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Integrated bioinformatics analysis in left atrial appendage of patients with atrial fibrillation for better understanding of atrial fibrillation associated thrombus formation

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Abstract
Atrial fibrillation (AF) is the most common tachycardia in clinic. However, the potential pathogenesis of AF associated thrombus formation is limited. Due to left atrial appendage (LAA) is the main source of thrombus formation. we identified the differentially expressed genes (DEGs) in LAA between AF and SR (sinus rhythm), which were obtained from 43 left atrial appendage (LAA) samples from 20 cases of AF and 23 cases of SR in the expression profiles of GSE79768 and GSE115574 downloaded from the Gene Expression Omnibus (GEO) database. The DEGs were analyzed by bioinformatics methods, including gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichments by DAVID and KOBAS online analyses. A total of 96 DEGs containing 27 downregulated and 69 upregulated genes were identified between AF and SR. GO analysis showed that the biological processes of DEGs included cellular responses to cytokine, antigen processing and presentation, and basement membrane disassembly. The main cellular components focused primarily on cell surface and the molecular functions focused primarily on IgG binding. KEGG pathway analysis indicated that these DEGs were enriched in 8 KEGG pathway, including staphylococcus aureus infection, tuberculosis, phagosome, asthma, leishmaniasis, antigen processing and presentation, salivary secretion and gap junction. which mainly involved in immune response and inflammation. A network of DEGs was confirmed including 46 nodes and 84 edges, which presented the interaction of DEGs. Collectively, DEGs and pathways were screened in LAA of AF, which is beneficial to understand the molecular mechanisms underlying AF associated thrombus formation.

Keywords: atrial fibrillation, left atrial appendage, GEO data, integrated bioinformatics, differentially expressed genes

Introduction

Atrial fibrillation (AF) is the most common tachycardia in clinic with high mortality and morbidity. By 2010, there were more than 33 million people diagnosed with AF worldwide. A recent study has confirmed that lifetime risk for developing AF was quantified to be more than 30% in Europe. Patients with AF are at high risk for stroke, heart failure and hospitalizations, and exhibit variable comorbidities that largely increase economic burden on society[1].

AF is an independent risk factor for thromboembolic events, including stroke and systemic thromboembolism. It has been reported that 57% of thrombi in valvular AF and 91% in nonvalvular AF (NVAF) originate in the LAA[2]. The mechanisms underlying the phenomenon that the LAA is the main source of thromboembolism are consisted by following areas: the LAA has a complex anatomy and neurohormonal properties; AF can induce blood stasis with reduced LAA peak flow velocities caused by decreasing contractility of left atrium and the LAA; AF is also associated with endothelial damage[2-4]. Research showed that genes related to thrombogenesis, including leptin (LEP), prostaglandin-endoperoxide synthase-1 (PTGS1), kinase insert domain receptor (KDR), complement factor-B (CFB), and platelet-derived growth factor receptor, alpha polypeptide (PDGFRA), were upregulated in the LAA of patients with AF. Multiple reasons make the LAA susceptible to thrombus formation[5, 6].

Recent years, the cellular and molecular mechanisms underlying AF have been studied extensively with atrial-tissue samples from AF patients and animal models. These studies provide support for multiple patterns of electrical, structural, and Ca^{2+}-handling remodeling producing a vulnerable substrate for AF genesis and maintenance[7, 8]. Recent biotechnological advances in gene expression microarrays have made it possible to investigate the changes in the mRNA of thousands of genes and been widely used to study gene expression profiles in many human diseases. It provides promising prospects for molecular prediction, molecular diagnosis and molecular therapy by large-scale technique for studying disease-related genes. At present, some studies have been performed on atrium gene expression profiles and a large amount of data have been published on public database platforms[9-11]. However, the results for the identification of significantly expressed mRNAs are inconsistent among different studies due to different experiment platforms, sample processing methods and sample heterogeneity. The integration and analysis of microarray data from several gene expression profiles may overcome these limitations and enable to find effective and reliable molecular markers, and are heavily employed in cancer. However integrated bioinformatics analysis is rarely used in tachycardia. Therefore, we propose to study more effective early diagnostic techniques and more reliable molecular markers for monitoring recurrence and evaluating prognosis, as well as to explore a more effective way to treat AF by integrated bioinformatics analysis. Moreover, we aim to identify the cellular and molecular mechanisms underlying thrombus formation in LAA and the way to prevent and treat it.

Methods

Data retrieval

Affymetrix Macroarray Data. Utilizing the keywords“atrial fibrillation,” we screened the GEO database. The gene expression profiles of GSE79768, GSE115574 (Gunseli et al. unpublished data, 2019) were downloaded. The platform for GSE79768 is GPL570, Affymetrix Human Genome U133 Plus 2 Array, which includes 6 LAA samples obtained from patients with AF and 7 LAA samples obtained from patients with SR. The platform for GSE115574 is GPL570, Affymetrix Human Genome U133 Plus 2 Array, which includes 14 LAA samples obtained from patients with AF and 16 left LAA samples obtained from patients with SR. Platform and series matrix file(s) were downloaded as TXT files. The dataset information is shown in ***Table 1***. Through the R software package, the download files were handled.

Table 1 Details for GEO AF data

Reference	Sample	GEO	Platform	AF	SR
Tsai F et al. (2016)	LAA	GSE79768	GPL570	6	7
Gunseli et al. (unpublished data, 2019)	LAA	GSE115574	GPL570	14	16

Abbreviation: LAA, left atrial appendage. GEO, Gene Expression Omnibus. AF, atrial fibrillation. SR, sinus rhythm.

DEG identification

In order to find out DEGs of each GEO dataset, we using the R language software and annotation package to convert the downloaded platform and series of matrix file(s). The ID corresponding to the probe name was converted into an international standard name for genes (gene symbol) and saved in a TXT file. the DEGs in AF and SR samples of the two microarray datasets were analyzed by utilizing the limma package[12] in R. fold change (FC) > 1.2 and a corrected $P < 0.05$ were used as the cut-off criteria of DEGs samples.

Integration of microarray data

The list of DEGs from the two microarray datasets was obtained through limma packet analysis. A list of genes that were up- or downregulated in the two chips were used for subsequent analysis. By using the UpSetR and VennDiagramR package, the common and unique gene lists were identified.

Functional enrichment analysis

The functional and pathway enrichment of the proteins encoded by candidate genes that are the common DEGs from the two microarray datasets were analyzed, and these genes were annotated by utilizing the DAVID database[13]. GO annotations were performed using the DAVID online tool. KEGG pathway analysis of DEGs was performed using the KOBAS online tool, and a P-value of < 0.05 was considered statistically significant.

PPI network integration

Interactions and pathway relationships between the proteins encoded by DEGs in AF were identified by STRING database, a software system in which the results are obtained from experimental data, databases, text mining and predictive bioinformatics data[14]. The highest confidence of the argument of interactions was set at > 0.4. To draw an interaction of DEGs, the Cytoscape (version 3.7.2) software was used to visualize and analyze the PPI network.

Results

Identification of DEGs in expression microarray datasets of GSE79768 and GSE115574

Two expression microarray datasets, including microarray datasets GSE79768 and GSE115574 were standardized by the limma package. When the GSE79768 dataset was screened by the limma package (corrected $P < 0.05$, FC > 1.2), 4,087 DEGs were obtained. Among them, 1,740 downregulated genes and 2,347 upregulated genes were identified. Additionally, 574 DEGs were screened in AF compared with SR from the GSE115574 dataset. Among them, 212 down-regulated genes and 362 upregulated genes were identified. DEGs in two sets of sample data of each microarray, two microarrays in total, are shown in ***Figure 1***. The cluster heatmaps of the top 100 DEGs are shown in ***Figure 2***.

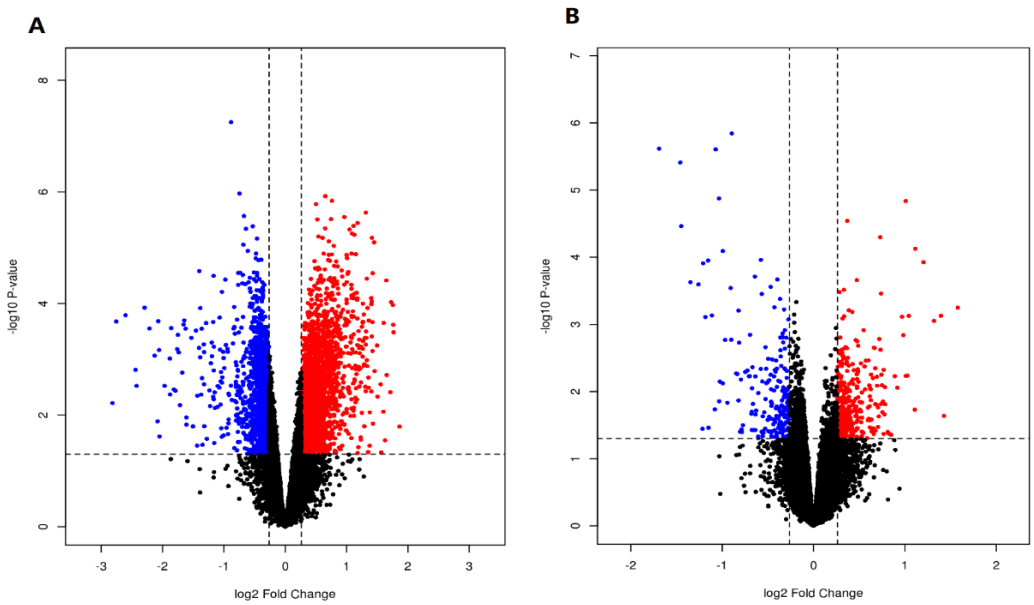
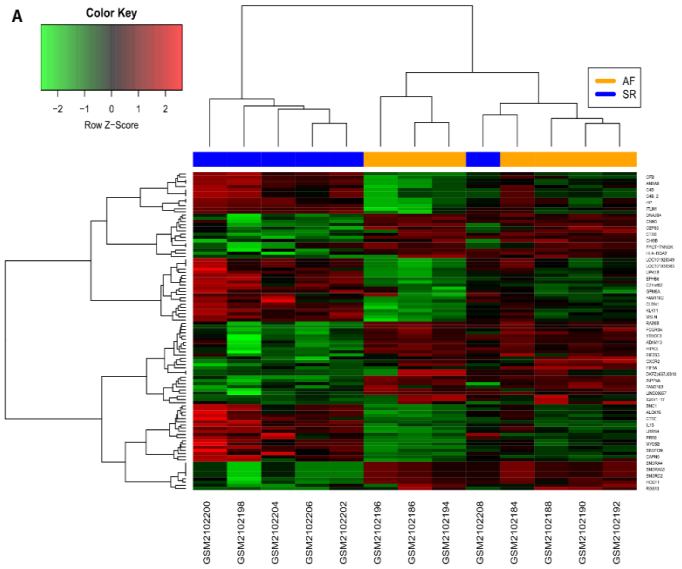


Figure 1. Differential expression of data between two sets of samples.

Notes: (A) GSE79768 data, **(B)** GSE115574 data. The red points represent upregulated genes screened on the basis of |fold change| > 1.2 and a corrected P-value of < 0.05. The blue points represent downregulation of the expression of genes screened on the basis of |fold change| > 1.2 and a corrected P-value of < 0.05. The black points represent genes with no significant difference. FC is the fold change.



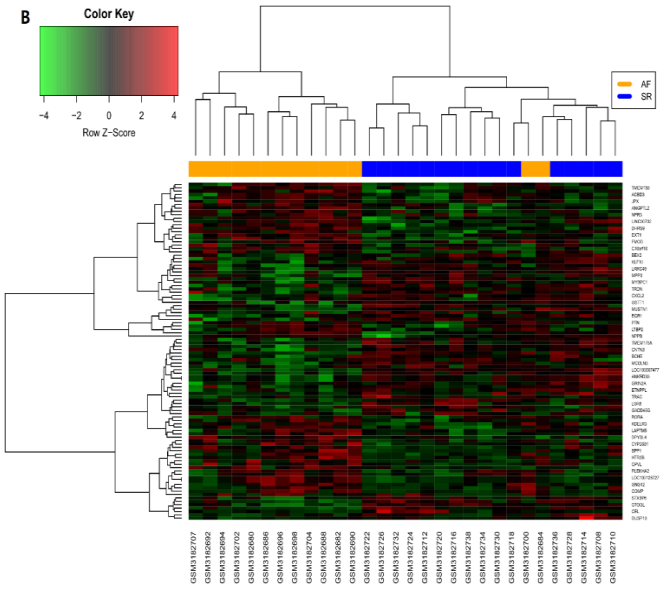


Figure 2. Hierarchical clustering heatmap of the top 100 DEGs in two sets of sample data of each microarray.

Notes: (A) GSE79768 data, **(B)** GSE115574 data. Red indicates that the expression of genes is relatively upregulated, green indicates that the expression of genes is relatively downregulated, and black indicates no significant changes in gene expression; gray indicates that the signal strength of genes was not high enough to be detected.

Abbreviation: DEGs, differentially expressed genes.

Identification of DEGs in AF Utilizing Integrated Bioinformatics Analysis

Using the VENN analysis, the list of DEGs of the two microarray datasets were analyzed. A total of 132 common DEGs were determined, and 96 of these DEGs were the same expression changes, including 27 down- and 69 upregulated genes, as shown in ***Figure 3*** and ***Table 2***. GO functional enrichments of GEGs with a P-value of < 0.05 were obtained by using the DAVID online analysis tool. GO analysis of DEGs was consisted by three functional groups, including biological processes, molecular function, and cell composition. Significant results of the GO enrichment analysis of DEGs in AF are shown in ***Table 3*** and ***Figure 4***. In the biological process group, the DEGs were mainly enriched in cellular responses to cytokine, antigen processing and presentation and basement membrane disassembly. In the molecular function group, these DEGs were mainly concentrated on IgG binding. In the cell composition group, the DEGs were mainly enriched in cell surface, junctional sarcoplasmic reticulum membrane, plasma membrane and junctional membrane complex. Furthermore, as shown in ***Tables 4*** and ***Figure 5***, KEGG pathway analysis suggested the signaling pathways of DEGs were mainly enriched in phagosome pathways, and the results indicated these DEGs were involved in signaling pathways as staphylococcus aureus infection, tuberculosis and asthma.

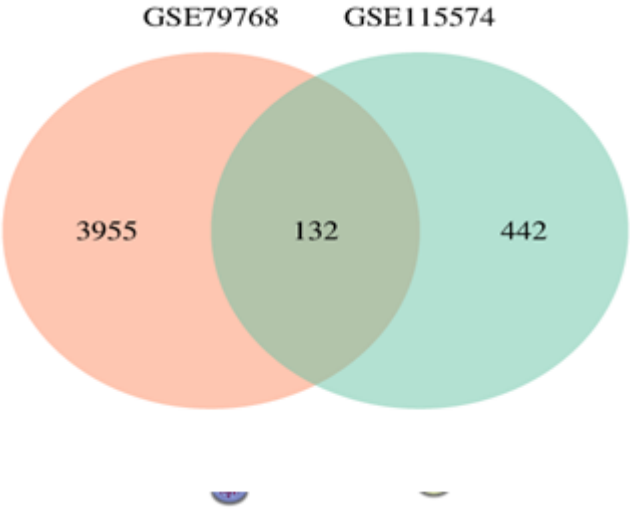


Figure 3. Venn diagram of the same expression changed DEGs based on the two microarray datasets.

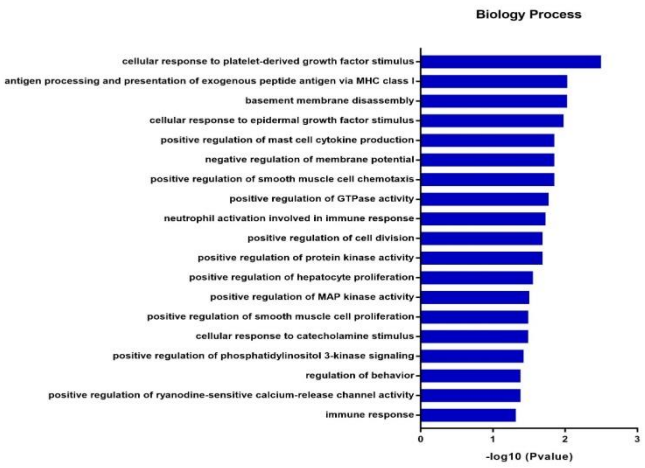
Table 2 screening DEGs in AF by integrated microarray

DEGs	**Gene names**
Downregulated	JPH1 FAM212B LOC101928718 ANKRD23 HPN MARCH11 USP54 BEX2 HOOK1 ANKRD39 MUSTN1 LIF LRRC49 PCP4 SLC26A9 LOC100129129 TADA2B CPNE5 NR4A3 SYT13 AAMDC DAPK2 TRDN SMTNL2 C1orf105 PSMG3-AS1 BCHE
Upregulated	HLA-DMA RELN PDE8B DPYSL4 FCGR2B FCGR2A VILL GPNMB TMEM159 MTURN NDUFAF7 SRPX HCST PTN FCER1G ZFP36L2 LPXN EVI2A DIS3 SMARCC1 AKAP6 C3AR1 DHRS9 SNAI2 COLQ MRPS18B OIP5-AS1 CTSS UAP1L1 LYZ RNASE6 TYW3 BNIP3 LOC101928304 MBOAT1 ALG11 RRAD MPHOSPH8 IQGAP1 SLC6A6 LOC101927990 ADCY6 COL21A1 TRIP11 SNX9 HTR2B TYROBP BRCC3 SNORD19B POPDC3 LBH ATP1B4 UTP14C SNX4 IRF8 CLEC10A CSF2RB HLA-DPB1 GATA6-AS1 NIN SNX13 CHGB KMT2E HEXB DENND5A AIF1 RGS10 CD53 PDGFD

Abbreviation: DEGs, differentially expressed genes

Term	Description	Gene count	P-value
GO:0009986	cell surface	10	6.05E-04
GO:0014701	junctional sarcoplasmic reticulum membrane	3	8.37E-04
GO:0019864	IgG binding	3	0.0011
GO:0036120	cellular response to platelet-derived growth factor stimulus	3	0.003184
GO:0005886	plasma membrane	29	0.007781
GO:0019886	antigen processing and presentation of exogenous peptide antigen	4	0.009322
GO:0034769	via MHC class II	2	0.009387
GO:0071364	basement membrane disassembly	3	0.010499
GO:0032765	cellular response to epidermal growth factor stimulus	2	0.014048
GO:0045837	positive regulation of mast cell cytokine production	2	0.014048
GO:0071673	negative regulation of membrane potential	2	0.014048
GO:0043547	positive regulation of smooth muscle cell chemotaxis	8	0.016918
GO:0002283	positive regulation of GTPase activity	2	0.018688
GO:0051781	neutrophil activation involved in immune response	3	0.020605
GO:0045860	positive regulation of cell division	3	0.020605
GO:2000347	positive regulation of protein kinase activity	2	0.027902
GO:0030314	positive regulation of hepatocyte proliferation	2	0.030332
GO:0043406	junctional membrane complex	3	0.031458
GO:0048661	positive regulation of MAP kinase activity	3	0.032445
GO:0071870	positive regulation of smooth muscle cell proliferation	2	0.032477
GO:0014068	cellular response to catecholamine stimulus	3	0.03756
GO:0050795	positive regulation of phosphatidylinositol 3-kinase signaling	2	0.041563
GO:0060316	regulation of behavior	2	0.041563
GO:0005887	positive regulation of ryanodine-sensitive calcium-release channel	12	0.044512
GO:0006955	activity	6	0.048242
	integral component of plasma membrane		
	immune response		

A



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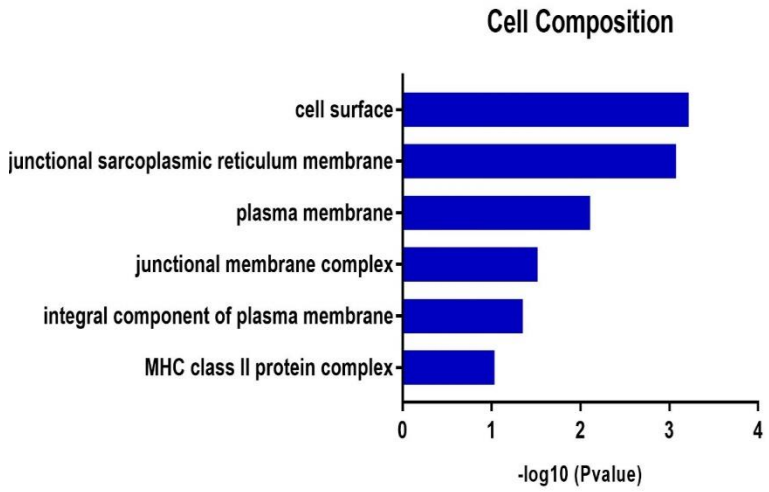

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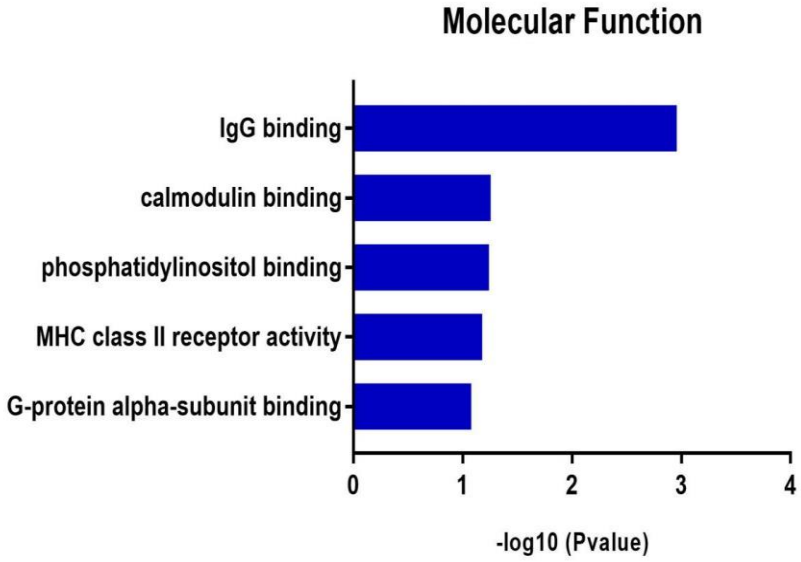

Figure 4. GO enrichment analysis of DEGs in AF.

Notes: GO analysis divided DEGs into three functional groups: **(A)** biological processes, **(B)** cell composition, and **(C)** molecular function.
Abbreviations: DEGs, differentially expressed genes; GO, gene ontology.

Table 4 KEGG pathway analysis of DEGs associated with AF

Pathway	ID	Database	Gene count	P-value	Genes
Staphylococcus aureus infection	hsa05150	KEGG Pathway	5	1.66E-04	C3AR1, FCGR2B, FCGR2A, HLA-DPB1, HLA-DMA
Tuberculosis	hsa05152	KEGG	6	0.002104	

Phagosome	hsa04145	Pathway KEGG	5	0.007439	FCGR2B, FCER1G, FCGR2A, CTSS, HLA-DPB1, HLA-DMA
Asthma	hsa05310	Pathway KEGG pathway	3	0.010566	FCGR2B, FCGR2A, CTSS, HLA-DPB1, HLA-DMA FCER1G, HLA-DPB1, HLA-DMA
Abbreviations: DEGs, differentially expressed genes; KEGG, Kyoto Encyclopedia of Genes and Genomes.					

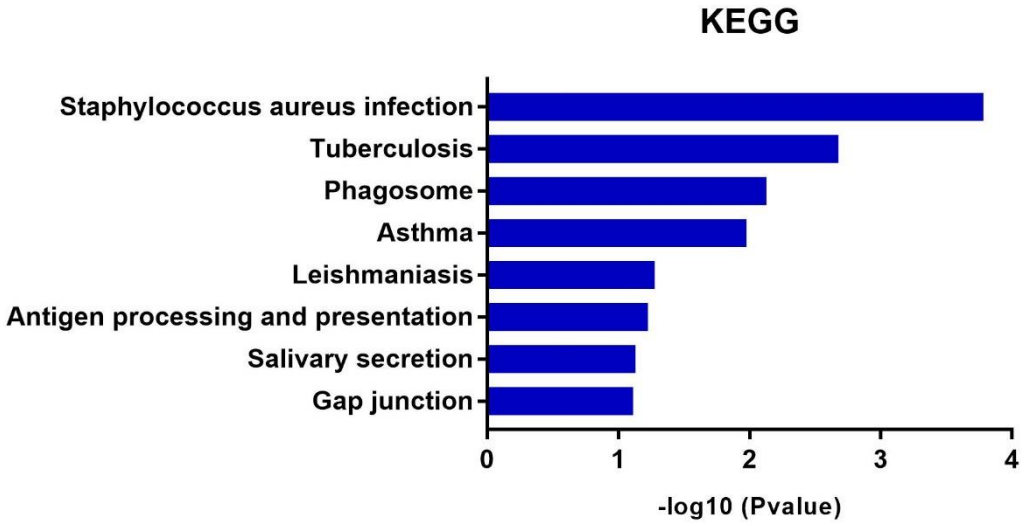

Figure 5. KEGG pathway enrichment analyses of DEGs in AF.

Establishment of the PPI Network and modules analysis

In order to further explore the biological characteristics of these DEGs, a PPI network was constructed by using the STRING database. There were 96 DEGs, including 27 downregulated genes and 69 upregulated genes between LAA of AF and SR patients. As presented in ***Figure 6***, after removing the isolated and partially connected nodes, a network of DEGs was confirmed including 46 nodes and 84 edges, TYROBP, CD53, CTSS, FCGR2B, FCER1G, AIF1, CSF2RB, IRF8, C3AR1, FCGR2A, LYZ, RNASE6, CLEC10A, ADCY6, HCST, HLA-DPB1, HLA-DMA, HEXB, SNX4 and TRDN were the most significant genes in PPI network.

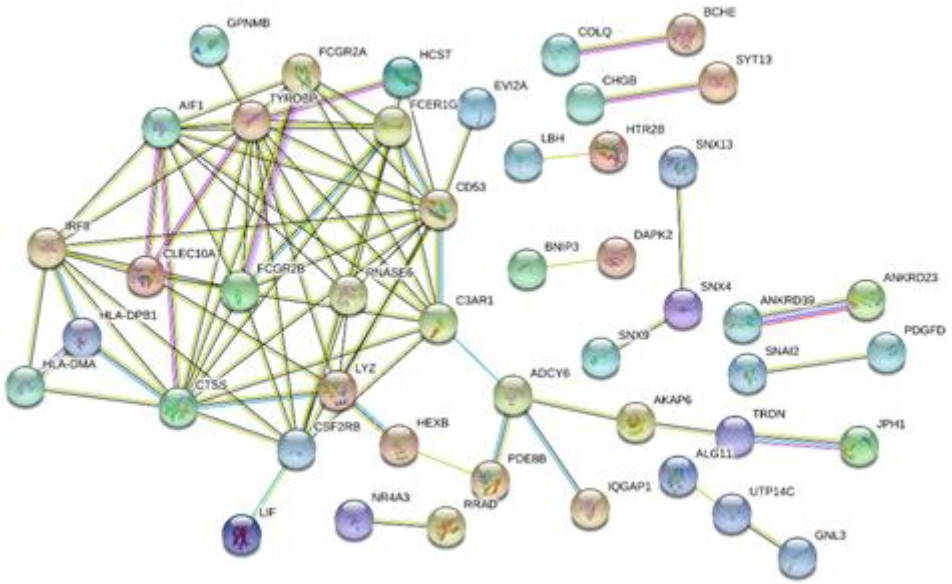

Figure 6. PPI network of DEGs in AF.

Notes: Circles represent genes, lines represent the interaction of proteins between genes, and the results within the circle represent the structure of proteins. Line color represents evidence of the interaction between the proteins.
Abbreviation: PPI, protein–protein interaction.

Discussion

In the study, integrated bioinformatics analysis in left atrial appendage of patients with atrial fibrillation was performed for the first time for better understanding AF associated thrombus formation. We found that a total of 96 DEGs, including 27 down- and 69 upregulated genes, were identified from the GSE79768 and GSE115574 database. From GO functional enrichment analysis, these DEGs were identified and mainly enriched in the following categories, including cellular responses to cytokine, antigen processing and presentation, basement membrane disassembly, and IgG binding. The PPI network was constructed, and 20 key genes were filtered out the module analysis, including TYROBP, CD53, CTSS, FCGR2B, FCER1G, AIF1, CSF2RB, IRF8, C3AR1, FCGR2A, LYZ, RNASE6, CLEC10A, ADCY6, HCST, HLA-DPB1, HLA-DMA, HEXB, SNX4 and TRDN. Moreover, by KEGG pathway enrichment analysis, we identified that these DEGs were mainly concentrated on phagosome pathways, and were involved in signaling pathways as staphylococcus aureus infection, tuberculosis and asthma.

AF remains one of the major causes of stroke, heart failure, sudden death and cardiovascular morbidity in the world. Furthermore, some studies have reported that AF prevalence is approximately 3% in adults aged 20 or older and AF is contributed to 20-30% of all strokes[15, 16]. The diagnosis of AF relies on electrocardiogram (ECG), with the typical pattern of AF. Many AF patients are asymptomatic and paroxysmal, and are often missed in clinic[17]. Its occurrence and development are complex biological processes. Therefore, in the present study we tried to use R software and integrated bioinformatics analysis to explore the molecular mechanism of AF.

Many studies have revealed that inflammation and its associated immune response play a key role in the pathophysiology underlying AF, which contributes to both electrical and structural remodeling of the atria. Furthermore, AF results in inflammation during atrial remodeling, which further aggravate AF[18-20]. The sources of inflammation in patients with AF mainly arise from systemic diseases, atrial myocardial

injury and immune diseases with autoantibody[21, 22]. In turn, AF can subsequently generate an inflammatory response, which suggest further promote atrial remodeling and perpetuate AF. Accumulated evidences have indicated that markers of inflammation and immune response including CRP, HSP27 and TNF are elevated during the arrhythmia, compared with individuals in sinus rhythm who have a history of AF[23-26].

AF results in an increase in inflammatory signals, some of which are associated with thrombogenesis. Soluble CD40 ligand is a critical mediator in the pathogenesis of numbers of cardiovascular setting and it plays an important role in activation of platelets. It has been demonstrated that Scd40L levels increased in AF patients, which provides further evidence of link between inflammation and thrombogenesis[27, 28].

Phagosome involved in enzymatic digestion of infectious agents and apoptotic cells into component parts has been determined to play an important role in inflammation and immune response. On the surface of phagocyte, many receptors that recognize particles or pathogenic agents such as bacteria and apoptotic, thus, necrotic cells are distributed widely. After the particles or pathogenic agents become opsonized with antibodies, they are recognized by Fc receptors (FcR). Cross-linking of Fc gamma receptors initiates a variety of signals mediated by tyrosine phosphorylation of multiple proteins, which leads through the actin cytoskeleton rearrangements and membrane remodeling to the formation of phagosomes[29-31]. Our study found that low affinity immunoglobulin gamma Fc receptor II-b (FCGR2B) was one of the key genes from PPI network analysis. FCGR2B and low affinity immunoglobulin gamma Fc receptor II-a (FCGR2A) were identified as DEG in our study and enriched in phagosomes pathways from KEGG pathway analysis.

Phagocytes provide antigenic ligands to stimulate clonal expansion of T and B cells which play a pivotal role in inflammation and immune response. It has been reported that cytotoxic lymphocytes are involved in the fibrosis of subepicardial fatty infiltrates, a process which is associated with AF[32]. Antigen processing and presentation are associated with initiation of immune responses. APCs present phagocytosed antigen on MHC II to activate CD4 T cells and cathepsin S plays the leading role in MHC II presentation[33]. MHC II can be encoded by a series of genes, including HLA-DPB1, HLA-DMA, HLA-DOA, HLA-DOB and so on. In our study, we proved cathepsin S (CTSS), HLA-DPB1and HLA-DMA were the key genes from PPI network analysis, and they were enriched in phagosomes pathways from KEGG pathway analysis.

TYROBP (TYRO protein tyrosine kinase binding protein), the highest score, was identified from the modules analysis. TYROBP is cytotoxicity-associated gene, which may be a novel biomarker for the diagnosis and prognosis of AF. As mentioned above, during the pathogenesis of AF, cytotoxicity from immune cells is the key process[32]. TYROBP exerts an important role in natural killer cell mediated cytotoxicity[34, 35]. From GO functional enrichment analysis, we found that TYROBP mainly enriched in neutrophil activation involved in immune. It has been reported that elevated NLR (Neutrophil to Lymphocyte Ratio) was associated with a higher incidence of AF[36, 37]. Our results might indicate a potential role of natural killer cells and Neutrophils in the pathogenesis of AF which needs to be confirmed in future studies.

Apoptosis can be present in various cardiomyopathies, which seems to be involved in multiple pathways. Researches provided support for the significant role in apoptosis of cardiac myocytes in the patients with chronic AF[38-41]. In our study, we demonstrated that CSF2RB (cytokine receptor common subunit beta) was obviously differentially expressed in AF, a gene which was the key gene identified from PPI analysis. CSF2RB was mainly involved in apoptosis and its associated JAK-STAT signaling pathway. we speculated that CSF2RB may play an important role in AF. Thus, further studies are needed in order to verify it.

From KEGG pathways, the results indicated these DEGs were involves in signaling pathways as staphylococcus aureus infection, tuberculosis and asthma. However, the patients enrolled in our study were

not reported with these diseases. We concluded that due to the related genes were associated with inflammation and immune response, they were enriched in these signaling pathways.

Compared with other studies of AF, innovation point and merit of our study was that the VENN method was utilized for the first time in exploring DEGs of LAA in AF study. Our study has important clinical significance for the treatment and prevention of AF and its complication. The results of this study provided the evidences that inflammation and immune response may be closely related with the pathophysiology underlying AF and thrombus formation in LAA. This finding improves our understanding of the pathogenesis of AF and the occurrence and development of the underlying molecular mechanisms. However, further molecular biological experiments are required to confirm the function of the identified genes.

Conclusion

In conclusion, our study provides an integrated bioinformatics analysis of DEGs in LAA of patients with AF. We have identified 96 GEGs in total, in which 69 genes were upregulated and 27 genes were downregulated. By analyzing the GO and KEGG pathways, we found that numbers of DEGs involved in inflammation and its associated phagocytosis.

Funding:

Disclosures:

None.

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