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Identification of oxidative stress-related genes associated with immune cells in Aortic Valve Stenosis based on bioinformatics analysis

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ABSTRACT

Aortic valve stenosis (AS) is the most common clinical valvular heart disease. Without effective pharmaceutical therapy at present, identifying effective therapeutic targets is critical. However, the pathological and molecular mechanisms of aortic stenosis are complex, including inflammatory infiltration, oxidative stress and so on. In this study, we investigated how oxidative stress interacts with immune cell infiltration in aortic stenosis using bioinformatics analysis, and provide a better understanding of aortic valve stenosis at the pathophysiologic level. After obtaining the datasets, including GSE153555, GSE51472 and GSE12644, from the Gene Expression Omnibus (GEO) database, the package 'limma' was applied to identify the differentially expressed genes (DEGs) in GSE153555. The GeneCards database searched for oxidative stress-related genes. We evaluated the expression of 22 immune cells using Cibersort. Clustering differentially expressed genes into different modules via Weighted gene correlation network analysis (WGCNA) and exploring the relationship among modules and immune cell types. The genes in modules intersected with oxidative stress-related genes to find oxidative stress genes related to immune infiltration. Finally, the GSE51472 and GSE12644 datasets were used to initially verify oxidative stress-related genes in aortic valve stenosis. A total of 1213 differentially expressed genes were identified in the GSE153555 dataset, and 279 of them were oxidative stress-related genes. Increased infiltration of B cell navie and Macrophages M1 in aortic stenosis was found. Using WGCNA, we clustered 15 modules. The brown module was identified as the most significant template positively correlated with T-cell regulatory Treg, and the magenta module was identified as a critical module associated with M1 macrophages with the highest negative correlation coefficient. The results verified by other datasets showed that in comparison to normal people, the aortic stenosis patients exhibited dramatically high IGFBP2 and SPHK1 expression. Both IGFBP2 and SPHK1 may be significantly involved in the mechanism of aortic stenosis pathophysiologically and can be used for aortic stenosis early detection, therapy, and therapeutic targets.

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Introduction

Aortic Valve stenosis (AS) is a serious progressive disease. With the acceleration of aging, the disease burden of AS is increasing (1). AS is a complex disease. Pathophysiological processes associated with the most prevalent valvular disorders include basement oxidative stress (OS), membrane rupture, inflammatory cell infiltration, lipid deposition, neuromodulator effects, and endothelial dysfunction (2,3). In addition, significant connections between elevated low-density lipoprotein cholesterol, hypertension, and age to AS were also observed (4). However, early treatment with statins and angiotensin II receptor antagonists in clinical trials failed to delay the progression of mild to moderate aortic stenosis, the underlying mechanism resulting in aortic stenosis is completely unknown (5). Currently, the important roles of immune system dysregulation in the pathogenesis of AS have been discovered (6). For example, more circulating regulatory T cells were found in the valve tissue and blood of AS patients (7). Inflammatory cells such as macrophages and mast cells infiltrate and release inflammatory mediators to participate in the progression of the disease (8). Other studies suggest that hypoxia is one of the important factors in the progression of advanced valvular disease (9) and OS also plays an important role in the process of aortic stenosis, NAD (P) H oxidase expression increased in the stenosis of the aortic valve, and contribute to the production of reactive oxygen species (10). While the immune cell-associated OS and AS have no recognized connections. As a system biology method for describing the patterns of gene association between different samples, weighted gene-related network analysis (WGCNA) was applied to obtain highly synergistic gene sets and determine the potential biomarkers and targets for therapy. It has been used in the study of other cardiovascular diseases (11).

We explored OS-related genes associated with immune cells in Aortic Valve Stenosis through comprehensive

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bioinformatics analysis. Meanwhile, the gene pathway enrichment was also analyzed in the hub module. Furthermore, we identified the central gene modules and accessed the composition of immune cells using WGCNA and Cibersort. Meanwhile, we also analyzed and new research validated these identified central genes. Our study offers novel ideas and provides candidate targets for AS treatment.

Materials and Methods

Datasets and data processing

The GSE153555 dataset, which includes the aortic valve leaflets from aortic stenosis and non-matched transplant donor hearts was obtained from the GEO database. For the identification of DEGs in AS, the package "Limma" was employed. Then, using the keyword OS, we searched and collected the OS-associated genes from the GeneCards database (https://www.genecards.org/). The genes identified both in GeneCards and GSE153555 were defined as the OS-related genes (OS genes) in AS. The differentially expressed OS genes were defined as OS-DEGs. The downloaded platform and matrix files of GSE51472 and GSE12644 were processed by R and annotation, which was used for validation.

Enrichment analyses of function and pathway

The significant biological processes, molecular functions, cellular components and vital pathways of the OS-DEGs were performed by utilizing a database for Integrated Discovery, Visualization, and Annotation (https://david.ncifcrf.gov/). *P* values were set to 0.05 for statistical significance.

Infiltration analysis of immune cell

Using GIBERSORT to evaluate the differences in 22 types of immune cell infiltration. We filtered out blank and missing data and obtained the immune cell proportions. Using the "vioplot" package in R, we got each immune cell infiltrating in the 2 groups presented with violin diagrams. Using the Pearson correlation coefficient to calculate the correlation of immune cells.

WGCNA and module screening

The R package WGCNA was used to investigate immune cell-related modules associated with AS. The DGEs were colored after being clustered into modules using the average linkage hierarchical clustering approach. The module merging threshold and the genes minimum number were set as 0.25 and 20 respectively. Next, we calculated the correlation of immune cells with the modules and screened highly correlated modules for further analysis.

Screening of immune cell infiltration-related OS genes

The association of the given gene expression profile with the eigengene of the given model. was represented by module membership. The higher absolute value of MM represents the more significant association of the given gene expression profile with module eigengene positively or negatively. We selected modules with an absolute value of correlation greater than 0.7 and obtained brown, green, yellow, and pink as hub modules. The genes in hub modules intersected with OS genes to obtain OS genes related to immune cell infiltration.

Validation

The key genes were validated with the GSE51472 and GSE12644 datasets from the GEO database. The SPSS 24.0 software was carried out to validate the key genes, and a sample t-test was conducted to evaluate the differences between the two groups.

Results

The DEGs of AS

There are 1,213 DEGs between aortic stenosis and normal samples identified through annotation in the GSE153555, which includes 704 up-regulated genes and 509 down-regulated genes. Meanwhile, we selected 3202 OS-associated genes from the gene card database. In the OS-associated genes and DEGs, 279 genes were co-expressed, with 169 being up-regulated and 110 being down-regulated. (Figure 1A-B)

Functional enrichment analyses

Next, we conducted the enrichment analysis of the KEGG function for the OS-DEGs in AS and using David's online tool evaluated their biological effects. Significant enrichment of BPs including inflammatory response, si-

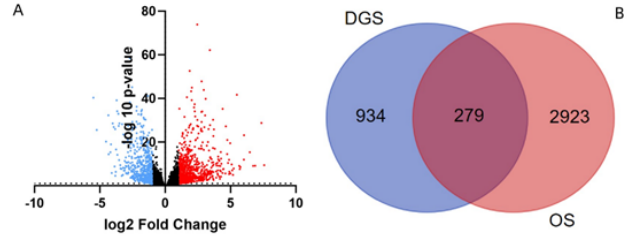

Figure 1. Identification of differentially expressed oxidative stress-related genes (OS-DEGs). (A). Volcano plot showing the differential gene expression between AS and normal groups. (B). Venn diagram illustrating the overlap between DEGs and OS-related genes, resulting in 279 overlapping genes (OS-DEGs).

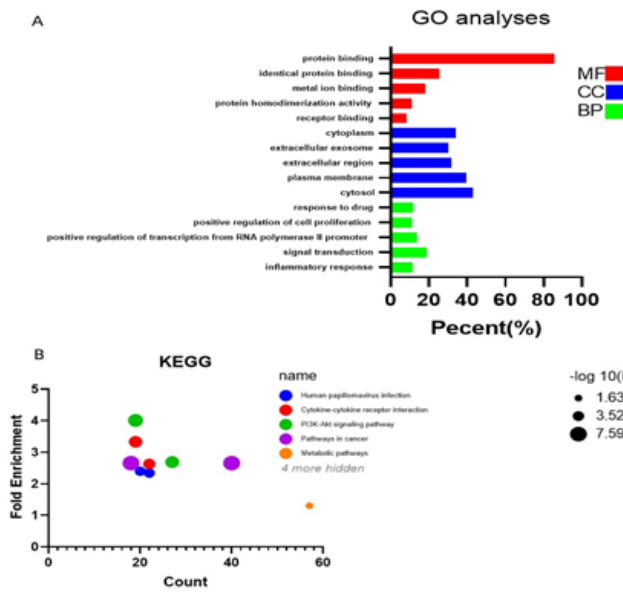

Figure 2. Functional enrichment analysis of OS-DEGs. (A). Bar plot showing the results of Gene Ontology (GO) pathway analysis, including Biological Processes (BP), Cellular Components (CC), and Molecular Functions (MF). (B). Bubble plot displaying the results of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis.

gnal transduction, positive regulation of transcription from RNA polymerase II promoter, positive regulation of cell proliferation, response to the drug, and cytosol, plasma membrane, extracellular region, extracellular exosome, and cytoplasm in CC was observed. The most critical factors for MF included receptor binding, protein homodimerization activity, metal ion binding, identical protein binding, and protein binding. In addition, significant enrichment of OS-DEGs in the Human papillomavirus infection, Cytokine-cytokine receptor interaction, PI3K-Akt signaling pathway, Pathways in cancer, and Metabolic pathways, was discovered by the enrichment analysis of the KEGG pathway. (Figure 2A-B)

Relative expression of immune cells

For the evaluation of the presumed relative immune cell expression, the Cibersort with 22 types of immune cells was conducted. Significant differences in the expression of Mast cells resting, Macrophages M1, NK cells activated, T cells gamma delta, T cells follicular helper, CD4 memory activated T cells and CD8 T cells were observed. Additionally, due to no expression in most of the samples, we eliminated neutrophils, eosinophils, activated mast cells, M0 macrophages, resting NK cells, gamma delta T cells, CD4 memory activated T cells, CD4 naïve T cells, memory Naïve B cells, CD4 naïve T cells, and memory B cells. A strong positive association of T cells follicular helper with NK cells activated (r = 0.767, P = 0.001) was discovered using the Pearson correlation coefficient. And negative association of B cell Naïve with Macrophages M2 (r = -0.699, P = 0.001) was also observed. (Figure 3A-C)

Construction of the weighted coexpression networks

According to our identified DEGs, we conducted WGCNA using 1213 genes. Then, a scale-free (scale-free R2 > 0.85) co-expression network was constructed and the

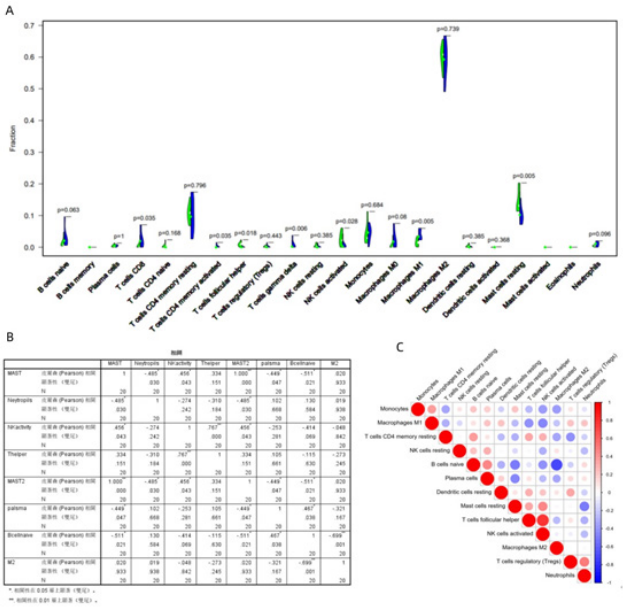

Figure 3. Immune cell landscape in AS. (A). Violin plot depicting the infiltration of 22 immune cell types between AS and normal samples, analyzed using the CIBERSORT algorithm. (B). Network plot illustrating the relationships between immune cell types, analyzed using the Spearman correlation algorithm. (C). Heatmap showing the correlation matrix of immune cell types.

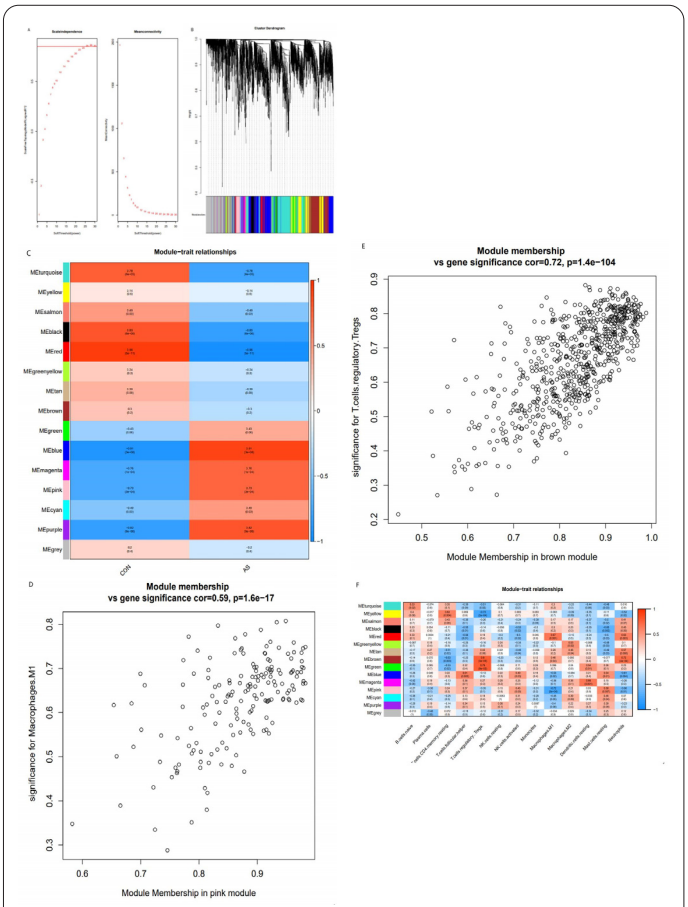

Figure 4. Construction of coexpression networks for DEGs. (A). Selection of the optimal soft-threshold power for WGCNA. (B). Hierarchical clustering dendrogram of genes, assigning them into 15 different modules. (C). Correlation heatmap displaying the correlation between 15 modules and phenotypes, with each module represented by a distinct color. (D). Scatter plot showing the significantly positive correlation of feature genes in the pink module with M1 macrophages (P-value < 0.01). (E). Scatter plot showing the significantly positive correlation of feature genes in the brown module with regulatory T cells (P-value < 0.01). (F). Relationship between the 15 modules and immune cell types.

soft-thresholding power was defined as β = 8. Next, we clustered the DEGs into 15 modules and showed with different colors, including grey, purple, cyan, magenta, blue, green, brown, tan, green, yellow, red, black, salmon, yellow, and turquoise. The correlation between each module and the selected immune cells was evaluated. The significantly positive correlation of the brown module with T cells regulatory Tregs (r = 0.8, P < 0.001) and Neutrophils (r = 0.72, P < 0.001), a green module with T cells regulatory Tregs (r = 0.76, P < 0.001), as well as the negative correlation of yellow module (r = −0.73, P < 0.001) with T cells regulatory Tregs and pink module with M1 macrophages were observed (r = 0.72, P < 0.001). (Figure 4A-E)

Verification of database

The standardization of the data sets The GSE51472 and GSE12644 confirmed the expression levels of IGFBP2 and sphingosine kinase 1 (SPHK1) genes in normal aortic valve and aortic stenosis. The results showed that in comparison to non-AS patients, AS patients exhibited significantly high IGFBP2 and SPHK1 expression. (Figure 5A-B)

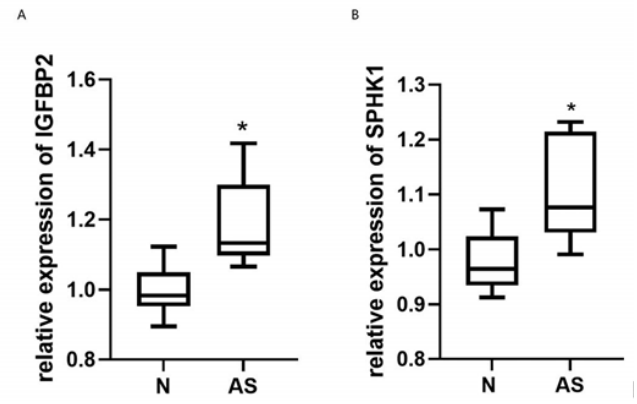

Figure 5. Validation of Key Gene Expression Levels. (A, B). Boxplots displaying the differential expression levels of IGFBP2 and SPHK1 genes between AS and normal samples. Both key genes showed upregulated expression (* $P < 0.05$).

Discussion

Aortic stenosis is a chronic inflammatory disease (12,13), mainly caused by extensive immune cell infiltration (such as T cells, macrophages, etc.) with each stage of aortic stenosis (14). It is suggested that congenital and adaptive immunity are involved in the progression of aortic stenosis. Evidence shows that AS progression is an actively regulated process. The most important cells in the aortic valve are valve interstitial cells, which exist in all valve layers (15). When valve interstitial cells trigger inflammatory cytokines, mechanical stress, and OS, they will cause endothelial dysfunction (16,17). Studies have shown that enhanced OS in the aortic valve could significantly promote aortic valve calcification development. Overexpression of MI polarization markers in CAVD shows that MI macrophage polarization plays a major role in calcified aortic stenosis, according to research (18). When OS in the inflammatory microenvironment exceeds anti-oxidation, immune cells will undergo autophagy or apoptosis (19). Studying immune cell-related OS helps to identify heart disease targets. Design new treatment strategies to prevent the effects of aortic stenosis and improve the long-term prognosis of patients.

In this study, we found significant enrichments of OS-DEGs genes in the cytoplasm, plasma membrane, extracellular region, extracellular exosomes, and cytoplasm. The identified proteins identified by our analysis were closely involved in an inflammatory response and signal transduction, and the main pathways were the metabolic pathway, cancer pathway, and cytokine-cytokine pathway. This is similar to the bioinformatics analysis results of other researchers (20). Furthermore, immune cell infiltration data revealed that B cell naive, Macrophages M1 immune cell infiltration increased. However, in healthy active valves, tissue innate immune cells are composed of macrophages and dendritic cells, while T cells or B cells are absent. It has been confirmed that the increased infiltration of macrophages into M1 phenotype macrophages in calcified aortic valves, and MI-type macrophages can secrete pro-inflammatory factors to enhance the calcification of valve interstitial cells (21,22). The positive correlation of the brown module with T cells regulatory Tregs, Neutrophils, and green module with T cells regulatory Tregs, as well as a negative correlation of the yellow module with T cells regulatory Tregs and pink module with Macrophages M1, were observed by WGCNA study. Other studies have identified regulatory T cells to be related to the advancement of aortic stenosis, implying that early management of circulating T cell levels in individuals with aortic stenosis may be required (7). It has also been found in other cardiovascular diseases that targeted therapy of regulatory T cells may provide new methods and directions for the prevention and treatment of cardiovascular diseases, such as atherosclerosis (23,24), postischaemic neovascularization, and heart failure (25). Both neutrophils and macrophages M1-type immune cells are involved in OS and promote inflammatory response (26,27).

We found more than 100 immune-related OS genes in the modules with a high correlation of immune cell infiltration and found that IGFBP-2 and SPHK1 were significantly increased in aortic stenosis. IGFBP-2 is reported to be a “dark horse” physiologically and pathologically. IGFBP-2 expressions have been shown to change in a variety of diseases, such as malignancies (28,29), those related to metabolism (30) immune-related diseases (31), and cardiovascular disease (32,33). Increased expression of IGFBP-2 increased intracellular hydrogen peroxide activity (34). IGFBP-2 was considered an independent risk factor for heart failure, all-cause mortality, and cardiovascular disease-related death, proving that it may play an important role in the prediction of cardiovascular disease (35). In this study, IGFBP-2 was found in Macrophages M1, T cells regulatory Tregs, and Neutrophils genes associated with OS. Through other database verification, it was found that its expression was significantly increased in aortic stenosis, which proved that it played an important pathophysiological role in the aortic valve.

SPHK1, sphingosine kinase 1, is the protein that catalyzes the phosphorylation of sphingosine to form sphingosine-1-phosphate (S1P). SPHK1 and its product S1P have been identified as having a key role in inflammation (36), anti-apoptosis (37), and inflammatory immune-related diseases (38). A previous study showed that SPHK1 played a key role in the activation of NLRP3 inflammasome (39). Another study discovered that the SPHK1 product S1P is important in OS-mediated renal injury (40). Moreover, the signaling pathway mediated by SphK1/S1P is significantly involved in inflammation and myocardial fibrosis after MI (41). Additionally, reduced myocardial injury was observed in the myocardial infarction rats after blocking of SphK1/S1P signaling pathway, and subsequent analysis showed that SPHK1 plays a central role in the correlation between S1P signaling and immune cell protein expression (42). However, the role of SPHK1 has never been reported in AS.

However, numerous limitations should be noted. First, because all the data analyzed and the results obtained in our present study are collected from public databases, studies for further external validation are also needed. Second, although the expression levels of IGFBP-2 and SPHK1 genes have been analyzed in other cohorts, in vitro and in vivo verification using experiments is also needed.

In conclusion, IGFBP-2 and SphK1 are significantly associated with the molecular mechanism of AS. These conclusions us to understand the OS in AS and other pathophysiological processes that may be involved. Unfortunately, these datasets do not include information about the cause of aortic stenosis. Most crucially, these findings

require confirmation through larger-scale research.

Conflict of interest

The authors have no potential conflicts of interest to report relevant to this article.

Author contributions

SL, ZB and CC designed the study and performed the experiments, SL, ZB and JQ collected the data, CC and JQ analyzed the data, SL, ZB and CC prepared the manuscript. All authors read and approved the final manuscript.

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