MicroRNA Profiling of Plasma Extracellular Vesicles in Bicuspid Aortopathy

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Abstract

Background: Bicuspid aortic valve (BAV) is the most common congenital cardiovascular disease and highly associated with ascending aortic dilation, *i.e.*, bicuspid aortopathy. When the maximal aortic diameter achieves a critical threshold, prophylactic surgical intervention is recommended to prevent potential aortic dissection or rupture. However, the aortic diameter threshold cannot precisely predict the aortopathy progression and should be supplemented by more non-size parameters to provide more information for surgical decision. There is an urgent need for identification of a stable prognostic biomarker for BAV patients with increased risk of aortopathy progression.

Methods and Results: In total, 24 patients with BAV and 6 healthy volunteers with tricuspid aortic valve (TAV) were involved in this study. Quantitative proteomics were employed to identify protein expressions in plasma. Sorted by the ratio of transforming growth factor-β1 (TGF-β1) to soluble endoglin (sENG) (T/E), which has been recognized as a plasma protein biomarker for aortopathy progression, BAV patients were divided into two groups with high ($\geq$ 0.5, n = 6) or low (T/E < 0.5, n = 18) ratio of T/E. The mcroiRNA (miR) profiling of plasma extracellular vesicles (EVs) was performed using small RNA sequencing. The results revealed that low ratio of EV-miR324 to miR145 ($\leq$ 0.1) was closely correlated to high ratio of T/E.

Conclusions: The ratio of plasma miR324 to miR145 from EVs may identify the risk of progression of BAV aortopathy.

Introduction

Bicuspid aortic valve (BAV) is the most common congenital heart malformation affecting 1-2% population. [1] Approximately more than 50% of BAV patients develop a dilated proximal aorta, *i.e.*, bicuspid aortopathy, which is associated with a high risk of acute aortic events like aortic dissection and rupture. [2, 3] Prophylactic surgical intervention based on aortic diameter threshold is recommended to avoid potential aortic emergencies from BAV aortopathy. The guideline for management of BAV aortopathy with concomitant aortic valve disorders recommends surgical treatment when aortic root or ascending aorta diameter is greater than 45 mm. [4] However, the current aortic diameter threshold in guideline is not a predictor for aortopathy progression. The patients whose aortic diameters are under the threshold cannot realize their disease progression. [5, 6] Therefore, there is an urgent clinical need for better identifying the high risk BAV patients who may present rapid progression of ascending aortic diameter and may benefit from intensive surveillance and prophylactic surgery to prevent life-threatening aortic complications.

Recently, novel imaging biomarkers have been explored for the identification of aortopathy progression. [7-9] For instance, several studies found wall shear stress (WSS) alterations in BAV patients by 4D-flow imaging. The hemodynamic alterations indicate extracellular matrix (ECM) dysregulation and elastic fiber degeneration in the ascending aorta of BAV patients. [10] However, the correlation between WSS magnitude

increase and growth of aortic diameter remain undetermined. [11] Recently, studies found that some circulating biomarkers including proteins, microRNAs (miRs) and long noncoding RNAs, could be more feasible to detect the presence of aortopathy. [12-14] Involved in tissue fibrosis, ECM remodeling and cell proliferation, transforming growth factor β1 (TGF-β1) pathway is attracting increasing attention in studies focusing on ascending aorta aneurysms. [15, 16] Endoglin (also known as CD105) has been recently reported to play a role in vascular remodeling. [17] Endoglin is an integral membrane glycoprotein that serves as a co-receptor for members of the TGF-β1 superfamily of proteins. It has a major role in regulating TGF-β1-dependent vascular remodeling. [18] In a clinical trial, Forte *et al.* presented the prognostic value of the ratio between TGF-β1 to soluble endoglin (TGF-β1/sENG, T/E), which is correlated to the aortic diameter growth rate in BAV patients (r = 0.66, $P < 0.001$). [19] A higher T/E ratio indicated a propensity to aortopathy progression in BAV patients with nondilated ascending aorta over a 3-year follow-up, showing its potential prognostic value.

Extracellular vesicles (EVs), including exosomes and microvesicles, are detectable in most body fluids and involved in multiple cell-to-cell communication both in normal or diseased states.[20] [21] EVs contained natural cargo molecules including small noncoding RNA as well as coding RNA (such as miRNA and mRNA, respectively) and proteins, and these cargos were transferred to neighboring cells or distant cells through circulation. [22, 23] Most of the miRNAs are protected from endogenous RNase activity and concentrated in EVs, and they can be detected in early stage of the disease. Although miRNAs have been widely validated for diagnosis and prediction of cardiovascular diseases and cancer, individual differences in samples and batch errors in experiments cannot be avoided. In the present study, we used the ratio of EV-miRNAs to compare the expression among different groups for normalization, so that the values from each sample were measured under the same conditions. Thus, the ratio of paired EV-miRNAs could be an early and stable biomarker for predicting aortopathy progression in BAV patients. In this study, we employed the plasma EV-miRNA profiling and correlated to the plasma proteomic data of BAV patients. The schematic workflow of this study is shown in **Figure 1**.

Methods

Study population

The BAV patients included in this study underwent surgical treatment and follow-up in Zhongshan Hospital Fudan University. According to severity of aortic dilatation, patients were defined as severe dilation (BAV-dilated, aortic diameter ≥ 45 mm, n = 13) and non-severe dilation (BAV-non, aortic diameter < 45 mm, n =11) groups, respectively. Patients in BAV-non group underwent aortic valve replacement (AVR) surgery, and those in BAV-dilated underwent AVR and concomitant ascending aorta replacement. Patients with impaired systolic ventricular function, significant coronary artery disease or aortic dissection were excluded in this study. Healthy donors with tricuspid aortic valve and normal aortic diameter (20mm-37mm), namely TAV-non, were employed as a control group (n = 6). Aortic specimens

were obtained from patients with a tricuspid aortic valve but without aortic dilation who underwent coronary artery bypass graft were used as control group (n=3).

Echocardiography

Transthoracic echocardiography was performed for all patients preoperatively. All echocardiographic studies were conducted according to standard techniques by experienced echocardiographers.

Plasma sample and aortic specimen collection

Written informed consent was obtained from all patients before participation. Human aortic specimens were utilized under approvals of Zhongshan Hospital, Fudan University Ethics Committee (NO. B2020-158) in accordance with the Declaration of Helsinki. The plasma samples involved in this study were collected in vacuum blood tubes with anticoagulant before operation and handled within one hour after collection. Aortic tissues were obtained from control patients with a tricuspid aortic valve but without aortic dilation who underwent coronary artery bypass graft, and patients from BAV-dilated and BAV-non groups (BAV-non and BAV-dilated) (n = 3, respectively). The sampling and clinic study were approved by the Human Research Ethics Committee of Shanghai Zhongshan hospital (B2018-285R). Written informed consent was obtained from all the patients according to the Declaration of Helsinki, and the methods were carried out in response to relevant guidelines.

EV RNA isolation and small RNA sequencing

The EVs were isolated by a precipitation method using ExoQuick Isolation Kit (SBI, CA, USA) according to the manufacturer's instruction. Total RNA was isolated from plasma EVs with Trizol reagent (Invitrogen, USA). Then, quantity and purity of the total RNA were defined by Nanodrop (Thermo, USA) and 1% agarose gel electrophoresis. Library was prepared with 1μg total RNA for each sample. Total RNA was purified by magnetic beads and among them small RNA regions corresponding to the 18-30 nt could be enrichment. Then the 18-30 nt small RNAs were ligated to adenylated 3’ adapters. After adding the unique molecular identifiers (UMI) labeled RT primer and performing reverse transcription, we synthesized the first and second strand respectively to amplify the cDNA. Finally, the quantitative and qualitative small RNA libraries were sequenced on BGISEQ-500 platform (BGI, China).

Proteomics with mass spectrometry

Human plasma was depleted using the high abundance top 14 mini column (Thermoscientific A36370). After measuring the depleted plasma protein concentration (BCA method, Pierce, Protein Quantification Assay Kits), 50 μg of proteins for each sample were reduced with 10 mM DTT (final concentration) for 30 min at 37 ℃ and alkylated with 55 mM iodoacetamide (final concentration) for an additional 30 min in dark at room temperature. Remaining detergent was removed by acetone precipitation. Briefly, acetone (-20 ℃) was added to 50 μg of proteins to a final concentration of 80% v/v and the proteins were precipitated overnight at -20℃. After centrifugation (15 min, -4℃, 16,000 g), the detergent-containing supernatant was removed, and the protein pellet was washed with 80% acetone (-20 ℃). Protein pellets

were then resolved in 50 µl 6M urea (in 10mM HEPES, pH = 8.0) and digested with 0.5µg of LysC for 3h at room temperature. After adding four volumes of 50 mM ammonium bicarbonate, 0.5 µg of trypsin was added and tryptic digestion carried out overnight. The next day, digestion was stopped by adding 1% TFA. Peptides were finally desalted on C18 StageTips and kept at -80°C until mass spectrometry analysis.

qPCR of aortic specimens

Aortic specimen miRNAs were isolated by using PureLinkTM miRNA Isolation Kit (Invitrogen, USA) and all the steps were followed according to the guideline. qPCR for miRNA was performed using TaqManTM Small RNA Assays and protocol was as mentioned in the guideline.

Screening of differential expression (DE) miRNAs and identification of its target genes prediction and novel miRNAs

Differential expression analysis was performed using DESeq2 (v1.16.139) for the reads count expression result with the parameters “adjusted p-value < 0.05, $|Log_2(foldchange)| \geq 1$”, and NOIseq (v3.18.140) [24] for the UMI expression result with the parameters “Probability > 0.8, $|Log_2(foldchange)| \geq 1$”. We used miRWalk 2.0 to predict the potential target genes of the differentially expressed miRNAs.

Statistical analysis

Data was analyzed with SPSS 19.0 (SPSS Inc., Chicago, IL, USA) and presented as a style of mean ± standard deviation (SD). Student’s t test was used for comparison of two groups. A Chi squared test or a Fisher’s exact test was used to compare the frequencies of the categorical variables when appropriate. Two-way ANOVA was used for comparison among more than two groups. $P < 0.05$* was considered as statistically significant.

Results

Clinical characteristics of the study groups

Clinical and demographic characteristics of BAV-non/dilated and TAV-non plasma donors were summarized in **Table 1**. BAV-dilated group showed a relatively older in age (58.77 ± 11.42) compared to control and BAV-non (50.50 ± 4.04, 49.91 ± 16.46, respectively) groups. Furthermore, individuals with hypertension, hyperlipidemia, sinus expansion, aortic insufficiency showed no difference between each other, while there was a significant difference between these groups among the index of ascending aorta diameter ($P < 0.0001$***), aortic valve gradient ($P = 0.006$*), aortic flow jet ($P = 0.004$*).

Plasma EV-miRNA sequencing and proteomics analysis to determine a candidate

The raw small RNA-seq data were normalized, after filtering and optimizing with FastQC, the modified reads were mapped to various small RNA databases or sequences. The miRNAs that are discriminate between BAV-non and BAV-dilated were identified as shown in the volcano plot, with the criteria $|Log_2$(fold

change) | > 1 and false discovery rate (FDR) < 0.05 (**Figure 2A**), which means the significant difference. Accordingly, 180 upregulated and 229 downregulated miRNAs in BAV patients. The top 20 miRNAs were shown in the (**Table S1**). To further understand the mechanism of BAV-aortic dilatation, we performed proteomic analysis of the patient's plasma. KEGG analysis revealed that protein functions were enriched in protein digestion and absorption as well as a range of other cellular functions (**Figure 2B**). There were 99 kinds of proteins involved in aortic aneurysm, among which 48 were located in extracellular space (**Figure 2C**). As mentioned before, TGF-β1 and ENG are closely related to the pathological process of aortic diseases, and ENG has been proved to be regulated by MMP-14. Based on the results of proteomics with mass spectrometry, we screened the miRNA targeted TGF-β1 (n = 14) and MMP-14 (n = 5) by miRWalk 2.0 and compared these candidates (n = 70 pairs) with the plasma T/E ratio for further study. After calculation, ratio of miR324/145 showed the highest linear correlation with T/E (**Figure 2D**).

Validation of the candidate miRNA ratio

We first predicted gene targets of miR145 and miR324 in **Figure 3A** and **B.** Then, we further verified the miRNA expression levels on the aortic tissues of the two groups (BAV-non and BAV-dilated, n = 3) of patients (**Table S2**) separately and found that the tissues also showed similar changes to those on plasma EVs (**Figure 3C, D**). To prove miRNA-targeted potential function, we applied GO enrichment analysis for miR145 and miR324, which showed their function enriched in the TGF-β pathway (**Figure 3E, S1**). Based on these, we divided BAV patients into low ratio of miR324/145 group (≤ 0.1, n = 7) and high ratio of miR324/145 group (> 0.1, n = 17), and performed the KEGG analysis (**Figure 3F**).

Expression level of miRNAs candidates associated with T/E

As shown in the **Figure 4A**, BAV-non showed a lower ratio of miR324/145, which was even lower than that in TAV-non, and the difference is statistically significant. However, there was no significant difference in the ratio of T/E (**Figure 4B**). According to the reports of related literature, the samples were divided into T/E-low group (n = 18) and T/E-high group (n = 6) to reflect the risk of dilations. Compared to T/E-high and control, the ratio of miR-324/145 in T/E-low increased significantly (**Figure 4C**).

Expression level of essential proteins in different groups

In quantitative proteomic analysis, the increased proteins including TGF-β1, MMP2 and MMP9, and decreased proteins including sENG, SOD3 and VWF were compared in groups classified by different criteria (**Figure 4D-F, S2**). TGF-β1 and MMP9 were significantly higher in BAV-T/E-high with low miR324/145 ratio, while ENG showed a significant lower expression compared to other BAV samples (**Figure 4D-F**).

Discussion

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Bicuspid aortic valve is the most common congenital cardiovascular malformation in population and usually accompanied by aortic dilatation, which significantly increases the risk of aortic dissection or rupture. The search for potential biomarkers to predict aortic diameter growth becomes even more crucial.

In the analysis of the clinical characteristics and echocardiography data from the included patients, BAV-dilated patient all had a large ascending aorta diameter with a lower aortic valve gradient and flow jet when contrasted to the BAV-non and TAV-non. According to hemodynamics, excessive aortic diameter causes thinning of the vessel wall, which further causes wall tension and eventually triggers dilatation and rupture of the ascending aorta. In addition, the valve malformation in the BAV patients would cause an increase in the shear force on the ascending aorta. [25-27]

Due to the hemodynamic and structural risks of valvular malformations in BAV patients, the identification of a gene or miRNA related to their aortic dilation process and a biological marker capable of predicting the rate of dilation becomes even more essential. We sequenced miRNAs from plasma extracellular vesicles of three groups and then screened them according to adjusted *P*-value < 0.05, $|Log_2(foldchange)| \geq 1$. Intriguingly, there were a total of 180 upregulated miRNAs and 229 downregulated miRNAs between BAV-dilated and BAV-non groups. Further screening targeted mRNAs by miRWalk 2.0, 14 of them were associated with TGFβ-1 and 5 of them were associated with MMP14 (closely related to the pathological process of aortic dilatation, as previously described). This further indicates that the selected candidate miRNA can be used as an important indicator of disease occurrence and development for further research.

To further understand the mechanism of aortic dilatation and its potential risk in patients with BAV, we performed proteomic analysis of plasma from three groups. Intriguingly, several proteins that have been validated to be associated with aortic dilatation or aneurysm also showed significant changes in plasma, further demonstrating that circulating biomarkers are a promising predictor of disease (**Figure 2B, C**). Since the histology of thoracic aortic aneurysms (TAA) (and abdominal aortic aneurysms) is characterized by loss of smooth muscle cells (SMC) in the aortic media and destruction of the extracellular matrix (ECM), matrix metalloproteinases (MMPs) have been implicated in the pathogenesis of aortic aneurysms[28]. It is believed that the balance of MMPs (especially MMP2 and MMP9) and the tissue inhibitors of metalloproteinases (TIMPs) play a critical role in aneurysm formation[29]. Superoxide dismutase (SOD) is associated with oxidative stress biology of the aortic wall and was proved to have be newer diagnostic techniques to adjudicate aortic catastrophe risk[30]. All proteins above together with TGF-β1 and sENG showed significant difference between the BAV-dilated/non and TAV-non. The activity and expression of MMPs in the aorta of BAV patients with ascending aortic aneurysm were higher than those of normal leaflet aortic aneurysm patients while sENG, SOD3 and VWF was lower. [31]

Compared with the protein biomarkers, the EV-miRNA biomarker could be advantageous due to its intrinsic stability and detectability at the early stage of disease. The study by Forte *et al.* identified a protein biomarker that could be tested in non-aneurysmal BAV patients for predicting the propensity to develop aortic lesions[19]. TGF-β and sENG (which can be cleaved by MMP14) have been shown to be

strongly associated with aortic dilatation and aneurysms, while T/E has also been shown to respond to aortic diameter growth in follow-up. In the present study, the best correlation between ratio of EV-miRNAs and proteins in the plasma were explored. We aligned 14 up-regulated miRNAs and 5 down-regulated miRNAs and performed linear analysis with T/E separately. After calculation, ratio of miR324/145 showed the highest linear correlation with T/E, which has the potential for further study to determine if it could be used to estimate the size of the aorta in patients with BAV. We first compared miRNA ratios between all three groups and found that this ratio was statistically different between all three groups. This implies that miR324/145 can indeed be used to respond to the diameter size of the aorta. More interestingly, when we grouped the BAV patients by T/E ratio, this ratio still demonstrated a statistical difference. This suggests to us that miR324/145 may not only reflect the current aortic diameter size, but also somehow suggest the growth rate of the aortic diameter.

Recent studies led to the assumption that circulating miRNAs are involved in the development and progression of aortic diseases. miR17, which plays an important role in cardiac matrix remodeling, [32] was proved to participate the process of aorta dilation in BAV patients by regulating TMIP-1/2 expression. [33] And miR145 is also responsible for the media remodeling through TGF pathway. [34] In addition, others including miR21, miR26, miR122 are associated with BAV and aortic dilation principally by the activation of TGF-β pathway and ErbB signaling pathways. [35] In this study, we detected the expression of miR324/145 from tissue by qPCR and the expression showed the same tendency as the expression in serum. To further confirm the function of our candidates, we performed mRNA prediction by miRWalk and analyzed its function using GO Analysis. We found that miR145 could indeed be enriched to the TGF-β1-associated pathway while miR324 did not exhibit a significant function associated with vasodilation. Most intriguingly, the expression of MMP9 was significantly increased in T/E-high with low miR324/145 ratio, which showed speculative risk for early intervention. No matter how the BAV patients sorted, we found that the pathway of focal adhesion showed significant difference from proteomic data (**Figure 2B, 3F and S3**). These findings indicate that focal adhesion could be a potential factor of aortic dilation.

ENG is one of the TGF-β1 receptors that regulates TGF-β1 signaling by binding to the TGF-β1 receptor. MMP-14 cleaves the extracellular region of ENG, allowing it to enter the serum as sENG. TGF-β1-mediated non-classical signaling pathways can lead to extracellular matrix degradation (especially MMP-2 expression). It eventually exists in serum as TGF-β1/sENG complex (**Figure 5A**)[19]. Further combined with our study, we suggest that TGF-β1 and ENG from the aortic wall directly or indirectly affect the expression of miR324 and miR145, which caused a decrease in the plasma EV-miRNA ratio. This decrease could respond to the altered metalloprotease activity and the oxidative stress within the aorta wall (**Figure 5B**). The miRNA ratio could be a potential marker with a negative correlation with the risk of disease development. Although it is the first study that provided an insight into possibility of EV-miRNA as biomarkers of aortopathy progression. However, future long-term clinic follow-up study is needed to verify the predictor value of the ratio of EV-miRNAs.

Conclusion

This study showed that plasma EV-miR324/145 ratio has the best correlation with plasma T/E ratio, which could be a potential predictor for the growth of aortic diameter, implying the need of closer clinical surveillance or earlier surgical treatment.

List Of Abbreviations

BAV: Bicuspid aortic valve; EVs: extracellular vesicles; TAV: tricuspid aortic valve; TGF-β1:transforming growth factor-β1; sENG: soluble endoglin; miR: mcroiRNA; WSS: wall shear stress; ECM: extracellular matrix; AVR: aortic valve replacement; UMI: unique molecular identifier; DE: differential expression; SD: standard deviation; SMC: smooth muscle cells; MMP: matrix metalloproteinase; TIMP: tissue inhibitors of metalloproteinase; SOD: superoxide dismutase;

Declarations

Ethics approval and consent to participate

Human aortic specimens were utilized under approvals of Zhongshan Hospital, Fudan University Ethics Committee (NO. B2020-158) in accordance with the Declaration of Helsinki. The sampling of plasama and clinic study were approved by the Human Research Ethics Committee of Shanghai Zhongshan hospital (B2018-285R). Written informed consent was obtained from all the patients according to the Declaration of Helsinki, and the methods were carried out in response to relevant guidelines.

Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

KZ, CW and HZ contributed to conceive and design the project. SZ, YZ and MA contributed to draft the manuscript and preform the experiments and data analysis. DL, WM and SB contributed to collect the samples. SL and SY contributed to draw figures and perform data analysis tools. YS, JL and HL

contributed to clinical data collection and analysis. All authors contributed to revision of the manuscript. All authors read and approved the final manuscript.

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Table

Table 1. Clinical Characteristics of the study groups

Characteristic	TAV-non (n=6)	BAV-non (n=11)	BAV-dilated (n=13)	*P* value (BAV-non VS BAV-dilated)
Male, %	67%	27%	46%	0.423
Age, years	50.50±4.04	49.91±16.46	58.77±11.42	0.135
Patient height, cm	164.17±7.90	163.4±8.5	163.2±7.7	0.968
BMI, Kg/m^2	24.70±2.39	24.51±3.87	23.82±3.27	0.639
Body surface area, m^2	1.74±0.18	1.80±0.19	1.78±0.19	0.765
BAV morphotype, RL/RN%	0	73%	69%	1.000
Ascending aortic diameter, mm	33.33±2.36	37.09±4.21	49.77±2.59	<0.001
Hypertension, %	0	27%	38%	0.679
Diabetes, %	0	9%	0%	0.458
Hyperlipidemia, %	0	9%	8%	1.000
Sinus dilation, %	0	9%	15%	1.000
Aortic regurgitation (>moderate), %	0	0%	0%	-
Aortic stenosis (>moderate), %	0	55%	54%	1.000
Aortic stenosis + regurgitation, %	0	45%	23%	0.391
Aortic valve gradient, mean, mm Hg	N/A	57.36±18.26	37.23±14.12	0.006
Aortic flow jet, m/s	N/A	4.92±0.76	3.93±0.72	0.004

Figures

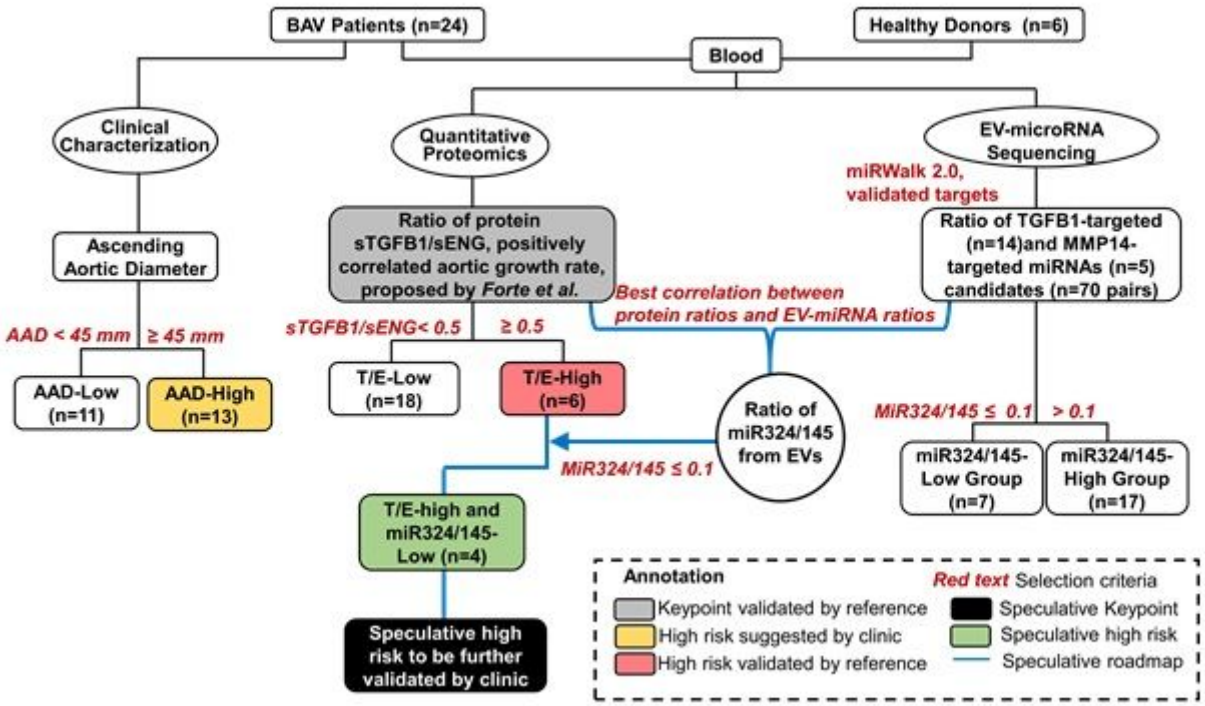

Figure 1

Workflow of exploring a prognostic biomarker for patients with BAV aortopathy.

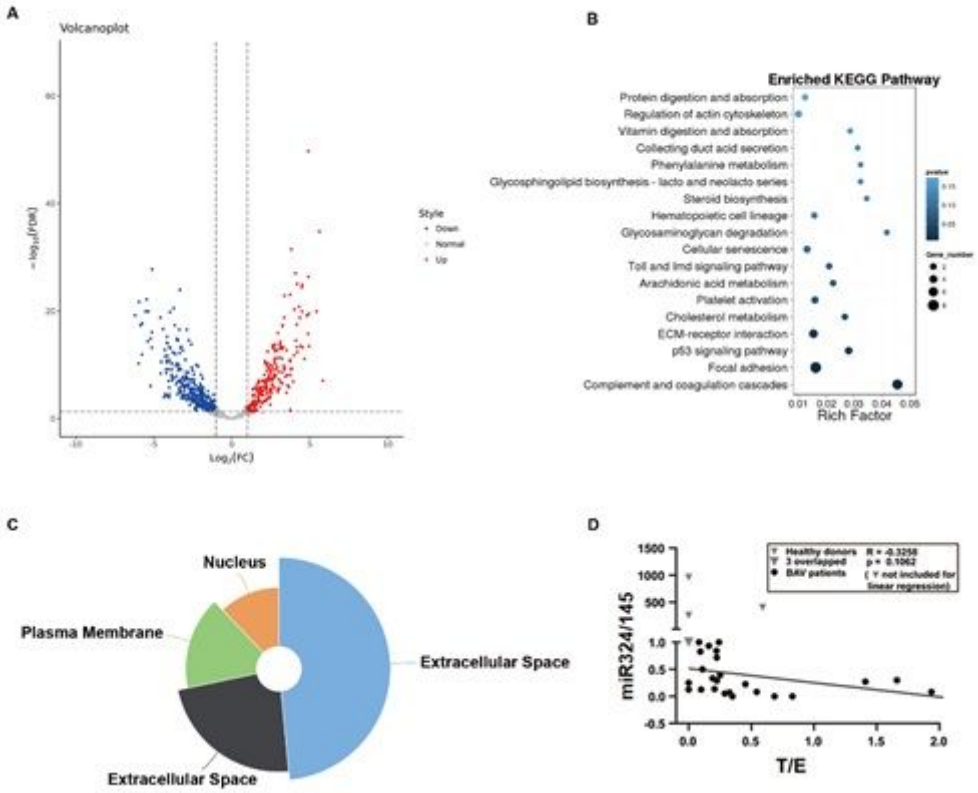

Figure 2

A, B. Results of RNA sequencing of Log2 mean expression intensity and KEGG analysis of proteomics in BAV-dilated and BAV-non. C. Location of aortic-aneurysm-related proteins. D. T/E was linearly related to miR324/145. (R = -0.3258)

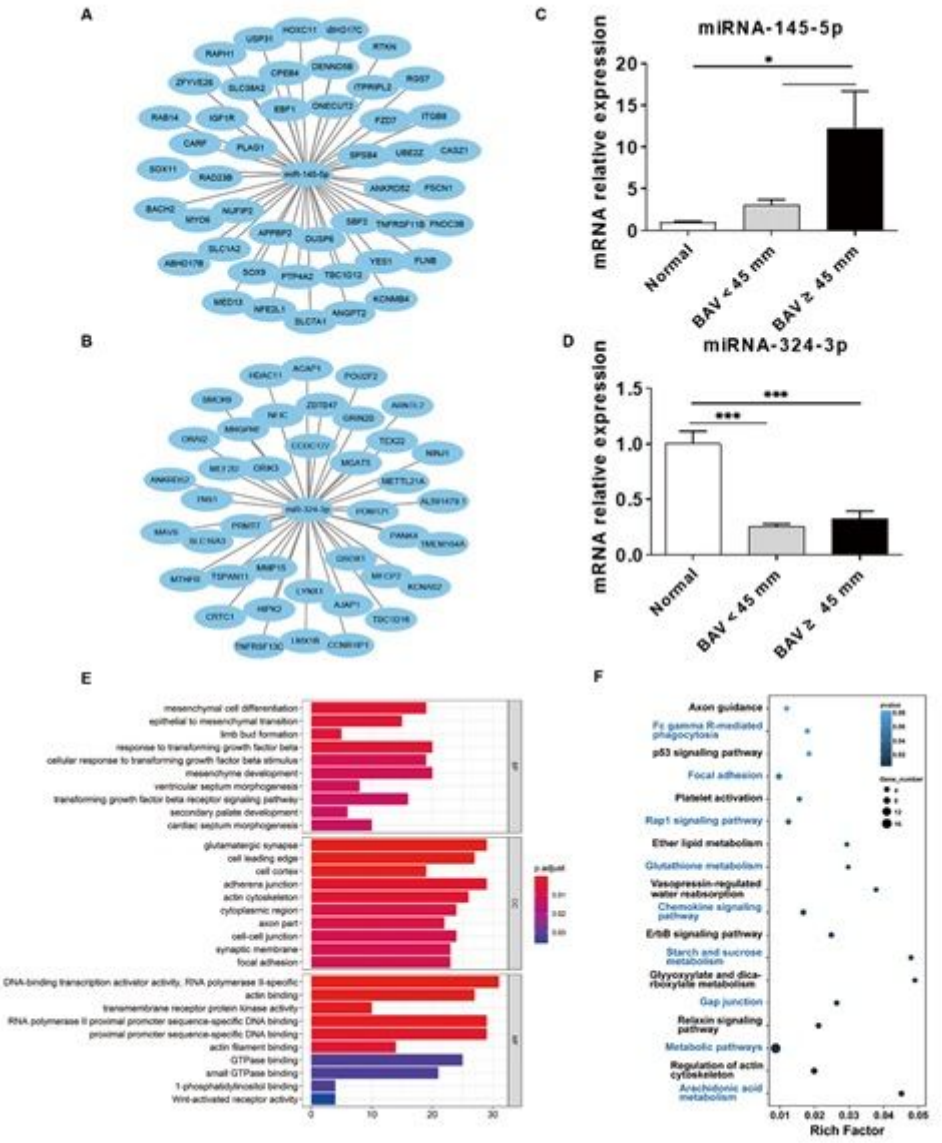

Figure 3

A, B. Predicted gene targets of miR145 and miR324. C, D. Expression of miR324 and miR145 in aortic tissues showed that miR-324 was decreased in both BAV-non and BAV-dilated groups and miR-145 was increased significantly in BAV-dilated group (*P < 0.05, ***P < 0.001). E. Go analysis of miR145 by miRWalk. F. KEGG analysis of proteomics between miR324/145-low and miR324/145-high groups.

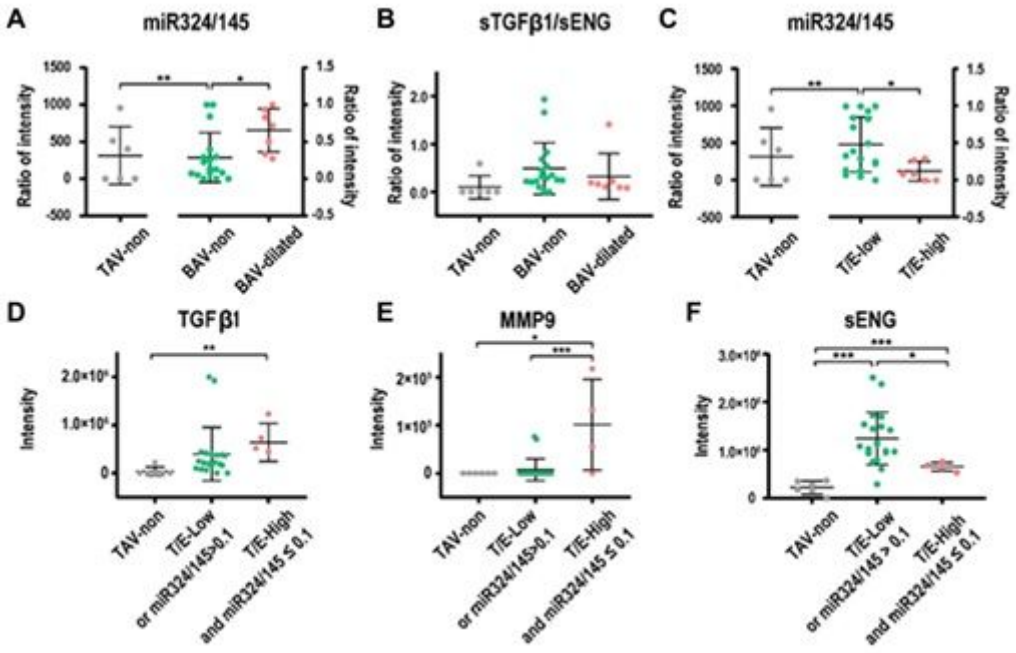

Figure 4

A-C. Ratio of miR324/145 and T/E in TAV-non, BAV-non and BAV-dilated or in TAV-non, BAV-T/E-low and BAV-T/E-high. D-F. The expression of increasing proteins including TGF-β1, MMP9, sENG in TAV-non, BAV-T/E-high and miR324/145 ≤ 0.1 and others.

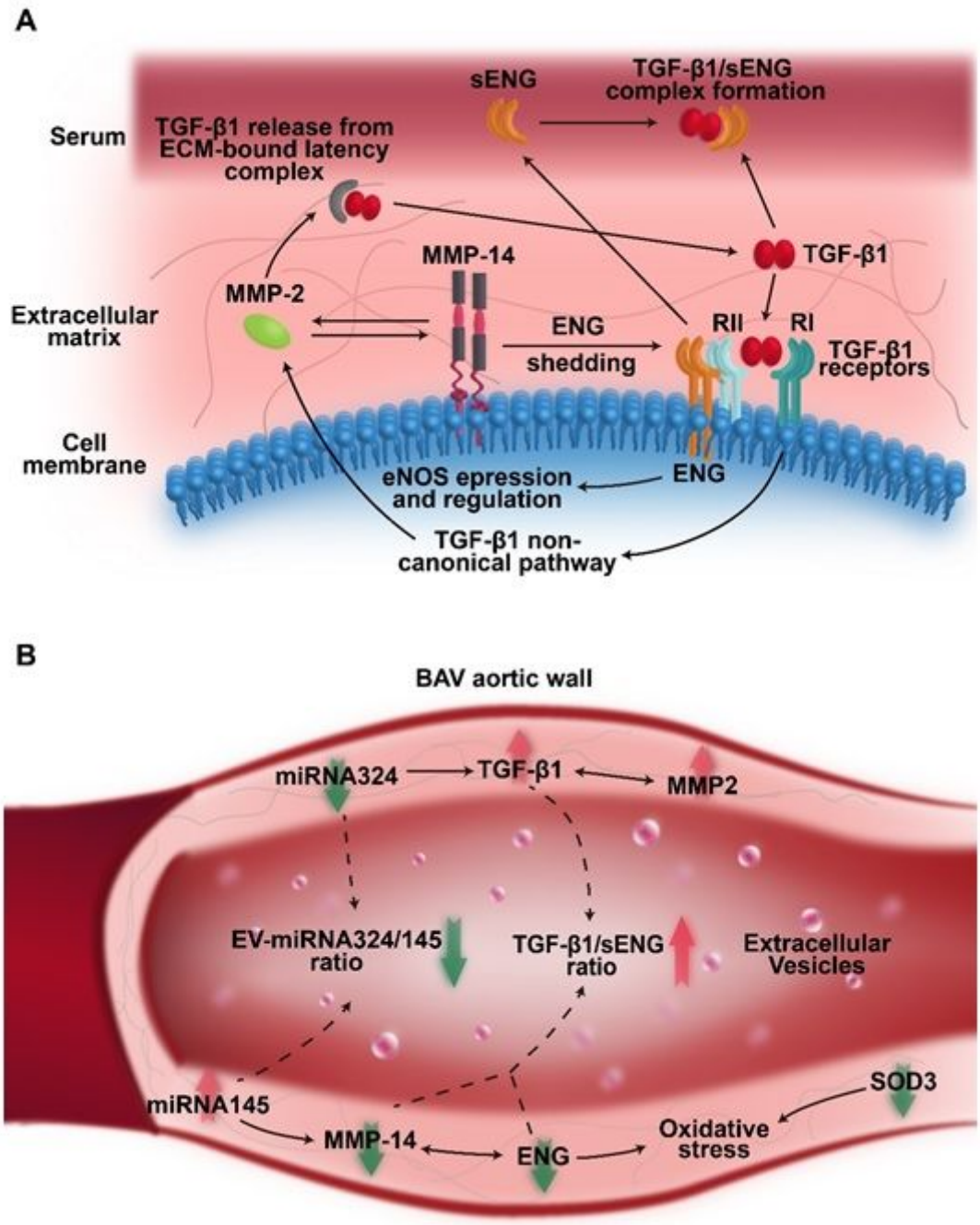

Figure 5

Possible mechanisms involved in BAV aortopathy. A, TGF-β1/sENG signaling pathways in BAV aortic wall. B, Plasma EV-miRNAs regulating BAV aortopathy.

Supplementary Files

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