Hindawi
Computational and Mathematical Methods in Medicine
Volume 2021, Article ID 5527240, 10 pages
https://doi.org/10.1155/2021/5527240

*Research Article*

# Construction of mRNA Regulatory Networks Reveals the Key Genes in Atrial Fibrillation

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Received 27 January 2021; Revised 17 March 2021; Accepted 29 March 2021; Published 25 May 2021

Academic Editor: Tao Huang



Atrial fibrillation (AF), the most familiar heart rhythm disorder, is a major cause of stroke in the world, whereas the mechanism behind AF remains largely unclear. In the current study, we used the RNA-seq method to identify 275 positively regulated mRNAs and 117 negatively regulated mRNAs in AF compared to healthy controls. Through bioinformatic analysis, it indicated that these distinctively expressed genes took part in regulating multiple AF-related biological processes and pathways, such as platelet aggregation, platelet activation, pri-miRNA transcription, and transforming growth factor-beta (TGF-$\beta$) receptor signaling pathway. Protein-protein interaction (PPI) network analysis identified ITGB5, SRC, ACTG1, ILK, ITGA2B, ITGB3, TUBB4B, CDK11A, PAFAH1B1, CDK11B, and TUBG1 as hub regulators in AF. Moreover, the quantitative real-time PCR (qRT-PCR) assay was conducted and revealed that these hub genes were remarkably overexpressed in AF samples compared to normal samples. We believed that this study would enrich the understanding of the pathogenesis of AF and enable further research on the pathogenesis of AF.

## 1. Introduction

Atrial fibrillation (AF) currently belongs to lists of the commonest causes of heart rhythm disease around the world [1]. The incidence and mortality caused by AF are increasing in recent years. In the past decades, some pathogenic factors were related to the progression of AF. For example, miR-31 produces arrhythmias in AF by depletion of neuronal nitric oxide synthase and dystrophin [2]. PVT1 regulates the progression of AF through the miR-128-3p-SP1-TGF-$\beta$1-Smad axis [3]. However, the mechanism behind AF remains to be further studied.

As technologies with high throughput developed, such as NGS (Next Generation Sequencing) and gene expression chip, it is helpful to further understand the potential mechanism of the development and progress of AF. Multiple groups show that the abnormal expression of coding and noncoding genes is associated with AF. For example, Marina et al. identified 112 differently expressed miRNAs and 40 dysregulated circRNAs in AF using the RNA-sequence method [4]. Similarly, Wang et al. found multiple miRNAs, such as miR-155, miR-146b-5p, and miR-19b, that were dysregulated in AF samples using RNA-seq analysis [5]. Of note, long uncoded RNAs have also been demonstrated to participate in regulating multiple biological processes in AF progression. Mei et al. identified 117 downregulated and 65 upregulated lncRNAs in AF patients compared with control samples [6]. LncRNA ENST00000559960 and uc004aef.3 are validated to be dysregulated in AF samples, which are revealed by microarray analysis [7]. However, further understanding of pathogenic mechanisms related to AF was still urgent that needs to decrease the morbidity of this disease.

In this research, we adopted RNA-seq technology to identify differently expressed mRNAs and checked the functional

significance of these dysregulated genes in AF. PPI networks analysis was used to identify hub genes in AF. We thought this study could provide novel potential diagnostic and therapeutic biomarkers for AF.

## 2. Material and Methods

*2.1. Adult Heart Sample Collection.* The blood specimens were gathered from 3 healthy samples and 5 AF patients' samples. The research conformed to the Declaration of Helsinki guidelines. The consent to donate to research was obtained through the Transfer of Tissue Agreement of our institution. This study was authorized by the hospital ethics committee and obtained written consent from all patients. Collection of venous blood samples was through EDTA tubes and PAXgeneTM blood RNA tubes (PreAnalytiX). EDTA tube was immediately centrifuged to separate the plasma, and all tubes were kept at -80°C.

*2.2. RNA Isolation and RNA Sequencing.* Trizol Reagent was used to segregate total RNA from a venous blood specimen. Total RNA samples' number and integrity were surveyed by a NanoDrop 2000 spectrophotometer. The integrity of the target RNA was tested using 1.5% agarose gel electrophoresis. Agilent 2100 Bioanalyzer was adopted to surveyed the RIN value (RIN > 7). The mRNA was sublimated through polygo-d ($T$) probe to select a target with polyA. The size of the fragment of distilled mRNA was 200 bp. The cDNA (complementary DNA) is produced. End repair was carried out after refinement through the QIAquick PCR kit, and the adaptors were ligated. Gel purification (2% TAE) was carried out to segregate the 300 nt fragments. An mRNA library was built. Besides, 18-30 nt RNA was gained from total RNA. The cDNA was bound chemically and reverse transcribed. At last, the consequent cDNA library was sequenced by adopting the HiSeq 2500 platform.

*2.3. Sequencing Data Analysis.* Initially, all raw data of RNA sequencing of every sample were manufactured by Fastqc [8] to manicure the adaptor and dislodge reads with low quality. The residual clean reads were shining upon the genomes by adopting Tophat [9]. The alignment results were input into cufflinks [10]. The unit of the expression level used in our study was FPKM. The Cuffdiff was applied to calculate differential expression. We found differentially expressed genes with the following criteria: FPKM > 10 and FDR < 0.05.

*2.4. Differential Expressed Genes (DEGs) in AF.* Differences in the expression between AF and healthy control samples were compared by $t$-test. DEGs of AF in comparison with controls were calibrated with $P$ value <0.05 and |log2 fold change| ≥1. The cluster analysis and plotting heat map of DEGs were performed by cluster package and heat map package in $R$ software.

*2.5. Bioinformatic Analysis.* To further explore the potential function and signaling pathways regulated by DEGs in AF, Gene Ontology (GO) [11] terminology analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway [12] analysis were performed based on the target by the DAVID (http://david.abcc.ncifcrf.gov/) [13]. The biological processes and pathways with $P$ value <0.05 were regarded as significant.

*2.6. Quantitative Real-Time PCR.* TB Green™ Premix Ex Taq™ II (TaKaRa, Dalian, China) and an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) were used to perform quantitative real-time PCR (qRT-PCR). Primers implemented in the experiments (listed in Table S1) were synthesized by Sangon Biotech. The normalization of Ct values was calculated by $\beta$-actin or RNU6 as inner control to evaluate the expression level of the genes. The expression of relative mRNA was performed by adopting the $2^{-\Delta\Delta Ct}$ method. Every sample was repeated three times.

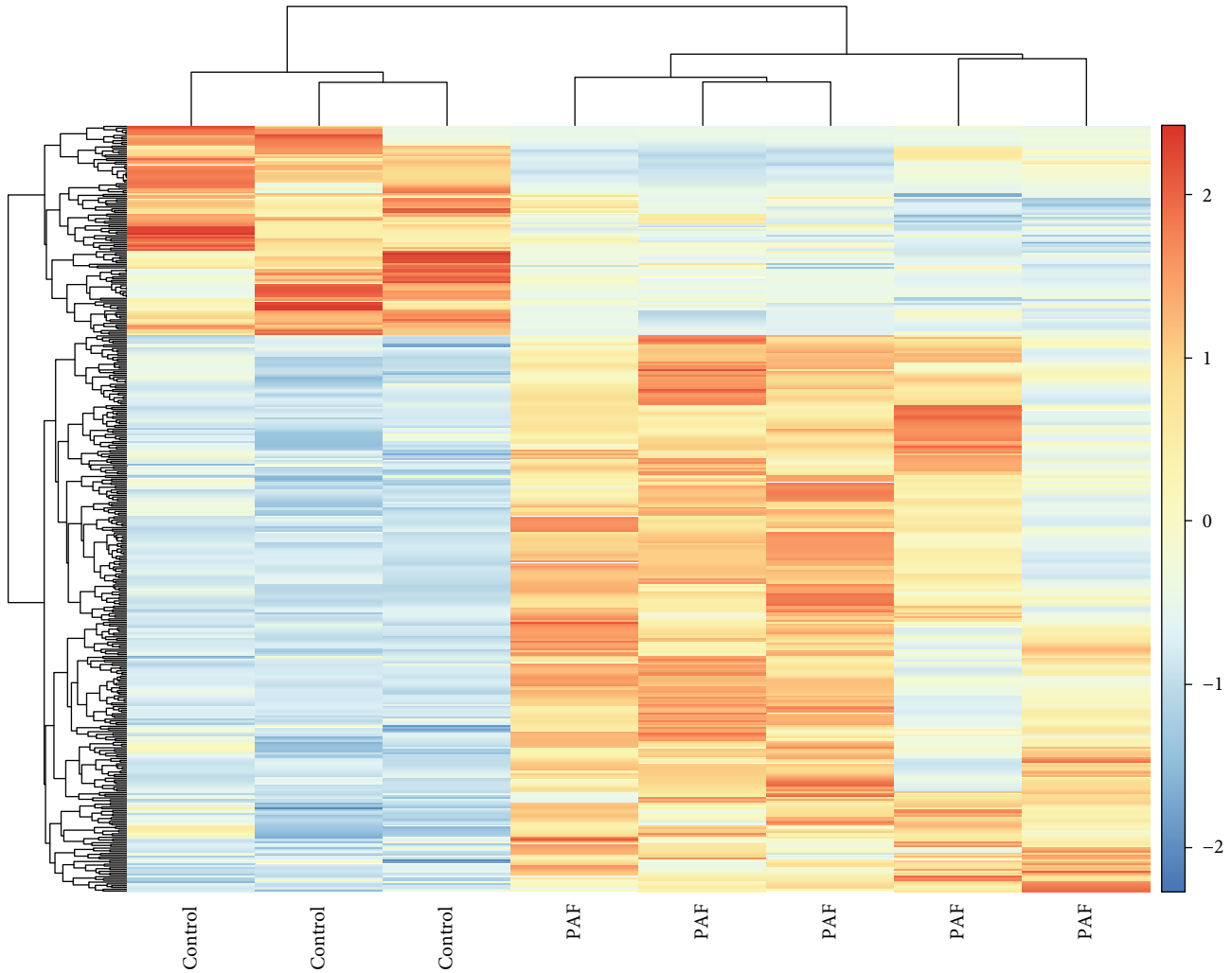
*2.7. Analysis of Protein-Protein Interaction.* Aiming at investigating protein interactions between differentially expressed mRNAs in AF, the Search Tool for the Retrieval of Interacting Genes (STRING) database [14] was performed to pick protein pairs with reciprocal actions. The protein-protein interaction (PPI) web was built with Cytoscape 3.4.0 (http://cytoscape.org/) [15]. In this network, each node characterizes a protein, and the edge represents the interrelationship between the two proteins.

*2.8. Analysis of Statistics.* DEGs were analyzed by adopting the DESeq2 package in $R$ Studio (Version: 3.6.1). |log2 fold change| ≥1 and $P$ adjust value <0.05 were selected as the cut-off value for recognizing DEGs. The experimental data were shown as mean ± SD (standard deviation) of no less than three replicate measurements. Student's $t$-test was used between groups. The $P$ value of <0.05 was regarded to be important to statistics with a confidence level of 95%.

## 3. Results

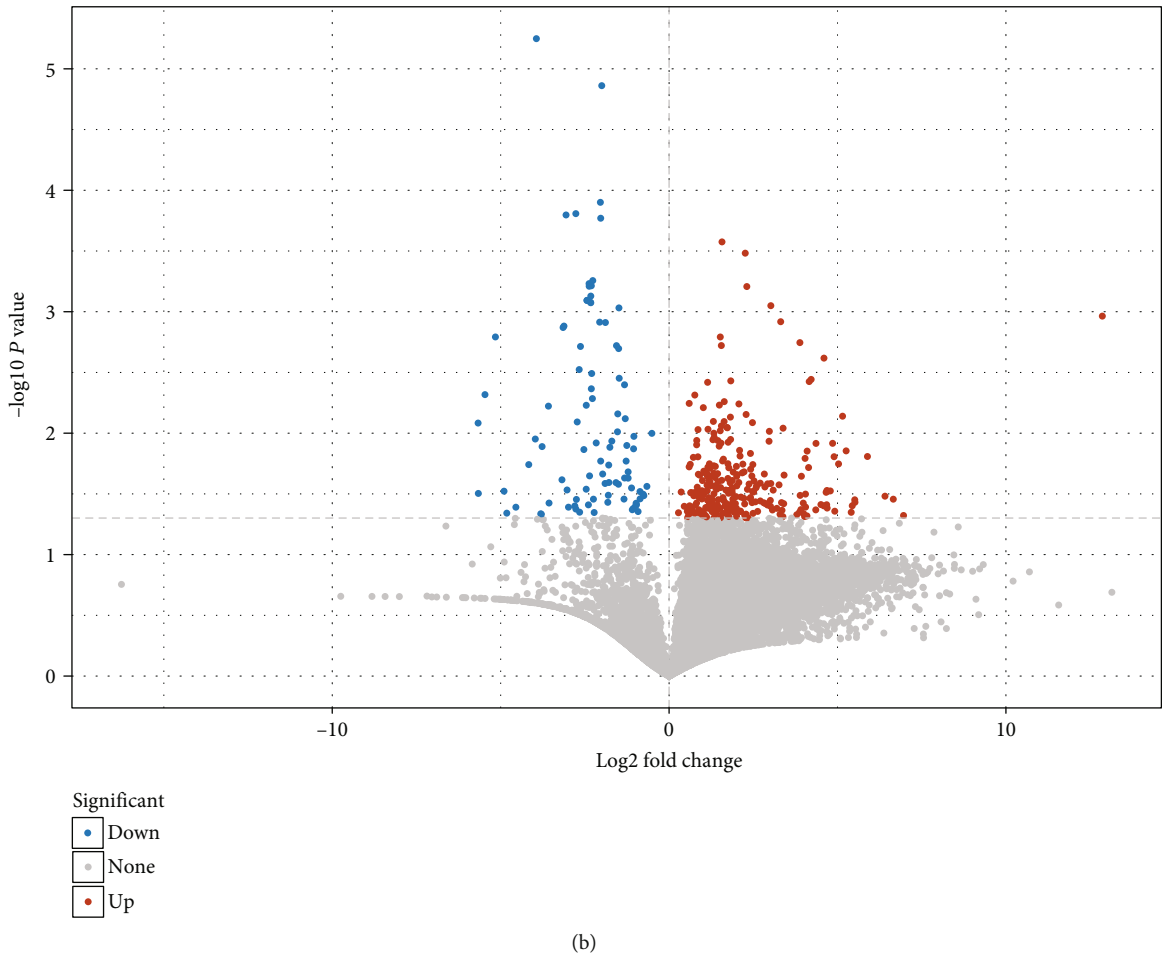
*3.1. Recognition of Distinctively Expressed mRNAs in AF.* By comparing the whole genome gene expression change between AF and healthy control samples using the RNA-seq method, a total of 392 DEGs were obtained, including 275 positively regulated mRNAs and 117 negatively regulated mRNAs in AF compared to healthy controls. Differential expression hierarchical clustering of mRNA was shown in Figures 1(a) and 1(b).

*3.2. GO and KEGG Pathway Analyses of DEGs in AF.* We next conducted the GO and KEGG analysis aiming at projecting the potential functions of distinctively expressed mRNAs in AF. As shown in Figure 2, the top 10 enriched biological process (BP) terms regulated by DEGs included platelet aggregation, integrin-mediated signaling pathway, platelet degranulation, iron ion homeostasis, inner ear development, regulation of cell growth, protein phosphorylation, positive regulation of pri-miRNA transcription, transforming growth factor-beta (TGF-$\beta$) receptor signaling pathway, and intracellular estrogen receptor signaling pathway (Figure 2(a)). The significantly enriched cellular component (CC) terms regulated by DEGs included endoplasmic reticulum, mitochondrial membrane, platelet alpha granule membrane,




(a)

Figure 1: Continued.




(b)

Figure 1: (a) Heat map and (b) volcano plot show differential expression of mRNAs between atrial fibrillation (AF) and healthy control samples. Red indicates high relative expression, and green indicates low relative expression.

melanosome, and integrin complex (Figure 2(b)). The significantly enriched molecular function (MF) terms regulated by DEGs included protein binding, cyclin-dependent protein serine/threonine kinase activity, ATP binding, pyridoxal phosphatase activity, protein kinase activity, and integrin binding (Figure 2(c)).

Meanwhile, KEGG pathway analysis showed that the differentially expressed genes were related to the regulation focal adhesion, platelet activation, dilated cardiomyopathy, estrogen signaling pathway, hypertrophic cardiomyopathy (HCM), dopaminergic synapse, ECM-receptor interaction, osteoclast differentiation, GnRH signaling pathway, and circadian entrainment (Figure 2(d)).

*3.3. Protein-Protein Interaction Network Analysis of DEGs in AF.* Next, we explored the relationship between these DEGs in AF by constructing a PPI network based on the STRING database. As shown in Figure 3, the network contained a total of 126 proteins and 265 edges. Hub proteins were highly connected to other proteins. In the PPI web, SRC (extent = 16), ACTG1 (extent = 14), ITGB3 (extent = 11), BMP2 (extent = 9), H2AFX (extent = 6), and H2BFS (extent = 6) were the hub genes.

*3.4. Recognition of Hub Gene Networks in AF.* Subsequently, hub gene network analysis was applied by the MCODE plugin. The cutoff degree was ≥2. Two hub networks were identified in the above PPI web. As shown in Figure 4(a), hub network 1 included ITGB5, SRC, ACTG1, ILK, ITGA2B, and ITGB3. And hub network 2 included TUBB4B, CDK11A, PAFAH1B1, CDK11B, and TUBG1 (Figure 4(b)).

*3.5. Validation of the Expression of Key DEGs in AF by qRT-PCR.* We validated the expression of key DEGs in 5 AF samples and 3 normal samples using the qRT-PCR assay. As present in Figures 5(a)–5(j), the results of RNA-seq analysis indicated that TUBG1, CDK11B, PAFAH1B1, CDK11A, TUBB4B, ITGA2B, ILK, ACTG1, SRC, and ITGB5 were upregulated in 5 AF samples compared to 3 normal samples. Then, the expression of these key DEGs was confirmed higher in AF samples than that in healthy control samples using the qRT-PCR assay (Figure 5(k)).

## 4. Discussion

Of note, the mechanism regulating AF progression remained to be further investigated. As RNA-sequence developed, this

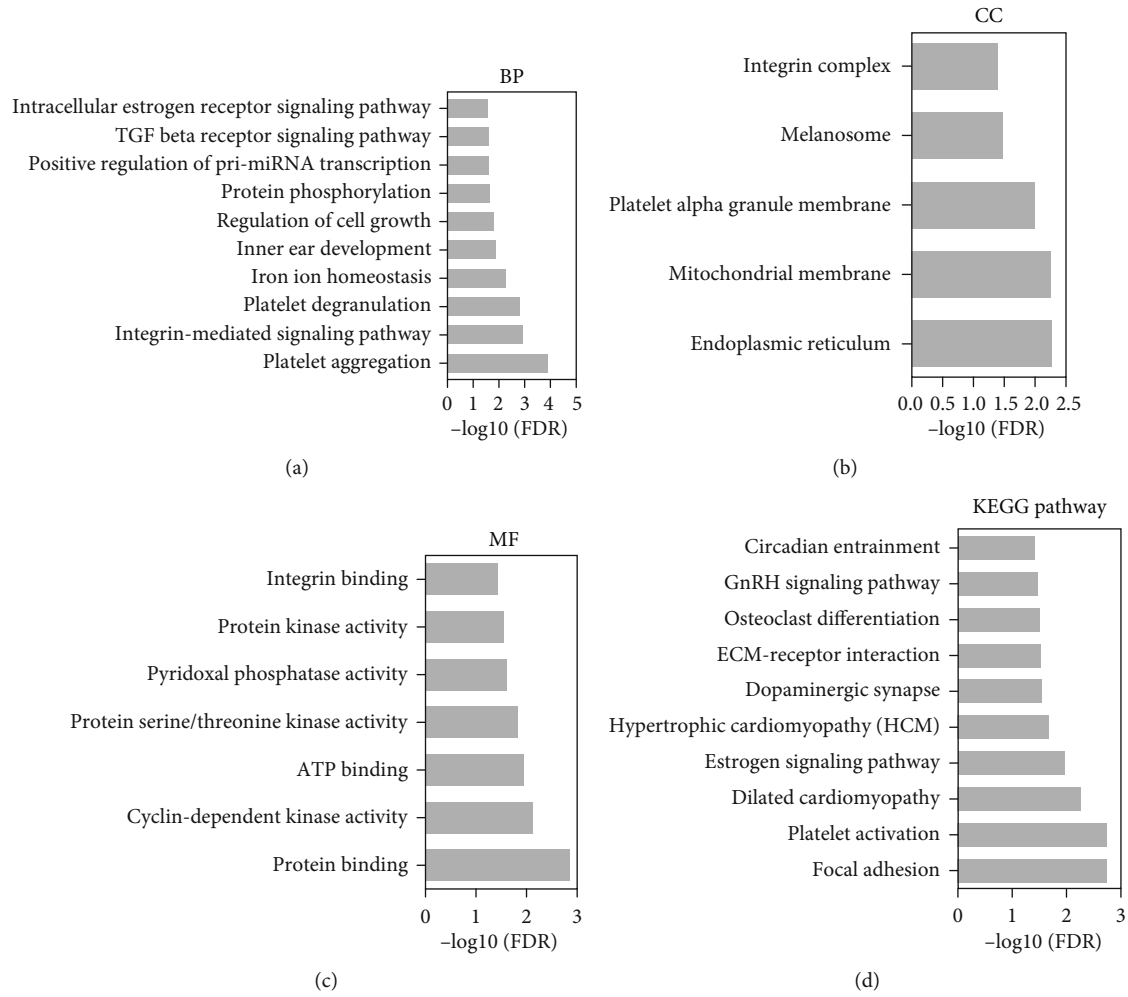



Figure 2: (a) Biological process (BP) analysis of DEGs in AF. (b) Cellular component (CC) analysis of DEGs in AF. (c) Molecular functions (MF) analysis of DEGs in AF. (d) KEGG pathway analysis of DEGs in AF.

method had been widely applied in exploring mechanisms related to the pathology of multiple human diseases, including cancers and AF. Based on the whole knowledge that we had, researches on the expression profiling of mRNA in the AF tissue by RNA sequencing are rare. In this experiment, we recognized 275 positively regulated and 117 negatively regulated mRNAs in 5 AF samples compared to 3 control samples.

Bioinformatic analysis revealed that these DEGs were involved in some biological processes and pathways, for example, platelet aggregation, platelet activation, pri-miRNA transcription, and TGF-$\beta$ receptor signaling pathway, which had been demonstrated to be associated with AF in previous studies. Pourtau's group found that AF pathology was in relation to platelet aggregation abnormalities and acute AF led to enhancive platelet aggregation [16]. Besides, it also reported that the stage of platelet activation is a crucial regulator for stroke risk associated with AF [17]. Interestingly, we found that DEGs in AF were significantly involved in regulating pri-miRNA transcription. MicroRNAs (miRNAs) have been demonstrated to be related to AF progression. For instance, the overexpression of miR-31 in AF results in arrhythmia through exhausting dystrophin and neuronal nitric oxide synthase [2]. MiR-328 regulates adverse electrical remodeling in AF [18]. Emerging studies show that multiple downstream targets of TGF-$\beta$ signaling contribute to the development of AF, including CD44/STAT3 and SMAD2/3 [19].

In the past decades, high-throughput technologies had been developed to help researchers to identify differentially expressed genes related to the progression of diseases. However, a big challenge in understanding the mechanisms of diseases is how to identify the core regulators from hundreds of differently expressed genes. Recent studies demonstrate that PPI analysis is an efficient tool for the identification of hub regulators. For example, Zou et al. identified that CD19, FGF9, SOX9, GNGT1, and NOG were hub genes associated with stroke in AF [20]. Zhong et al. found ACLY played a key regulator in type 2 diabetes using PPI network analysis [21]. The present study constructed PPI networks to reveal the interactions among DEGs in AF. A total of 126 proteins and 265 edges were included in this network. Among these proteins, SRC, ACTG1, ITGB3, BMP2, H2AFX, and H2BFS were identified as the hub genes. SRC was reported to be dysregulated in atrial remodeling owing to tachypacing using

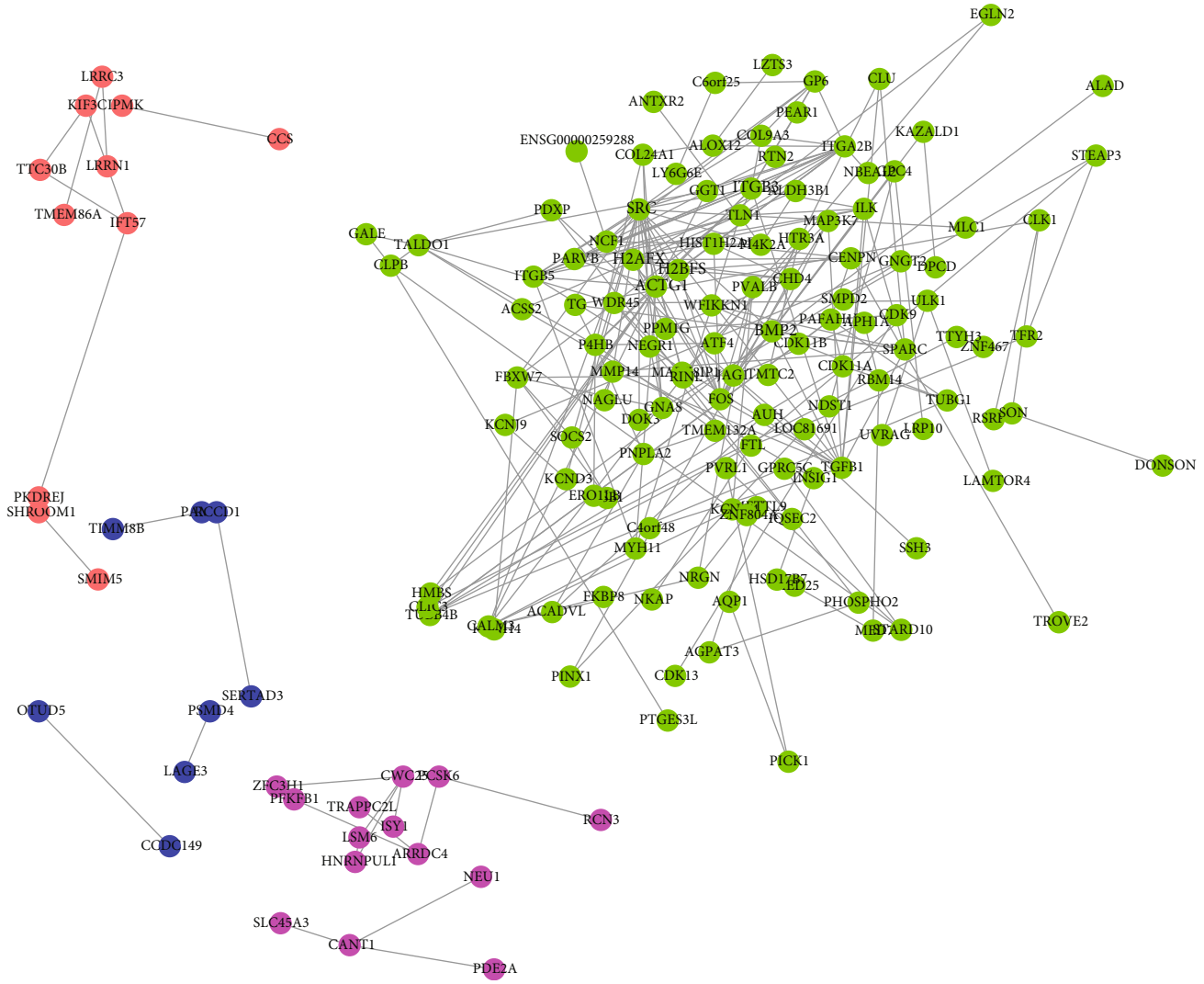
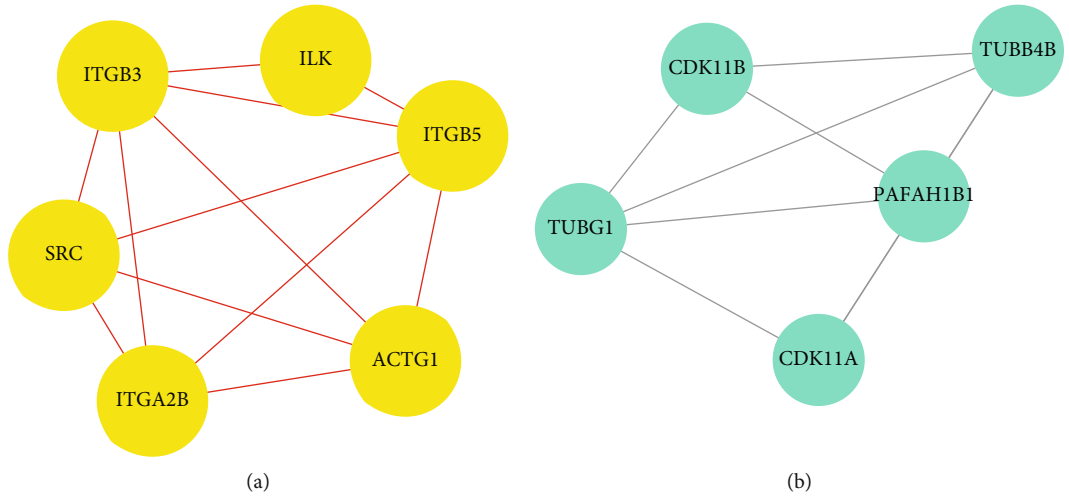



Figure 3: We constructed a PPI network for DEGs in AF. The network contains a total of 126 proteins and 265 edges.




(a) (b)

Figure 4: (a, b) Two hub networks were identified using the MCODE plugin. The degree cut-off was ≥2.

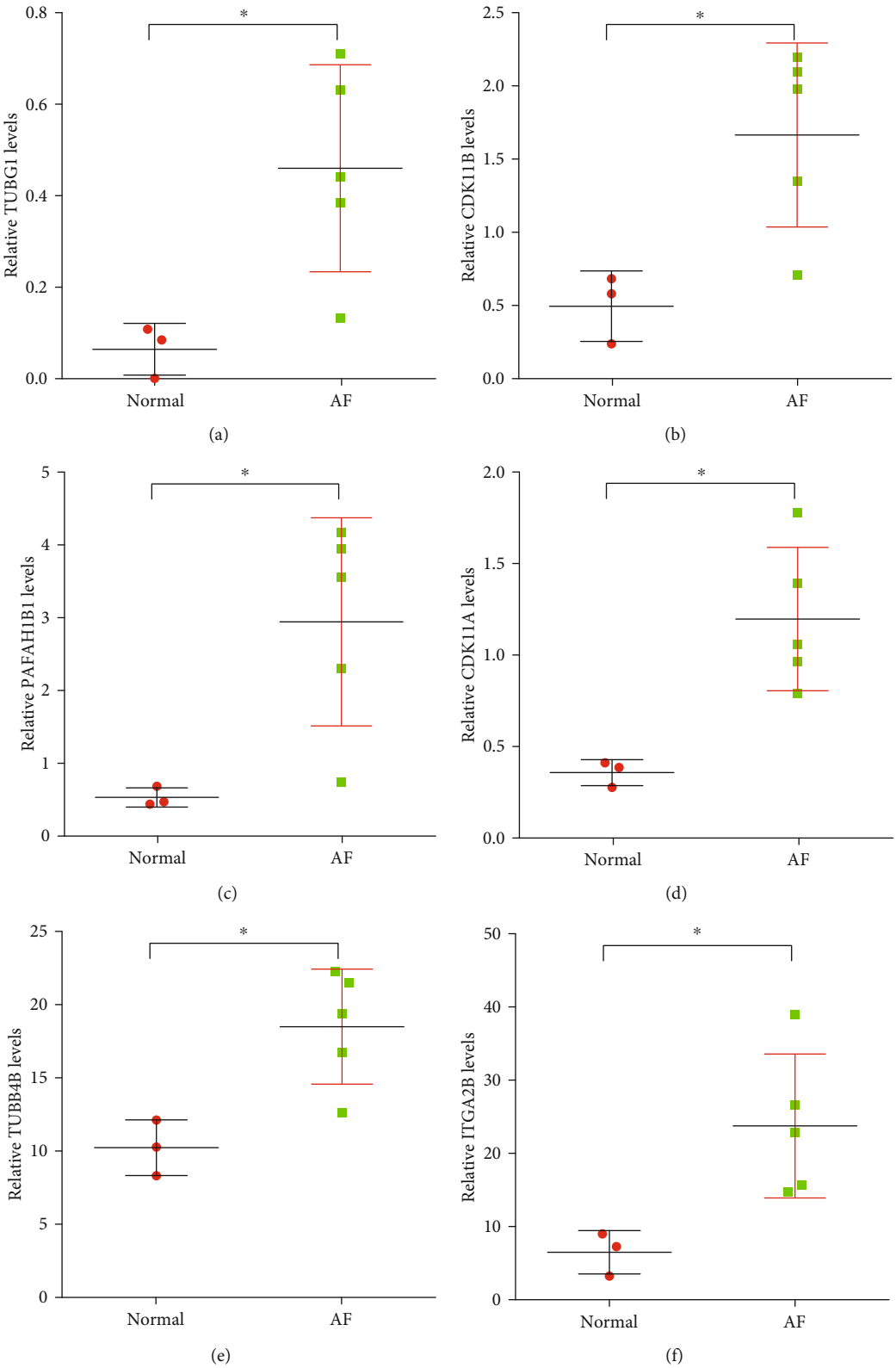



Figure 5: Continued.

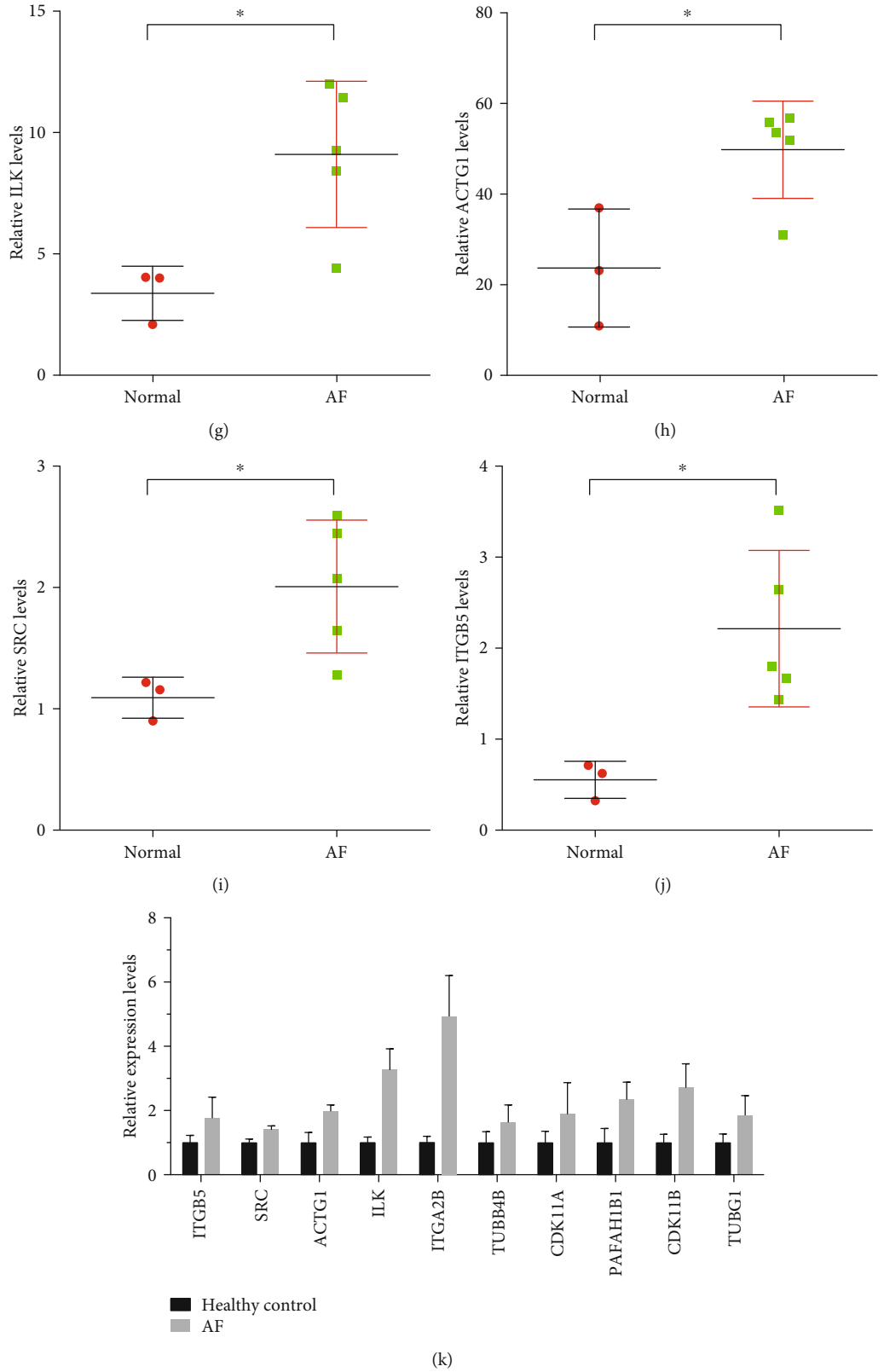



Figure 5: (a)–(j) The RNA-seq data indicated that TUBG1, CDK11B, PAFAH1B1, CDK11A, TUBB4B, ITGA2B, ILK, ACTG1, SRC, and ITGB5 were upregulated in 5 AF samples compared to 3 normal samples. (k) The qRT-PCR assay demonstrated that these key DEGs were upregulated in 5 AF samples compared to 3 normal samples.

kinomic array analysis [22]. ACTG1 is a component of the cytoskeleton and associated with hearing loss and skin cancer cell proliferation [23, 24]. Our study showed that ACTG1 was related to AF for the first time. A previous study showed that BMP2 was involved in cardiac development and may influence responses to hypoxia [25]. Moreover, we identified two hub networks, which included 6 proteins and 5 proteins, respectively. QRT-PCR validation showed that these hub genes ITGB5, SRC, ACTG1, ILK, ITGA2B, TUBB4B, CDK11A, PAFAH1B1, CDK11B, and TUBG1 were significantly overexpressed in AF samples compared to normal samples. These proteins have been reported to play various roles in human diseases. For example, ILK is a diffusely showed serine/threonine-protein kinase existing in focal adhesions, playing a core role as a multipurpose effector of growth factor signaling and cell-matrix interactions [26]. TUBB4B and TUBG1were microtubules proteins, which played a key role in keeping the cell shape and regulating cell-matrix adhesion, cell-cell interaction, protein transport, and cell movement [27]. CDK11A and CDK11B were cyclin-dependent kinases, which were involved in regulating the cell cycle [28]. We thought these reports together with our findings would provide a novel insight to understand the mechanism regulating AF.

## 5. Conclusion

Overall, we screened 275 mRNAs positively regulated and 117 mRNAs negatively regulated in AF compared to healthy controls. The bioinformatic analysis indicated these DEGs took part in regulating multiple AF-related biological processes and pathways, such as platelet aggregation, platelet activation, pri-miRNA transcription, and TGF-$\beta$ receptor signaling pathway. PPI network analysis identified ITGB5, SRC, ACTG1, ILK, ITGA2B, ITGB3, TUBB4B, CDK11A, PAFAH1B1, CDK11B, and TUBG1 as hub regulators in AF. Moreover, the results of the qRT-PCR assay showed that these hub genes were significantly overexpressed in AF samples compared to normal samples. We believed that this study would enrich the understanding of the pathogenesis of AF and lay the foundation for further research.

## Data Availability

The data that support the findings of this study are available with approval from the author.

## Conflicts of Interest

The author(s) declare(s) that they have no conflicts of interest.

## Authors' Contributions

Nannan Chen and Mu Qin performed the conception and design. Nannan Chen and Qunlin Gong performed the development of methodology. Nan Xu and Yu Lin performed the sample collection. Jiahong Wang and Qunlin Gong contributed to the analysis and interpretation of data. Nannan Chen, Jiahong Wang, and Pengxiang Zheng contributed to the writing, review, and/or revision of the manuscript. Nannan Chen and Mu Qin contributed equally as joint first authors.

## Acknowledgments

The study was supported by the Shanghai Health and Family Planning Commission Project (grant no. 201940057).

## Supplementary Materials

Supplement Table S1: quantitative real-time PCR primers. *(Supplementary Materials)*

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