



Identification of key genes and pathways in calcific aortic valve disease by bioinformatics analysis

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Background: Calcific aortic valve disease (CAVD) is the most common type of valvular heart disease in the elderly. This study is aimed to explore molecular mechanism of CAVD via bioinformatics analysis.

Methods: The gene expression profiles of GSE51472 (including 5 normal aortic valve and 5 calcified aortic valve) and GSE83453 (including 8 normal aortic valve and 19 calcified aortic valve) were downloaded from the Gene Expression Omnibus (GEO) database. Differentially expressed genes (DEGs) were screened using the MetaDE package in R software. Functional and pathway enrichment analysis were performed based on Gene ontology (GO) and KEGG pathway database. Then, STRING database, Cytoscape and MCODE were applied to construct the protein-protein interaction (PPI) network and screen hub genes. Pathway enrichment analysis was further performed for hub genes and gene clusters identified via module analysis.

Results: A total of 107 DEGs were identified in CAVD (53 up-regulated genes, and 54 down-regulated genes), and they were mainly enriched in the terms of immune response, extracellular matrix organization, leukocyte transendothelial migration, cell adhesion molecules (CAMs), and fatty acid metabolism. Five hub genes including VCAM1, MMP9, ITGB2, RAC2, and vWF were identified via PPI network, which were mainly enriched in terms of leukocyte transendothelial migration and cell adhesion. An independently down-regulated protein cluster containing ALDH2, HIBCH, ACADVL, ECHDC2, VAT1L, and MAOA was also identified via PPI network.

Conclusions: The present study identified VCAM1, MMP9, ITGB2, RAC2, vWF and ALDH2 as key genes in the progression of CAVD. Immune cells infiltration might play a key role in the progression of CAVD, while ALDH2-mediated detoxification effect might play a protective role in CAVD. Further studies are needed to elucidate the pathogenesis of CAVD.

Keywords: Calcific aortic valve disease (CAVD); Gene Expression Omnibus (GEO) data; differentially expressed genes (DEGs); integrated bioinformatics analysis

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Introduction

Calcific aortic valve disease (CAVD) is the most common type of valvular heart disease in the elderly (1). Calcific aortic valve stenosis occurred in 2% of people above 65 years old; while the incidence of aortic valve sclerosis, the early stage of CAVD without hemodynamic consequence,

was as high as 26% (2). Even without hemodynamically significant obstruction of left ventricular outflow, CAVD still can be an independent risk factor for cardiovascular mortality, all-cause mortality and myocardial infarction (1,3). Currently, the only effective therapeutic option to improve clinical outcomes in CAVD patients is early surgery (4).

Histological studies suggested that the early lesion of CAVD was an active inflammatory process shared some similarities with atherosclerosis, such as lipid deposition, macrophage and T-cell infiltration, and basement membrane disruption (5). While the end-stage of CAVD usually involved heterotopic ossification consisting of mature lamellar bone formation and active bone remodeling (6). Several serum biomarkers such as lipoprotein(a) (2,7), LDL-C (2), and serum phosphate (8) had been found to be associated with the prevalence of CAVD. Various molecular, cellular, and biomechanical mechanisms had been found involved in the progression of CAVD, such as bicuspid aortic valve, osteogenic mechanisms, endocrine mechanisms, dysregulated mineral metabolism, osteoclast deficiency, and developmental signaling (9). Growing evidences suggested that the progression of CAVD was a actively regulated process, which might be suspended or reversed via potential pharmacologic intervention (10). However, thus far, there have been no identified pharmacologic interventions or presumably underlying pathways that affect the CAVD progression process. Besides, the key genes and signaling pathways underlying the progression of CAVD remain poorly understood.

Gene expression microarrays have been widely used to study gene expression profiles in many human diseases, which provide an effective way for studying disease-associated genes and discovering novel therapeutic targets and biomarkers (11). Recently, several studies had compared the mRNA expression profile of CAVD (12,13). However, the results were inconsistent due to small sample size, different technological platforms, different data processing and sample heterogeneity. The disadvantage of these single studies can be overcome by integrated bioinformatics analysis, which combines different microarray dataset to obtain a more steady result (14). MetaDE package developed by Wang *et al.* contains more than 12 popular genomic meta-analysis methods, which is an effective integrative analysis solution (15).

In the present study, we applied integrated bioinformatics methods to identify key genes and pathways in CAVD. Two microarray profile datasets [GSE51472 (12) and GSE83453 (13)] were downloaded from the Gene Expression Omnibus (GEO) database. Differentially expressed genes (DEGs) were screened using the MetaDE package in R software. Gene ontology (GO) and pathway enrichment analysis of DEGs were then performed. A protein-protein interaction (PPI) network was established,

and key genes and pathways associated with CAVD were analyzed.

Methods

Microarray data

The present study was a bioinformatics study based on publicly accessible microarray datasets. Thus, an ethics approval was not required. The gene expression profiles of GSE51472 (12) and GSE83453 (13) were downloaded from the GEO database (<http://www.ncbi.nlm.nih.gov/geo>). The platform for GSE51472 is GPL570, Affymetrix Human Genome U133 Plus 2.0 Array, which includes 5 normal aortic valve samples, 5 fibrotic aortic valve samples and 5 calcified aortic valve samples. The control group contained aortic valves from patients underwent surgery due to ascending aortic aneurysm or dissection, while the valves were macroscopically smooth, pliable and opalescent, and without visible calcifications. The fibrotic group contained valves that were macroscopically thickened and stiff, while the patients had no significant transvalvular pressure gradient. The calcified group contained aortic valves from patients with severe aortic valve stenosis, and the valves were presented with varying degrees of calcification. Only the normal and calcified aortic valve samples were used for our bioinformatics analysis. The platform for GSE83453 is GPL10558, Illumina HumanHT-12 V4.0 expression beadchip, which consists of 8 normal tricuspid aortic valve samples, 10 calcified bicuspid aortic valve samples and 9 calcified tricuspid aortic valve samples. The calcified bicuspid and tricuspid aortic valve samples were merged to build the calcified aortic valve group in our bioinformatics analysis. The dataset information is shown in *Table 1*. The data were normalized and log₂ transformed.

Data preprocessing

The probe IDs was converted into international standard names for genes (gene symbol). For multiple probes that mapped to a single gene, interquartile range (IQR) of each probe was calculated with MetaDE.match package in R software, and the probe with largest IQR was selected to represent the expression level of the gene. Next, MetaDE.merge package was used to merge the gene expression profiles to obtain the commonly profiles genes between the two datasets. Finally, either 30% un-expressed genes (small mean intensity) or 30% un-informative genes (small

Table 1 Details of GEO datasets

GEO accession	Platform	Reference	Sample	Normal	Calcified
GSE51472	GPL570	Ohukainen <i>et al.</i> [2015]	Aortic valve	5	5
GSE83453	GPL10558	Guaque-Olarte <i>et al.</i> [2016]	Aortic valve	8	19

GEO, Gene Expression Omnibus.

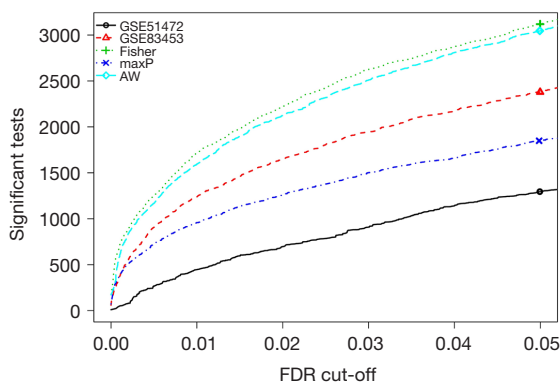


Figure 1 Detection competency curves of individual and integrated analysis. In each individual dataset, moderated-*t* statistics was used to generate P values while AW, maxP, and Fisher's methods were utilized to combine these P values for integrated analysis. This figure was generated using the MetaDE package in R. AW, adaptive weight; maxP, maximum P value method; FDR, false discovery rate.

standard deviation) were filtered out by MetaDE.filtering package, and the remaining 7,946 genes were remained for further analysis.

Integration of microarray data

MetaDE package was used to perform the integrate analysis (15). Moderate *t* test was used to determine the gene expression difference in each dataset, and the maximum P value method (maxP) was used to combine the P values from moderate *t* test. An adjusted P value of <0.0001, based on the false discovery rate (FDR) using Benjamini-Hochberg method was used to select DEGs.

GO and pathway enrichment analysis of DEGs

GO annotations were performed using DAVID database (<https://david.ncifcrf.gov/>) (16). KEGG (17) pathway enrichment analysis was performed using the KOBAS online analysis database (<http://kobas.cbi.pku.edu.cn/>).

Up- and down-regulated DEGs were subjected to GO and pathway enrichment analysis separately. P value <0.05 was used as threshold value.

PPI network analysis

The STRING database (<http://string-db.org/>) (18) was used to identify the interactions between proteins encoded by DEGs based on experimental data, databases, text mining, and predictive bioinformatics data. A combined score of >0.4 was set as threshold value. PPI networks were constructed with Cytoscape software (19). The genes with higher degrees of interaction were considered as hub genes, and pathway enrichment analysis was performed for these hub genes. The plug-in Molecular Complex Detection (MCODE) was used to screen the modules of PPI network in Cytoscape software. MCODE score >3 and number of nodes >4 were considered as threshold value. Pathway enrichment analysis were performed for genes in the modules.

Results

Identification of DEGs

Three method were used to detect common DEGs between the two microarray datasets: the maximum P value method (maxP), the Fisher's method (Fisher), and the adaptive weight method (AW). The detection competency curves (Figure 1) suggested all the three methods were useful to detect common DEGs between the two datasets. The maxP method was chosen in this study. Under the threshold of adjusted P value of <0.0001, 107 DEGs were identified in CAVD. Among the 107 DEGs, 53 were up-regulated genes, and 54 were down-regulated genes (Table 2). Heatmap showed the expression pattern of these DEGs (Figure 2), suggesting the consistency of the 107 DEGs in each dataset.

GO analysis

GO terms were divided into three functional groups:

Table 2 Screening DEGs in CAVD by integrated analysis of microarray

DEGs	Gene names
Up-regulated	<i>TAGLN, SMOC2, A2M, VCAM1, MFAP5, TNFRSF11B, SH3KBP1, MMP9, IBSP, STMN2, IL7R, HTRA1, THBS2, THY1, CTHRC1, SCG2, CCL19, PLOD2, SLC20A1, ARL4C, VWF, IGJ, SYTL2, PTGER4, FCGR1B, SERPINA1, ITGB2, SLAMF8, CA12, CD53, PLAUR, FCGR2A, CD93, ENPP2, HLA-DPA1, HLA-DRA, HLA-DMB, CD14, HLA-DMA, RGS19, PLAU, SPP1, RAC2, CD74, CYBA, WAS, ARHGAP30, CXCL16, C5AR1, LAPTM5, CORO1A, TYROBP, NCF4</i>
Down-regulated	<i>F5, AFF1, KLF4, TMEM100, RHOA, HIBCH, CDH19, FOXO1, LIMCH1, MEIS3P1, SNX1, MEIS2, TMOD1, LPAR1, IL17D, SPON1, IGSF10, ECHDC2, MXI1, TSPAN8, SCARA5, EHP1, PRTFDC1, LOC729680, CLDN11, MASP1, MAOA, ALDH2, TCEAL2, PLCE1, PTGDS, SHC4, VAT1L, GPR83, DCLK1, ITM2A, PGM5, ABCA8, PPAP2B, ABLIM1, NFIA, ANGPTL7, COL6A6, COL4A3, RHOB, PMP22, NTRK2, GPM6B, RBMS3, SESTD1, GSN, SPTBN1, FEZ1, ACADVL</i>

DEGs, differentially expressed genes; CAVD, calcific aortic valve disease.

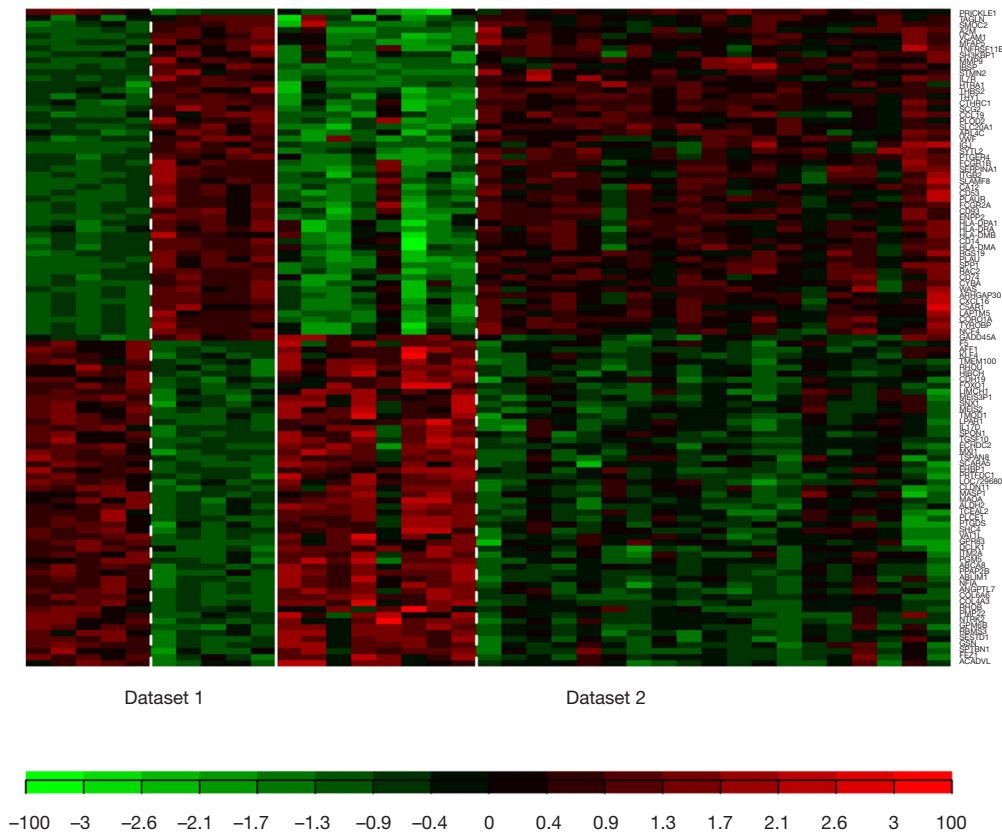


Figure 2 The heatmap of the actual expression profiles for the 53 up- and 54 down-regulated DEGs. The heatmap was generated using MetaDE package in R. The expression profiles greater than the mean are colored in red and those below the mean are colored in green. 0, normal aortic valve; 1, calcified aortic valve; DEG, differentially expressed gene.

biological processes (BP), cellular component (CC), and molecular function (MF). The top 5 enriched GO terms in each category were listed in *Table 3*. For BP, the up-regulated DEGs were mainly enriched in GO terms of immune response, extracellular matrix organization, antigen

processing and presentation via MHC class II, positive regulation of T cell proliferation, and chemotaxis; while the down-regulated DEGs were mainly enriched in GO terms of fatty acid beta-oxidation, cell adhesion, cytoskeleton organization, cellular response to hydrogen peroxide. For

Table 3 GO analysis of DEGs

GO ID	Term	No. of genes	P value
Up-regulated			
GO-BP terms			
GO:0006955	Immune response	14	1.97×10 ⁻¹⁰
GO:0030198	Extracellular matrix organization	8	1.73×10 ⁻⁶
GO:0002504	Antigen processing and presentation of peptide or polysaccharide antigen via MHC class II	4	1.64×10 ⁻⁵
GO:0042102	Positive regulation of T cell proliferation	5	3.00×10 ⁻⁵
GO:0006935	Chemotaxis	6	3.04×10 ⁻⁵
GO-CC terms			
GO:0042613	MHC class II protein complex	5	3.83×10 ⁻⁷
GO:0005886	Plasma membrane	29	5.61×10 ⁻⁷
GO:0009986	Cell surface	11	2.13×10 ⁻⁶
GO:0070062	Extracellular exosome	21	3.29×10 ⁻⁵
GO:0005615	Extracellular space	14	6.22×10 ⁻⁵
GO-MF terms			
GO:0023026	MHC class II protein complex binding	4	1.25×10 ⁻⁵
GO:0032395	MHC class II receptor activity	3	8.46×10 ⁻⁴
GO:0004872	Receptor activity	5	0.004
GO:0001948	Glycoprotein binding	3	0.015
GO:0019864	IgG binding	2	0.031
Down-regulated			
GO-BP terms			
GO:0006635	Fatty acid beta-oxidation	3	0.007
GO:0007155	Cell adhesion	6	0.011
GO:0007010	Cytoskeleton organization	4	0.012
GO:0070301	Cellular response to hydrogen peroxide	3	0.012
GO:0032060	Bleb assembly	2	0.029
GO-MF terms			
GO:0003779	Actin binding	5	0.007

GO, gene ontology; DEGs, differentially expressed genes; BP, biological process; CC, cellular component; MF, molecular function.

CC, the up-regulated DEGs were mainly enriched in GO terms of MHC class II protein complex, plasma membrane, cell surface, extracellular exosome, and extracellular space; while no GO-CC term was enriched from down-regulated DEGs. For MF, the up-regulated DEGs were mainly enriched in GO terms of MHC class II binding and receptor activity.

Pathway enrichment analysis

The most significantly enriched pathways of DEGs analyzed by KEGG analysis were listed in *Table 4*. The up-regulated DEGs were enriched in phagosome, complement and coagulation cascades, leukocyte transendothelial migration, cell adhesion molecules (CAMs), and antigen

Table 4 KEGG pathway enrichment analysis of DEGs

ID	Description	No. of genes	P value	Genes
Up-regulated				
hsa04145	Phagosome	11	2.47×10 ⁻¹⁶	FCGR2A, THBS2, CORO1A, HLA-DMB, HLA-DRA, HLA-DPA1, ITGB2, NCF4, CD14, CYBA, HLA-DMA
hsa04610	Complement and coagulation cascades	7	2.11×10 ⁻¹¹	SERPINA1, VWF, ITGB2, A2M, PLAUI, C5AR1, PLAUR
hsa04670	Leukocyte transendothelial migration	7	3.02×10 ⁻¹⁰	ITGB2, MMP9, THY1, NCF4, VCAM1, CYBA, RAC2
hsa04514	CAMs	6	4.94×10 ⁻⁸	ITGB2, HLA-DMA, HLA-DMB, HLA-DRA, HLA-DPA1, VCAM1
hsa04612	Antigen processing and presentation	5	8.39×10 ⁻⁸	HLA-DRA, HLA-DMA, HLA-DPA1, CD74, HLA-DMB
Down-regulated				
hsa04933	AGE-RAGE signaling pathway in diabetic complications	3	0.003	COL4A3, FOXO1, PLCE1
hsa00340	Histidine metabolism	2	0.0005	ALDH2, MAOA
hsa00410	beta-Alanine metabolism	2	0.0009	ALDH2, HIBCH
hsa00380	Tryptophan metabolism	2	0.0014	ALDH2, MAOA
hsa00071	Fatty acid degradation	2	0.0017	ALDH2, ACADVL

DEGs, differentially expressed genes; CAMs, cell adhesion molecules.

processing and presentation. It was noteworthy that four HLA molecules (HLA-DRA, HLA-DMA, HLA-DPA1, HLA-DMB) presented in three pathways associated with immune response, while ITGB2 and VCAM1 presented in two pathways associated with cell adhesion and migration. The down-regulated DEGs were enriched in AGE-RAGE signaling pathway, metabolism of amino acid (histidine, beta-Alanine, tryptophan) and fatty acid. ALDH2 presented in metabolism of amino acids and fatty acid.

PPI network analysis

A total of 61 nodes and 143 protein pairs were obtained with a combined score of >0.4 based on the STRING database (Figure 3A). The top 5 hub genes a degree of interaction ≥10 were VCAM1 (degree =16), MMP9 (degree =14), ITGB2 (degree =14), RAC2 (degree =13), and vWF (degree =10). Pathway enrichment analysis suggested that these hub genes mainly involved in leukocyte transendothelial migration and cell adhesion (Figure 3B). MCODE detected a significant protein cluster containing HLA-DPA1, HLA-DRA, HLA-DMA, HLA-DMB, and CD74 (Figure 4A). Pathway enrichment analysis showed that these genes mainly involved in antigen processing and presentation and

CAMs (Figure 4B). We also conducted pathway enrichment analysis to the independently down-regulated protein cluster containing ALDH2, HIBCH, ACADVL, ECHDC2, VAT1L, and MAOA (Figure 4C), which suggested that these genes mainly involved in metabolism of fatty acid and various amino acids (Figure 4D).

Discussion

In the present study, a total of 107 DEGs (53 up and 54 down-regulated genes) were identified in CAVD by integrated analysis of microarray datasets (GSE51472 and GSE83453). Further bioinformatics analysis suggested that VCAM1, MMP9, ITGB2, RAC2, and vWF had a high degree of interaction in the PPI network and were key genes in CAVD. These key genes were significantly enriched in pathway of leukocyte transendothelial migration. The gene cluster containing HLA-DPA1, HLA-DRA, HLA-DMA, HLA-DMB, and CD74 detected by MCODE also suggested the role of immune response in CAVD. Moreover, the down-regulated gene ALDH2 might play a protective role in CAVD.

Inflammation is involved in the development and progression of CAVD (20), and calcification of the aortic valve

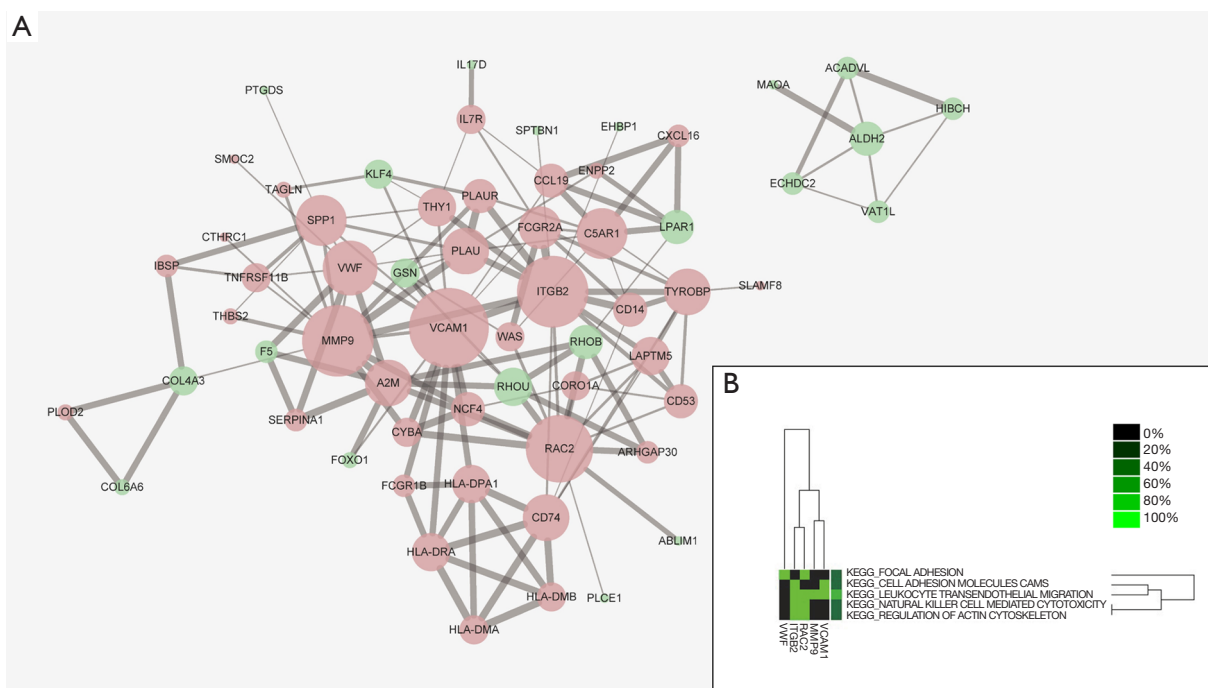


Figure 3 PPI network analysis. (A) Protein-protein interaction network of DEGs. Red nodes represent up-regulated genes and green nodes represent down-regulated genes. The size of each node is positively correlated with the degree of interaction. The width of each edge is positively correlated with the combined score; (B) pathway enrichment analysis of the top 5 hub genes. DEG, differentially expressed gene; PPI, protein-protein interaction.

was considered an inflammation-dependent process (21). T lymphocytes (22) and macrophages (5) had been identified in early aortic valve lesions. In the present study, 4 hub genes (*VCAM1*, *MMP9*, *ITGB2*, and *RAC2*) involved in leukocyte transendothelial migration, which was the first step of the immune cells infiltration. Aortic valve endothelial cells cover the surface of the aortic valve, and provide a protective function on aortic valve (23,24). High expression of *VCAM1* had been found in the endothelium of calcified human aortic valves (24). *VCAM1* on the endothelial cells are involved in the firm adhesion of leukocytes (25), and it also activates intracellular calcium release and NADPH oxidase Nox2, which further promotes the migration of leukocytes (26). Increase in *MMP-9* had been found in aortic stenosis (27). *MMP9* is synthesized by macrophages, which regulates the activation of T lymphocytes (28). *MMP9* also can regulate leukocyte migration by processing chemokines and cytokines (29). *ITGB2* normally expressed only in leukocytes, which promote leukocyte adhesion to endothelium (30). *RAC2* is a member of GTPase signaling, which had been proven involved in regulation of leukocyte transendothelial migration (31). However, the role of

ITGB2 and *RAC2* in CAVD has not been illuminated yet. *HLA-DPA1*, *HLA-DRA*, *HLA-DMA*, *HLA-DMB*, and *CD74* are MHC class II molecules, which mainly expressed in antigen presenting cells such as macrophages and dendritic cells (32). It had been previously reported that the proportion of circulating CD8+ T cell expressing *HLA-DR* was elevated during CAVD, which suggested that besides leukocytes infiltration of the aortic valve, a systemic adaptive immunity also play a role during CAVD (33).

vWF plays a major role in platelet adhesion and hemostasis (34). An *in vitro* study conducted by Balaoing *et al.* showed that *vWF* stimulated by histamine from porcine aortic valve endothelial cells significantly increased valvular interstitial cell calcification *in vitro*, which suggested a possible role of *vWF* in progression of CAVD (35).

ALDH2 is a detoxifying enzyme for the removal of toxic acetaldehyde such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) (36). More than 40% of the East Asians population carries a common *ALDH2*2* mutant allele (Glu504Lys), which results in reduction of the enzymatic activity (37). Recently, various studies had highlighted the relationship between *ALDH2* and

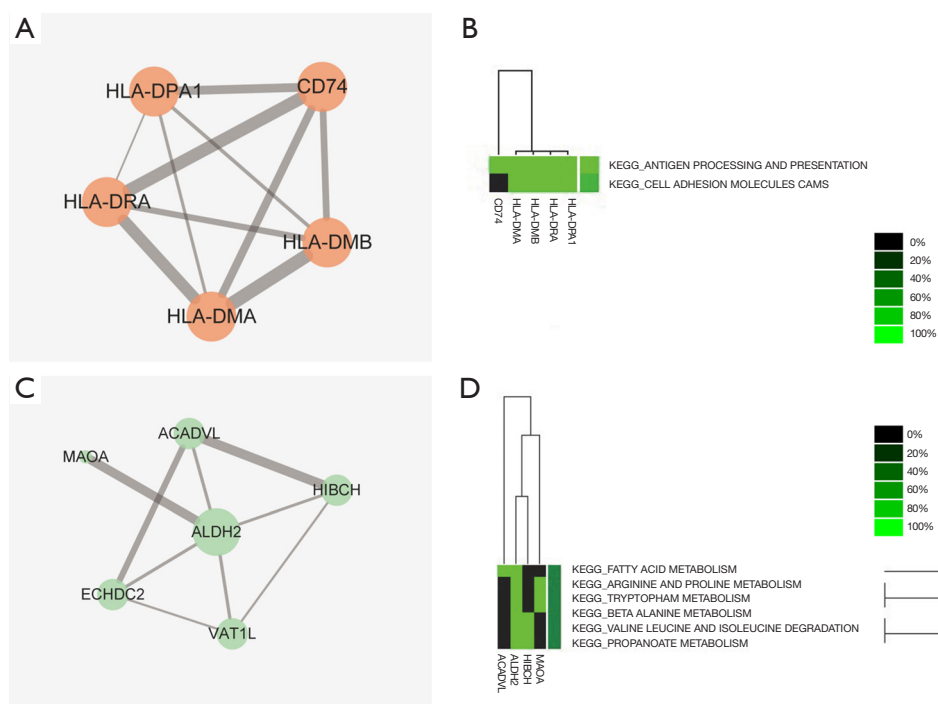


Figure 4 Modules from the protein-protein interaction network. The significant up-regulated gene cluster (A), and pathway enrichment analysis of this cluster (B); the significant down-regulated gene cluster (C), and pathway enrichment analysis of this cluster (D).

cardiovascular disease. Epidemiological studies suggested that the inactive East-Asian variant of ALDH2 (ALDH2*2) increased the risk of coronary artery disease (38). Animal study suggested that Alda-1, an activator of ALDH2, inhibited atherosclerosis in apolipoprotein E-knockout mice (39). *In vitro* study also suggested that activation of ALDH2 significantly attenuated the oxygenized LDL induced endoplasmic reticulum stress and apoptosis in smooth muscle cells (40). The present study suggested that the expression of ALDH2 was down-regulated in CAVD, however, the role of ALDH2 in CAVD had not been reported yet. It has been demonstrated that dysregulation of antioxidant mechanisms led to increased oxidative stress, which contributed to the progression of CAVD (41). We speculated that ALDH2-mediated detoxification effect reduced the oxidative stress in aortic valve, which might play a protective role in CAVD.

There are several limitations of the present study. The sample sizes of the microarray datasets used in the integrated analysis were relatively small. Besides, due to the very small sample sizes, subgroup analysis was not able to be performed to distinguish the possible different

mechanisms involved between bicuspid and tricuspid valves. Although the integrated bioinformatics approach obtained a steadier result than single microarray, further studies with larger sample sizes covering wider range of races are still needed.

In conclusion, the present study identified VCAM1, MMP9, ITGB2, RAC2, vWF and ALDH2 as key genes in the progression of CAVD by integrated analysis of microarray datasets. Further bioinformatics analysis suggested that immune cells infiltration might play a key role in the progression of CAVD, while ALDH2-mediated detoxification effect might play a protective role in CAVD. The study provides a set of useful targets for further investigation into the molecular mechanisms, biomarkers and treatment targets of CAVD. Further molecular biological experiments are needed to confirm the function of the identified genes associated with CAVD.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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