

Identification of microRNAs and genes as biomarkers of atrial fibrillation using a bioinformatics approach

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Abstract

Objective: We aimed to explore potential microRNAs (miRNAs) and target genes related to atrial fibrillation (AF).

Methods: Data for microarrays GSE70887 and GSE68475, both of which include AF and control groups, were downloaded from the Gene Expression Omnibus database. Differentially expressed miRNAs between AF and control groups were identified within each microarray, and the intersection of these two sets was obtained. These miRNAs were mapped to target genes in the miRNet database. Functional annotation and enrichment analysis of these target genes was performed in the DAVID database. The protein-protein interaction (PPI) network from the STRING database and the miRNA-target-gene network were merged into a PPI-miRNA network using Cytoscape software. Modules of this network containing miRNAs were detected and further analyzed.

Results: Ten differentially expressed miRNAs and 1520 target genes were identified. Three PPI-miRNA modules were constructed, which contained miR-424, miR-15a, miR-542-3p, and miR-421 as well as their target genes, *CDK1*, *CDK6*, and *CCND3*.

Conclusion: The identified miRNAs and genes may be related to the pathogenesis of AF. Thus, they may be potential biomarkers for diagnosis and targets for treatment of AF.

Keywords

MicroRNAs, atrial fibrillation, computational biology, microarray analysis, cyclin D kinase, cyclin D3

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Introduction

Atrial fibrillation (AF) is one of the most common types of cardiac arrhythmia.¹ It is an important risk factor for consequences such as stroke and atrial appendage thrombus formation,^{1,2} which may increase the risk of mortality in patients with AF.^{3,4} Therefore, revealing the mechanism of pathogenesis of AF is important and could indicate pathways to prevent and treat AF.

MicroRNAs (miRNAs) are small, non-coding RNA molecules involved in RNA silencing and posttranscriptional regulation of gene expression.^{5,6} Recent studies have revealed important roles for miRNAs in various physiological and pathological cardiac processes, such as the regulation of cardiac excitability and formation of arrhythmia,^{7,8} cardiac hypertrophy,⁹ myocardial infarction,^{9,10} and diabetic cardiomyopathy.¹¹ An increasing number of tissue and circulating miRNAs have been identified as biomarkers of AF, involved in the development,¹² progression,^{12–14} and complications¹⁵ of the disease.

Microarray analysis has become a powerful tool in the characterization of many pathophysiological processes. In this study, we used publicly available miRNA microarray data to perform *de novo* analyses. We focused on complementary bioinformatics to explore the data more deeply and aimed to construct an updated prediction of new miRNAs and genes as biomarkers of chronic AF to supplement currently available data.

Materials and methods

The data used in this study were obtained from open access databases on the internet and did not require ethical permission or patient consent for use.

Microarray data and differentially expressed miRNAs

We searched the Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo/>), using key words atrial fibrillation, microRNA, and miRNA, and selected microarrays with data of good cross-comparability. Microarray data for GSE70887 and GSE68475 were selected and downloaded; the corresponding platforms were GPL19546 (Agilent-021827 Human miRNA Microarray; Agilent Technologies, Santa Clara, CA, USA) and GPL15018 (Agilent-031181 Unrestricted_Human_miRNA_V16.0_Microarray 030840; Agilent Technologies), respectively. GSE70887 included four atrial tissue samples from patients with chronic AF (AF group) and four from patients without AF (control group). GSE68475 included 10 atrial tissue samples from patients with persistent or permanent (≥ 6 months) AF (AF group) and 11 atrial tissue samples from patients with normal sinus rhythm (control group).

Differentially expressed miRNAs between the AF group and control group were screened within each microarray using the online tool GEO2R in the GEO database. GEO2R is an R-programming-based language for analysis of gene expression datasets by *t*-test or analysis of variance (ANOVA). It can help identify differentially expressed miRNAs between two groups of samples under the same conditions.¹⁶ Differentially expressed miRNAs were selected by using cut-off values of $P < 0.05$ and $|\log_2 FC| > 0.5$, where FC = fold change. After these two sets of differentially expressed miRNAs were selected, we obtained their intersection for the following analyses.

MiRNA-target-gene mapping

All differentially expressed miRNAs were uploaded to the miRNet database (<http://www.mirnet.ca/>)¹⁷ to map their corresponding target genes. The miRNA-target-gene network was constructed based on this mapping.

Functional enrichment analysis

The target genes were then annotated and enriched in the Database for Annotation, Visualization, and Integrated Discovery (DAVID; <https://david-d.ncifcrf.gov/>).^{18,19} Then, gene ontology: biological process (GO-BP) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were obtained. *P*-values < 0.05 were considered statistically significant.

Prediction of protein-protein interaction

We then applied Search Tool for the Retrieval of Interacting Genes (STRING, <http://string-db.org/>)²⁰ to construct a protein-protein interaction (PPI) network based on the target genes detected above. Interactions of proteins in the STRING database were screened by using a confidence score criterion; PPI pairs with a confidence score >0.9 were considered significant in this analysis.

PPI-miRNA network construction and module clustering analysis

The PPI network and miRNA-target-gene network were merged into a whole PPI-miRNA network by using the Cytoscape software (<https://cytoscape.org>). This network was calculated by using the Cytoscape plugin "MCODE" to explore potential functional modules. Degree cutoff value was set to 2 and the node score cutoff to 0.2 as the criteria in this process. Modules containing miRNAs were selected.

Results

Identification of differentially expressed miRNAs and miRNA-target-gene mapping

Data cross-compatibility was assessed (Figure 1) and 10 significant differentially expressed miRNAs were identified (Table 1). To explore the possible targets of the 10

miRNAs identified above, miRNA-target gene mapping was implemented in miRNet. A total of 1520 genes and 2235 miRNA-target-gene pairs were identified.

Functional enrichment analysis

The 1520 genes identified were uploaded to DAVID for functional enrichment analysis. Numerous GO-BP terms and KEGG pathways were enriched, and those with the smallest *P*-values are listed in Tables 2 and 3. Specifically, biological processes related to cell growth and apoptosis were highly enriched.

PPI pairs and PPI-miRNA network

The genes targeted by the differentially expressed miRNAs were imported to the STRING database, and 2543 PPI pairs were obtained. Using the Cytoscape software, we merged the PPI network and miRNA-target-gene network into a whole PPI-miRNA network. Module clustering analysis of this network was then performed. Three significant modules containing miRNAs (miR-424, miR-421, miR-15a, and miR-542-3p) were obtained (Figure 2). Genes linking to miR-424 with the highest node scores (*CDK1*, *CDK6*, and *CCND3*) in module A (Figure 2a) were involved in biological processes related to cell growth and apoptosis, as mentioned above.

Discussion

AF is the leading cause of stroke and atrial appendage thrombus formation worldwide and is a major medical problem.^{1,2} In the present study, we used publicly available miRNA microarray data and novel bioinformatic approaches to predict the potential key miRNAs and genes associated with the pathogenesis of AF.

In our functional enrichment analysis, biological processes related to cell growth and apoptosis were highly enriched.

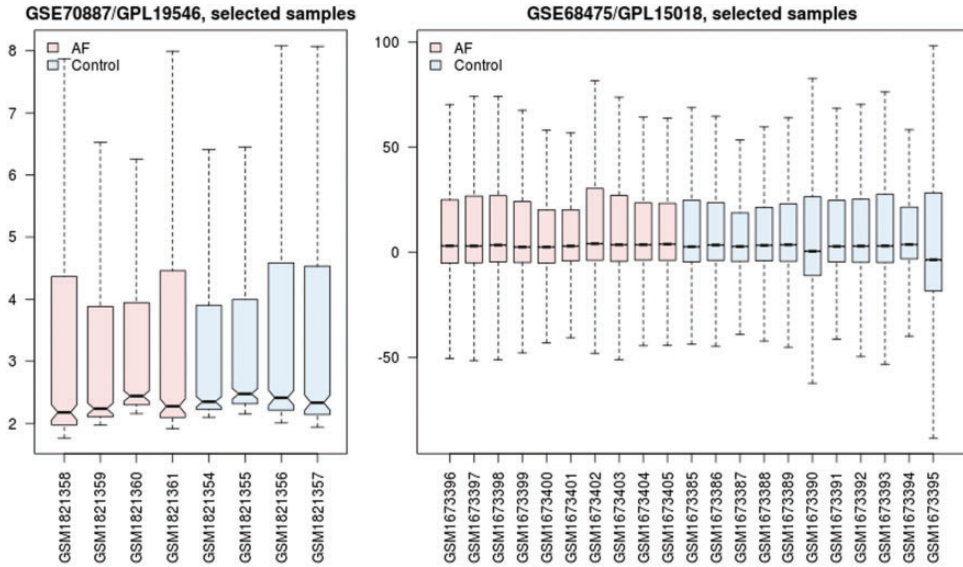


Figure 1. Cross-comparability assessment of microarray data of GSE70887 and GSE68475 (<http://www.ncbi.nlm.nih.gov/geo/>). GSE70887 included four atrial tissue samples from patients with chronic atrial fibrillation (AF) and four from patients without AF (control). GSE68475 included 10 atrial tissue samples from patients with persistent or permanent (≥ 6 months) AF (AF) and 11 from patients with normal sinus rhythm (control).

Table 1. A list of differentially expressed miRNAs.

miRNA	P-value	Log ₂ FC
hsa-miR-503	0.000628	0.828181
hsa-miR-542-3p	0.00223	0.607736
hsa-miR-208b	0.004467	1.729022
hsa-miR-421	0.016152	0.501949
hsa-miR-22*	0.022647	0.675245
hsa-miR-126*	0.023377	0.595347
hsa-miR-424	0.031095	0.604423
hsa-miR-224	0.035734	0.665672
hsa-miR-1285	0.043129	-0.504033
hsa-miR-15a	0.047701	0.530578

The miR* nomenclature indicates a miRNA that originates from the same hairpin structure of the corresponding predominant miR (without *) and is thought to be complementary to the predominant miR. FC (fold change) values are expressed as the comparison of AF versus controls.

Moreover, in the PPI-miRNA network, we showed that genes *CDK1*, *CDK6*, and *CCND3*, linked to miR-424, were involved in these biological processes, suggesting their role in the pathogenesis of AF.

The role of abnormal cell growth and apoptosis in AF and other heart diseases has been studied previously. Expression of certain genes associated with cell growth and the cell cycle has been found to be altered in patients with AF, especially in fibroblasts of the heart.^{21,22} AF is associated with structural remodeling of the atria, the hallmark of which is development and progression of atrial fibrosis, in which growth of fibroblasts is stimulated.²³ In contrast, dedifferentiation of atrial myocytes has been found in AF, and this process is mediated by atrial fibroblast activation.²⁴

CDK1 (Cyclin-dependent kinase 1) is a highly conserved protein that functions as a serine/threonine kinase and is a key player in cell cycle regulation.²⁵ In an animal study, isoproterenol-induced cardiac fibrosis was shown to be associated with increased levels of CDK1 in fibroblasts exclusively in the adult mouse heart; thus,

Table 2. Functional enrichment analysis of target genes.^{18,19}

GO ID	Term	Gene counts	P-value
GO:0046907	Intracellular transport	103	2.75E-10
GO:0007049	Cell cycle	114	1.42E-09
GO:0045449	Regulation of transcription	294	2.22E-09
GO:0045184	Establishment of protein localization	109	2.51E-08
GO:0043067	Regulation of programmed cell death	113	3.66E-08
GO:0010941	Regulation of cell death	113	4.47E-08
GO:0015031	Protein transport	107	5.61E-08
GO:0010608	Posttranscriptional regulation of gene expression	43	7.31E-08
GO:0042981	Regulation of apoptosis	111	7.51E-08
GO:0043069	Negative regulation of programmed cell death	61	1.12E-07

GO, gene ontology.

Table 3. KEGG pathway enrichment analysis of target genes.^{18,19}

KEGG Pathway	Gene counts	P-value
hsa05200: Pathways in cancer	76	1.00E-14
hsa05215: Prostate cancer	29	2.20E-09
hsa05210: Colorectal cancer	28	2.52E-09
hsa05220: Chronic myeloid leukemia	23	4.79E-07
hsa05212: Pancreatic cancer	22	9.72E-07
hsa04115: p53 signaling pathway	21	1.53E-06
hsa05213: Endometrial cancer	18	1.85E-06
hsa05221: Acute myeloid leukemia	19	2.17E-06
hsa05218: Melanoma	20	1.29E-05
hsa04722: Neurotrophin signaling pathway	28	1.44E-05

KEGG, Kyoto Encyclopedia of Genes and Genomes.

targeting CDK1 in the diseased heart may inhibit fibrosis and confer cardioprotection.²⁶ Another study showed that estrogen loss is associated with cardiac fibrosis and diastolic dysfunction. GPR30 (G protein-coupled estrogen receptor) is expressed in rat cardiac fibroblasts, and activation of GPR30 limits proliferation of these cells by suppressing cell cycle proteins including CDK1.²⁷

CDK6 is a member of the family of serine/threonine kinases and a key regulator during the G₁/S cell cycle transition.²⁸ Aberrant CDK6 expression has been reported in various cancer types,^{29–32} suggesting its involvement in aberrant cell cycle and uncontrolled cell growth. CDK6 has

also been reported to be involved in certain heart diseases. For example, inflammation is associated with various cardiovascular diseases, of which C-reactive protein is a marker. C-reactive protein treatment of cardiac myocytes results in a significant reduction in the levels of CDK6 in a concentration-dependent manner.³³ In turn, depression of CDK6 contributes to the effect of attenuation of miR-1 on provoking cardiomyocyte hypertrophy.³⁴

CCND3, the gene encoding cyclin D3, is a regulator of progression through the G₁ phase of the cell cycle. It is an oncogene in various B-cell lymphoma subtypes.^{35,36} Rho-associated coiled-coil kinases play a role in cardiac cell physiology and are also

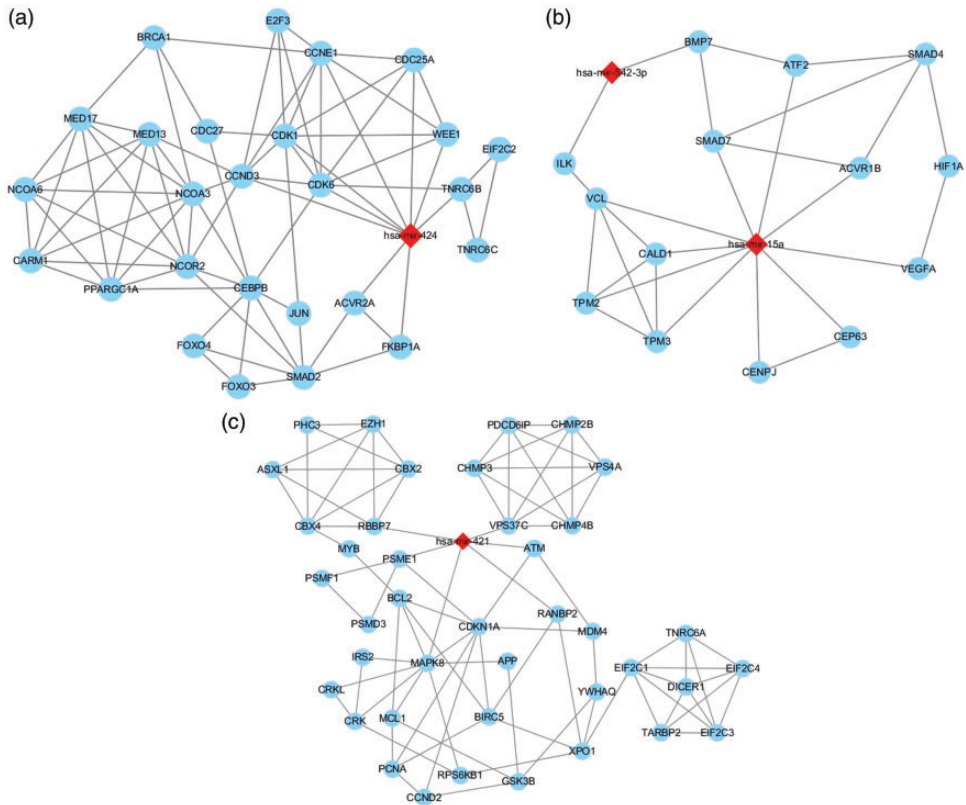


Figure 2. Three protein-protein interaction (PPI)-miRNA modules were constructed using Cytoscape software. Modules A, B, and C involved miR-424, both miR-542-3p and miR-15a, and miR-421, respectively. Red diamonds represent up-regulated miRNAs, and blue ellipses represent target genes.

expressed in the developing heart, the blockade of which decreases expression of cell cycle proteins, including cyclin D3, in cardiomyocytes.³⁷

MiR-424 plays a role in cell cycle regulation.^{38,39} It is reported to be involved in the differentiation of cardiac myocytes, and altered expression of miR-424 is associated with certain heart diseases, such as heart failure⁴⁰ and tetralogy of Fallot.⁴¹ In our PPI-miRNA module A (Figure 2a), CDK1, CDK6, and CCND3 were co-regulated by miR-424. Hence, miR-424 may be a central regulator in the pathogenesis of AF.

Other miRNAs, such as miR-15a, miR-542-3p, and miR-421 (Figure 2b and 2c),

were shown by our results to be possible key regulators in AF, although there was little evidence of direct associations with AF.

Circulating miR-15a has been found to be up-regulated in diffuse myocardial fibrosis.⁴² In addition, two genes (*SMAD7* and *VEGFA*) directly linked to miR-15a in our module B (Figure 2b) are reported to be involved in the pathogenesis of AF. Degradation of Smad7 protein (possibly mediated by miR-21) may decrease the inhibitory feedback regulation of transforming growth factor (TGF)- β 1/Smad signaling and serves as a key mechanism of AF-induced atrial fibrosis.^{43,44} In contrast, miR-15a has

been shown to down-regulate the level of Smad7 in other diseases.^{45,46} Hence, miR-15a may play a role in AF-induced atrial fibrosis via the Smad7 pathway.

VEGFA is a member of the platelet-derived growth factor (PDGF)/vascular endothelial growth factor (VEGF) family. It induces proliferation and migration of vascular endothelial cells and is essential for both physiological and pathological angiogenesis. It has been reported that the plasma level of VEGF is higher in patients with AF and that the interaction among this angiogenic marker, platelets, and tissue factor (TF) may alter endothelial integrity, thereby contributing to the pro-thrombotic state in AF.^{47,48} In contrast, miR-15a level has been reported to be positively correlated with VEGFA level^{49,50} in other pathologic conditions. Thus, an elevated miR-15a level may predispose AF patients to thromboembolic events via the VEGFA pathway, and early changes in miR-15a level could be a new predictor of prognosis.

Finally, miR-542-3p and miR-421 have been shown to be related to certain cardiovascular diseases. MiR-542-3p displays antiangiogenic characteristics in endothelial cells.⁵¹ In a genetic mouse model of diabetes, miR-542-3p is down-regulated in the heart with diabetic cardiomyopathy.⁵² MiR-542-3p is significantly down-regulated in ischemic stroke and may be an important regulator in its pathological processes and development.^{53,54} Transcription factor E2F1-dependent miR-421 regulates mitochondrial fragmentation and myocardial infarction by targeting Pink1.⁵⁵ Moreover, ACE2 (angiotensin converting enzyme 2) plays critical roles in several pathologies, including cardiovascular disease. Moreover, miR-421 seems to have the ability to down-regulate ACE2, which reveals a novel potential therapeutic target for cardiovascular diseases.⁵⁶

In summary, our results suggest that miRNAs including miR-424, miR-421, miR-15a, and miR-542-3p as well as genes including *CDKI*, *CDK6*, and *CCND3* may have potential as new biomarkers for AF. These miRNAs and genes may be involved in the pathogenesis of AF via cell cycle or apoptotic pathways and may be potential therapeutic targets for AF. However, this study has some limitations. The microarray data included only a small number of patients. The results are not yet supported by confirmatory laboratory experiments. Hence, further studies are needed to verify the clinical applications of these findings.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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