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The Regulating Network and Significance of Neutrophils in Aortic Valve Stenosis

Hanghang Du[1,2#], Peian Cai[1,3#], Xuan Jiao[1,2], Chang Liu[2], Hai Tian[1,2*], Wei Chen[1,2,4*]

[1]Department of Cardiovascular Surgery, The Second Affiliated Hospital of Harbin Medical University, Harbin, 150000, China.
[2]Future Medical Laboratory, The Second Affiliated Hospital of Harbin Medical University, Harbin, 150000, China.
[3]Department of Thoracic Surgery, The Affiliated Cancer Hospital of Zhengzhou University & Henan Cancer Hospital, Zhengzhou, 450008, China.
[4]Key Laboratory of Preservation of Human Genetic Resources and Disease Control in China, Ministry of Education, Harbin Medical University, Harbin, 150000, China.
*Correspondence to: Hai Tian and Wei Chen (E-mail: doctor_tianhai@163.com; cw19820120@163.com)
#Hanghang Du and Peian Cai contributed equally to this work.
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Abstract

Objective: Valves replacement is the only strategy for aortic valve stenosis (AVS) treatment. A comprehensively understanding about pathogenesis of AVS would be helpful for individualized treatment of AVS in the future.

Methods: The mRNA profiles of Normal and AVS samples were harvested from the Gene Expression Omnibus database. The differently expressed genes (DEGs) were identified via limma package. Among the DEGs, the least absolute shrinkage and selection operator (LASSO) logistic regression and random forest (RF) analysis were utilized to identify the biomarkers for AVS. Cibersort package were used to assess the difference of infiltration levels of 22 types of immune cells between Normal and AVS groups. Besides, the most significant immune cell was also evaluated by RF analysis. The relationships between the identified biomarkers and immune cells were assessed via correlation analysis. Set Enrichment Analysis (GSEA) of single genes was conducted to reveal the potential mechanisms involved by the biomarkers. And the DGIdb database was utilized for the drug prediction for the crucial biomarkers.

Results: There was a total of 543 DEGs including 315 up-regulated and 228 down-regulated DEGs. Among them, CXCL5, COL4A3 and EPB41L4B were the significant biomarkers of AVS. The T cells CD4 memory resting and activated, plasma cells, M0 macrophages, T cells regulatory (Tregs) and neutrophils were the significantly infiltrative immune cells in which neutrophils was the most important immune cell type in AVS. CXCL5 could regulate all significantly infiltrative immune cells involved in AVS development, while COL4A3 and EPB41L4B only mediated the neutrophils in AVS. Moreover, ECM receptor interaction, focal adhesion, chemokine signaling pathways and insulin signaling pathway were the main mechanisms involved by the biomarkers. Collagenase clostridium histolyticum and Ocriplasmin was the potential drug for COL4A3.

Conclusion: The infiltration of neutrophils mediated by CXCL5, COL4A3, and EPB41L4B may be a pivotal mechanism of AVS.

Keywords: Aortic valve stenosis, machine learning, immune infiltration, biomarker, neutrophils

Introduction

With the aging of the population, aortic valve stenosis (AVS) has become one of the most frequent valve heart diseases.[1] Besides, on the account of the decline of the incidence rate of rheumatic aortic stenosis, aortic valve calcification has become the most common pathogeny for AVS.[2] According to the statistics, the incidence of aortic valve calcification reached 26% in 5,201 volunteers aged 65 and older, most of whom were in the early stages without hemodynamic disturbances, but increased to 48% in older adults aged 85 and older.[3] Although the open surgery and transcatheter intervention for valve replacement could be administrated for AVS,[4] there are no satisfactorily clinic strategies to prevent or control AVS.[5] Reveling the biological progress of AVS is still in the need.

At the beginning of AVS, a certain amount of calcium deposition in the aortic cusps would first lead to the aortic valve sclerosis without obstruction to left ventricular outflow.[6] However, as the disease progresses, calcified nodules would gradually invade the valve leaflet and annulus, leading to valve stenosis and hemodynamic changes.[7,8] Although the specific mechanism from normal valve to stenosis is still unclear, most scholars believe that the inflammatory response on the valve is an important link.[9,10] It is reported that a large number of activated pro-inflammation cells could be detected on the stenosis aortic valve, which could release numerous inflammatory factors, chemokines, as well as proinflammatory prostaglandins and leukotrienes to mediate inflammatory response.[11-14] Besides, the increased mechanical stress could also enhance local inflammation response of valve endothelial cell and further propagate the influx of inflammatory cells.[15] The inflammatory environment of the valve could also lead to impaired cell function, which mainly includes of the breakdown of extracellular matrix (ECM) synthesis and degradation.[16] Some studies believed that the imbalance of ECM degradation and synthesis was the initial stage of valve calcification.[17,18] And with the stimulation of inflammatory, the bone morphogenic protein (BMP2), RUNX family transcription factor 2 (Runx2), as well as intracellular adhesion molecule 1 (ICAM-1) of valvular interstitial cells could be activated and exerted a tight link between inflammation and calcification.[19] Some anti-inflammation strategies for have also proved the significance of inflammation in AVS. It is reported that hydrogen sulfide, evogliptin could exhibit a potent inhibition on calcification of aortic valve in animal model via suppressing inflammatory response.[20,21]

The process of inflammation in AVS was quite complex in which the various immune cells exert different roles in the inflammatory process.[22,23] The macrophages, mast cells, CD8+ T cells had been largely confined to the lesions of AVS.[5] Functionally, macrophages change local proinflammatory levels by releasing inflammatory factors of Interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)-α.[24] The infiltration of mast cells would promote the angiotensin II synthesis that is contributing

to ECM remodeling.[25] And CD8+ T cell accumulation close to endothelial cells that produced interferon-γ and the damage of valve in AVS.[26] However, the more specific mechanism of immune regulation in AVS is still unclear. Therefore, an in-depth understanding of the inflammatory and immune regulation mechanism of AVS may provide a new perspective for the early intervention and treatment for AVS.

In the present study, the array profiles of normal and AVS aortic valve were harvested from the database of Gene Expression Omnibus (GEO) and utilized for screening the significant biomarker via the techniques of bioinformation and machine learning. Moreover, the landscape of immune infiltration in AVS was also investigated and the key cells type in AVS was determined via the machine learning method. Sequentially, the synergistic relationship among different immune cells and the correlation relationship between biomarker and different immune cells were revealed. Finally, the potential target drugs for biomarker were also predicated based on a specific online database. The results of this study were expected to provide some theoretical references for the prevention and treatment of AVS in the future.

Materials and Methods

Data Acquisition and Processing

The dataset of GSE51472 and GSE12644 were downloaded from GEO database (https://www.ncbi.nlm.nih.gov/geo/). GSE51472 concluded 5 cases of Normal, sclerotic aorta valve and AVS respectively.[27] And GSE12644 included 10 cases of Normal and AVS respectively.[28] The above datasets were both originated from the GPL570 platform of the Affymetrix Human Genome U133 Plus 2.0 Array. The raw matrix of both datasets was all performed the $\log2^{(x+1)}$ transformation. And every probe ID was converted into the official gene symbol according to the annotation of GPL570 platform. The maximum expression value of the same gene symbol was determined as its expression value in corresponding sample. With the aim for exploring the mechanism of AVS, the 5 case of sclerotic aorta valve were exclude and a total of 30 sample (including 15 case Normal and 15 case AVS) was integrated and were eliminated the batch effects via the "SVA" R package.[29]

The Differentially Expressed Genes (DGEs) Analysis

The DEGs analysis were performed with the online tool based on "limma" R package and developed by Sangerbox (http://www.sangerbox.com/home.html) between the Normal and AVS groups.[30,31] And the DEGs were determined with the threshold of |Fold Change (FC)| > 2 and P value < 0.05.

Functional Enrichment Analysis for DEGs

With the aim for investigating the potentially biological function of DEGs, the function enrichment analyses were conducted with the DEGs via the "clusterProfiler" package.[32] The potential biological functions of DEGs were mainly analyzed from Biological Progress (BP), Cellular Component (CC), Molecular Function (MF) and Kyoto Encyclopedia of Genes and Genomes (KEGG). The terms with the threshold of the adjusted P value < 0.05 were thought to be significance. And the Top 10 terms ranged by Count were visualize by bubble chart.

Screening the Hub Biomarkers for AVS Diagnosis

To determine the potential diagnostic markers for AVS, the andom forest (RF) and least absolute shrinkage and selection operator (LASSO) logistic regression methods were conducted for determining the significant genes for AVS.[33,34] The LASSO regression prediction model was performed with R package "glmnet"[35] where the alpha and nlambda were set as 1 and 1,000 respectively. The lambda.min was selected as the optimal lambda. The "randomForest" R package was utilized for RF analysis.[36] The common genes that were identified with LASSO and RF were considered as the hub markers for AVS diagnosis. The diagnostic receiver operating characteristic (ROC) analysis and the area under the curve (AUC) of ROC analysis were performed with online tool based on "pROC" R package and developed by Sangerbox (http://www.sangerbox.com/home.html) for evaluation of the diagnostic efficiency of biomarkers.[31,37]

Immune Cell Infiltration Analysis

In order to compare the level of immune cell infiltration between the Normal and AVS groups, the proportion of 22 types immune cell were calculated using the "Cibersort" R package[38] with a reference set of LM22.[39] And the result of immune cell infiltration between different groups was visualized by violin chart and $P < 0.05$ was thought to be significant. The correlation relationship among the different cells was analyzed with "corrplot" R package via Spearman's rank test and visualized by heatmap.[40] Moreover, the hub type of immune cell was also been determined by RF analysis. Finally, the correlation relationship between the amounts of immune cells and the levels of biomarkers were analysis Spearman's rank correlation.

Gene Set Enrichment Analysis (GSEA)

With the aim for identifying the potential function and mechanism of the biomarkers, the GSEA for the biomarkers was performed with the GSEA function of an online tool of sangerbox (http://www.sangerbox.com/home.html) with a reference C2: KEGG gene sets.[31]

Potential Therapeutic Drugs for AVS

In order to screen the potential drugs for AVS treatment, the biomarkers were utilized as the targets for drugs prediction via DGIdb database (https://www. dgidb.org/) in which the gene–drug interaction data were recorded.[41] And the relationships among drugs, genes and corresponding immune cells were displayed as sankey diagram.

Collection of Clinical Samples

Three stenosis valve samples were collected from patients with AVS, diagnosed by transthoracic echocardiography and CT, undergoing valve replacement surgery. Normal and sclerosis valves were all collected from patients undergoing prosthesis ascending aortic replacement with aortic valve due to type I aortic dissection. Valves obtained from type I aortic dissection were classified as sclerosis if yellowish calcification spots were found and the valve were slightly thickened without obstruction to left ventricular outflow examined by echocardiography and CT. Valves from type I aortic dissection were classified as normal if the valves were transparent and thin, without adhesion and calcified nodules. None of the patients had a history

of rheumatoid disease, congenital heart disease, or infectious disease. All patients were informed and signed consent forms. The clinical characteristics of the included patients are summarized in Table 1. And the statistically differences of clinical characteristics among the groups were performed with one-way ANOVA. And the study was approved by the Ethics Committee of the Second Affiliated Hospital of Harbin Medical University and conducted following the guideline of Declaration of Helsinki.

Immunohistochemical (IHC) Assay

The expression of CD11b, CXCL5, COL4A3 and EPB41L4B were detected on the normal, sclerosis and stenosis valves. Firstly, the tissues were fix in 4% paraformaldehyde at 4˚C for 24 h and were dehydrated with gradient alcohol to make the 4-μm paraffin sections. The sodium citrate buffer (0.01 mol/l) was used for antigen recovery of sections at 100˚C for 20 min, followed by blocking the endogenous peroxidase with 3% H_2O_2 at room temperature for 20 min. After being blocking with 10% goat serum at room temperature for 30 min, the sections were incubated with primary antibodies against CD11b (ab52478, abcam), CXCL5 (KHC1246, Proteintech), COL4A3 (ab85103, Abcam), EPB41L4B (GTX81687, GeneTex) prediluted according to the manufacturer's instructions overnight at 4˚C. Next, the HRP conjugated secondary antibody incubated the sections at room temperature for 1 h. The DAB and hematoxylin solution were utilized for visualization and nuclear staining, respectively. Finally, the sections were observed under a light microscope (Leica, Germany).

Table 1. **Clinical characteristics of patients for the tissue harvest**

Characteristics	Normal (3)	Sclerosis (3)	Sclerosis (3)	*P* value
Age (year)	55 ± 6.2	60.7 ± 2.1	65.3 ± 8.4	n.s.
Male (%)	100	100	100	n.s.
Smoking (%)	100	100	100	n.s.
Diabetes mellitus (%)	0	0	0	n.s.
BMI (kg/m^2)	25.5 ± 2.9	24.28 ± 3.3	25.36 ± 3.2	n.s.
Triglycerides (mmol/L)	1.1 ± 0.3	1.3 ± 0.4	1.5 ± 0.2	n.s.
LDL (mmol/L)	2.8±0.2	2.9 ± 0.2	3.0 ± 0.4	n.s.
HDL (mmol/L)	1.6±0.1	1.4 ± 0.1	1.5 ± 0.1	n.s.
Cholesterol (mmol/L)	3.9±0.6	4.7 ± 0.3	4.3 ± 0.8	n.s.

Results

DEGs Analysis between Normal and AVS Groups

A total of 30 samples including 15 cases of Normal and 15 cases of AVS were collected for DEGs analysis. Because all samples from two datasets (GSE126444 and GSE51472) had batch effect impact DEGs analysis (Fig. 1A), the difference between batches in the merged dataset was eliminated firstly before screening DEGs (Fig. 1B). Next, the DEGs analysis was performed with "limma" package. Based on the threshold of *P* value < 0.05 and |Fold Change (FC)| > 2, there were a total of 543 DEGs including 228 down-regulated and 315 up-regulated (Fig. 1C). These results suggested AVS was a complex pathological process regulated by multiple genes.

Enrichment Analysis for DEGs

With the aim for exploring the potential mechanism involved by DEGs, the GO and KEGG enrichment analyses were conducted. In the BP category, the leukocyte migration, positive regulation of cytokine production, leukocyte mediated immunity, cell chemotaxis, leukocyte chemotaxis were the top 5 enriched terms (Fig. 2A). As for CC category, collagen-containing extracellular matrix, external side of plasma membrane, secretory granule membrane, secretory granule lumen and cytoplasmic vesicle lumen were the top 5 terms (Fig. 2B). Meanwhile, the terms of receptor ligand activity, signaling receptor activator activity, cytokine activity, G protein-coupled receptor binding and glycosaminoglycan binding were the top 5 MF terms (Fig. 2C). In the KEGG enrichment results, in addition to inflammatory or viral related progress, cytokine-cytokine receptor interaction, chemokine signaling pathway, ECM-receptor interaction, IL-17 signaling pathway, as well as complement and coagulation cascades were the main enrichment pathways involved by the DEGs (Fig. 2D). Combine the above results, it could be concluded that the development of AVS was involved in biological processes and signal mechanisms of inflammation or immune response, as well as ECM changes or remodeling.

Screening the Hub Biomarkers for AVS

In order to screen the hub biomarkers in AVS development, two methods of machine learning containing of LASSO logistic regression and RF analyses were utilized to identify the biomarkers from the DEGs. With LASSO regression analysis, there were total of 8 biomarkers identified in the DEGs (Fig. 3A and Table 2). At the same time, with the RF analysis (Fig. 3B), the top 10 genes ranked by importance were identified as

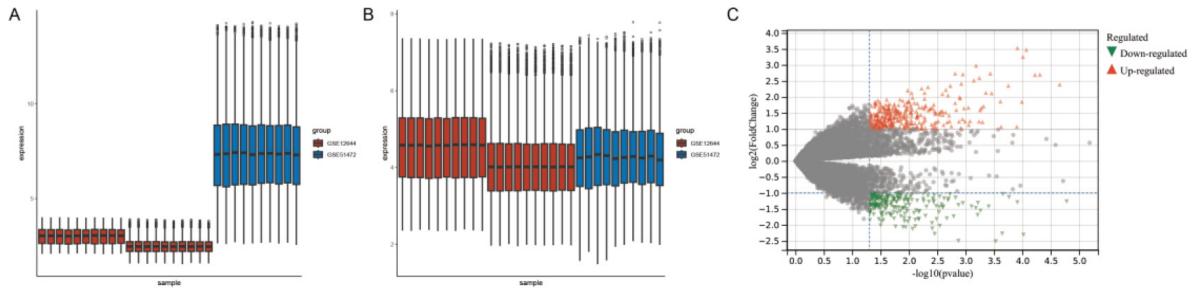

Fig. 1 **DEGs analysis between Normal and AVS groups. A. Boxplot for GSE12644 and GSE51472 profiles without batch correction. B. Boxplot for GSE12644 and GSE51472 profiles with batch correction. C. The volcano map of DEGs between AVS and Control groups with the thresholds of P < 0.05 and |FC| > 1. DEGs: Differentially Expressed Genes. AVS: Aortic Valve Stenosis. FC: Fold Change.**

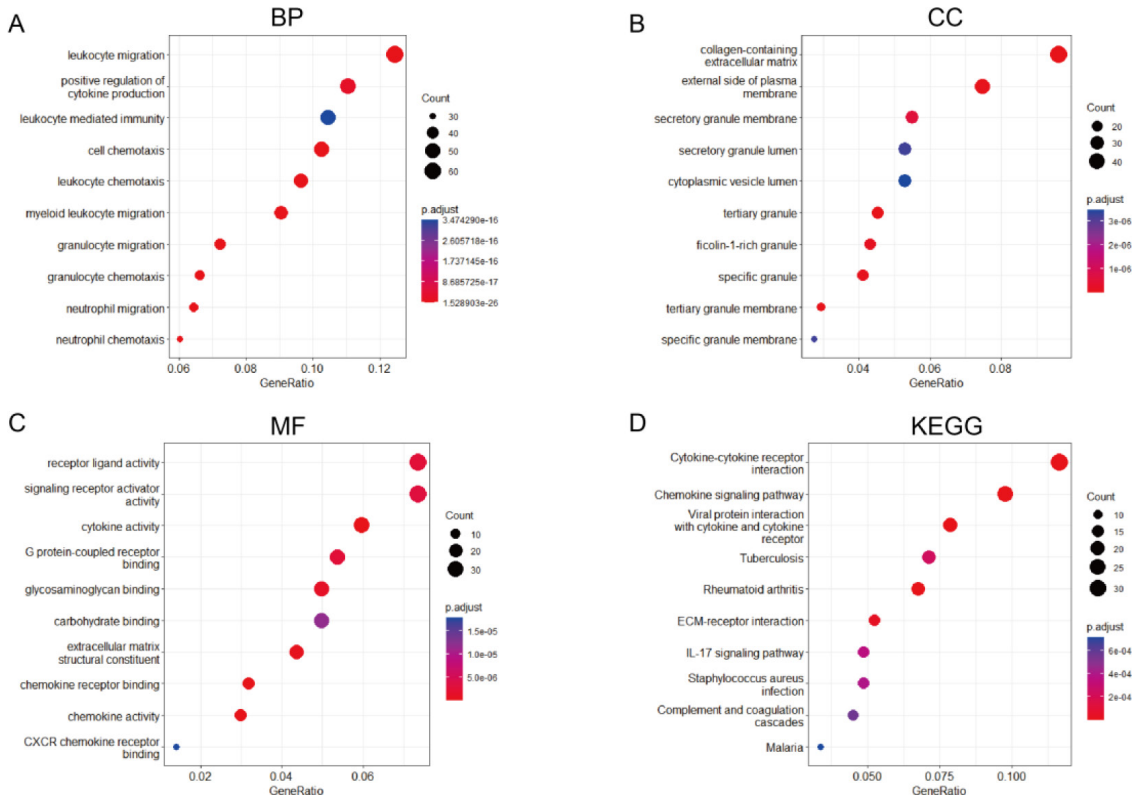

Fig. 2 **Enrichment analysis for DEGs. A. The GO terms of BP involved by DEGs between AVS and Control groups. B. The GO terms of CC involved by DEGs between AVS and Control groups. C. The GO terms of MF involved by DEGs between AVS and Control groups. D. The GO terms of KEGG involved by DEGs between AVS and Control groups. The thresholds set as Adjust P < 0.05. DEGs: Differentially Expressed Genes. AVS: Aortic Valve Stenosis. GO: Gene Ontology. BP: Biological Progress. CC: Cellular Component. MF: Molecular Function. KEGG: Kyoto Encyclopedia of Genes and Genomes.**

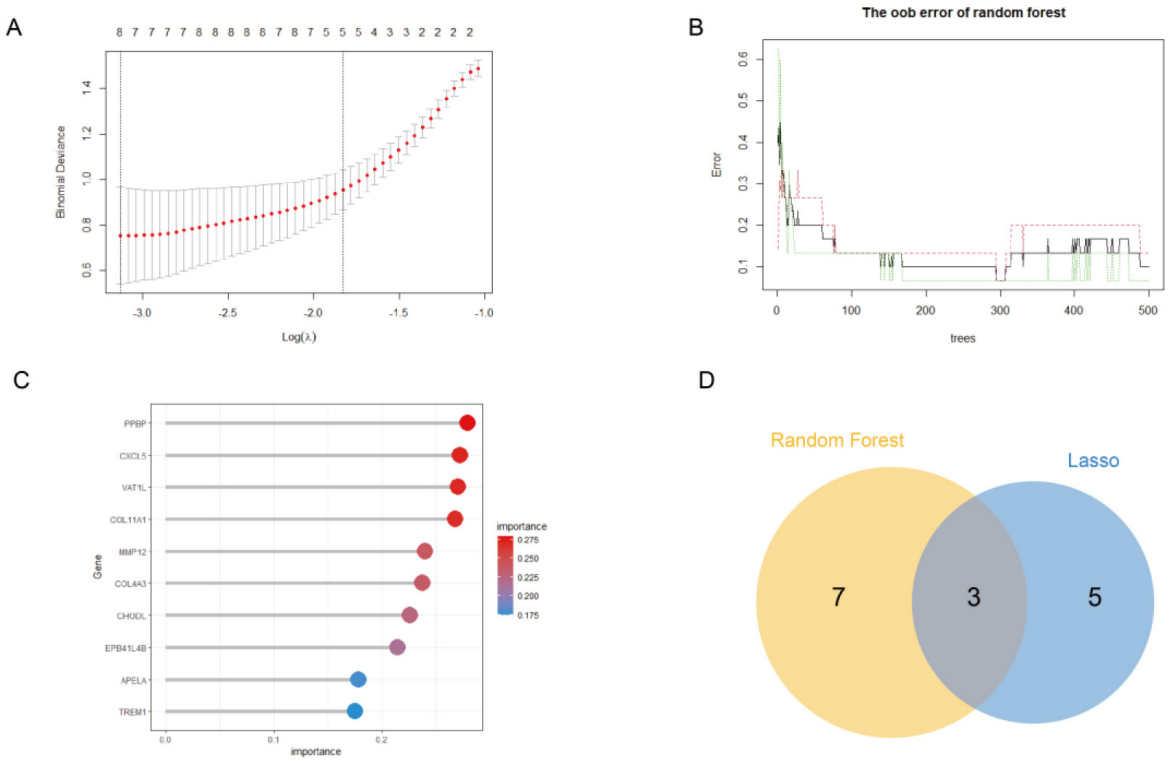

Fig. 3 **Screening the hub biomarkers for AVS. A. Biomarkers detection with LASSO regression analysis, and the optimal lambda was set as lambda.min. B. Biomarkers detection with RF analysis. C. The top 10 biomarkers determined with RF analysis. D. The common biomarkers determined by LASSO regression and RF analyses. LASSO: the Least Absolute Shrinkage and Selection Operator logistic regression. RF: Random Forest.**

Table 2. **The hub gene list identified with LASSO logistic regression**

Gene	Coef
EPB41L4B	–0.357
CXCL5	0.371
COL4A3	–0.243
SPP1	0.071
ATP1A2	–0.466
SCG2	0.485
PTGDS	–0.283
AGTR1	–0.036

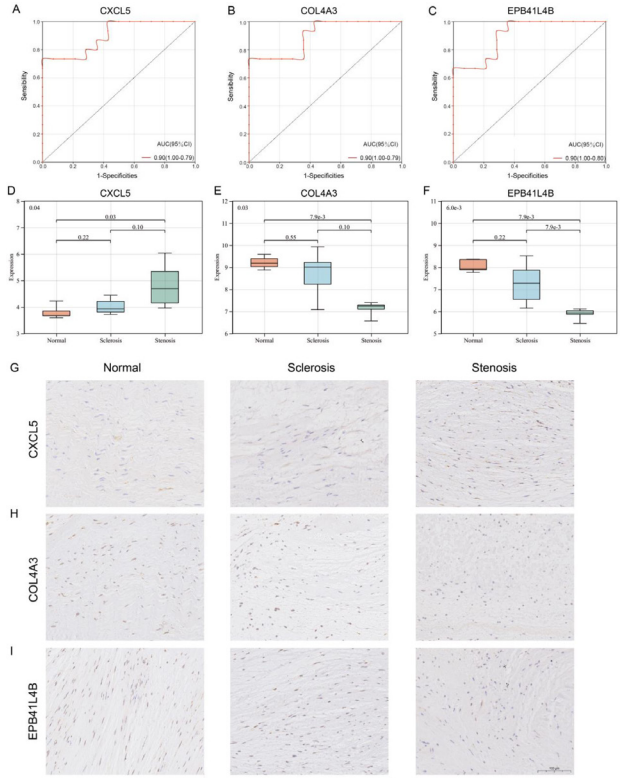

Fig. 4 **Diagnostic assessment for hub biomarkers. A-C. The ROC curve and AUC values for every biomarker of CXCL5 (A), COL4A3 (B) and EPB41L4B (C). D-F. The expressions of every biomarker of CXCL5 (D), COL4A3 (E) and EPB41L4B (F) among the Normal, Sclerosis and Stenosis groups that the significances were determined by non-parametric test. G-I. The expression of CXCL5 (G), COL4A3 (H), EPB41L4B (I) in human normal, sclerosis and stenosis valves detected by IHC (Sclar bar = 100 μm). ROC: Receiver Operating Characters. AUC: Area Under the Curve. CXCL5: C-X-C Motif Chemokine Ligand 5. COL4A3: Collagen Type IV Alpha 3 Chain. EPB41L4B: Erythrocyte Membrane Protein Band 4.1 Like 4B. IHC: Immunohistochemical.**

biomarkers in DEGs (Fig. 3C). As expected, 3 genes (CXCL5, COL4A3, EPB41L4B) were the common hub biomarkers identified by both LASSO regression and RF analysis (Fig. 3D).

Diagnostic Assessment for Hub Biomarkers

ROC analysis was performed to evaluate the specificity and sensitivity of CXCL5, COL4A3, EPB41L4B in AVS diagnosis. As expected, all of them had a high diagnostic efficacy (AUC = 0.90) (Fig. 4A-C). Moreover, the expressions of CXCL5, COL4A3, EPB41L4B in the AVS development (healthy valve from Normal, Sclerosis to Stenosis) were compared to investigate them effect on the calcification degree of valve. As expected, the expressions of CXCL5 showed an elevating, and COL4A3, EPB41L4B exhibited a decreasing trend in the development of AVS (Fig. 4D-F). These results suggested CXCL5, COL4A3, EPB41L4B could not only be used as diagnostic markers of AVS, but also involved in the calcification development of valve. At the same time, the expression of the identified biomarkers was detected in the normal, sclerosis and stenosis valves. As expected, the expression of CXCL5 showed a gradually increasing trend as it progressed towards AVS, while COL4A3 and EPB41L4B showed opposite trends (Fig. 4G-I).

Immune Infiltration Assessment between AVS and Normal Groups

Subsequently, the differences of immune infiltration between Normal and AVS groups were investigated. It was obvious that the proportions of M2 macrophages, T cells CD4 memory resting, T cells CD8 and mast cells resting were the largest among the 22 types of immune cells in both group (Fig. 5A). Besides, the abundances of plasma cells, T cells CD4 memory resting and activated, T cells regulatory (Tregs), M0 macrophages and neutrophils had significant difference between Normal and AVS groups (Fig. 5B). Compared with Normal group, plasma cells, T cells CD4 memory activated, M0 macrophages and neutrophils had a higher proportion in AVS group, while T cells CD4 memory resting and Tregs had a less abundances in AVS group (Fig. 5C). Furthermore, the relationships among the different immune cells in AVS were also explored to explore the synergistic and antagonistic effects. Obviously, among the above significant types of immune cells, with the threshold of COR > 0.6, the neutrophils had positive correlation with plasma cells while had a negative correlation with B cell naive, T cells CD4 memory resting and Tregs. Tregs was positively correlated with B cells naive, T cells CD8 and T cells follicular helper, as well as negatively correlated with the plasma cells. And plasma cells were negatively correlated with B cells naive. Lastly, the importance of the 22 types of immune cells in AVS was calculated via RF, and the neutrophils had the highest score (Fig. 5D), suggesting the infiltration of neutrophils might exert a crucial effect on AVS development. Similarly, we selected CD11b as a marker for neutrophils,[42,43] and detected a gradual increase in neutrophil infiltration in different valve tissues during the progression of AVS (Fig. 5E).

The Correlation Relationship between the Biomarkers and Immune Cells

With the aim of exploring the regulatory mechanism of immune microenvironment of AVS, the correlated relationship between immune cells and biomarkers were investigated. As shown in Fig. 6A, with the threshold of $P < 0.05$, CXCL5 had a positive effect on neutrophils, T cells CD4 memory activated, M0 macrophages, plasma cells, as well as a negative effect on T cells CD4 memory resting, Tregs in the significantly infiltrated immune cells. For COL4A3, it was only negatively correlated with neutrophils in the significantly infiltrated immune cells (Fig. 6B). The results of EPB41L4B were the same as those of COL4A3, which was only negatively

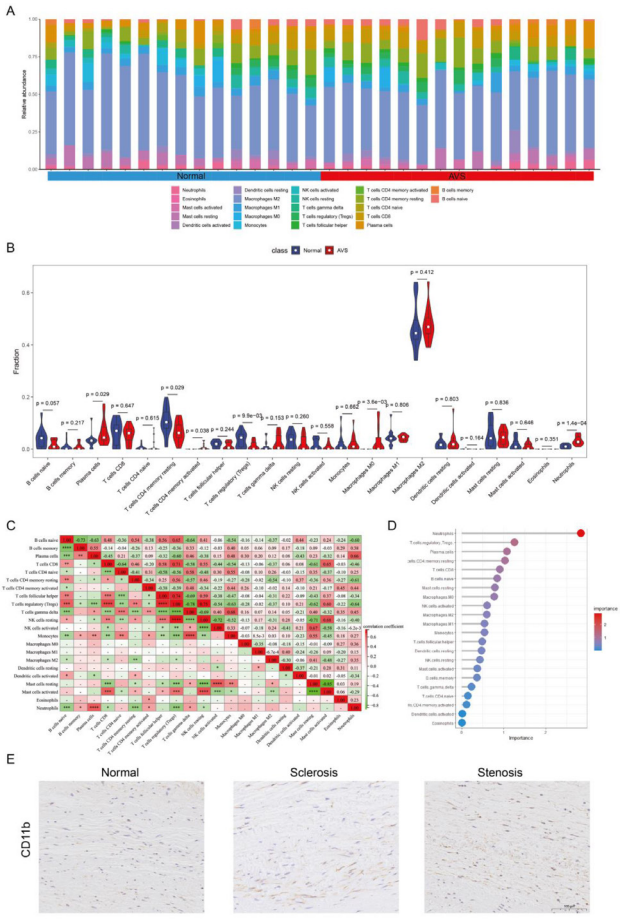

Fig. 5 **Immune infiltration assessment between AVS and Normal groups. A. The proportion of 22 types of immune cells between AVS and Normal groups. B. The vioplot for comparison regarding the proportion of 22 kinds of immune cells between AVS and Normal groups determined by wilcox test. C. Correlation of 22 immune cell type compositions determined by Spearman's rank test. D. The importance value for each immune cells in AVS determined by RF analysis. E. The expression of CD11b in human normal, sclerosis and stenosis valves detected by IHC (Sclar bar = 100 μm). *P<0.05, **P<0.01, ***P<0.001. AVS: Aortic Valve Stenosis. RF: Random Forest. IHC: Immunohistochemical.**

correlated with the infiltration of neutrophils (Fig. 6C). Therefore, CXCL5 might be the most crucial gene with a regulation role of all significantly infiltrated immune cells in AVS.

The Correlation Relationship Between the Biomarkers and Calcified Related Genes

Considering BMP2, Runx2 and ICAM-1 were the tight link between inflammation and calcification in AVS,[19] the correlation relationship between the biomarkers and calcified related genes was explored. Perhaps due to the limited number of samples, BMP2 and ICAM-1 were not significantly overexpressed in the AVS group (Fig. 6D). Therefore, the correlation analysis with Runx2 and biomarkers were performed. In the result, CXCL5 had significant correlation with RUNX2 (Fig. 6E) while did not with COL4A3 and EPB41L4B (Fig. 6F and 6G). The results suggested CXCL5 and RUNX2 might have some interactive mechanisms in AVS.

The Potential Mechanism and Target Drugs of Biomarkers

Finally, the GSEA with the biomarkers were performed for exploring the potential mechanisms involved by them in AVS. With the threshold of $P < 0.05$, CXCL5 was mainly enriched in the pathways of ECM receptor interaction, focal adhesion, chemokine signaling pathways (Fig. 7A). COL4A3 was mainly involved in the insulin signaling pathway, neuroactive ligand receptor interaction, focal adhesion, complement and coagulation cascades (Fig. 7B). And EPB41L4B was enriched in the insulin signaling pathway, excepted the pathways in cancer (Fig. 7C). At the same time, the potential drug for biomarkers was also investigated via DGIdb database. However, there were only two drugs for COL4A3, Collagenase clostridium histolyticum (CCH) and Ocriplasmin (Fig. 7D). The above results revealed the potential signaling mechanism involved by the biomarkers and their target drugs.

Discussion

The prevalence of AVS is gradually increasing especially in high-income countries.[5] The main strategies for AVS treatment include surgical aortic valve replacement and transcatheter aortic valve implantation (TAVI) that were effectively improve the outcome of AVS patients.[44,45] As there is no satisfactory ways and means to prevent or control the progress of AVS, the high cost of surgery and strict postoperative monitoring would also bring great burden to society and families with the progress of social aging.[5,46] Exploring the pathogenesis of AVS would be helpful to provide new ideas for developing prevention strategies for AVS.

As reported, calcification of the aortic valve is responsible for the valve stenosis.[2] The risk factors of valve calcification were similar to atherosclerosis, such as hypertension, chronic inflammation, dyslipidemia, male, diabetes and renal failure.[47] These results suggest that AVS is a biological process involving multiple gene regulation. In the present study, there was a number of 543 DEGs including 228 down-regulated and 315 up-regulated DEGs, compared between AVS and Normal groups. Moreover, these DEGs were mainly enriched in the terms of immune response and immune cell migration, collagen-containing extracellular matrix, receptor ligand activity, and chemokine/cytokine receptor interaction. These results suggested that the DEGs involved in AVS lesions mainly focused on immune response, chemokine/cytokine interaction, and extracellular matrix remodeling.

Screening the hub biomarkers of AVS would help to develop targeted gene/drug therapy for AVS and provide more personalized treatment for AVS patients. In the previous study, Chen et al had identified as the fatty acid metabolism-related HIBCH gene was a significant biomarker for aortic valve calcification.[48] And metabolic syndrome related genes of BEX2, SPRY2, CXCL16, ITGAL and MORF4L2 were a better diagnostic biomarker for aortic valve calcification.[49] However, the above studies focused on the hub biomarkers in the metabolic-related genesets, which did not filter the crucial genes from the whole DEGs regulation network. In our present study, we screened the hub biomarkers from the DEGs for AVS with machine learning of LASSO regression and RF analysis. In our results, CXCL5, COL4A3, EPB41L4B were identified and exhibited a better diagnostic value for AVS. More importantly,

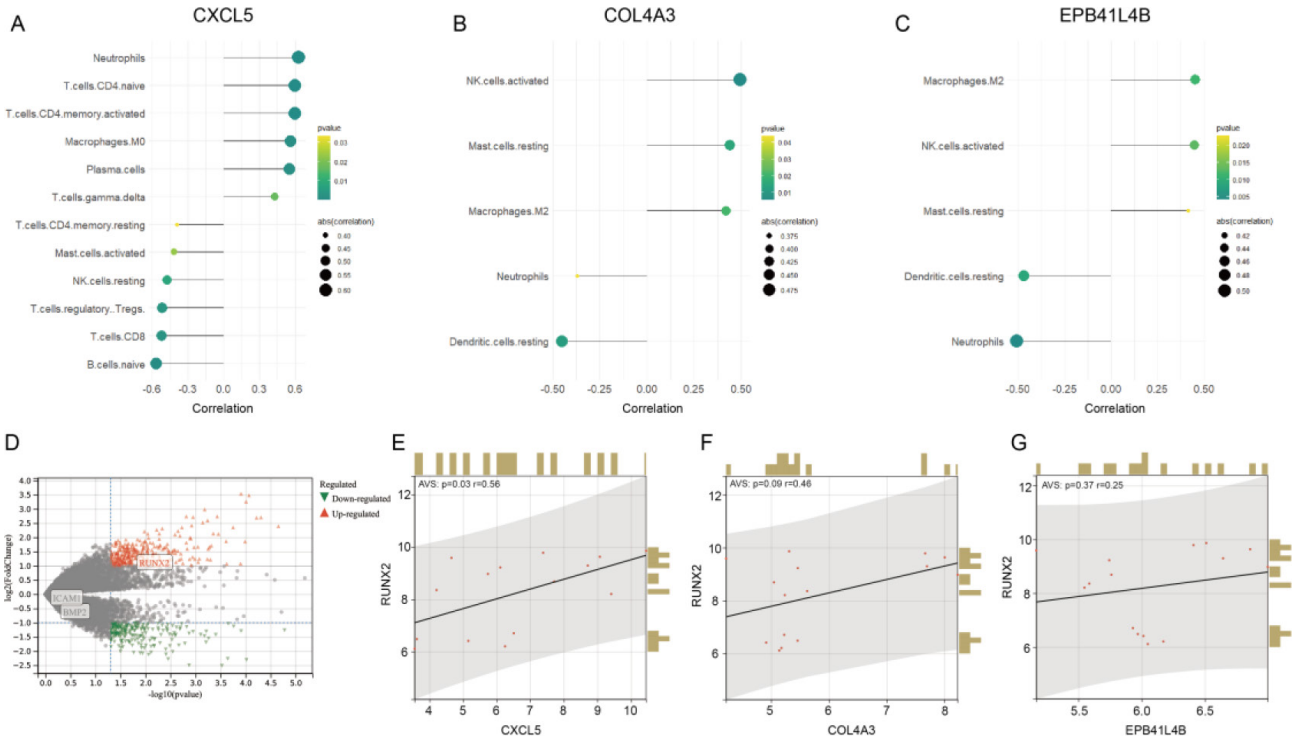

Fig. 6 **The correlation relationship among the biomarkers, immune cells and calcified related genes. A. The correlation between the CXCL5 expression and immune cell in AVS with the threshold of P < 0.05. B. The correlation between the COL4A3 expression and immune cell in AVS with the threshold of P < 0.05. C. The correlation between the EPB41L4B expression and immune cell in AVS with the threshold of P < 0.05. D. The expressions of RUNX2, ICAM1 and BMP2 between AVS and Normal groups. E. The correlation between the CXCL5 and RUNX2 expressions. F. The correlation between the COL4A3 and RUNX2 expressions. G. The correlation between the EPB41L4B and RUNX2 expressions. AVS: Aortic Valve Stenosis. RUNX2: RUNX Family Transcription Factor 2. ICAM1: Intercellular Adhesion Molecule 1. BMP2: Bone Morphogenetic Protein 2. CXCL5: C-X-C Motif Chemokine Ligand 5. COL4A3: Collagen Type IV Alpha 3 Chain. EPB41L4B: Erythrocyte Membrane Protein Band 4.1 Like 4B.**

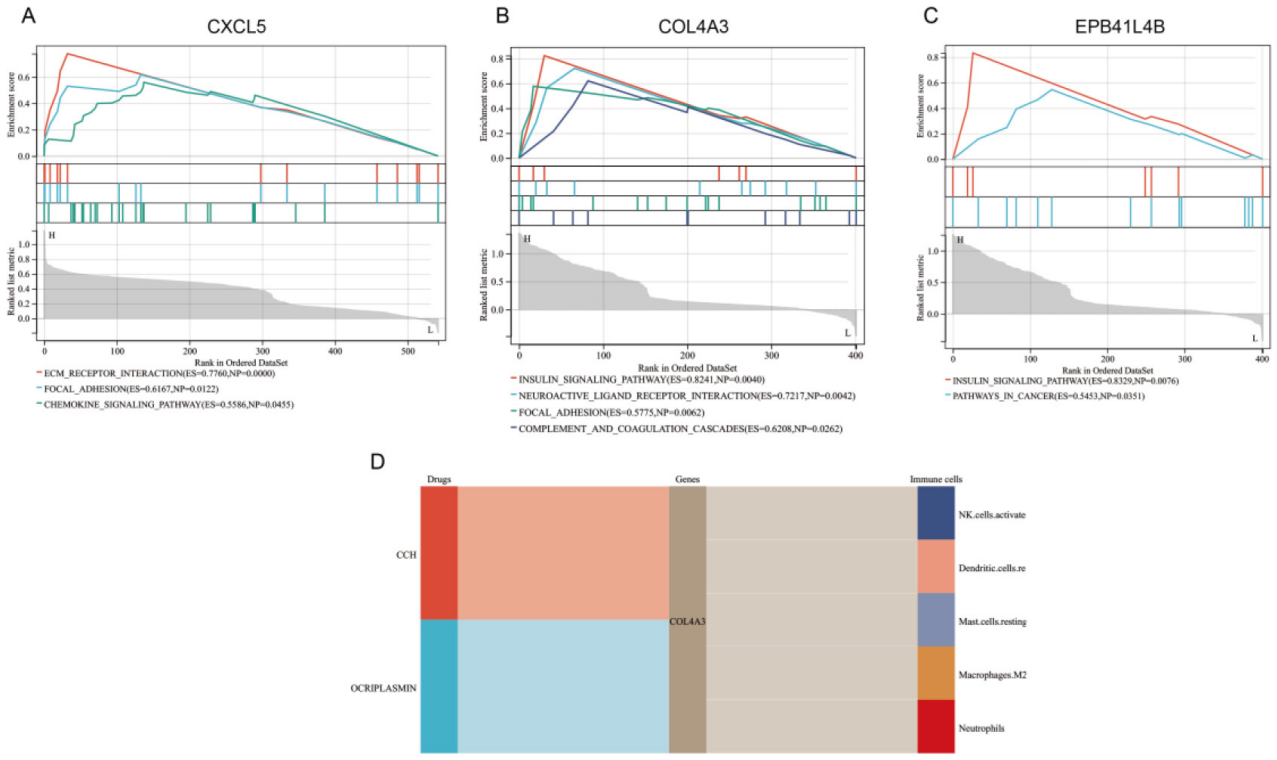

Fig. 7 **The potential mechanism and target drugs of biomarkers. A-C. The single gene GSEA for CXCL5 (A), COL4A3 (B) and EPB41L4B (C) with the threshold of P < 0.05. D. The sankey diagram for display the relationship among the drugs, biomarkers and immune cells. CXCL5: C-X-C Motif Chemokine Ligand 5. COL4A3: Collagen Type IV Alpha 3 Chain. EPB41L4B: Erythrocyte Membrane Protein Band 4.1 Like 4B.**

CXCL5 increased gradually from normal valve to sclerotic valve, and finally to stenosis valve, while COL4A3 and EPB41L4B decreased gradually in the progress. These results suggested CXCL5, COL4A3, EPB41L4B expressions were related to the degree of calcification of the valve and might be involved in the AVS development.

It is a well-accepted concept that inflammation response is the main mechanism involved in the AVS development.[22,23] And there was study revealed the different levels about immune cell infiltration between the calcified and normal aortic valve where the proportion of B cells memory was significantly elevated in aortic valve calcification while NK cells activated was significantly decreased in the aortic valve calcification.[49] In our results, compared with Normal group, plasma cells, T cells CD4 memory activated, M0 macrophages and neutrophils had a higher proportion in AVS group, while T cells CD4 memory resting and Tregs had a less abundances in AVS group. More than this, neutrophils were identified as the most crucial immune cells for AVS determined by the RF analysis. The neutrophils exerted a synergies effect with plasma cells while an antagonism relationship with Tregs, T cells CD4 memory resting and B cell naïve. These results revealed the immune microenvironment of AVS which would be contributed for immunotherapy development for AVS.

Next, we also investigated whether the AVS immune microenvironment could be affected by the hub biomarkers. CXCL5 (C-X-C motif chemokine ligand 5), as a member of the CXC subfamily of chemokines, plays a synergistic role with other inflammatory factor like IL-6, IL-8 in some inflammatory disease.[50-52] In cardiovascular disease, the inhibition on CXCL5 could reduce aortic matrix metalloproteinase 9 expression and protect against acute aortic dissection.[53] Besides, CXCL5 interacts with the G protein-coupled receptor CXCR2 to induce chemotaxis and activation of neutrophils.[54] Coincidently, in our study, CXCL5 was significantly up-regulated in AVS and was positively correlated with neutrophils. Moreover, CXCL5 also shows a positive correlation with T cells, CD4 memory activated, M0 macroscopic, and plasma cells, and a negative correlation with T cells, CD4 memory resting, and Tregs. These results suggested CXCL5 might have the regulatory and chemotactic effects on the above immune cells in AVS. COL4A3 (collagen type IV alpha 3 chain) is the main component of basement membranes. It is reported that COL4A3 expression was decreased in the asthmatic lung tissue,[55] suggesting the expression of COL4A3 is related to matrix remodeling in inflammatory environment. In our results, COL4A3 was significantly down-regulated in AVS, and exhibited a negative correlation with neutrophils. The results suggested COL4A3 might be an important biomarker of AVS and affect local immune microenvironment. EPB41L4B (erythrocyte membrane protein band 4.1 like 4B) is a member of the Four.1 protein, ezrin, radixin, moesin superfamily and serves as a structural constituent of cytoskeleton.[56] It is reported that the abnormal expression of EPB41L4B is related to tumor metastasis and plays a tumor suppressor role in lung adenocarcinoma,[56,57] suggesting the down-regulation of EPB41L4B expression easily leads to cell proliferation and migration, and could mediate the local immune microenvironment. Similar to our results, EPB41L4B expression was decreased in the AVS and was negatively correlated with infiltration of neutrophils.

In addition, the potential mechanisms of the hub biomarkers were also explored. CXCL5 was mainly involved in the ECM receptor interaction, focal adhesion, chemokine signaling pathways. COL4A3 was mainly involved in the insulin signaling pathway, focal adhesion, complement and coagulation cascades. And EPB41L4B was enriched in the insulin signaling pathway. From the results, we could conclude that hug biomarkers were mainly involved in inflammation/immune response, ECM remodeling, and these mechanisms have been reported to be related to the development of AVS.[21,58] Inhibiting the activation of insulin signaling pathway could induce aortic valve calcification.[59] In our results, the genes involved in regulating insulin signaling pathway were significantly down-regulated in AVS, suggesting an activation of insulin signaling pathway in AVS.

Moreover, we still predicted molecular drugs that could target these biomarkers. Unfortunately, only COL4A3 had potential drug treatment, namely CCH and Ocriplasmin. CCH is the collagenase of the bacterium *Clostridium histolyticum,* and is approved by the U.S. Food and Drug Administration (FDA) on clinical practice for Peyronie disease, Ledderhose disease, Dupuytren contracture treatment.[60-63] Moreover, in an animal study, application of CCH could dissolve the fibrous capsule surrounding the silicone implant.[64] The main mechanism of CCH in treating the above-mentioned diseases is the degradation of abnormal collagenous fiber proliferation and inhibition of adverse ECM remodeling, which is also related to the pathogenesis of AVS.[17] Similarly, Ocriplasmin is approved by U.S. FDA for the treatment of symptomatic vitreomacular adhesion.[65] Essentially, Ocriplasmin is a type of plasma, derived from yeast *Pichia pastoris* by a recombinant DNA technology.[65] Ocriplasmin exerts therapeutic effects on symptomatic vitreomacular adhesion by acting on collagen, laminin and fibronectin in the vitreous structure and vitreo-retinal interface.[66] Thus, considering that the progress of AVS also involves disturbances in the ECM microenvironment, we believe that CCH and Ocriplasmin might also be a promising approach to drug therapy for AVS. The findings might provide more adequate personalized treatment for AVS patients.

However, our research also had some limitations. For example, the restriction of sample size may miss the discovery of some key genes. Whether the biomarkers are AVS phenotype genes or pathogenic genes has not been verified in the corresponding cell or animal experiments. And whether the screened drugs could exert therapeutic effects on AVS in animal models is worth further exploration.

In conclusion, CXCL5, COL4A3 and EPB41L4B were the hub biomarkers of AVS. The plasma cells, T cells CD4 memory resting and activated, Tregs, M0 macrophages and neutrophils was the significantly infiltrative immune cells in which neutrophils was the most important immune cell type. CXCL5 could regulate all the above significantly infiltrative immune cells involved in AVS development, while COL4A3 and EPB41L4B only mediated the neutrophils in AVS. Moreover, ECM receptor interaction, focal adhesion, chemokine signaling pathways and insulin signaling pathway were the main mechanisms involved by the biomarkers. CCH and Ocriplasmin was the potential drug for COL4A3.

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Author Contributions

P. C., X. J., H. T., W. C. substantially were responsible for the experiment conception and design. P. C., X. J., H. D. were contributed to data acquisition and statistical analysis. P. C. and C. L. were responsible for interpretation of experimental data, drafting the manuscript. P. C. and W. C. confirmed the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

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Availability of Data and Materials

The original data for this study are available from publicly available databases (https://www.ncbi.nlm.nih.gov/geo/).

Ethics Approval and Consent to Participate

Not applicable.

Conflict of Interest

The authors declare no conflict of interest.

Patient Consent for Publication

Not applicable.

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