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Using integrative bioinformatics approaches and machine–learning strategies to identify potential signatures for atrial fibrillation

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ABSTRACT

Atrial fibrillation (AF) is the most common tachyarrhythmia and seriously affects human health. Key targets of AF bioinformatics analysis can help to better understand the pathogenesis of AF and develop therapeutic targets. The left atrial appendage tissue of 20 patients with AF and 10 patients with sinus rhythm were collected for sequencing, and the expression data of the atrial tissue were obtained. Based on this, 2578 differentially expressed genes were obtained through differential analysis. Different express genes (DEGs) were functionally enriched on Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG), mainly focusing on neuroactive ligand-receptor interactions, neuronal cell body pathways, regulation of neurogenesis, and neuronal death, regulation of neuronal death, etc. Secondly, 14 significant module genes were obtained by analyzing the weighted gene co-expression network of DEGs. Next, LASSO and SVM analyzes were performed on the differential genes, and the results were in good agreement with the calibration curve of the nomogram model for predicting AF constructed by the weighted gene co-expression network key genes. The significant module genes obtained by the area under the ROC curve (AUC) analysis were analyzed. Through crossover, two key disease characteristic genes related to AF, HOXA2 and RND2, were screened out. RND2 was selected for further research, and qPCR verified the expression of RND2 in sinus rhythm patients and AF patients. Patients with sinus rhythm were significantly higher than those in AF patients. Our research indicates that RND2 is significantly associated with the onset of AF and can serve as a potential target for studying its pathogenesis.

1. Introduction

Atrial fibrillation(AF) is the most widespread cardiac arrhythmia that results in significant reduction in cardiac output、left atrial thrombosis and cerebral infarction. Palpitations and chest tightness are the most common symptoms of AF[1].AF is believed to occur through two events that is the formation of an arrhythmogenic atrial substrate and an arrhythmogenic trigger. Atrial remodeling is the primary proarrhythmic substrate[2]. AF may be triggered through multiple factors that perturb normal electrical conduction such as hypokalemia, hypomagnesemia, hypovolemia, and alterations in parasympathetic and sympathetic activity[2–4]. While previous research has identified several factors that contribute to the development and progression of AF. However, the role of genetic factors of AF remains largely unexplored.

Rnd2, encoding a member of the Rho GTPase family, localizes to chromosome 17(q21)[5]. Unlike classical GTPases, Rnd2 have altered sequences at residues critical for GTP hydrolysis and are permanently in the GTP-bound form with no detectable GTPase activity[6]. Rnd2 is present in the cytoplasm and endosomes and directly binds to vacuolar protein sorting 1-A (Vps4A), the central protein regulating early endosome trafficking[7,8] and abundantly expressed in testis and in the brain [9]. Rnd2 regulates neuronal actin dynamics during cortical neuronal migration[10]. Thus, cortical malformations caused by Rnd2 overexpression in brains may be related to the development of epilepsy and focal cortical dysplasia (FCD)[11,12]. Furthermore, in the vasculature, the sex hormone steroids, which induce vascular smooth muscle contraction, increase Rnd2 expression in muscles[13]. Finally, Rnd2 is a

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centromeric neighbor of the breast and ovarian cancer susceptibility gene BRCA1[14]. Although Rnd2 has extremely important physiological and pathological roles, its function in atrial fibrillation still needs to be further explored.

In recent years, with the popularization of bioinformatics, key genes for disease occurrence can be quickly and accurately screened. Therefore, bioinformatics analysis may help to better understand the molecular mechanisms of atrial fibrillation, paving the way for the discovery of biomarkers and new treatment options. In this context, our study aimed to conduct a comprehensive analysis of RNA sequences in patients with atrial fibrillation. Our study attempts to integrate differential analysis, weighted correlation network analysis(WGCNA)、least absolute shrinkage and selection operator (LASSO) and support vector machines(SVM) analysis to produce a more accurate analysis of AF causative genes[15]. Our findings may provide reference for the diagnosis and treatment of atrial fibrillation. Ultimately, we hope our research will help develop more effective treatments for atrial fibrillation.

2. Materials and methods

2.1. Data acquisition

Fig. 1 shows the data analysis procedures of our study. In this study, we obtained left atrial appendage tissue from 26 patients with atrial fibrillation as the experimental group and left atrial tissue from 15 patients with sinus rhythm as the control group. Table 1 shows clinical and demographic characteristics of the study groups. Among them, 10 samples were taken from the control group and 20 samples were taken from experimental group for mRNA transcriptome sequencing. The RNA-seq data of the experimental group and the control group were obtained, and subsequent data analysis was performed based on this. The remaining samples were used for experimental verification.

2.2. Differential gene expression analysis

Normalize the downloaded matrix data obtained from R. The R language “Limma” package was used to screen the differentially expressed genes (DEGs) of the normalized spectrum. Genes with |Log2 fold-change (log2FC) |≥1 and P value < 0.05 were defined as differentially expressed genes. Then draw the volcano map and heat map respectively according to the R voice “ggplot2” package and “pheatmap” package[16].

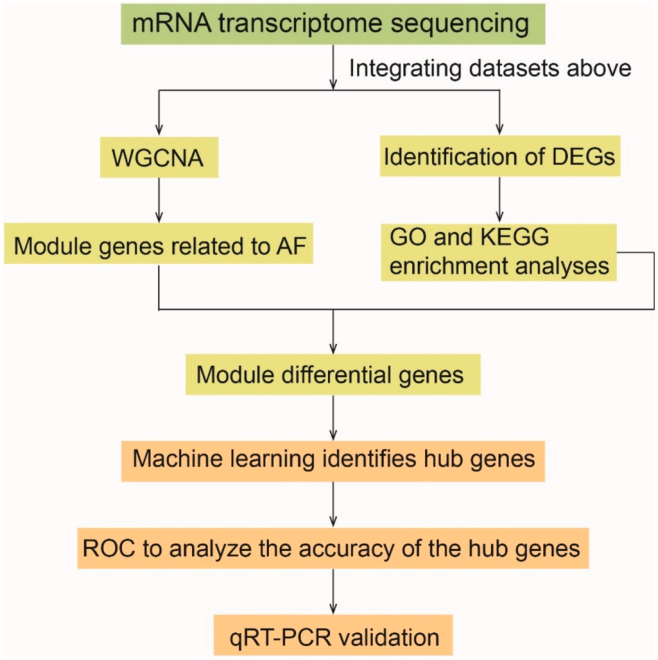

Fig. 1. Flowchart of the study.

Table 1
Baseline demographic and clinical characteristics of patients with AF and controls.

Characteristics	Controls(n = 15)	AF Patients(n = 26)	P-value
Age(years), mean (p25, p75)	39.64(28.75, 50.50)	57.65(48.75, 68.00)	< 0.001
Sex			0.273
Male, n (%)	10(66.7 %)	12(46.2 %)	
Female, n (%)	5(33.3 %)	14(53.8 %)	
BMI (kg/m2), mean(p25, p75)	24.33(22.225, 27.807)	23.604(21.900, 26.000)	0.759
Systolic BP (mm Hg), mean ± SD	137.79 ± 50.140	114.62 ± 13.799	0.112
Diastolic BP (mm Hg)	85.57 ± 31.090	75.88 ± 14.049	0.285
Diabetes, n (%)	0(0 %)	2(7.7 %)	0.287
cerebral apoplexy, n (%)	6(40 %)	4(15.4 %)	0.056
Creatinine (mmol/L), mean(p25, p75)	88.379 (45.600,122.450)	80.308 (52.000,92.750)	0.777
Total cholesterol (mmol/L)		4.1179 ± 0.73405	
LDLC (mmol/L) mean (p25, p75)		1.0607(0.8275, 1.3300)	
HDLC (mmol/L) mean (p25, p75)		2.1086(1.6350, 2.6100)	
Triglyceride (mmol/L) mean(p25, p75)		1.6400(0.9200, 1.8650)	

SD: standard deviation; AF: Atrial fibrillation; LDLC: Low density lipoprotein cholesterol; HDLC: High density lipoprotein cholesterol.

2.3. Identification of co-expression modules by WGCNA

To explore the interactions between genes, we used the systems biology method WGCNA to construct a gene correlation network. The specific steps are as follows[17]: (1) Import data sets containing more than 25 % of mutated genes in the samples into WGCNA; (2) Eliminate outlier samples to ensure the reliability of the network construction results; (3) Use the “pick-Soft-Threshold” function to calculate adjacent degree, and obtain a soft threshold (β) based on the co-expression similarity; (4) Convert the adjacency relationship into a topological overlap matrix (TOM), and calculate the corresponding dissimilarity (1-TOM); (5) Through hierarchical clustering and linking The cut tree function detects modules. To divide genes with similar expression profiles into gene modules, average linkage hierarchical clustering is performed on the gene tree diagram, and the “tom-based” difference measurement method is used; (6) For modules related to clinical attributes, calculate module membership (MM, correlation between specific genes and module characteristic genes (ME)) and gene significance (GS, correlation between specific genes and clinical variables); (7) Visualize the signature gene network. Conduct further analysis of the genetic information in the module.

2.4. Functional enrichment analysis of DEGs

To explore the functions and pathways of overlapping DEGs, gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) were used to functionally enrich the intersection genes. In the enrichment results, both P < 0.05 and adjusted P < 0.05 terms are expressed.

2.5. Screening and validation of hub genes

This study uses three algorithms to screen key genes of AF, namely support vector machines (SVM), LASSO, and WGCNA. To reduce the risk of overfitting, both the LASSO logistic regression model and the SVM model were validated using 5-fold cross-validation. For LASSO, the

hyperparameter lambda (λ) was optimized by minimizing the cross-validated mean squared error (MSE). For SVM, the radial basis function (RBF) kernel was used, and hyperparameters (C and γ) were tuned using a grid search approach combined with 5-fold cross-validation. This iterative validation ensures that the performance metrics are robust and not overly influenced by the dataset's small size. Then, a Venn diagram is used to obtain the intersection of the three selected genes to obtain the hub genes for further analysis. Finally, this study used" ggpubr "package to calculate the receiver operating characteristic curve (ROC) and area under the curve to evaluate the diagnostic value of key genes for atrial fibrillation.

2.6. q-PCR

The transcriptome sequencing samples used in the discovery phase and the qPCR validation samples used in the validation phase were sourced from different donors, ensuring independence between the two datasets. The qPCR validation was conducted on an entirely separate cohort, addressing potential concerns regarding overfitting. Total RNA was extracted and reverse transcribed into cDNA using a reverse transcription kit (Vazyme, Nanjing, China). According to the instructions of the qPCR kit (Yeason, Shanghai, China), the reaction system (20uL) was prepared and qPCR was performed using LightCycler® 96 SW 1.1. 2^(−ΔΔCT) was used to calculate the relative expression of mRNA.

2.7. Statistical analysis

Data were analyzed by GraphPad software and expressed as mean ± SD. P values < 0.05 were considered as statistically significant. Statistical differences between two groups were compared using a t-test.

3. Results

3.1. Identify DEGs

By using transcriptome sequencing, we generated gene expression data for patients with atrial fibrillation and those with sinus rhythm, which has been uploaded to the GEO database under the accession number GSE282504.After raw data preprocessing, using $|\log2FC| \geq 1$ and P value < 0.05 as the standard, 2578 DEGs were obtained from the RNA-seq data of 10 control groups and 20 atrial fibrillation samples, of which 1875 genes were down-regulated, and 703 genes were Upregulated. The distribution of these DEGs is shown in the volcano plot (Fig. 2A).

3.2. GO and KEGG enrichment analysis of DEGs

Next, we performed GO and KEGG pathway functional enrichment on differentially expressed genes. The results showed that significantly enriched pathways included neuroactive ligand-receptor interaction, regulation of neuron differentiation, neuronal cell body, regulation of neurogenesis and neuron death (Fig. 2B).

3.3. WGCNA and module identification

All genes were sorted from large to small in variance, and genes with the top 25 % of variance were selected for analysis. After removing outliers, we obtain a sample clustering tree. The soft threshold is set to 14 to construct a scale-free network(Fig. 3A and B). Subsequently, we establish the adjacency matrix and construct the TOM(Fig. 3C). Functionally similar modules were identified based on average hierarchical clustering and link cutting trees. Black modules and purple modules have the highest correlation with AF(Fig. 3D).

3.4. Screening of hub genes

This study used LASSO logistic regression algorithm, WGCNA analysis and SVM algorithm to screen key marker genes. The results showed that a total of 31 genes were identified by LASSO(Fig. 4B), a total of 8 genes were identified by the SVM algorithm (Fig. 4C), and a total of 31 genes were identified by WGCNA. Next, the Venn diagram shows that under the three algorithms, RND2 and HOXA2 is an overlapping gene (Fig. 4D). Both of them are down-regulated genes.

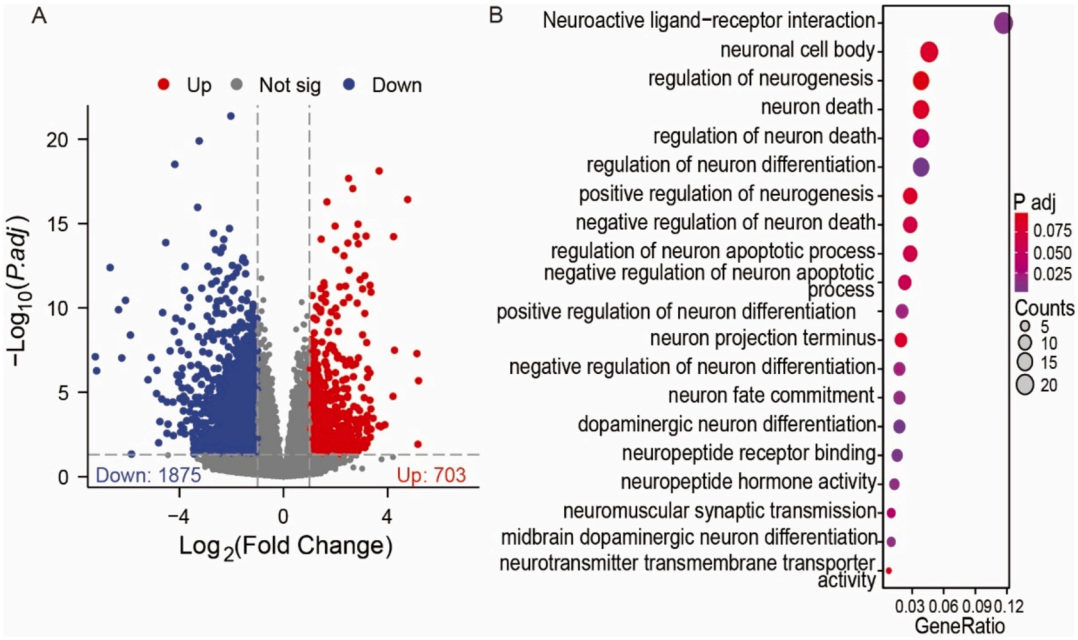

Fig. 2. Identification of differentially expressed genes (DEGs) and enrichment analysis of DEGs. (A)Nodes in red represent 703 upregulated genes, nodes in blue represent 1875 downregulated genes, and gray dots represent no significantly changed genes. (B)The Bubble chart show enriched items of DEGs. The x-axis labels represent gene ratios and y-axis labels represent Gene ontology (GO) and Kyoto encyclopedis of genes and genomes(KEGG) pathway. Different colors of circles represent different adjust P-values. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

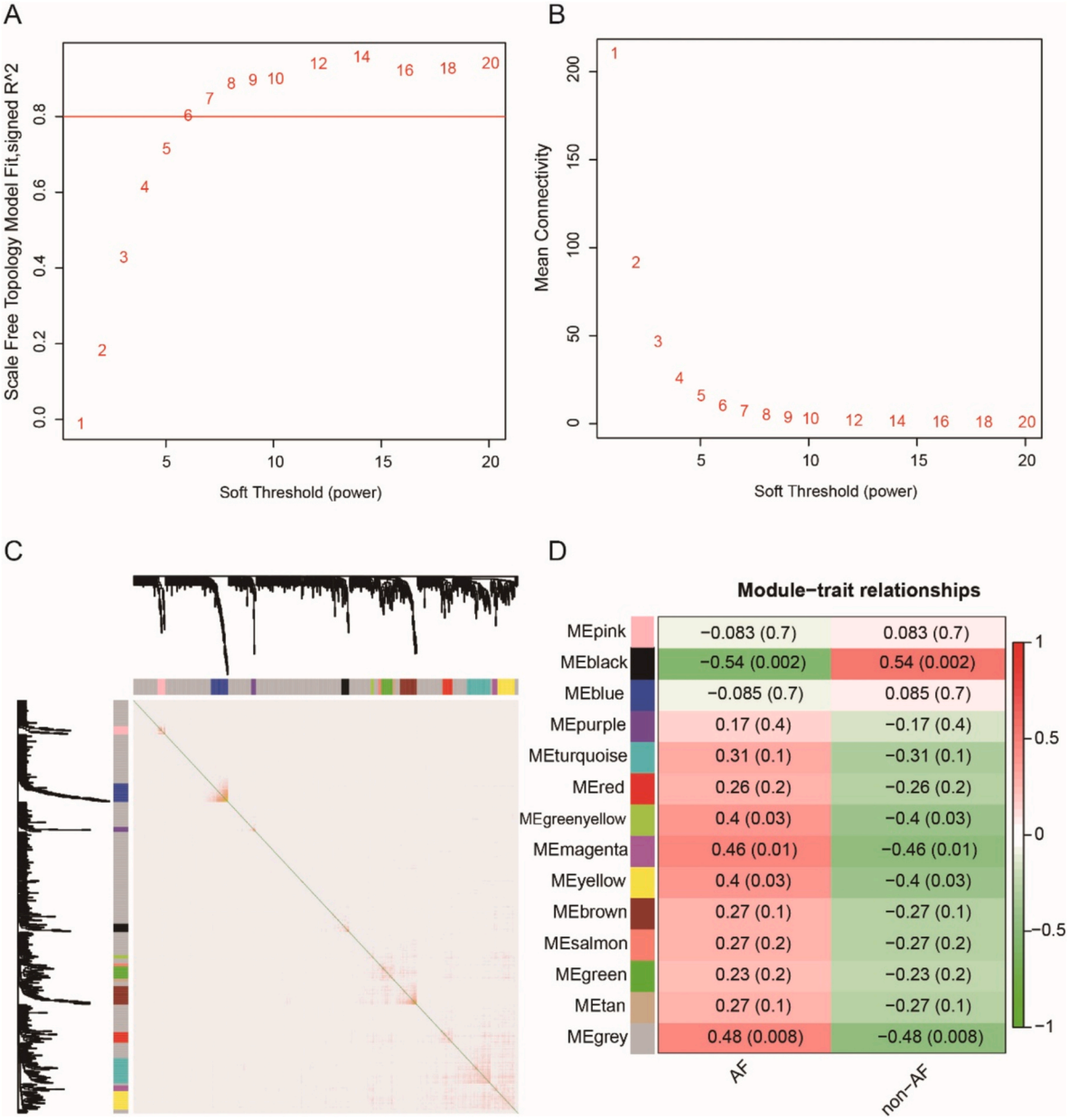

Fig. 3. Construction of weighted gene co-expression network analysis (WGCNA) modules in AF. (A,B) Analysis of network topology for various soft thresholding powers. (C) Visulustering alizing the gene network using a heatmap plot. (D) Clustering dendrogram of differentially expressed genes related to AF.

3.5. Validation of RND2 in AF

To evaluate the potential predictive value of RND2 and HOXA2 in key gene signatures in AF, ROC curves were generated. The AUC of RND2 is 1, and the AUC of HOXA2 is 0.995.

(Fig. 5A). Because RND2 has a higher diagnostic value for AF than HOXA2, further verification of the expression level of RND2 in tissues will be conducted.

3.6. Expression of RND2 in AF patients

For further verification, we used qPCR to measure RND2 expression in 6 cases of atrial fibrillation and 5 cases of sinus rhythm. The results showed that the expression of RND2 in patients with sinus rhythm was higher than that in those with AF ($P < 0.001$) (Fig. 5B).

4. Conclusion

In summary, we have shown for the first time using bioinformatics and qPCR that RND2 is significantly associated with atrial fibrillation. This provides a possible target for further elucidating the pathogenesis of atrial fibrillation in the future, allowing people to have a deeper understanding of atrial fibrillation.

5. Discussion

In this study, we integrated RNA-seq data from 20 AF patients and 10 persons in sinus rhythm to identify Hub genes associated with the onset of AF. After merging and normalizing the data sets, 2578 DEGs were obtained. Through GO enrichment analysis and KEGG enrichment analysis, DEGs were significantly related to neuroactive ligand-receptor interactions and neuronal cell body pathways, respectively. Then, the module with the highest correlation with AF is obtained through

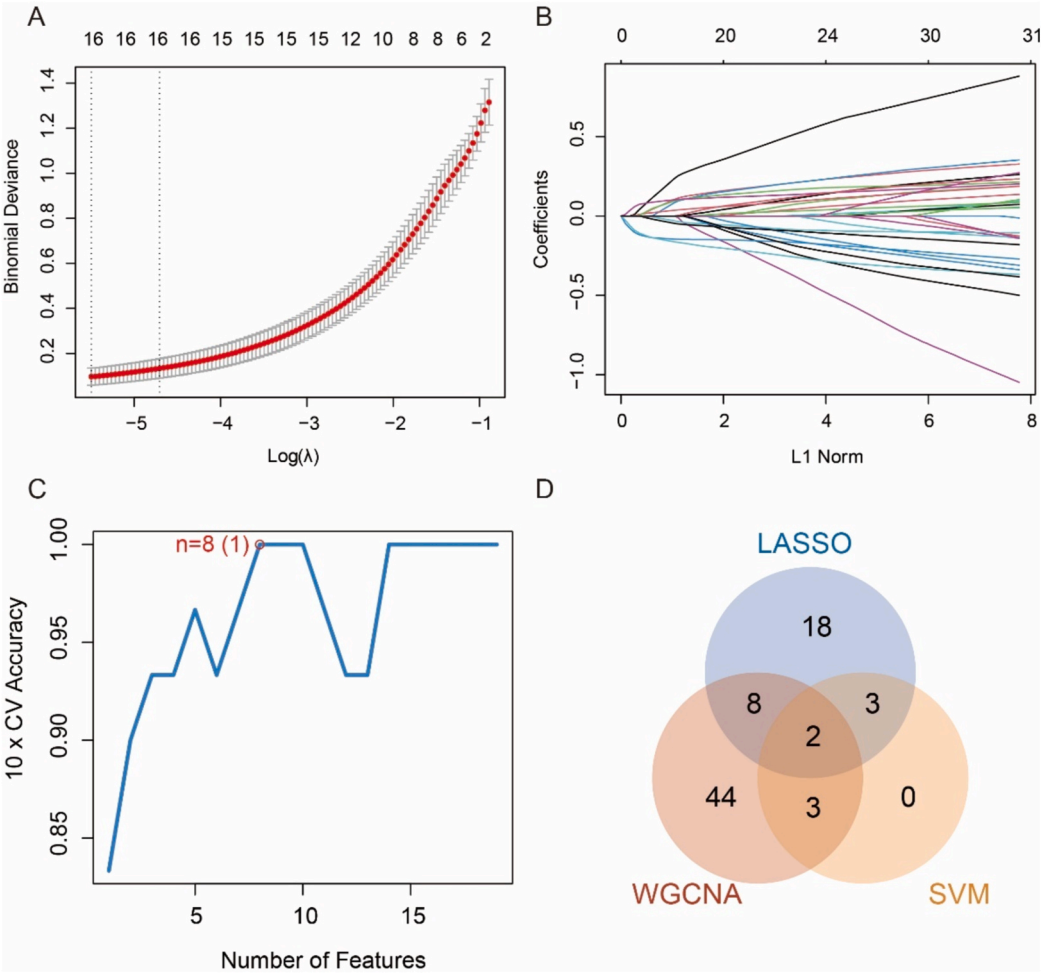

Fig. 4. Screening of critical signatures via multiple machine-learning algorithms. (A) Selection of the optimal param-eter(lamda) in the least absolute shrinkage and selection operator (LASSO) model, and generation of a coefficient profile plot. (B) LASSO coeffcient profile of the 31 genes, and different colours represent different genes. (C) The abscissa represents the numbre of features and the ordinate is 10 x CV Accuracy, which represents the accuracy of the curve change after 10 times cross-validation. In the figure, n = 8(1) indicates that there are 8 features, and the accuracy is 1. (D) Venn gram shows the intersection of critical signatures obtained by the three strategies.

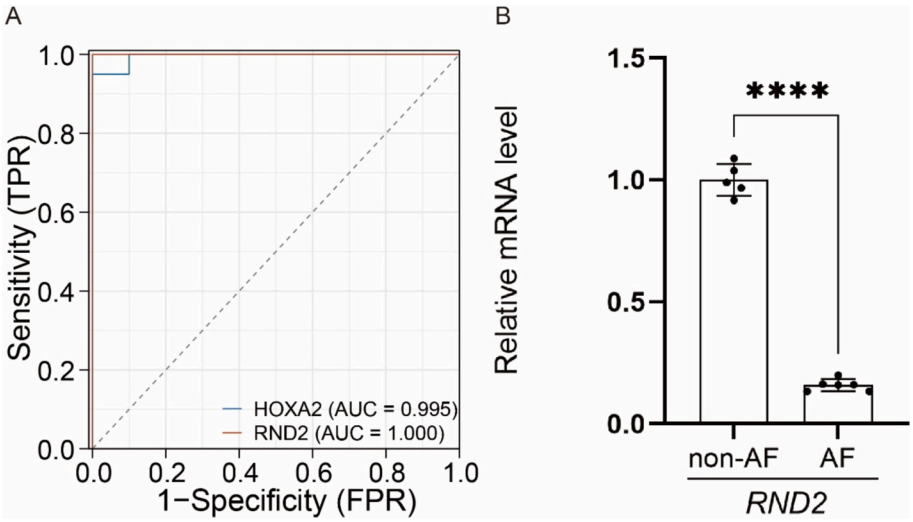

Fig. 5. (A) The diagnostic power of RND2 and HOXA2, and combined in atherosclerosis by ROC curve. (B) RND2 expression level in human with or without AF.

WGCNA. The genes in the module were then analyzed through the LASSO logistic algorithm and SVM algorithm. The results of the Venn diagram showed that RND2 and HOXA2 may be Hub genes. Gene investigation showed that HOXA2 is mainly related to the development of the nervous system, so further inflammation is not required. In addition, we detected the expression of RND2 by qPCR. By utilizing independent cohorts for discovery and validation phases, our study minimizes the risk of overfitting and enhances the robustness and generalizability of the identified biomarkers (RND2 and HOXA2). We found that the expression level of RND2 was increased in patients with sinus rhythm compared with those with AF. Taken together, our results provide a potential target for elucidating the pathogenesis of atrial fibrillation.

A total of 31,203 genes were identified using RNA sequencing. After conducting differential analysis, we found that 1,493 of these genes were differentially expressed, which is 4.7 percent of the total. This indicates the use of a standard and rigorous filtering criterion. The choice of log2FC ≥ 1 or $\leq$ -1 as the threshold has important biological implications. It indicates that the RNA expression level of a gene in the AF group is upregulated to at least 2-fold or downregulated to 50 % relative to the control group. Changes of this magnitude are highly likely to impact cellular processes, tissue functions, and even organism-level phenotypes. This threshold is widely accepted in molecular biology as a meaningful and reasonable cutoff for identifying genes with substantial biological significance. While stricter thresholds (e.g., log2FC ≥ 1.5 or 2) might further refine the results, they would exclude many genes that could still play critical roles in the pathophysiology of AF.

AF is the most common tachyarrhythmia, accounting for approximately 30 % of all patients with arrhythmias[18]. According to estimates from the American Heart Association, more than 33 million people worldwide suffer from atrial fibrillation. In the United States, 2.3 million people have atrial fibrillation, a number expected to increase to 5.6 million by 2050, in part due to an aging population and increased prevalence of cardiovascular disease[18]. Because the atria cannot effectively pump blood into the ventricles and the atria fibrillate irregularly, blood will remain in the atria and form turbulence, forming thrombi. These blood clots may break off and enter the systemic circulatory system, causing blockage in the brain or other organs[19]. In addition, atrial fibrillation can also make the ventricular rate extremely irregular, resulting in reduced cardiac output and damage to the function of organs sensitive to blood supply, such as the brain. There are many causes of atrial fibrillation, including hypertension, coronary heart disease, heart valve disease, cardiomyopathy, hyperthyroidism, excessive alcohol consumption, and after heart surgery. Age is also an important factor in the development of atrial fibrillation, and the prevalence of atrial fibrillation increases with age. The incidence of atrial fibrillation increases significantly in people over 65 years of age. The occurrence and maintenance of atrial fibrillation are mainly related to structural remodeling, electrical remodeling and autonomic nerve remodeling. Although there have been a lot of studies on the pathogenesis and treatment of atrial fibrillation, its pathogenesis has not been fully elucidated, and the treatment effect is unsatisfactory. Relying on a large amount of evidence-based medical data, the current treatment of atrial fibrillation mainly includes drug treatment and surgical treatment. Drug therapy is the basic treatment for atrial fibrillation, which means actively preventing thromboembolism, converting and restoring sinus rhythm, and controlling ventricular rate on the basis of treating the primary disease and triggering factors. However, its effect is not ideal and it is difficult to cure AF. Although surgical treatment methods mainly including maze surgery and Wolf Mini-maze surgery can cure atrial fibrillation, there are some complications. Therefore, in view of the problems that the pathogenesis of atrial fibrillation has not been fully elucidated and the treatment effect is unsatisfactory, we plan to use bioinformatics analysis methods to conduct research based on omics data to find the key gene RND2 in the pathogenesis of atrial fibrillation.

In recent years, biomarkers have gradually emerged in the study of atrial fibrillation, becoming an important tool for assessing the risk of atrial fibrillation, predicting disease progression, and guiding clinical decision-making. Biomarkers such as cardiac troponin T (cTnT) and N-terminal pro brain natriuretic peptide (NT-proBNP) have been widely studied to assess the risk and prognosis of atrial fibrillation[20,21]. Inflammatory response plays an important role in the occurrence and maintenance of atrial fibrillation. Research has shown that patients with atrial fibrillation typically have a low-grade inflammatory state, with elevated levels of inflammatory factors such as tumor necrosis factor alpha (TNF − α) and interleukin-6 (IL-6) in atrial fibrillation patients. These inflammatory factors further exacerbate the occurrence and persistence of atrial fibrillation by promoting atrial fibrosis and electrophysiological remodeling[22,23]. Metabolic abnormalities are also an important risk factor for atrial fibrillation, especially in patients with diabetes and obesity. Research has found that metabolic syndrome is closely related to the occurrence of atrial fibrillation, and metabolic abnormalities increase the risk of atrial fibrillation by causing electrophysiological changes and structural remodeling in the atria. For example, in patients with diabetes, the late sodium current in atrial myocytes increases, which leads to the prolongation of action potential, thereby increasing the susceptibility to atrial fibrillation[24,25]. In addition, obesity and metabolic syndrome are also associated with atrial dilation and fibrosis, further exacerbating the occurrence of atrial fibrillation[26,27].

Rnd2 encodes a member of the Rho GTPase family and is mapped to chromosome 17 (q21). Unlike classical GTPases, Rnd2 changes its sequence at residues critical for GTP hydrolysis and is permanently in its GTP-bound form with no detectable GTPase activity. Rnd2 is present in the cytoplasm and endosomes and binds directly to vacuolar protein sorting 1-A (Vps4A), a central protein that regulates early endosomal trafficking and is abundantly expressed in the testis and brain. Rnd2 migration in cortical neurons. Thus, cortical malformations caused by Rnd2 overexpression in the brain may be associated with the development of epilepsy and focal cortical dysplasia (FCD). In addition, in the vasculature, sex hormone steroids induce vascular smooth muscle contraction, increasing the expression of Rnd2 in muscles. Finally, Rnd2 is a centromeric neighbor of the breast and ovarian cancer susceptibility gene BRCA19. Our study confirms that RND2 plays an important role in the pathogenesis of AF, but further research is needed on the specific regulatory mode.

Several shortcomings of our study are worth mentioning. First, our study lacked relevant clinical data and could not conduct prognostic analysis. Although this study provides valuable insights into atrial fibrillation biomarkers, the relatively small sample size (20 AF patients and 10 controls) may limit its statistical power. Power analysis suggested that at least 26 samples per group would be required to achieve optimal reliability. However, obtaining left atrial tissue samples, particularly from sinus rhythm donors, poses significant ethical and logistical challenges, which is a well-recognized limitation in clinical research. Despite this limitation, our study offers important preliminary findings that contribute to the understanding of atrial fibrillation mechanisms and lay the groundwork for future studies with larger cohorts. Secondly, the AF sample size of the studies involved was limited, and the analysis failed to cover the impact of cardiovascular risk factors on the overall data analysis and results, which may affect gene expression in AF patients. To address these shortcomings, we will conduct further clinical studies with more detailed clinical data and larger sample sizes to confirm the study results. Finally, this study has preliminarily screened the key marker genes of AF. We need to further confirm these findings in in vitro and in vivo studies, and further study to elucidate the pathological mechanism of RND2 in the pathogenesis of AF, which may contribute to the application of RND2 in the diagnosis and treatment of AF.

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Ethics approval and consent to participate

The present study received approval from the ethics committee of The First Affiliated Hospital of The University of Science and Techology of China (Reference number 2024KY-248). Prior to participation, written informed consent was obtained from the patient involved in the study.

CRediT authorship contribution statement

Shihao Fu: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Zian Feng:** Methodology, Formal analysis, Data curation. **Ao Li:** Software, Methodology, Investigation. **Zhenxiao Ma:** Methodology, Investigation, Formal analysis. **Haiyang Zhang:** Writing – review & editing. **Zhiwei Zhao:** Writing – review & editing, Software, Resources, Methodology, Funding acquisition, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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