P<0.05$). **Conclusions:** TNF signaling pathway, IL-17 signaling pathway and rheumatoid arthritis are most closely related to the occurrence and development of PAD, and immune differential genes may be the key molecules of PAD, which provides a new idea for further exploring the pathogenesis of PAD.

Keywords: Peripheral arterial disease (PAD), Immune differential genes, IL-17, TNF, Rheumatoid arthritis, Immune cell

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Introduction

Peripheral arterial disease (PAD) is a disease in which atherosclerotic plaques are formed in arteries outside the heart and brain, and the vascular stenosis is greater than 50%^[1]. It often leads to obstruction of the arterial blood supply of the lower or upper extremities. The typical symptoms are intermittent claudication and resting pain. Data from the Global Burden of Disease Study 2013 showed that PAD caused more than 40,000 deaths, an increase of 155% from 1990^[2]. In addition, as a manifestation of a widespread systemic atherosclerotic disease, about 60% of PAD patients will suffer from ischemic heart disease, and 30% from cerebrovascular disease. The cardiovascular disease (CVD) mortality rate of PAD patients is 2-3 times higher than that of the general population^[3]. PAD has become the third leading cause of CVD in the world after coronary artery disease and stroke, which has seriously affected people's health, and its treatment is limited to anti-platelet, anti-thrombotic, and intravascular interventions. On the other hand, because about 50% of PAD patients are asymptomatic, diagnosis and treatment are often seriously inadequate. hence, studying the pathogenesis of PAD is particularly important to reduce mortality.

PAD is reported to be a complex disease influenced by cardiovascular risk factors (smoking, high blood pressure, diabetes and dyslipidemia) and population aging, which are important risk factors for PAD in all countries^[4]. Scholars have conducted a great deal of exploration on diagnostic and prognostic markers of PAD, and found many new markers. Studies have found that many genes can be used as susceptibility sites for PAD^[5,6], and gene therapy also plays a certain role in the treatment of PAD^[7,8]. Atherosclerosis is a chronic inflammatory disease, and inflammation is closely related to immunity. Some scholars^[9] have shown that immunological analysis may provide new insights into the pathogenesis of PAD. On this basis, the functional enrichment and immune cell analysis of immune differential genes of PAD were carried out through bioinformatics method in this paper, and the important pathways and immune differential genes involved in the development process of PAD were obtained, which provided bioinformatics basis for the research on the development mechanisms of PAD and might be potentially helpful for diagnosis and prognosis of PAD.

Materials and methods

Microarray Data

The gene chip dataset GSE27034 was obtained from the GEO database

(<https://www.ncbi.nlm.nih.gov/geo/>). The expression profile included a total of 37 peripheral blood mononuclear cell samples, of which 19 PAD patients with an ankle-brachy-index (ABI) ≤ 0.9 and 18 age- and sex-matched healthy controls with ABI > 1.0 . The chip platform is GPL570[HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array.

Obtaining differentially expressed genes (DEGs)

The R language was used to conduct background correction, add missing value and remove duplicate of the original data of GSE27034. The probe name of the chip GSE27034 matrix data was converted into the gene name through the platform file GPL570. The R language Limma package was used to screen DEGs, the screening criterion was set as $|\log_2FC| > 1$ and adjusted $P < 0.05$. The R software was used to draw heatmap and volcano plot of DEGs.

Screening immune differential genes

To further understand the immune genes related to PAD, we obtain immune-related genes by IMMPort database (<https://www.immport.org/>)^[10], which consists of four components-Private Data, Shared Data, Data Analysis, and Resources for data archiving, dissemination, analyses, and reuse. The PAD immune differential genes were screened in PAD DEGs. The screening criterion was set as $|\log_2FC| > 1$ and adjusted $P < 0.05$. The R software was used to draw heatmap and boxplot of immune differential gene.

GO and KEGG pathway enrichment analysis of immune differential gene

Gene Ontology (GO) includes biological process (BP), cellular component (CC), and molecular function (MF). In order to explore the function of immune differential gene, GO enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were performed by using R software. A adjusted $P < 0.05$ was considered statistically significant.

Protein-protein interaction (PPI) network construction and module selecting

STRING database (Search Tool for the Retrieval of Interacting Genes, <https://string-db.org/>)^[11] can construct protein-protein interaction network to evaluate functional genomics data. We used STRING online database to construct PPI network of immune differential gene, the obtained source files were imported into Cytoscape

3.7.0 software for visual analysis. The key modules were identified by using Molecular Complex Detection (MCODE) plug-in with both MCODE score and node counts number more than 5.

Establishment of immune differential gene network

Coexpedia database (<https://www.coexpedia.org/>)^[12] is distinctive co-expression databases by the following three aspects: 1. all co-expression links were evaluated for functional association by statistical assessment. 2. all co-expression links are associated with particular biomedical contexts. 3. all co-expression links have associated medical subject heading (MeSH) terms, which provide anatomical or disease context information.

Obtaining immune cells

CIBERSORT database (<http://cibersort.stanford.edu>)^[13] provides a method to calculate cell composition quantitatively from gene expression profiles of a large number of tissues. It can estimate the immune composition of the sample. The DEGs profile data were converted into the corresponding 22 immune cell proportion data of the samples by using the CIBERSORT. The immune cell proportion data was calculated with 100 permutations and a threshold of $P < 0.05$, and filtered by Perl to delete the sample with $P > 0.05$. Then, R software pheatmap and vioplot were used to draw the heatmap and violin map of the distribution of immune cells in the samples.

Results

Identifying the DEGs in PAD patients

In order to investigate the inherent differences between PAD and control group, we performed Bayesian test on GSE27034 microarray data, and obtained 89 DEGs (PAD group/normal control group), among which 79 genes were up-regulated and 10 genes were down-regulated. The expression of different genes in the two groups was shown in Figure 1.

Fig.1 DEGs in the PAD and control group. **(a)** Heatmap analysis of differential genes. The red and green represent the significantly upregulated and downregulated DEGs. **(b)** Volcano plot of the differentially expressed genes. These genes consist of 79 upregulated genes and 10 downregulated genes. The screening criterion was $|\log_2FC| > 1$ and adjusted $P < 0.05$.

Functional and pathway enrichment analysis of the DEGs

We used R language to analyze the GO and KEGG pathway enrichment of 89 DEGs. The GO BP analysis indicated that the DEGs were enriched in regulation of leukocyte and lymphocyte migration, mucosal immune response, organ or tissue special immune response, T cell migration, innate immune response in mucosa. DEGs were significantly enriched in the MF related to receptor ligand activity, receptor regulator activity, protein heterodimerization activity, G protein-coupled receptor binding, growth factor activity, chemokine regulator binding, cytokine activity, CXCR chemokine receptor binding (Figure 2(a)). KEGG pathway analysis indicated that immune differential genes were mainly enriched in TNF signaling pathway, IL-17 signaling pathway, colorectal cancer, and rheumatoid arthritis (Figure 2(b)).

(a)

(b)

Fig.2 Enrichment analysis of the DEGs. **(a)** GO enrichment of the DEGs. **(b)** The pathways enriched for the DEGs in PAD. The size of the circle indicates the number of genes. The screening criterion was set as adjusted $P < 0.05$.

Screening the immune differential genes in PAD patients

Due to the DEGs were significantly enriched in the BP related to immunity, we urgently wanted to clarify the relationship between PAD and immune genes. From the selected DEGs and the immune genes of IMMPORT database, we have obtained 21 immune differential genes: ADM, BMP15, CAMP, CCL2, CCL20, CMTM2, CXCL10, CXCL8, EGF, EREG, FCGR3B, FGF17, FOS, GMFG, IL12RB2, NAMPT, NR4A2, PROK2, PTGS2, RSAD2, TRBV27. Compared with the control group, except for FGF17 and IL12RB2, the expression of other 19 genes in the PAD group was significantly overexpressed. The result is shown in Figure 3(a) and Figure 3(b).

(a)

(b)

Fig.3 The expression of immune differential genes in PAD group and control group. **(a)** Heatmap analysis of 21 immune differential genes. The red and green represent the significantly upregulated and downregulated DEGs. **(b)** The expression of 21 immune differential genes.

Functional and pathway enrichment analysis of the immune differential genes

We used R language to analyze the GO and KEGG pathway enrichment of 21 immune differential genes. GO analysis results indicated that immune differential genes were significantly enriched in the BP related to response to lipopolysaccharide, response to molecule of bacterial origin, response to extracellular stimulus, T cell migration, regulation of leukocyte and lymphocyte migration. For MF, the immune differential genes were significantly enriched in receptor ligand activity, receptor regulator activity, G protein-coupled receptor binding, chemokine regulator binding, cytokine activity, CXCR and CCR chemokine receptor binding (Figure 4(a)). KEGG pathway analysis indicated that immune differential genes were mainly enriched in TNF signaling pathway, IL-17 signaling pathway, cytokine-cytokine receptor interaction, viral protein interaction with cytokine and cytokine receptor, and rheumatoid arthritis (Figure 4(b)).

(a)

Fig.4 Enrichment analysis of immune differential genes. (a) GO function of immune differential genes. (b) The pathways enriched for immune differential genes in PAD. The size of the circle indicates the number of genes. The screening criterion was set as adjusted $P < 0.05$.

Protein-protein interaction network construction and key module screening of immune differential genes

We input 21 immune differential genes into STRING database. According to the information in the STRING database, a PPI network with 16 nodes and 37 edges was constructed by using Cytoscape software (Figure 5(a)). Furthermore, the MCODE plugin was used to identify possible key modules from the network, resulting module with score 6 points (Figure 5(b)). Pathway enrichment analysis revealed that the immune differential genes involved in module were related to TNF signaling pathway, IL-17 signaling pathway, viral protein interaction with cytokine and cytokine receptor, and rheumatoid arthritis (Table 1).

Fig.5 PPI network construction among immune differential genes and functional module diagram. **(a)** PPI network construction among immune differential genes. **(b)** Functional module diagram.

TABLE 1: KEGG pathway analysis of the genes in the functional modules

ID	Description	Count	<i>p.</i> adjust
hsa04657	IL-17 signaling pathway	6	1.16E-09
hsa04668	TNF signaling pathway	5	3.79E-07
hsa05323	Rheumatoid arthritis	4	1.48E-05
hsa04061	Viral protein interaction with cytokine and cytokine receptor	4	1.48E-05

Immune differential gene co-expression network

A total of 21 immune differential genes were introduced into Coexpedia database to obtain the co-expression network of 16 immune differential genes (Figure 6). The ranking of 16 immune differential genes is shown in Table2. In addition, GeneSet analysis of the disease ontology revealed gene-related diseases, and found that rheumatoid arthritis ranked first, as shown in Table 3.

Fig.6 Co-expression network of immune differential gene.

TABLE 2: Ranking of immune co-expression genes

Rank	Gene Symbol	Score
1	CXCL8	109.093
2	NAMPT	78.37
3	PTGS2	59.683
4	FCGR3B	46.75
5	CXCL10	35.255
6	NR4A2	34.31
7	CCL2	30.332
8	ADM	27.615
9	RSAD2	27.288
10	EREG	22.483
11	CCL20	18.832
12	FOS	14.959
13	PROK2	13.6
14	GMFG	13.011
15	CMTM2	5.42
16	CAMP	2.344

TABLE 3: GeneSet Analysis: Disease Ontology

Rank	MDO ID	Name	<i>p</i> -value
1	DOID:7148	rheumatoid arthritis	6.155E-11
2	DOID:848	arthritis	1.736E-10
3	DOID:824	periodontitis	3.894E-10
4	DOID:850	lung disease	9.984E-10
5	DOID:0050117	disease by infectious agent	7.25E-09
6	DOID:3083	chronic obstructive pulmonary disease	1.112E-08
7	DOID:1883	hepatitis C	2.459E-08
8	DOID:3770	pulmonary fibrosis	2.977E-08
9	DOID:2237	hepatitis	2.996E-08
10	DOID:10591	pre-eclampsia	5.285E-08

Immune cells in PAD and control samples

Converting the DEGs expression profile data into the proportion of immune cells corresponding to the sample, and filtering out the samples with $P > 0.05$, 14 PAD samples and 14 control samples are obtained. The distribution of the immune cells in the PAD and control group showed that neutrophils in the PAD group were present at higher fractions than in the control group ($P = 0.001$), while the macrophage M0 was significantly lower ($P = 0.027$). The results are shown in Figures 7.

Fig.7 Immune cells in PAD and control. **(a)** Heatmap analysis of the 22 immune cells. The horizontal axis shows the samples are divided into normal control and PAD group. **(b)** Significant changes in immune cells in PAD and control groups. The blue and red violin represent the normal control and the PAD group, respectively.

Discussion

As people's lifestyle, diet and exercise change, the incidence of PAD also increased significantly^[14]. PAD is estimated to affect the health of 10-15% of the population, with some 200 million people living with PAD worldwide suffering from PAD^[15,16]. As a result, it is very important to study the mechanism of PAD. Therefore, it is very important to study the mechanism of PAD. Based on this, this article uses bioinformatics methods to analyze the immune molecular level of PAD, hoping to provide new ideas for the prevention and treatment of PAD.

In this study, we analyzed the immune differential genes of PAD and found that except for FGF17 and IL12RB2, the other 19 immune genes were significantly overexpressed in PAD. Therefore, we speculated that FGF17 and IL12RB2 play a protective role in PAD. Most of the studies on FGF17 have focused on hypogonadism^[17] and tumor^[18]. IL12RB2 is mainly involved in the occurrence and development of Behcet's disease^[19]. And they have not been widely reported in the PAD. Furthermore, we analyzed the functional and pathway enrichment of the DEGs and immune differential genes, and found that functional and pathway enrichment of immune differential genes were basically consistent with that of DEGs. It can be seen that the occurrence and development of PAD is mainly related to 21 immune

differential genes. The 21 immune differential genes were mainly involved in TNF signaling pathway, IL-17 signaling pathway, cytokine-cytokine receptor interaction, viral protein interaction with cytokine and cytokine receptor, and rheumatoid arthritis. These inflammatory signaling pathways are roughly consistent with the "endothelial injury response theory" of atherosclerosis pathogenesis. There is evidence that anti-inflammatory therapy can alleviate atherosclerosis^[20], and these immune differential genes are involved in the inflammatory process, which provides a new therapeutic target for active and effective disease control and inflammation suppression. Rheumatoid arthritis is a chronic, systemic autoimmune disease. The basic pathological changes are synovitis and vasculitis, while vasculitis often involves small and medium arteries and veins, resulting in stenosis or blockage of the lumen, which is similar to the pathological manifestations of PAD. Some scholars have found that cardiovascular risk factors and disease characteristics are consistent with changes in vascular hemodynamics in RA^[21], which further supports this conclusion. Interestingly, GeneSet Analysis of disease ontology found that rheumatoid arthritis ranked first among the diseases related to PAD immune genes. From this, we speculated that rheumatoid arthritis is a risk factor for PAD. A cross-sectional study has shown that rheumatoid arthritis is associated with a higher risk of developing atherosclerotic vascular disease^[22]. The European League of Rheumatology (EULAR) working group assessed the risk of CVD in patients with inflammatory joint disease and found that RA patients had a higher risk of CVD^[23].

Immune differential gene co-expression network analysis found that chemokine ligand CXCL8 score was the highest, indicating that it plays an important role in the network. Studies have shown that cytokines and chemokines are involved in the development of atherosclerosis^[24]. Therefore, targeting them may be an effective strategy to inhibit the progress of atherosclerosis. NAMPT, ranked second, is also related to inflammation and can act as a cytokine. Intracellular and extracellular NAMPT plays a dual role in atherosclerosis^[25], and its role in PAD is worthy of further study.

The distribution of the immune cells indicated that neutrophils and macrophages were higher in number in the PAD group, while macrophage M0 showed the opposite results. Atherosclerosis is a chronic inflammatory disease. Neutrophils and macrophages can secrete pro-inflammatory mediators, including platelet-derived growth factor, fibroblast growth factor, TNF- α , IL-1, etc., which promote plaque

growth and inflammatory response. A growing number of studies have shown that the neutrophil-lymphocyte ratio (NLR) increases significantly with the severity of PAD disease^[26,27]. It has also been shown that activation of T cell is a marker of atherosclerosis^[28]. Systemic immune inflammation index (SII) as a new biomarker can better predict cardiovascular disease^[29]. In conclusion, PAD is an inflammatory and immune disease^[30].

Compared with previous studies, this article focused on the immunology of PAD, and found that immune differential genes and immune cells are closely related to the occurrence and development of PAD. Immune differential genes may be the key molecules of PAD, which provides a new way to further explore the pathogenesis and therapeutic treatments of PAD. Of course, this study also has certain limitations. First of all, the chip data used in this study is a single-center study, which is representative to some extent. Secondly, the molecular mechanism and therapeutic targets of PAD still need to be verified through a series of experiments.

Conclusions

In summary, this study found that TNF signaling pathway, IL-17 signaling pathway, cytokine-cytokine receptor interaction, viral protein interaction with cytokine and cytokine receptor, and rheumatoid arthritis play a crucial role in the occurrence of PAD. In addition, immune differential genes such as CXCL8 may be effective targets for prevention and treatment of PAD. The occurrence of PAD is also related to the abnormal expression of neutrophils and macrophage M0. It can be seen that the occurrence of PAD is related to inflammation and immune abnormalities.

Abbreviations

PAD: peripheral arterial disease; DEG: differentially expressed gene; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; BP: biological process; MF: molecular function; PPI: Protein-protein interaction, MCODE: Molecular Complex Detection.

Ethical Approval and Consent to participate

Not applicable.

Consent for publication

All the authors consent for publication.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Authors' Contributions

Wan-Xia Yang and Yun-Yan Pan conceived and designed the study. Wan-Xia Yang and Fang-Fang Wang made the diagrams and tables of the article. Wan-Xia Yang, Fang-Fang Wang and Fei-Fei Li made wrote the paper. Jian-Qin Xie, Chong Shi and Chong-Ge You revised the article. All the authors read and approved the manuscript.

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Figures

Figure 1

DEGs in the PAD and control group. (a) Heatmap analysis of differential genes. The red and green represent the significantly upregulated and downregulated DEGs. (b) Volcano plot of the differentially

expressed genes. These genes consist of 79 upregulated genes and 10 downregulated genes. The screening criterion was $|\log_2FC| > 1$ and adjusted $P < 0.05$.

Figure 2

Enrichment analysis of the DEGs. (a) GO enrichment of the DEGs. (b) The pathways enriched for the DEGs in PAD. The size of the circle indicates the number of genes. The screening criterion was set as adjusted $P < 0.05$.

Figure 3

The expression of immune differential genes in PAD group and control group. (a) Heatmap analysis of 21 immune differential genes. The red and green represent the significantly upregulated and downregulated DEGs. (b) The expression of 21 immune differential genes.

Figure 4

Enrichment analysis of immune differential genes. (a) GO function of immune differential genes. (b) The pathways enriched for immune differential genes in PAD. The size of the circle indicates the number of genes. The screening criterion was set as adjusted $P < 0.05$.

Figure 5

PPI network construction among immune differential genes and functional module diagram. (a) PPI network construction among immune differential genes. (b) Functional module diagram.

Figure 6
Co-expression network of immune differential gene.

Figure 7

Immune cells in PAD and control. (a) Heatmap analysis of the 22 immune cells. The horizontal axis shows the samples are divided into normal control and PAD group. (b) Significant changes in immune cells in PAD and control groups. The blue and red violin represent the normal control and the PAD group, respectively.