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Co-expression and interaction network analysis identifies neutrophil-related genes as the core mediator of atrial fibrillation

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Abstract. Atrial fibrillation (AF) is the most common cardiac arrhythmia and can cause serious complications. Several studies have shown that neutrophils may influence AF progression. However, the key genes related to neutrophils in AF have not been fully elucidated. Here, we downloaded microarray expression data of AF, and screened differentially expressed genes. Key immune cells in AF were identified by immune cell infiltration analysis. Weighted gene co-expression network analysis (WGCNA) and protein-protein interaction (PPI) analysis were used to construct gene co-expression modules and identify hub genes. The association between key genes and neutrophils was then verified. Our results showed that 303 differentially expressed genes (DEGs) were screened in AF and sinus rhythm (SR), of which 194 were up-regulated and 109 were down-regulated. DEGs were mainly enriched in functions and pathways of neutrophil activation and biological functions of neutrophil activation-mediated immune response. Immune infiltration analysis revealed elevated levels of neutrophil infiltration in AF. WGCNA analysis revealed that the modules in dark red were associated with neutrophils. PPI analysis of these modules yielded 10 hub genes. S100A12, FCGR3B and S100A8 are 3 potential key genes related to neutrophils in AF, which are significantly positively correlated with neutrophils. These genes deserve further investigation and may be potential therapeutic targets for AF.

Key words: Atrial fibrillation — Neutrophils — Hub genes — Immune cell infiltration — WGCNA

Introduction

Atrial fibrillation (AF) refers to the replacement of steady atrial electrical activity by abnormal fibrillation waves, resulting in palpitations, dizziness, shortness of breath and other symptoms (Brundel et al. 2022). Primary cardiovascular disease, metabolic syndrome, exercise and heavy drinking are important risk factors for AF (Lau et al. 2016). More than 590 000 people worldwide have AF (Kornej et al. 2021), and the prevalence of AF is increasing (Kornej et al. 2020). AF reduces the quality of life of patients, and may also cause serious complications such as thromboembolism, heart failure, and stroke (Carlisle et al. 2019). At present, the main treatment methods of AF include radiofrequency ablation, drug therapy and electrical cardioversion (Jost et al. 2021). Treatment limitations and high socioeconomic burden of AF make it a major clinical challenge (Heijman et al. 2018). However, the complex pathophysiology of AF is not fully understood. Therefore, it is essential to study the mechanism of its occurrence and excavate the key genes to lay the foundation for subsequent gene targeted therapy.

Neutrophils represent the most abundant effector cells of the human immune system and have antibacterial and pro-inflammatory functions (Skendros et al. 2018). Several recent studies have shown that neutrophils can exert crucial effects on the pathophysiology of AF. The formation of neutrophil extracellular traps was found to be enhanced in AF patients with left atrial dilatation (Mołek et al. 2023). Xiao et al. found that neutrophil levels were higher in persistent AF and that immune cells may interact with specific genes (Xiao et al. 2021). Clinical data show that an increase in the neutrophil-to-lymphocyte ratio leads to an increased risk

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of AF (Berkovitch et al. 2019; Wu et al. 2021). Although the role of neutrophils in AF has received some attention, the key genes related to neutrophils in AF are not fully understood and urgently need to be excavated.

The use of bioinformatics analysis to mine disease-related genes plays an important role and is more intuitive and effective. Moreover, weighted gene co-expression network analysis (WGCNA) becomes an important strategy in bioinformatics applications to identify potential mechanisms and therapeutic targets of diseases (Langfelder and Horvath 2008). Through our review of the literature, few researches have investigated specific gene and functional roles in the regulation of neutrophils in AF patients. Here, we aimed to identify potential key genes related to neutrophils in AF using the Gene Expression Omnibus (GEO) database by integrating multiple bioinformatics approaches. This study focuses on new findings in the molecular mechanisms of AF and may be beneficial facilitate new therapeutic targets to provide effective strategies for the diagnosis and remedy of AF.

Materials and Methods

Microarray data

We downloaded GSE31821, GSE41177, GSE79768 microarray expression matrices from the GEO database (www.ncbi.nlm.nih.gov/geo), and extracted the left atrial tissue expression file. All three datasets used the GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array. The data sets were combined to avoid the heterogeneity of results caused by the small number of samples in each data set. These microarray data were collected and combined using "combat" method in "sva" package in software R, which can remove batch effects. A total of 38 human atrial tissue and left atrial appendage samples from AF patients and sinus rhythm (SR) subjects were obtained, including 11 control data (SR group) and 27 experimental data (AF group), as shown in Table 1.

Data processing and identification of differentially expressed genes (DEGs)

The 3 raw datasets were preprocessed in R package affy, including background calibration, normalization, and log2 transformation. Differentially expressed genes were identified by "limma" package in software R. This package is a popular method for computing moderated t-statistics using a combination of the limma::lmFit and limma::eBayes functions. The selection criteria were |Fold change (FC) | ≥ 1.5, adjusted *p* value (adj*p*) < 0.05. The pheatmap R package was applied to create heat maps and the limma package was used to create volcano maps.

Table 1. Microarray data

	SR	AF
GSE31821	2	4
GSE41177	3	16
GSSE79768	6	7

AF, atrial fibrillation; SR, sinus rhythm.

Pathway enrichment analysis of Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG)

The R package clusterProfiler was used for GO and pathway enrichment analysis. GO functions include cellular composition (CC), biological process (BPs) and molecular function (MF). Bar plot, bubble plot and circle plot were generated using enrichplot, ggplot2 and GOplot, respectively. With adjusted $p < 0.05$ related GO terms and KEGG pathways were considered significantly enriched.

Immune cell infiltration analysis

To explore whether there were difference in immune cell infiltration between AF and SR samples, we used the R package CIBERSORT for analysis (Xiao et al. 2021). The filter condition was set to $p < 0.05$ filtered samples. The percentage of each immune cell type was calculated in both samples. The "vioplot" package was used to visualize the levels of 22 immune cell subtypes between AF and SR. Correlation heatmaps were performed using the "corrplot" package to reveal the correlation of the 22 infiltrating immune cell subtypes.

Construction and identification of WGCNA module

WGCNA was used to construct the gene co-expression network. First, genes with more than 25% variation between samples in the comprehensive dataset were imported. Outlier samples were removed to ensure results reliability. Then modules were detected by hierarchical clustering and dynamic tree cutting functions. Genes with similar expression patterns were divided into a module. The correlation between modules and traits and the association between modules were analyzed. The gene information in the module was used for subsequent analysis.

Protein-protein interaction (PPI) network analysis of differential genes

String database was used to analyze the PPI network of the two module genes significantly related to the central granulocyte, and Cytoscape (3.5.1) was used to draw the PPI co-expression network. The Degree algorithm of Cytoscape CytoHubbaE plugin was used to screen the top 10 hub genes in the PPI co-expression network. The R package pheatmap was used to map the expression of the 10 hub genes.

Neutrophils-related gene analysis in AF

To explore the association of the 10 hub genes and neutrophils, we used limma to calculate the correlation between the two in AF samples.

Result

Identification of DEGs

The overall flow chart of this study is presented in Figure 1. A total of 303 DEGs between AF and SR (FC ≥ 1.5, adj$p$ < 0.05) were screened. Among them, 194 DEGs were up-regulated and 109 DEGs were down-regulated in AF. The volcano map and heat map of DEGs are shown in Figure 2A and B, and it can be observed that there are obvious differences between the two groups.

Functional enrichment analysis of DEGs

To further investigate the biological functions of DEGs, GO and KEGG functional enrichment analyses were performed. GO enrichment showed that the functions of the DEGs were mainly related to neutrophil activation and biological functions of neutrophil activation-mediated immune response (Fig. 3A). KEGG enrichment showed that the DEGs were mainly involved in immune signaling pathways such as phagosome, Th17 cell differentiation, and intestinal immune network related to IgA production (Fig. 3B). The above indicated that AF-related DEGs may function through immune regulation.

Neutrophils are the potential core immune cells in AF

We further analyzed the differences of immune cell subsets between AF and SR samples. The percentage of the 22 immune cells in each sample is shown in bars (Fig. 4A). T cells CD8, macrophages and neutrophils accounted for a large percentage, compared with other immune cells (Fig. 4A). Differences in immune cell infiltration showed that the abundance of T cells gamma delta (γδ T cells, $p = 0.022$) and neutrophils ($p = 0.038$) was significantly increased in AF samples compared with SR group (Fig. 4B). However, the levels of T cells CD8, T cells regulatory (Tregs), macrophages M2 and mast cells activated were lower in AF samples than those in SR samples ($p < 0.05$, Fig. 4B). The correlation of the 22 immune cells showed a positive correlation between γδ

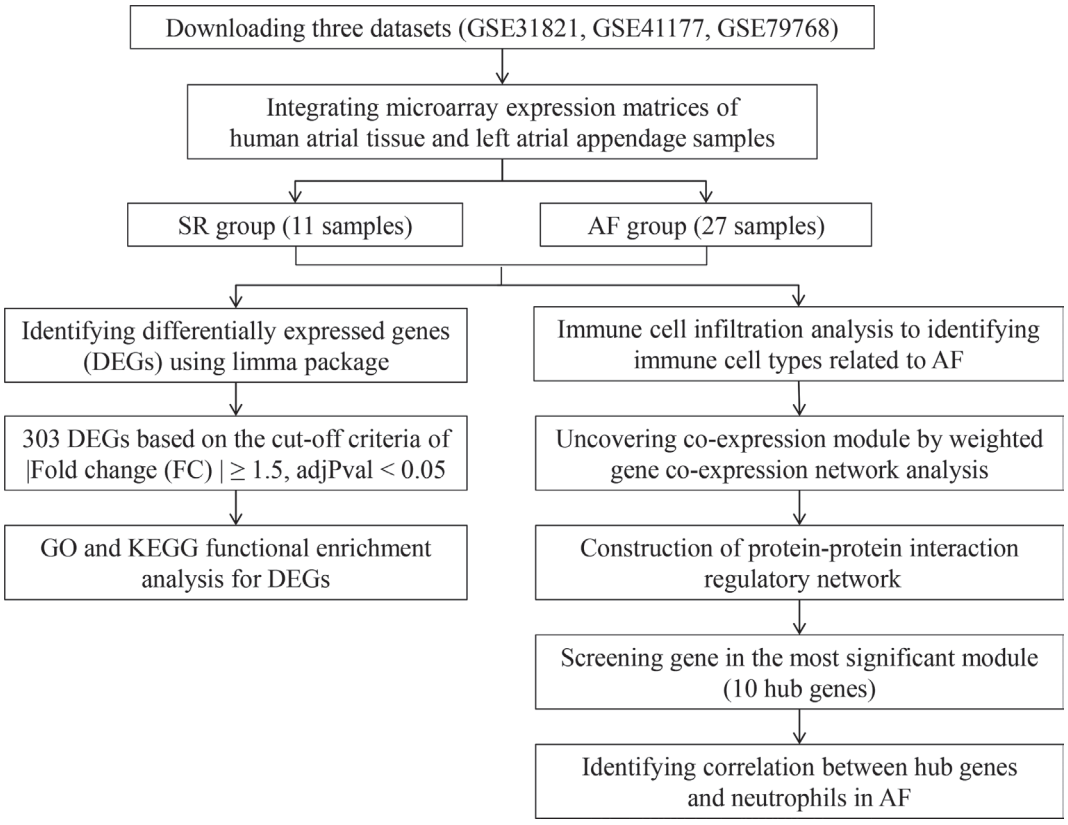

Figure 1. Flow chart of this study. AF, atrial fibrillation; SR, sinus rhythm.

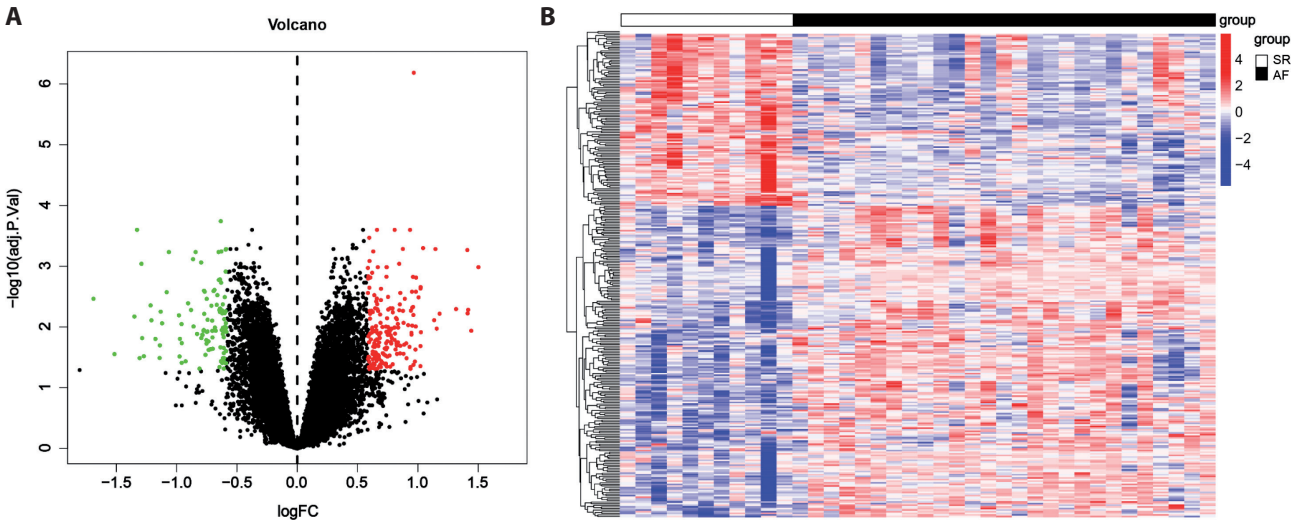

Figure 2. Volcano map (**A**) and heat map (**B**) of differentially expressed genes (DEGs) of AF and SR Atrial tissue and left atrial appendage samples. Red and green dots in the volcano plot represent up-regulated and down-regulated DEGs, respectively. Each row of the heat map represents a DEG and each column represents a sample. Red and blue indicate up-regulated and down-regulated DEGs. For abbreviations, see Figure 1; and for color figure, see online version.

T cells and neutrophils (r = 0.31) and a negative correlation between neutrophils and macrophages M0 (r = −0.38) (Fig. 4C). Therefore, neutrophils may be potential core immune cells associated with AF.

Modular characteristic of genes related to neutrophils in AF

We used WGCNA to determine neutrophil associated gene co-expression modules in AF. We performed a cluster analysis in which all samples were in the cluster and within the cutoff threshold (height < 1), so no outliers need to be removed. In our study, we chose $\beta = 8$ (scale-free $R^2 = 0.85$) as the soft threshold to ensure scale-free networks (Fig. 5A). The threshold was set to 0.25 to merge similar modules in the cluster tree, resulting in five modules with similar co-expression features of genes, as shown in Figure 5B. The correlation heat map showed that the module eigengene (ME) module_green (r = 0.72, $p < 0.001$) and module_turquoise (r = 0.51, $p = 0.001$) of neutrophils were both significantly and positively correlated with neutrophils (Fig. 5C). DEGs in the previous GO function were mainly related to immunity mediated by central granulocyte activation, so we selected the gene in green and turquoise modules which associated with neutrophils for further analysis (Table S1 in Supplementary material).

Identification of hub gene involved in neutrophils regulation

The correlation between gene modules of neutrophils and immune cell types was analyzed. The DEGs in the above modules with significant correlations were imported into the STRING online tool to evaluate the interactions. The results showed that 82 nodes and 157 edges were identified from the PPI network (Fig. 6A). CytoHubba from Cytoscope was then used to identify and select hub genes through five ranking algorithms. The top 10 hub genes included APOE, C1QC, CSF1R, CTNNB1, FCGR3B, HSP90AB1, S100A12, S100A8, SOD1, and TYROBP (Fig. 6B). These genes were considered as potential key genes for further analysis. The heat map showed the difference in the expression of 10 hub genes between AF and SR groups, with the hub genes being more significantly up-regulated in AF (Fig. 6C).

Correlation analysis between hub genes and neutrophils in AF

We further investigated the association of the 10 hub genes with neutrophils. The results showed that S100A12 (r = 0.41, $p = 0.0003$), FCGR3B (r = 0.47, $p < 0.0001$) and S100A8 (r = 0.41, $p = 0.001$) were significantly positively correlated with neutrophil abundance, while gene C1QC (r = −0.16, $p = 0.04$) was significantly negatively correlated with neutrophil abundance (Fig. 7). Both neutrophil (Fig. 4B) and hub genes including CIQC (Fig. 6C) were up-regulated in AF, but CIQC showed the negatively correlation with neutrophil, which was inconsistent with their both up-regulated expression, so it could be excluded. This suggests that S100A12, FCGR3B and S100A8 may be three potential key genes related to neutrophils in AF.

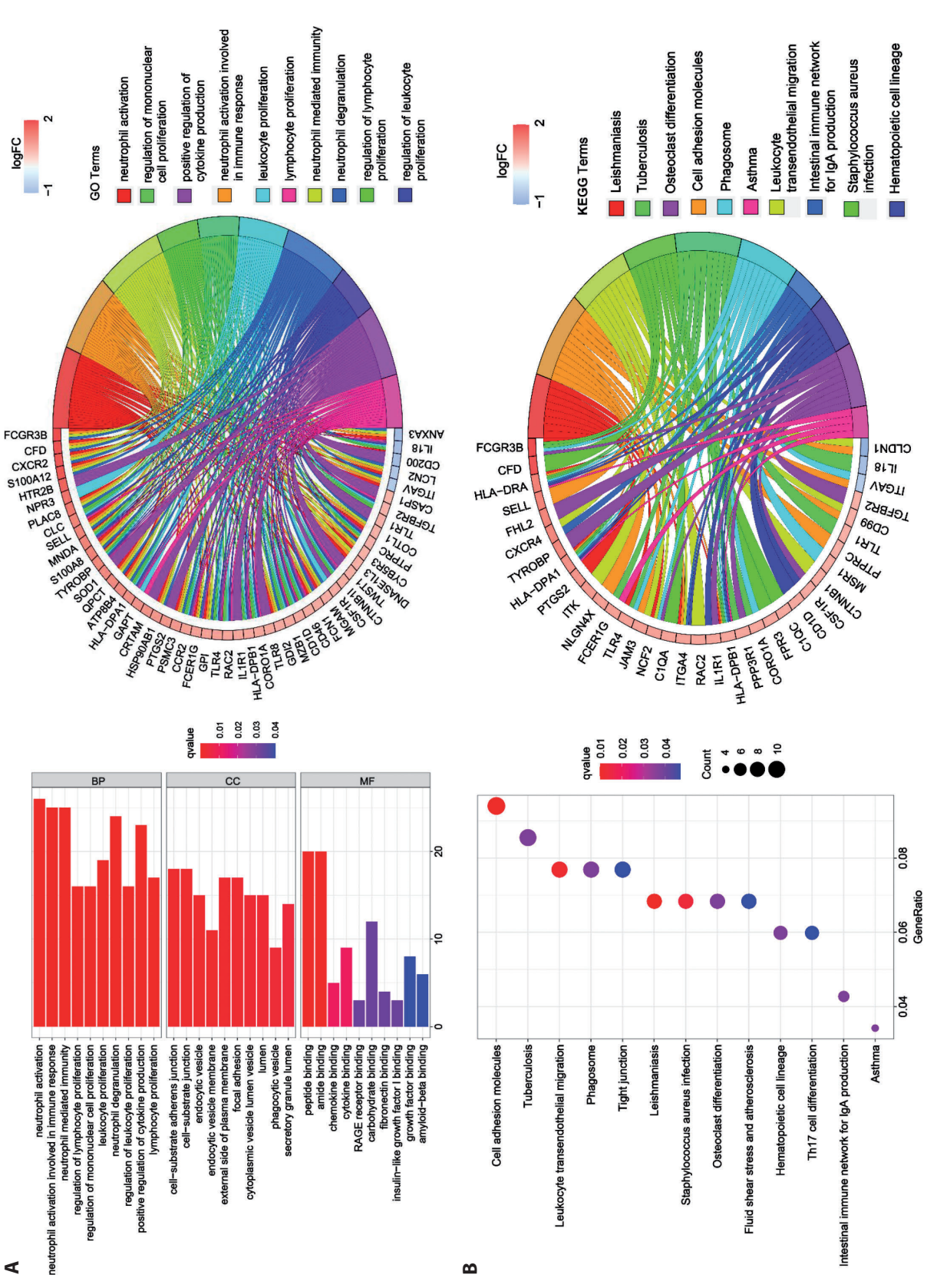

Figure 3. Functional and pathway enrichment analysis of differentially expressed genes (DEGs). **A.** Cellular components of Gene Ontology (GO) enrichment analysis of DEGs. **B.** Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of DEGs.

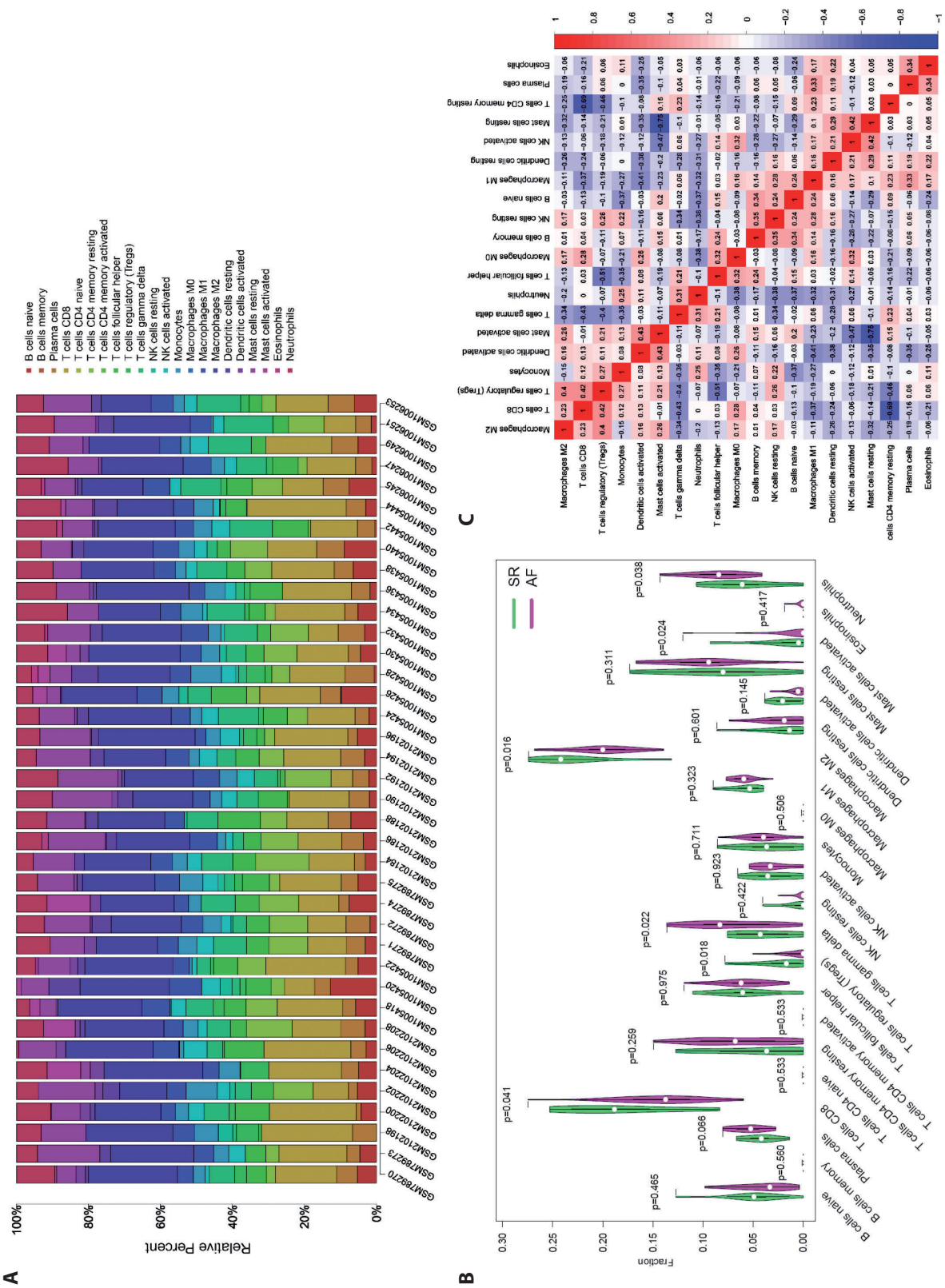

Figure 4. Distribution and visualization of immune-cell infiltration. **A.** Relative percentages of 22 types of immune cells in AF and SR samples. **B.** Comparison of 22 immune cell subtypes between AF and SR groups. **C.** Correlation matrices for all 22 immune cell subtypes. Immune cell subtypes are shown on both the horizontal and vertical axes. Red and blue indicate higher positive or negative correlation levels. For abbreviations, see Figure 1 and for color figure, see online version.

Discussion

AF is the most common cardiac arrhythmia in clinical practice. Multiple evidences suggest that AF may be associated with immune and inflammatory responses (Hu et al. 2015; Liu et al. 2018). Among them, the role of neutrophils in AF has attracted particular attention. In this study, we integrated the microarray expression matrices of 27 AF and 11 SR samples from three GEO datasets and identified significant pathways associated with AF risk. The relationship between neutrophils and AF was illustrated by immune cell infiltration analysis. WGCNA and PPI were used to screen the hub genes associated with neutrophils in AF. Three potential key genes associated with neutrophil, S100A12, FCGR3B and S100A8, were finally identified. These genes may play important functions in AF.

Neutrophils are important inflammatory cells, and their elevated activation is associated with various cardiovascular diseases (Gaul et al. 2017). Previous studies have confirmed the efficacy of neutrophil-to-lymphocyte ratio as a predictor or prognostic indicator of AF (Gibson et al. 2010; Guo et al. 2014). It has been found that the excess of neutrophil degranulation protein in the atrium of AF patients may promote myocardial cell remodeling and be more susceptible to fibrosis and thrombosis (Kawasaki et al. 2021). Wu et al. (2022) also found that atrial samples from AF group contained higher γδ T cells and neutrophils compared with SR by immune infiltration analysis, which is consistent with our results. Gan et al. (2023) found that neutrophils are dysregulated in AF, and immune cell disease (including neutrophils) caused by 7 hub immune-related genes may be a common pathogenesis of AF in dilated cardiomyopathy. Yan et al. (2021) published findings from the WGCNA analysis that involved specific datasets related to AF, GSE115574. The analysis revealed that among 22 kinds of immune cells, M1 macrophages exhibited the

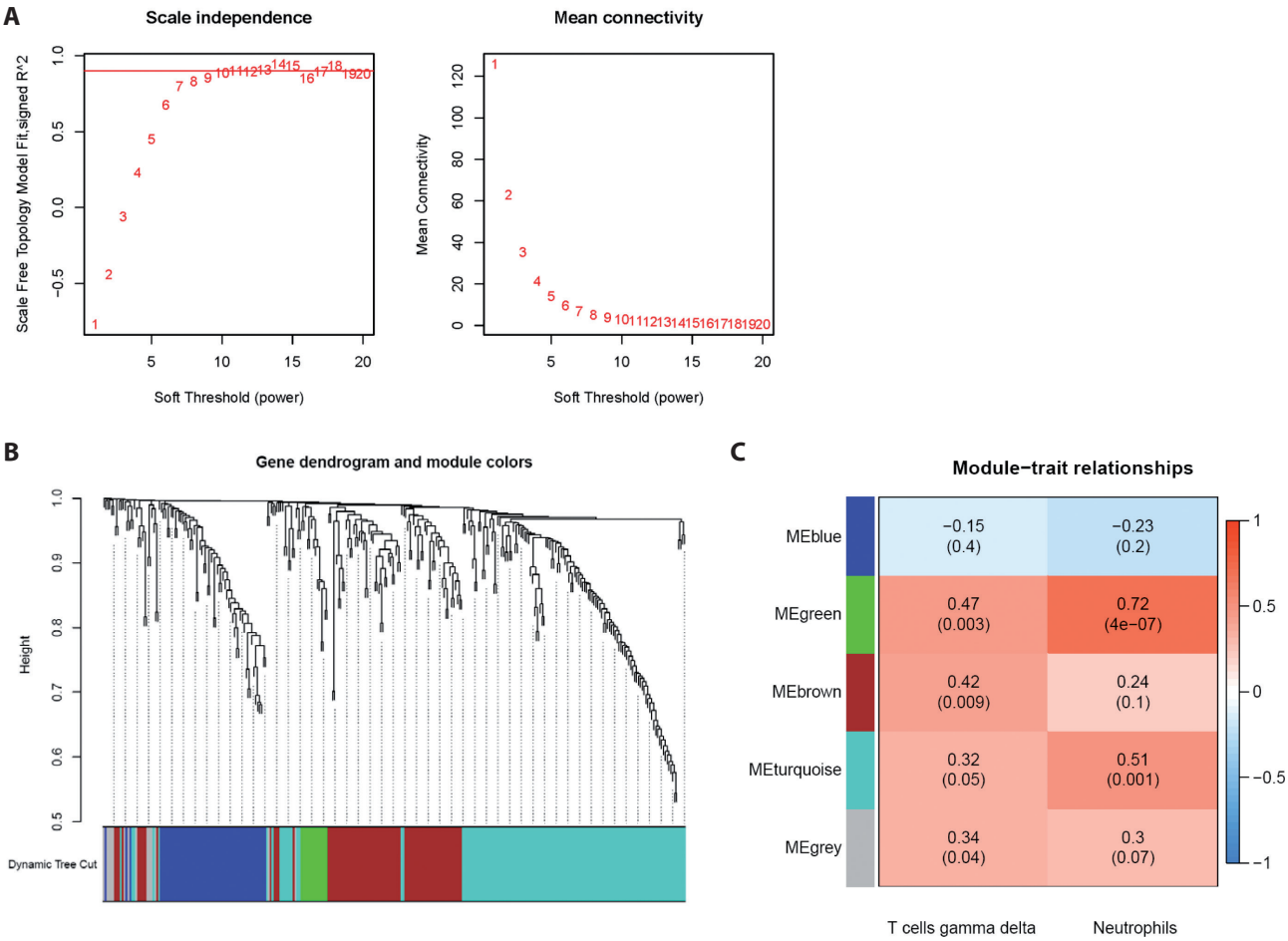

Figure 5. Weighted gene co-expression network analysis (WGCNA). **A.** Threshold screening plot. **B.** Dendrogram of hierarchical cluster of modules. **C.** Heat map showed that magenta modules were significantly associated with neutrophils. Numbers within and outside parentheses indicate *p*-values and correlation coefficients.

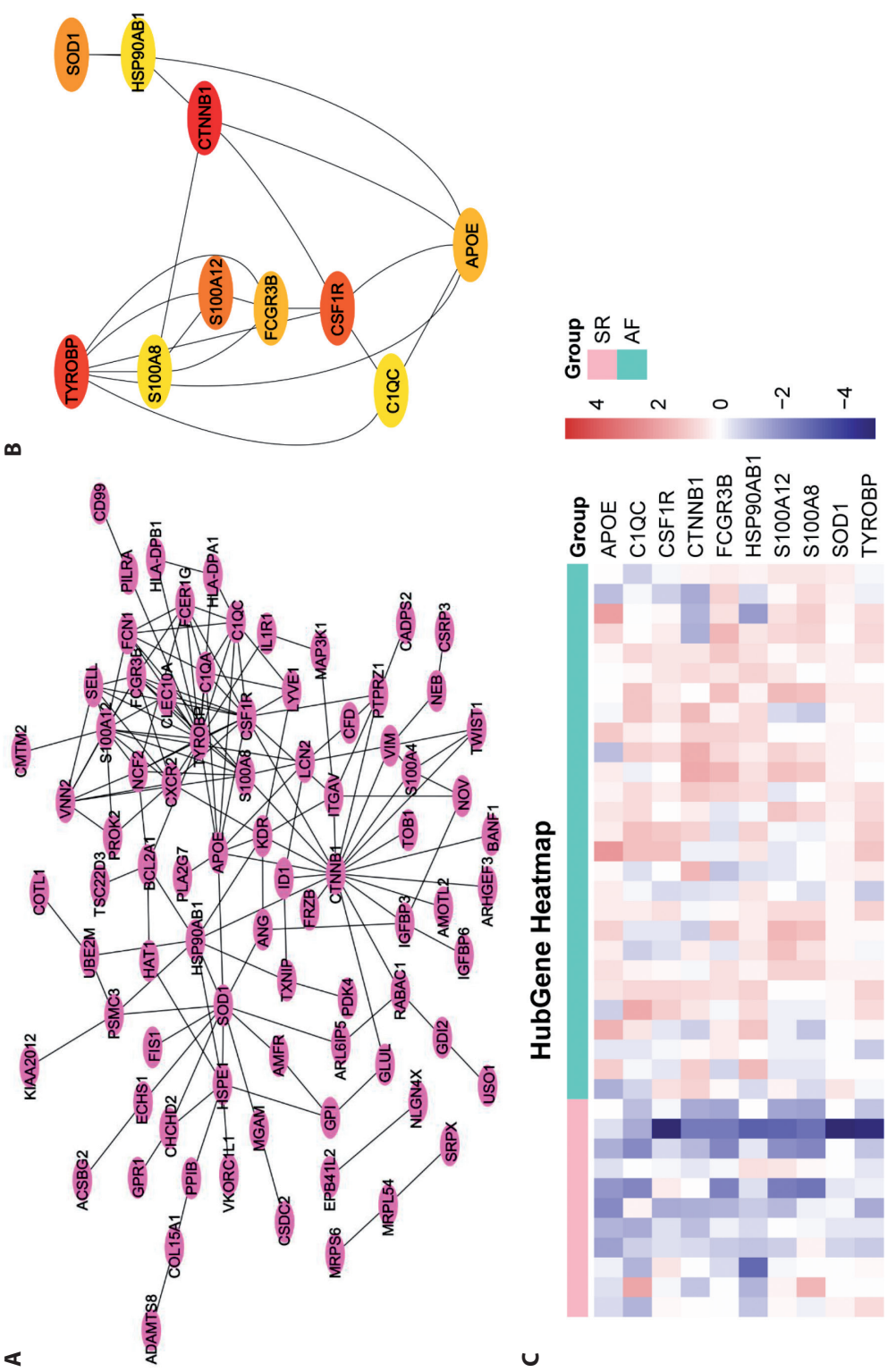

Figure 6. Protein-protein interaction (PPI) network. **A.** PPI network of genes in magenta module. **B.** 10 hub genes were screened out. **C.** Heat map of hub gene expression.

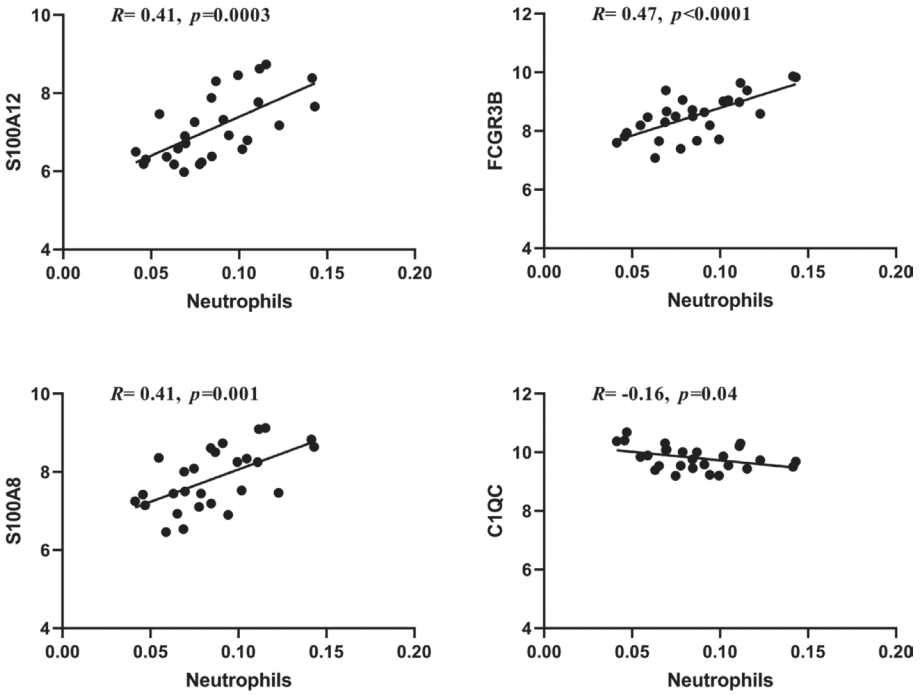

Figure 7. Association of four hub genes (S100A12, S100A8, FCGR3B and C1QC) with neutrophils.

highest correlation coefficient with AF (Yan et al. 2021). This has certain similarities to our study, suggesting that the relevant hub genes related to neutrophils may influence the mechanism of AF occurrence.

S100A12 is a protein-coding gene (Garcia et al. 2013). In humans, S100A12 is mainly expressed and secreted by neutrophils (Bagheri 2017). S100A12 expression on neutrophils induces pro-inflammatory responses by binding to the receptor for advanced glycation end products and subsequent activation of intracellular signal transduction pathways (Nazari et al. 2017). In addition, studies have shown that S100A12 may drive neutrophil infiltration by inducing inflammatory response and ultimately lead to remodeling of the atrium (Xiao et al. 2021). Therefore, these results imply that S100A12 may mediate AF by regulating the inflammatory response of neutrophils. However, no specific experimental study has been found to confirm that S100A12 promotes AF, which is worthy of further exploration. S100A8, structurally similar to S100A12, is a low molecular weight calcium and zinc binding protein (Xu et al. 2012). S100A8 can induce chemotactic and adhesion of neutrophils, and mediate intracellular inflammatory signal transduction (Xu et al. 2012). Neutrophil-derived S100A8 has been found to enhance granulopoiesis after myocardial infarction (Sreejit et al. 2020). In addition, S100A8 could induce neutrophil activation and regulate CD11b expression and neutrophil recruitment in chronic pulmonary tuberculosis (Scott et al. 2020; Sprenkeler et al. 2022). Therefore, these studies combined with our results suggest that S100A8 can activate neutrophils, thereby contributing to the development of AF.

FCGR3B belongs to the FCGR gene cluster, and its encoded protein is a low-affinity receptor for the Fc region of gamma immunoglobulin (IgG) (McKinney et al. 2012). FCGR3B is a newly identified gene involved in neutrophil regulation. Relevant researches have suggested that FCGR3B can affect cardiovascular inflammatory diseases. Reduced expression of FCGR3B, which may lead to impaired clearance of immune complexes, has been identified as a risk factor for lupus nephritis (Zheng et al. 2017). In addition, genetic polymorphisms of FCGR3B were found to predict the risk of recurrence of eosinophilic granulomatosis with polyangiitis. The mechanism is that FCGR3B deficiency may delay the clearance of immune complexes by neutrophils and leads to the formation of a pro-inflammatory state (Alberici et al. 2020). The role of FCGR3B in the specific mechanisms of neutrophil involvement in AF has not yet been seen. But combining these studies and our results, we speculate that FCGR3B influences AF by mediating the clearance of immune complexes by neutrophils.

In the current study, we have discussed the involvement of 3 potential key genes (S100A12, FCGR3B and S100A8) related to neutrophils in AF, suggesting that they may provide new strategies for AF treatment as potential therapeutic targets. At the same time, there are some limitations of the study that deserve further exploration. Firstly, the development of AF is caused by a variety of factors, and several important factors such as region, age, and genetic history are difficult

to be taken into account. In addition, we only focused on a few important enrichment results and related genes, and further interactions between immune cells and DEGs should be concerned for immune infiltration. Finally, the potential key genes need to be further validated in experimental studies to determine their mechanisms in AF.

Conclusion

In this study, we screened 303 DEGs in AF, which were mainly enriched in neutrophil-mediated immune-related pathways. We used immune cell infiltration to identify neutrophils as potential core immune cells in AF. WGCNA combined with PPI and correlation analysis finally identified 3 potential key genes of neutrophil regulation, S100A12, FCGR3B and S100A8. These genes deserve further investigation and may provide targets for the development of novel therapeutic strategies for AF.

Ethics approval. The data of this study were obtained from the public data-base and no ethical approval was required.

Conflict of interest. The authors declare that they have no competing interests.

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Data availability. The datasets analyzed in this study could be found in GSE31821, GSE41177, and GSE79768 datasets in GEO database.

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Supplementary Material

Co-expression and interaction network analysis identifies neutrophil-related genes as the core mediator of atrial fibrillation

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Supplementary Table

Table S1. Gene in green and turquoise modules

Module_green	Module_turquoise
LOC100652824	GLIPR2
FRZB	PILRA
FCGR3B	ID1
SELL	CNGA1
CMTM2	TRNP1
CXCR2	LOC100131303
S100A12	LINC00520
PROK2	PLEK2
CLC	COTL1
FCN1	TUFT1
VNN2	TSC22D3
BCL2A1	ADAMTS8
MGAM	KIAA0430
S100A8	EPB41L2
	ANG
	ARHGEF3
	LOC101926918
	CC2D2B
	CLEC10A
	HFE2
	PPIL1
	MSS51
	HSP90AB1
	NLGN4X
	GDI2
	COL15A1
	IL1R1
	KDR
	DRAP1
	IGFBP3
	RP11-399O19.9
	SLC35G3
	PPIB
	MRPS6
	TXNIP

(continued)

Table S1. (continued)

Module_green	Module_turquoise
	BAI2
	PIN1P1
	APOE
	GLUL
	FBLN5
	ZBED9
	FBXW12
	CADPS2
	SCN1B
	LIX1L
	UBE2M
	PSMC3
	CSDC2
	PDK4
	ETNPPL
	HLA-DPB1
	DUSP4
	LINC01018
	FMO2
	TYROBP
	PTPRZ1
	VIM
	IGFBP6
	AMOTL2
	HSPE1
	IBA57-AS1
	TWIST1
	ALX3
	LOC100131180
	S100A4
	C1QC
	CFD
	LINC00326
	GPR1
	CTNNB1
	MAP3K1
	NCF2
	RP11-90C4.2
	SLC16A9
	RP11-157B13.7
	CYB5R3
	RPA2
	SLC26A9
	TSPAN15
	ENPP5
	USO1
	RP11-382B18.4
	RNF128
	GNB2

(continued)

Table S1. (continued)

Module_green	Module_turquoise
	BANF1
	PLA2G7
	C1QA
	HLA-DPA1
	ECHS1
	LYVE1
	RABAC1
	VKORC1L1
	SUCO
	MRPL54
	GPI
	KRTAP17-1
	TRIM22
	ITGAV
	TMEM256
	ACSBG2
	SRPX
	CSRP3
	ARL6IP5
	LINC00622
	FAM96B
	BOD1
	SMS
	FCER1G
	TOB1
	EPHX1
	AMFR
	NOV
	RAI2
	NNT-AS1
	SEC11A
	COX16
	NEB
	NDUFAF3
	LINC00261
	COX8A
	SNX7
	MIR100HG
	SOD1
	CAPNS1
	CHCHD2
	FIS1
	HAT1
	CD99
	AP3S1
	OST4
	MPC2
	LCN2
	CSF1R
	EIF3G