Identifying miRNA-TF-mRNA Regulatory Network for Aortic Valve Calcification Diseases Through Bioinformatics Analysis

Mingdong Zhang

Shanghai General Hospital <https://orcid.org/0000-0001-7029-5682>

Zhexin Lu

Shanghai General Hospital

Yongliang Fan

Shanghai General Hospital

Hongbing Gu

Shanghai General Hospital

Sheng Shi

Shanghai General Hospital

Yizhou Ye (✉ yeyizhouvip@sina.com)

Shanghai General Hospital

Research

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Abstract

Background: Calcereous aortic valve disease(CAVD) is widespread in society, and its incidence seems to increase with the age of the global population. However, the specific pathogenesis of calcareous aortic valve disease remains unclear. The purpose of the research is to construct miRNA-TF-mRNA regulatory networks and find the key genes, microRNAs (miRNAs) and transcription factors (TFs) to clarify the pathogenesis of CAVD and provide new treatment methods for CAVD.

Methods: GSE51472, GSE87885 and GSE12644 datasets were downloaded from Gene Expression Omnibus (GEO) database. Differentially expressed genes (DEGs) and differentially expressed miRNAs(DEMs) were identified through integrative analysis by R software. Moreover, KEGG pathway analysis and Gene Ontology (GO) analysis were performed using the clusterProfiler R package. Then, STRING database, Cytoscape and Cytohubba were applied to construct the protein-protein interaction (PPI) network and screen hub genes. Finally, a miRNA-TF-mRNA network was constructed based on bioinformatics data based on the TRRUST, miRWalk and miRtarbase database. And through the verification of dataset GSE12644, we have determined the key regulatory factors.

Results: We have established a regulatory network of miRNA-TF-mRNA includes 17 genes, 17 miRNAs and 12 TFs. In the miRNA-TF-mRNA network, genes VCAM1, ITGB2, CD86, transcription factors HIF1A, KLF2 are identified as key regulators.

Conclusions: Our study has constructed a regulatory network of miRNA-TF-mRNA in CAVD, which may provide new insights about the interaction between genes, miRNAs and TFs in the pathogenesis of CAVD, and identify potential biomarkers or therapeutic targets for CAVD, which may reveal promising approaches for CAVD diagnosis and therapy.

Background

Calcereous aortic valve disease(CAVD) is a common disease that affects patients from middle age, but as individuals are older than > 80 years old, its incidence rapidly accelerates(1). The calcification of aortic valve is a slowly progressing process, involving aortic valve hardening, mild valve thickening, and significant valve calcification until the calcified aortic valve function is impaired.(2). During the progression of the disease, the thickened aortic valve has little effect on the mechanical performance of the valve, and its performance is basically asymptomatic, until it reaches a severe stage, which manifests as syncope, angina pectoris and heart failure, and even requires valve replacement(3). Despite growing knowledge, experience, and technological developments, the pathophysiology underlying CAVD remains incompletely defined, and there is no effective method to delay or change its progress. Surgical intervention and transcatheter aortic valve replacement remain the only available options to treat CAVD while efficient conservative therapy is lacking(4). However, neither mechanical nor bioprosthetic valves are ideal solutions. The bioprosthetic repair valve replacement surgery will face the possibility of biological valve failure and reoperation due to the limitation of biological materials. (5) On the other hand,

mechanical valve replacement requires lifelong anticoagulation therapy because of the increased risk of thrombosis(6).Therefore, it is of great significance to clarify the pathogenesis of CAVD and to find biomarkers for early diagnosis of CAVD and new therapeutic targets.

MicroRNA is an important type of non-coding endogenous RNA, which plays an important regulatory role in cell proliferation, differentiation, migration, apoptosis and other biological behaviors(7). Recent studies have shown that the susceptibility and progress of miRNAs and CAVD are closely related. MiRNAs are also involved in the regulation of the expression of inflammatory factors, fibrosis and calcification-related factors and the regulation of inflammatory cell function, which indicates that it is related to the inflammatory response in the valve. For example, down-regulation of MiR-939 expression in calcified valves may lead to endothelial nitric oxide synthase synthesis reduces and promotes the progression of aortic valve calcification(8).MiR-26a can enhance the expression of calcification suppressor gene JAG2 and inhibit the progression of CAVD(9). Therefore, the in-depth study of the interaction between miRNA and other mechanisms and the search for miRNA molecules that may serve as CAVD markers are of great significance for elucidating the pathogenesis of CAVD, early detection and intervention in the progress of CAVD.

In our study, we constructed the comprehensive miRNA-TF-mRNA co-regulatory network in CAVD and clarified five important regulatory genes. Firstly, we identified the differentially expressed CAVD-related mRNAs. Then we used multiple databases to predict related miRNAs and TFs, and looked for the interaction between them. Based on our findings, a miRNA-TF-mRNA network was then constructed to identify key genes, TFs and miRNAs associated with CAVD, which helps to reveal the complex regulatory mechanisms underlying CAVD and novel markers or targets for the diagnosis and treatment of CAVD. Finally, we used another independent dataset to verify that genes VCAM1, ITGB2, CD86, transcription factors HIF1A, KLF2 are good classifiers of CAVD and might play key roles in the progression of CAVD.

Results

weighted gene co-expression network analysis(WGCNA)

After preprocessing and filtering for the GSE51472 dataset, we selected the top 5000 genes sorted by median absolute deviation from largest to smallest for subsequent analysis. We used R package of "WGCNA" to build the correlation network , and selected the soft threshold power parameter as 16 to ensure a scale-free network(Figure 1A). Finally, Ten modules were identified with a merging threshold of 0.25 (Figure 1B). Then, we plotted the heatmap of module-trait relationships to evaluate the association between each module and clinical traits. Focusing on the status trait , the blue module exhibited the highest positive correlation ($r=0.83$; $P<0.001$) (Figure 1C). The gene significance for CAVD and module membership in the blue module showed a strongly significant correlation, which indicated that genes in the blue module were highly correlated with CAVD (Figure 1D). Subsequently, the genes in the blue gene were chosen for further analysis.

DEGs Screening

According to the filtering criterion described before, we identified the differentially expressed genes (DEGs). The distribution of DEGs between CAVD and healthy controls was intuitively illustrated by the volcano map (Figure 2A), where the red and light blue dots represented the up and downregulated genes, respectively. The heat map was also drawn to show the differences between CAVD and healthy groups (Figure 2B). Finally, we identified 189 overlapping mRNAs by intersecting the DEGs between calcified aortic valves and the normal cases and the mRNAs obtained from the blue module. (Figure 2C).

Functional enrichment and pathway analysis

Significant GO terms and KEGG pathways were ordered by p value and the top six GO terms and KEGG pathways are shown in figure 4. In the term of biological process (BP), the genes were mainly related to the regulation of lymphocyte activation, leukocyte migration, T cell activation (Figure 3A). In the cellular component (CC) group, the genes were mainly enriched for the external side of plasma membrane (Figure 3B). As for the molecular functions (MF) of these genes, they mainly affect the serine-type peptidase activity, antigen binding and immunoglobulin binding (Figure 3C). The chemokine signaling pathway, phagosome and natural killer cell mediated cytotoxicity were the most significant pathway (Figure 3D).

PPI network analysis

The PPI network of 189 DEGs which contained 177 nodes and 1093 edges was constructed and visualized using STRING database. Subsequently, the hub genes were identified by the CytoHubba in the PPI network of the DEGs. In total, 45 genes were identified with the degree > 20 (Figure 4A). Finally, the 45 genes were defined as hub genes. Furthermore, the functional enrichment pathway enrichment of the hub genes was also analyzed shown in Figure 4B. The results of functional enrichment analysis show that the hub gene enriched for Leukocyte Migration, T Cell Activation, Lymphocyte Differentiation, External Side of Plasma Membrane, C-C Chemokine Receptor Activity G Protein-Coupled Chemoattractant Receptor Activity and so on. And these hub genes were significantly associated with Natural Killer Cell Mediated Cytotoxicity, Chemokine Signaling Pathway, Viral Protein Interaction with Cytokine and Cytokine Receptor according to the pathway analysis result.

Data Processing of DEMs and TF-miRNA-mRNA co-expression network construction

We obtained 12 transcription factors through TRRUST. In the GSE87885, we have obtained 103 DEMs using the above filtering criteria and displayed them in the form of heat map (Figure 5A). Then take the intersection with miRNA predicted by miRTarBase and miRwalk, 17 miRNAs were defined (Figure 5B). Finally, a miRNA-TF-mRNA co-expression network was constructed, which concludes 17 genes, 17 miRNAs and 12 TFs (Figure 6).

Validation and Efficacy Evaluation of Hub Genes

The expression levels of the hub genes and TFs in the miRNA-TF-mRNA network were investigated in GSE12644 dataset. As shown in Figure 7, the expression of VCAM1, ITGB2, CD86 and HIF1A was also significantly up-regulated in CAVD patients compared to controls in dataset GSE12644. Similarly, HLF2

is significantly down-regulated. In addition, the receiver operating characteristic(ROC) curve shows that each area under curve(AUC) in the GSE12644 dataset was greater than 0.7, which can better distinguish CAVD from the control group (Figure 8).

Discussion

Calcific aortic valve disease (CAVD) refers to the fibrosis and calcification of the aortic valve and its surrounding tissues caused by various reasons(10). The disease manifests in the early stage of aortic valve sclerosis, punctate calcification of the valve and thickening of the valve leaflets, without hemodynamic changes (aortic valve flow rate < 2m/s), and no obvious clinical symptoms; as the disease progresses further, the annulus and valve tissue appear thickened, hardened and adhered, leading to fibrosis and calcification of the aortic valve, resulting in aortic stenosis (AS), which in turn leads to a series of hemodynamic changes in the body and Seriously endangers human health (11–13). CAVD is not only the result of aging, but an active pathophysiological process involving multiple factors such as lipid deposition, inflammatory cell infiltration, neovascularization, and cell apoptosis(14).

In the present study, we screened significant DEGs and DEMs between the CAVD and normal samples from the GEO database. Subsequently, through a series of bioinformatics analysis, the miRNA-tF regulatory network was constructed and the genes related to CAVD were determined. The GO analysis demonstrated that those DEGs were enriched in regulation of lymphocyte activation, leukocyte migration and T cell activation. KEGG analysis revealed that DEGs mostly related to the chemokine signaling pathway, phagosome and natural killer cell mediated cytotoxicity. Finally, three hub genes and two TFs(VCAM1, ITGB2, CD86, HLF2 and HIF1A) Identified as key regulators of CAVD.

First of all, In our study, the majority of the genes screened in the network were associated with chemokines, including CCL19, CCR1, CCR2, CCR7, CXCR4. At present, most scholars believe that inflammation is the central link in the pathogenesis of calcified aortic valve disease. Chemokines are a secreted small molecule heparin binding protein, belonging to a superfamily of cytokines, which play an important role in inflammatory response(15). It can chemoattract leukocytes for directional movement, reach the local immune response, and participate in immune regulation and immune pathological response(16). New data shows that CCL19 and CCL21 are involved in inflammatory responses and T cells homing in non-lymphoid tissues(17, 18). Now, more and more evidence shows that CCR is not only related to inflammation, but also related to cardiovascular calcification. Studies have found that SDF-1 can induce platelet aggregation and increase intracellular calcium by binding to the SDF-1 receptor CXCR4 expressed by platelets(19). A clinical study showed that CCR5 polymorphism is related to the degree of heart valve calcification and CCR2 was confirmed with the ability to promote the osteoblastic transformation of valvular interstitial cells(20–22). These strongly suggest that chemokines may regulate pathological cardiovascular calcification. More research is needed to prove the connection between CAVD and CCR and the underlying molecular mechanism.

Secondly, We found that VCAM1 and ITGB2 has important significance in the progress of CAVD. Studies have shown that inflammatory activation of endothelial cells is closely related to calcification in CAVD(23). At the same time, inflammation can induce the early and sustained expression of endothelial cell inflammatory adhesion molecules VCAM-1 and ICAM-1(24). And VCAM-1 has been reported with increased level in endothelium of CAVD, which lead to the main route for tissue leukocyte infiltration and inflammatory process(25–27). Meanwhile, the VCAM-1 interacts with very late antigen-4 on activated lymphocytes and leads to the extravasations of activated CD4 and CD8 lymphocytes into the valve tissue and aggravates inflammatory infiltration.(28). ITGB2 is the beta-2 subunit of the integrin LFA-1, which is also expressed on lymphocytes, especially leukocytes(29). Typically, macrophages gain access to the cardiovascular system via interacting with vascular endothelial cells. Adhesion molecules expressed on the surface of endothelial cells interact with a specific receptor expressed on the surface of macrophages allowing firm and sustained adhesion of macrophages to the vasculature initiating the pro-inflammatory response(30). Meanwhile, Past researchs suggest that MRTF-A may regulate the transport of macrophages by activating the transcription of ITGB2 to promote the pathogenesis of cardiac hypertrophy(31). So, we speculate that the interaction of VCAM1 and ITGB2 triggers an inflammatory response in the aortic valve tissue, which in turn leads to the occurrence of calcification.

Third, CD86 is also one of the very meaningful markers. CD86 is a glycoprotein expressed on antigen-presenting cells which provides costimulatory signals to T cells. Research shows that CD86 can activate regulatory T cells and memory effector T cells express a functional form of CD86 that can costimulate naive T cell responses(32). Studies have shown that there are T cell clonal proliferation and CD4 + and CD8 + T cell infiltration in the calcified aortic valve after surgery, it is confirmed that there is inflammation and damage mediated by adaptive immune response elements in calcified aortic valve(33). Choi et al first confirmed the existence of CD86-expressing dendritic cells in aortic valve(34). All these indicate that CD86 may play a role in CAVD, but its specific mechanism still needs to be explored in depth.

Finally, HIF1A and KLF2 are two important transcription factors discovered in this study. HIF is a hypoxia-inducible factor which is the main regulator of oxygen homeostasis. The increased expression of HIF-targeted genes is associated with many human diseases, including ischemic cardiovascular disease, chronic lung disease, and carcinoma(35). Treatment with the HIF1 α inhibitor PX478 significantly reduced calcification of aortic valves in both static and the fibrosa-flow conditions demonstrating their potential as novel anti-CAVD therapeutics(36). In our study, HIF1A is predicted to act as a transcription factor for ITGB2 which is a vital gene in CAVD. So, We speculate that HIF1A-ITGB2 may be one of the important pathways in the pathogenesis of CAVD. KLF2 is a transcription factor induced by laminar flow. Its expression is reduced in calcified human aortic valves and endothelial calcification models, which suggests that KLF2 downregulation may be involved in calcification. Similarly, in our study, we also found low expression of HLF2 in calcified aortic valves. Previous studies have shown that silencing the KLF2 gene can induce the endothelial-mesenchymal transition and lead to calcium phosphate deposition in endothelial cells, which ultimately leads to aortic calcification(37). The above findings indicate that HIF1A and KLF2 are the key molecules in the development of CAVD, which are worthy of our in-depth study.

The miRNA-TF-mRNA regulatory network includes 17 miRNAs, of which 9 miRNAs act on two key TFs respectively. Among them, miRNA-509-5p and miRNA-3121-3p both act on two vital transcription factors(HIF1A,KLF2), however, the interaction between them and the mechanism of action in CAVD have not been reported, and further studies are still needed. MiR-3926 inhibits the proliferation of synovial fibroblasts and the secretion of inflammatory factors by targeting toll-like receptor 5, which suggests that miRNA-3926 may have an impact on the progression of CAVD in the regulation of inflammatory cell function(38). Endothelial cell damage may be the initiating factor in the occurrence of calcified aortic valve stenosis, which is similar to the formation of atherosclerosis(39). miR-186-5p and miR-17-5p are diagnostic biomarkers of atherosclerosis and regulate the proliferation and migration of vascular smooth muscle,which may also mediate the occurrence and progression of CAVD.(40, 41)

Our study has some limitations. First, one limitation of the current research is that the number of samples was relatively small, the input data might still be insufficient to identify and validate key genes in the CAVD development. Second Our study only relies on bioinformatics analysis, and further molecular biology experiments are needed to verify the results of this research. In a follow-up study, the molecular verification experiment will be conducted to verify molecular markers which can be used as a new target for CAVD treatment.

Conclusion

Overall, bioinformatics analysis of mRNA and miRNA expression profiles identified TF-miRNA-mRNA regulation loops, in which VCAM1, ITGB2, CD86, HIF1A and KLF2 might be key genes associated with the development and progression of CAVD, and HIF1A-ITGB2 may be one of the key pathways. These results also contribute towards a deeper understanding of the pathogenesis of CAVD, and unveil potential targets for clinical treatment.

Methods

Dataset Collection

The datasets which contain expression profiles of miRNAs and mRNAs in aortic valves in this study were obtained from the public Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/gds/>) for subsequent analysis. The dataset GSE51472 contains mRNA expression profiles, which includes 5 normal aortic valve samples, 5 fibrotic aortic valve samples and 5 calcified aortic valve samples was based on the platform GPL570(Affymetrix Human Genome U133 Plus 2.0 Array). The miRNA expression profile GSE87885 based on the GPL22555 LC Sciences μ ParaFlo human miRNA array contains aortic valves from two healthy people and three CAVD patients. The dataset GSE12644, which includes 10 calcified aortic valve samples and 10 normal aortic valve samples, was used to verify the hub genes and perform ROC analysis. RAW datas from the GSE51472, GSE87885 and GSE12644 dataset were preprocessed and normalized using the R package limma.

Identification of differentially expressed mRNAs(DEGs)

The WGCNA package in the R package was used for WGCNA analysis to identify module that were significantly associated with CAVD, which was considered as the key module of CAVD. Then, We used the limma package in R studio to get the DEGs between the calcified aortic valves and normal aortic valves. P value < 0.01 and $|\log_2FC| \geq 2$ were considered as the cutoff criteria for the selection of DEGs. Finally, The overlapping genes in the key module and DEGs were defined as the final DEGs, which were significantly correlated with CAVD.

Functional annotation and pathway enrichment analysis

GO terms and signal pathways with significant enrichment were screened out with the threshold p value < 0.05 by the “clusterProfiler” package in R. Of the results that exceeded the threshold, we selected the top 6 KEGG pathways and top 6 terms of each GO domain as significant terms.

Construction of PPI Networks and Identification of Hub Genes

The String database (<http://www.string-db.org>) was applied to construct the protein-protein interaction (PPI) network with the threshold of minimum required interaction score > 0.7 for the DEGs. Cytoscape was used to perform PPI network analysis, the cytoHubba plug-in of Cytoscape was used to calculate the degree rank of DEGs, and genes with degrees greater than 20 were selected as hub genes. Finally, the hub genes were subjected to Pathway Analysis .

Identification of DEMs and miRNA-TF-mRNA co-expression network construction

The TRRUST(<http://www.grnpedia.org/trust>) analysis tool was used to predict the TFs that target at hub genes. The limma package in R studio was used to get the DEMs between the calcified aortic valves and normal aortic valves. The DEMs were screened by the P values < 0.05 and $\log_2FC \geq 2$. The miRWalk (<http://mirwalk.umm.uni-heidelberg.de/>) and miRTarBase(mirtarbase.cuhk.edu.cn) were used to predict the miRNAs which target at TFs gained above. After that, the predicted miRNAs were further filtered by matching the DEMs selected before, and then we got the relationship between DEMs and TFs. Finally, a miRNA-TF-mRNA co-expression network was constructed.

Hub Genes Validation and Efficacy Evaluation

The hub genes and TFs were further validated in GSE12644 datasets downloaded from GEO database. We compared the expression of these hub genes and transcription factors and drew a box diagram. Also, ROC curve was plotted and AUC was calculated with “pROC” package to evaluate the ability to distinguish CAVD patients and controls.

Abbreviations

CAVD:calcareous aortic valve disease

TF:transcription factor

GEO:gene expression omnibus

WGCNA:weighted gene co-expression network analysis

DEG:differentially expressed gene

DEM:differentially expressed miRNA

GO:gene ontology

KEGG:kyoto encyclopedia of genes and genomes

PPI:protein-protein interaction

CC:cellular component

BP:biological process

MF:molecular function

ROC:receiver operating characteristic

AUC:area under curve

Declarations

Ethics approval and consent to participate:

GEO belong to public databases. The patients involved in the database have obtained ethical approval. Users can download relevant data for free for research and publish relevant articles. Our study is based on open source data, so there are no ethical issues and other conflicts of interest.

Consent for publication:Not applicable.

Availability of data and materials:The datasets used and analysed during the current study are available from the public GEO database.

Competing interests:The authors declare that they have no competing interests.

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Authors' contributions: This research was conducted in collaboration with all authors. MZ designed the project. MZ,ZL and YF analyzed and interpreted the results. MZ,HG,SS and YY discussed the results. MZ and ZL wrote the manuscript. All authors reviewed the manuscript. All authors read and approved the final manuscript.

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Authors' information:

Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China

Mingdong Zhang

Department of Cardiovascular Surgery, Shanghai General Hospital, Shanghai Jiao tong University School of Medicine, Shanghai 201620, China.

Zhexin Lu, Yongliang Fan, Hongbing Gu, Sheng Shi and Yizhou Ye

Corresponding author

Correspondence to Yizhou Ye. Department of Cardiovascular Surgery, Shanghai General Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 201620, China. Email: yeyizhouvip@sina.com.

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Figures

Figure 1

A: The scale-free fit index was obtained from the analysis of the scale-free index and the mean connectivity for various soft-threshold powers. B: After dynamic tree cut and merging, 10 gene modules were detected. C: Heatmap of the correlation between module eigengenes and clinical traits. The blue module was significantly correlated with calcification. D: The scatter plot between the blue module membership and the gene significance for CAVD.

Figure 2

A:Volcano plots of the DEGs screened from GSE51472. The light blue plot points represent up-regulated DEGs and red plot points represent down-regulated DEGs. B:Heat map of the common DEGs. Each row represents the DEGs, and each column represents one of the samples of normal or calcified valves. The light blue and red color represent upregulated and downregulated DEGs, respectively. C:Venn diagram shows the common genes between the genes in the blue module and DEGs between CAVD and healthy controls.

Figure 3

A:Results of biological process enrichment analysis. B:Results of cellular component analysis. C:Results of molecular function analysis. D:Results of KEGG pathway enrichment analysis. Abscissa axis is the gene ratio of the enriched gene number. Size of round node represents the count of genes significantly enriched in each pathway or GO term.

Figure 4

A: 45 hub genes screened by CytoHubba. B: The results of CC, BP, MF and KEGG are respectively shown in red, light blue, green, and dark blue.

Figure 5

A:Heat map of DEMs. Each row represents miRNAs, and each column represents one of the samples of normal individuals or patients. Red regions denote that the expression of genes was relatively upregulated and light blue regions indicate that the expression of genes was relatively downregulated.

B:Venn diagram of the common miRNAs from the DEMs and miRNAs obtained from miRWalk and miRTarBase.

Figure 6

MiRNA-TF-mRNA regulatory network. Red circles represent genes, green triangles represent miRNAs and yellow diamonds represent TFs.

Figure 7

Validation of hub genes and TFs in dataset GSE12644. Expression levels of VCAM1 , ITGB2, CD86 and HIF1A was also significantly up-regulated were significantly upregulated in CAVD patients, and HLF2 is significantly down-regulated.

Figure 8

ROC curve of hub genes and TFs including VCAM1 , ITGB2, CD86 ,HLF2 and HIF1A in dataset GSE12644.