Possible key microRNAs and corresponding molecular mechanisms for atrial fibrillation

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ABSTRACT

**Objective:** We aimed to find crucial microRNAs (miRNAs) associated with the development of atrial fibrillation (AF), and then try to elucidate the possible molecular mechanisms of miRNAs in AF.

Methods: The miRNA microarray, GSE68475, which included 10 right atrial appendage samples from patients with persistent AF and 11 samples from patients with normal sinus rhythm, was used for the analysis. After data preprocessing, differentially expressed miRNAs were screened using limma. Target genes of miRNAs were predicted using miRWalk2.0. We then conducted functional enrichment analyses for miRNA and target genes. Protein-protein interaction (PPI) network and module analyses for target genes were performed. Finally, transcription factors (TFs)-target genes regulatory network was predicted and constructed.

Results: Seven genes, including *CAMK2D, IGF2R, PPP2R2A, PAX6, POU3F2, YWHAE*, and *AP2A2*, were targeted by TFs. Among these seven genes, *CAMK2D* (targeted by miR-31-5p), *IGF2R* (targeted by miR-204-5p), *PAX6* (targeted by miR-223-3p), *POU3F2* (targeted by miR-204-5p), *YWHAE* (targeted by miR-31-5p), and *AP2A2* (targeted by miR-204-5p) belonged to the top 10 degree genes in the PPI network. Notably, MiR-204-5p, miR-31-5p, and miR-223-3p had more target genes. Besides, *CAMK2D* was enriched in some pathways, such as adrenergic signaling in cardiomyocytes pathway and cAMP signaling pathway. *YWHAE* was enriched in the Hippo signaling pathway.

Conclusion: miR-31-5p played a crucial role in cardiomyocytes by targeting *CAMK2D* and *YWHAE* via cAMP and Hippo signaling pathways. miR-204 was involved in the progression of AF by regulating its target genes *IGF2R*, *POU3F2*, and *AP2A2*. On the other hand, miR-223-3p functioned in AF by targeting PAX6, which was associated with the regulation of apoptosis in AF. This study would provide a theoretical basis and potential therapeutic targets for the treatment of AF. *(Anatol J Cardiol 2020; 23: 324-33)*

Keywords: atrial fibrillation, microRNAs, differentially expressed genes, pathways

Introduction

Atrial fibrillation (AF) is an abnormal heart rhythm characterized by irregular and rapid beating (1). The most common alterable risk factors for AF are valvular heart disease and high blood pressure (2). The prevalence rate of AF is approximately 1% in the general population, and goes up to 8% for individuals older than 80 years of age (3). The morbidity rate of AF is high, causing significant losses to society and individuals (4). However, the precise molecular mechanisms underlying AF have not been elucidated (5). Moreover, medical intervention for this disease is relatively limited (6). Warfarin is currently the most prescribed oral anticoagulant for the prevention of venous thromboembolism and systemic embolism in patients with AF. However, therapeutic management with warfarin is a challenge because of its narrow therapeutic index and the extensive interindividual and interethnic differences in dose requirements (7). Therefore, understanding the molecular mechanisms underlying AF may provide clues for the treatment of the disease.

MicroRNA (miRNA), a small non-coding RNA molecule, plays a role in RNA silencing and post-transcriptional gene expression regulation. Recently, some studies reported the significant role of miRNAs in regulating arrhythmogenesis and cardiac excitability in cardiac diseases, such as diabetic cardiomyopathy (8) and AF (9). Li et al. (10) identified several miRNAs and their target genes involved in the pathogenesis of AF

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by analyzing microarrays data. Harling et al. (11) indicated that increased serum miR-483-5p levels might predict the risk of postoperative AF. The miR-29a-3p may play roles in the development of AF by downregulating L-type Ca^{2+} current (12). Furthermore, the significant roles of some miRNAs (e.g., miR328, miR-26, and miR-1) in the pathogenesis of AF have been validated (9, 13, 14).

Morishima et al. (15) generated the dataset GSE68475 to profile miRNA expression in human atrial appendages, and they determined that miR-30d was essential for the electrical remodeling of AF. However, the possible molecular mechanisms of the miRNA in AF have not been elucidated. Therefore, we aimed to find crucial miRNAs associated with AF development, and then try to interpret the possible molecular mechanisms of miRNA in AF. This study purposed to provide a theoretical basis and potential therapeutic targets for the treatment of AF.

Methods

Workflow of this study

In the current study, the miRNA microarray GSE68475, which included 10 samples from patients with persistent AF and 11 samples from patients with normal sinus rhythm, was used for the analysis. After data preprocessing, differentially expressed miRNAs were screened. The target genes of miRNAs were predicted. We then conducted functional enrichment analyses for miRNA and target genes. Subsequently, protein-protein interaction (PPI) network and module analyses for target genes were performed. Finally, transcription factors (TFs)-target genes regulatory network was predicted and constructed.

Microarray data

The miRNA microarray GSE68475 (15) was downloaded from the gene expression omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) database. It included 10 right atrial appendage samples from patients with persistent AF (AF group, with a documented record of sustained AF for 6 months or longer) and 11 right atrial appendage samples from patients with normal sinus rhythm (NSR group, with no documented history of AF). Human right atrial appendage samples were collected from male patients aged 60–79 years undergoing open-heart surgery at Oita University Hospital, who were included after excluding chronic heart failure, diabetes, inflammatory diseases, endocrine diseases, metabolic diseases, kidney diseases requiring hemodialysis, history of steroid treatment, and paroxysmal AF. Clinical data of patients, including blood pressure and history of medicine, are presented in Supplementary Table 1 [came from the original data of Morishima et al. (15), who generated the dataset GSE68475]. The platform was GPL15018 Agilent-031181 Unrestricted_Human_miRNA_V16.0_Microarray 030840 (Feature Number version).

Data preprocessing

For the original miRNA data, the limma (linear models for microarray data) (16) (Version 3.10.3, http://www.bioconductor.org/packages/2.9/bioc/html/limma.html) in R software (version 3.3.2) was used to perform background correction of expression values and normalized pre-processing of expression profile data, which included conversion of original data format, provision of missing value, background correction (MAS), and data standardization. Annotation was conducted using platform annotation files, and probes not matching to miRNAs were removed.

Screening of differentially expressed miRNA

Differentially expressed miRNAs between AF group and NSR group were screened using limma (16) (Version 3.10.3, http://www.bioconductor.org/packages/2.9/bioc/html/limma.html). For each significantly differentially expressed miRNA, adj. p value <0.05 and |log fold change (FC)| >0.585. The heatmap was drawn using the R software pheatmap (17) (Version 1.0.10, https://cran.r-project.org/web/packages/pheatmap/index.html).

Prediction of miRNA-target genes

We focused on analyzing the significantly differentially expressed top 10 upregulated and downregulated miRNAs. The miRNA-target genes were predicted using miRWalk2.0 (18) (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/). In the microRNA information retrieval system, the searching criteria were as follows: minimum seed length=7, p value <0.05, and input parameters=3'UTR. The searching databases were miRWalk (http://mirwalk.umm.uni-heidelberg.de/), miRanda (http://www.microrna.org/), miRDB (http://mirdb.org), miRMap (https://mirmap.ezlab.org/), Pictar2 (http://pictar.bio.nyu.edu), RNA22 (https://cm.jefferson.edu/rna22/Interactive/), and Targetscan (http://targetscan.org). The miRNA-target results were required to appear in seven databases at the same time. The miRNA-target genes regulatory network was constructed using Cytoscape (19) (version 3.2.0, http://www.cytoscape.org/).

Functional enrichment analyses for miRNA and target genes

We performed the Kyoto Encyclopedia of Genes and Genomes (KEGG, https://www.kegg.jp/) (20) pathway enrichment analyses for miRNA using R software clusterProfiler (21). Furthermore, gene ontology (GO, http://geneontology.org/)-biological process (BP) (22) and KEGG (20) pathway enrichment analyses were conducted for genes targeted by miRNAs. Significant threshold values were p value <0.05 and count value ≥2.

Protein-protein interaction network and module analyses for target genes

The interactions between gene coding proteins were predicted using the STRING database (23) (Version 10.0, http://www.

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Supplementary Table 1. The clinical data of included patients in microarray dataset GSE68475

Experimental group	Sample ID	Age (years)	Sex (M/F)	HR (beats/min)	BPs (mm Hg)	BPd (mm Hg)	BNP (pg/mL)	LAD (mm)	LVEF (%)	Surgical procedure	Underlying heart disease	History of medicine
NSR	21	77	M	72	110	66	–	27	62	AVR, CABG	AS, AP	ARB, CA
NSR	44	79	M	73	100	62	704.8	44	67	AVR	ASR	AC, CA, DU
NSR	58	69	M	59	117	72	176.3	31	49	AVR	AR	ARB, DU
NSR	59	67	M	72	148	66	92.9	48	55	Bentall	AAE	None
NSR	98	68	M	65	108	68	21.8	34	76	AAR, left vertebral artery reconstruction	TAA, aberrant left vertebral artery	ARB, CA, ST
NSR	116	76	M	90	109	59	963.5	55	70	DVR, TAP	MS, ASR, TR	AC, DI
NSR	122	67	M	74	90	50	251.7	34	66	AVR	AS	None
NSR	128	66	M	71	129	78	394.6	44	72	AAR, CABG	TAA, AP	ARB, CA, BB, ST
NSR	144	74	M	65	136	60	31.2	38	80	MVP	MR	ARB, DU, ST
NSR	174	74	M	49	129	59	48.7	25	73	AAR, CABG	TAA, AP	ARB, CA, ST
NSR	187	78	M	69	119	47	103.5	30	64	AVR	AS	AL, Asp, NO
AF	38	71	M	53	99	46	98.3	47	51	AVR, maze	AR	AC, AN
AF	82	71	M	65	103	63	208.9	48	69	MVP, maze	MR	ARB, DI
AF	106	61	M	93	137	82	237.3	56	64	MVP, maze, CABG	MR, AP	ARB
AF	129	71	M	76	121	66	–	30	67	AAR, AVR, TAP, maze	TAA, AR, MR, TR	ARB, CA, AN
AF	141	62	M	76	106	72	131.5	40	80	AAR, maze	TAA	BB, DI
AF	194	74	M	63	103	52	1412.5	45	64	DVR, TAP, maze	AR, MR, TR	AC, ARB, CA, BB, DU, Asp
AF	221	69	M	46	148	64	501.1	38	42	Maze, MVP, TAP, VSD closure	MR, TR, VSD, LA thrombus	AC, CA, DU
AF	226	72	M	70	104	60	92.6	34	64	MVP, maze	MSR	DU
AF	231	75	M	63	130	87	223.2	56	63	DVR, TAP, maze	AR, MR, TR	ARB, DU
AF	260	66	M	93	139	82	152.5	38	75	MVP, TAP, maze	AR, MR	ARB CA, AN

AAE - annulo-aortic ectasia; AAR - ascending aorta replacement; AC - angiotensin-converting enzyme inhibitor; AF - atrial fibrillation; AL - anti-aldosterone drug; AN - antiarrhythmic agent; AP - angina pectoris; AR - aortic regurgitation; ARB - angiotensin II receptor blocker; AS - aortic stenosis; Asp - aspirin; ASR - aortic stenosis and regurgitation; AVR - aortic valve replacement; BB - β-blocker; BNP - brain natriuretic peptide; BPd - diastolic blood pressure; BPs - systolic blood pressure; CA - calcium antagonist; CABG - coronary artery bypass graft; DI - digitalis; DU - diuretic; DVR - double valve replacement; HR - heart rate; LA - left atrial; LAD - left atrial diameter; LVA - left ventricular aneurysm; LVEF - left ventricular ejection fraction; MR - mitral regurgitation; MS - mitral stenosis; MVP - mitral valvuloplasty; NO - no donor; NSR - normal sinus rhythm; OMI - old myocardial infarction; SAVE - septal anterior ventricular exclusion operation; ST - statin; TAA - thoracic aortic aneurysm; TAP - tricuspid annuloplasty; TR - tricuspid regurgitation; VSD - ventricular septal defect

string-db.org/). The input gene sets were genes regulated by miRNAs, and the species were homo. PPI score was set as 0.4 (representing median confidence). The PPI network was constructed using Cytoscape (version 3.2.0).

Degree centrality was used to analyze the score of nodes in the network. The higher the node score, the more crucial the node was, and the more likely it was to be the key node.

The significantly enriched modules were analyzed using Cytoscape plug-in MCODE (24) (version 1.4.2, http://apps.cytoscape.org/apps/MCODE). The threshold value was score ≥5.

GO-BP and KEGG pathway enrichment analyses were performed for key nodes of the top 10 degree and module. The threshold value was score ≥5.

Prediction of transcription factors-target genes regulatory networks

Based on WebGestalt (25) (http://www.webgestalt.org/), TF-target enrichment was predicted for the top 10 degree and module genes using overrepresentation enrichment analysis (ORA). The TFs-target genes regulatory network was constructed using Cytoscape.

Results

Differential expression analyses

After preprocessing for miRNA data, according to sample dispersion, the samples 01, 05 in the AF group and 03, 08 in the

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NSR group were removed, and 8 AF samples and 9 NSR samples were obtained.

After screening, 49 (30 upregulated and 19 downregulated) significantly differentially expressed miRNAs were obtained. The heatmap for differentially expressed miRNAs is presented in Figure 1.

miRNA-target genes regulatory network

After the prediction through miRWalk2.0, overall 244 miRNA-target genes pairs, including 12 miRNAs (3 upregulated and
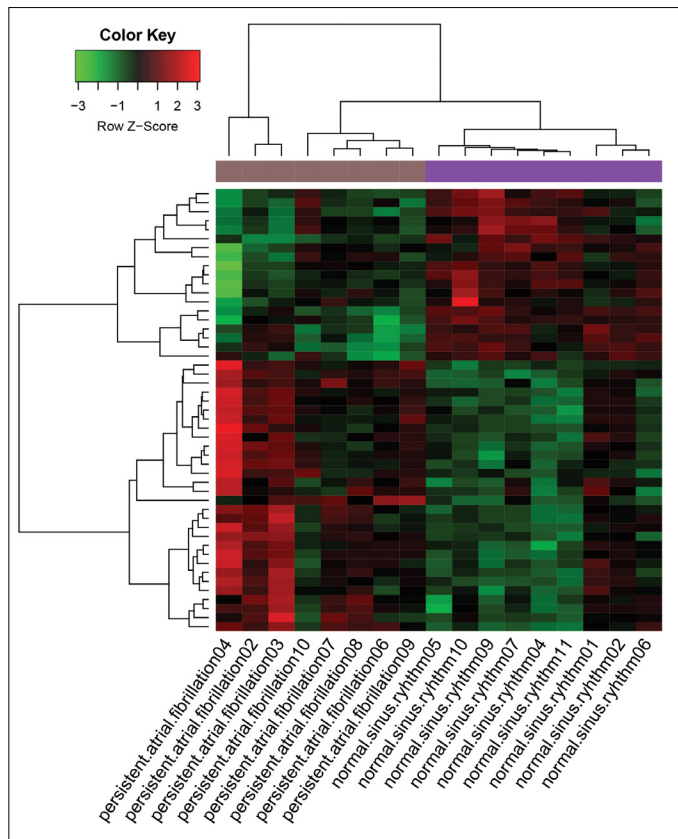

Figure 1. The heatmap for differentially expressed miRNAs

9 downregulated), were obtained. The miRNA-target genes regulatory network (244 nodes) is presented in Figure 2. For example, miR-204-5p targeted *IGF2R*, *POU3F2*, and *AP2A2*; miR-31-5p targeted *CAMK2D*, *PPP2R2A*, and *YWHAE*; and miR-223-3p targeted *PAX6*.

Functional enrichment analyses for miRNAs and target genes

KEGG pathway enrichment result for miRNA was obtained. Furthermore, target genes regulated by miRNAs were significantly enriched in 14 KEGG pathways and 40 GO-BP terms. The top 20 results are presented in Figure 3. For example, *CAMK2D* was enriched in amphetamine addiction, dopaminergic synapse, adrenergic signaling in cardiomyocytes, insulin secretion, circadian entrainment, cAMP signaling pathway, melanogenesis, oocyte meiosis, cholinergic synapse, and long-term potentiation. *PPP2R2A* was enriched in the dopaminergic synapse, adrenergic signaling in cardiomyocytes, and the Hippo signaling pathway. *YWHAE* was enriched in the Hippo signaling pathway and oocyte meiosis.

Protein-protein interaction network for target genes

The PPI network for target genes included 148 nodes and 229 relationship pairs (Fig. 4a). The module (score=5) included 5 nodes and 10 relationship pairs. The top 10 nodes with higher degrees and module nodes are presented in Table 1. The top 10 genes with higher degrees were enriched in four KEGG pathways (dopaminergic synapse, Hippo signaling pathway, adrenergic signaling in cardiomyocytes, and oocyte meiosis) and four GO-BP terms (G2/M transition of mitotic cell cycle, regulation of cellular response to heat, regulation of heart rate by cardiac conduction, and cellular response to calcium ion), and the module genes were significantly enriched in one KEGG pathway (synaptic vesicle cycle) and seven GO-BP terms (positive regulation of dendrite extension, clathrin-mediated endocytosis, calcium ion-regulated exocytosis of neurotransmitter, synaptic vesicle endocytosis, regulation of calcium ion-

Table 1. The top 10 degree nodes and module nodes in protein-protein interaction network

| Top 10 degree | | | Module | | |
Nodes	Description	Degree	Nodes	Description	Degree
UBE2I	Up-target	15	*SYT1*	Up-target	10
PAX6	Down-target	12	*AP2A2*	Down-target	9
SYT1	Up-target	10	*IGF2R*	Down-target	9
PPP1CB	Up-target	10	*FCHO2*	Down-target	7
AP2A2	Down-target	9	*SYT2*	Down-target	6
IGF2R	Down-target	9			
POU3F2	Down-target	9			
YWHAE	Down-target	8			
CAMK2D	Down-target	8			

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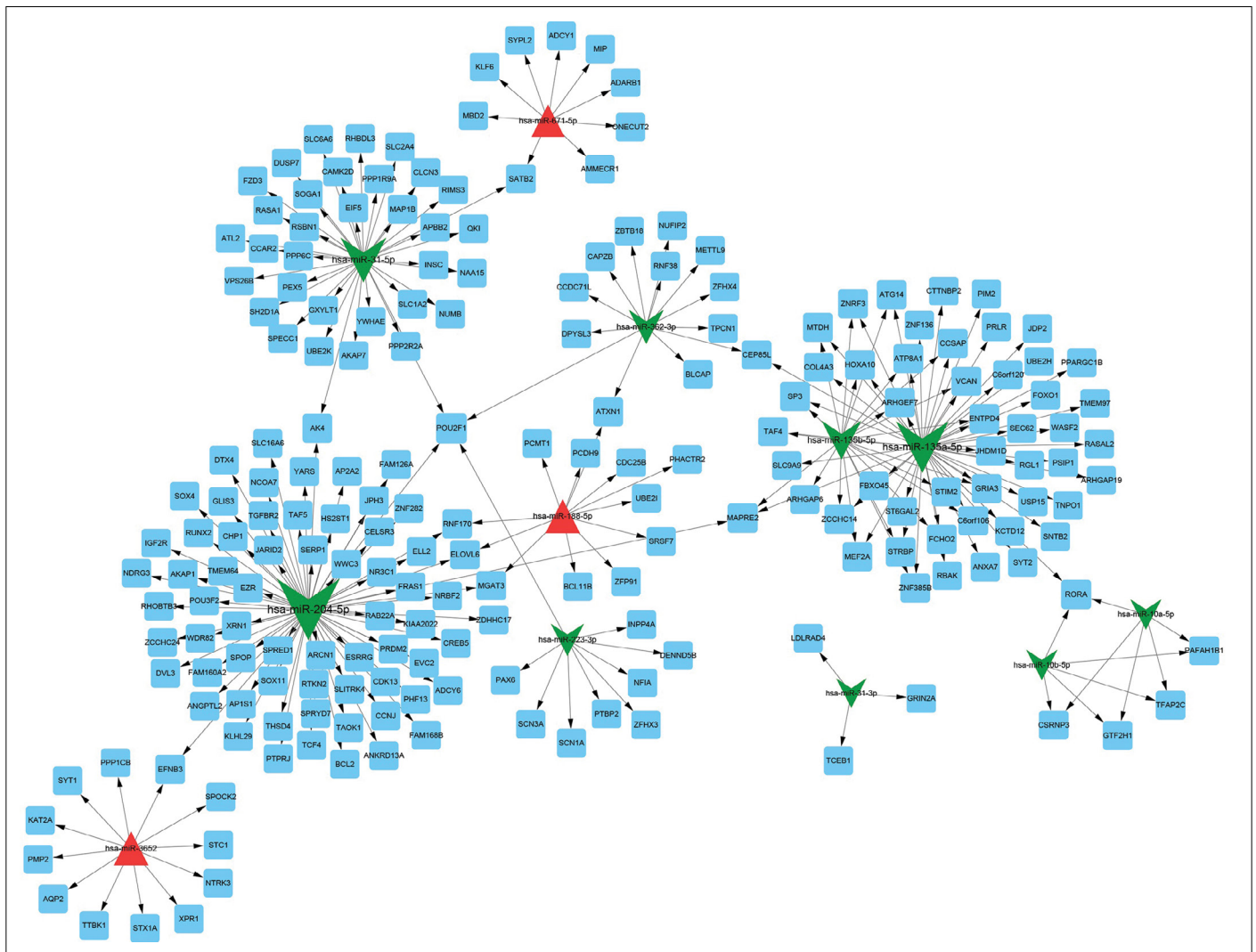DOI:10.14744/AnatolJCardiol.2020.39483

Figure 2. The miRNA-target genes regulatory network. Red triangle: upregulated miRNA; green arrow shape: downregulated miRNA; blue squares: target genes; arrowhead: regulatory direction

dependent exocytosis, neurotransmitter secretion, and vesicle fusion) (Fig. 4b).

Transcription factors-target genes regulatory network

Overall, eight TFs were predicted through WebGestalt. Totally, 22 regulatory relationship pairs were obtained through integration, of which 7 genes were regulated by downregulated miRNAs. TFs-target genes regulatory network is illustrated in Figure 5. *CAMK2D* was targeted by six TFs, *IGF2R* was targeted by four TFs, *PPP2R2A* was targeted by four TFs, *PAX6* was targeted by three TFs, *POU3F2* was targeted by two TFs, *YWHAE* was targeted by two TFs, and *AP2A2* was targeted by one TF.

Discussion

In this study, seven genes, including *CAMK2D*, *IGF2R*, *PPP2R2A*, *PAX6*, *POU3F2*, *YWHAE*, and *AP2A2*, were targeted by

TFs. Among these seven genes, *CAMK2D*, *IGF2R*, *PAX6*, *POU3F2*, *YWHAE*, and *AP2A2* belonged to the top 10 genes with higher degrees in the PPI network. Furthermore, miR-204-5p targeted *IGF2R*, *POU3F2*, and *AP2A2*; miR-31-5p targeted *CAMK2D* and *YWHAE*; and miR-223-3p targeted *PAX6*. MiR-204-5p, miR-31-5p, and miR-223-3p had more target genes. In addition, *CAMK2D* was significantly enriched in some pathways, such as adrenergic signaling in cardiomyocytes pathway and cAMP signaling pathway. *YWHAE* was significantly enriched in the Hippo signaling pathway.

Atrial electrical and structural remodeling is the foundation of AF (26), and CaMKII had been reported to mediate the processes associated with atrial contractile function and structural remodeling in AF (27). Yao et al. (28) suggested that in pathological states, *CAMK2D* was associated with heart failure and myocardial hypertrophy, and *CAMK2D* could play a role in arrhythmia. Activation of *PAX6* can result in apoptosis, premature neurogenesis, and mitotic arrest (29). Jesel et al. (30) indicated
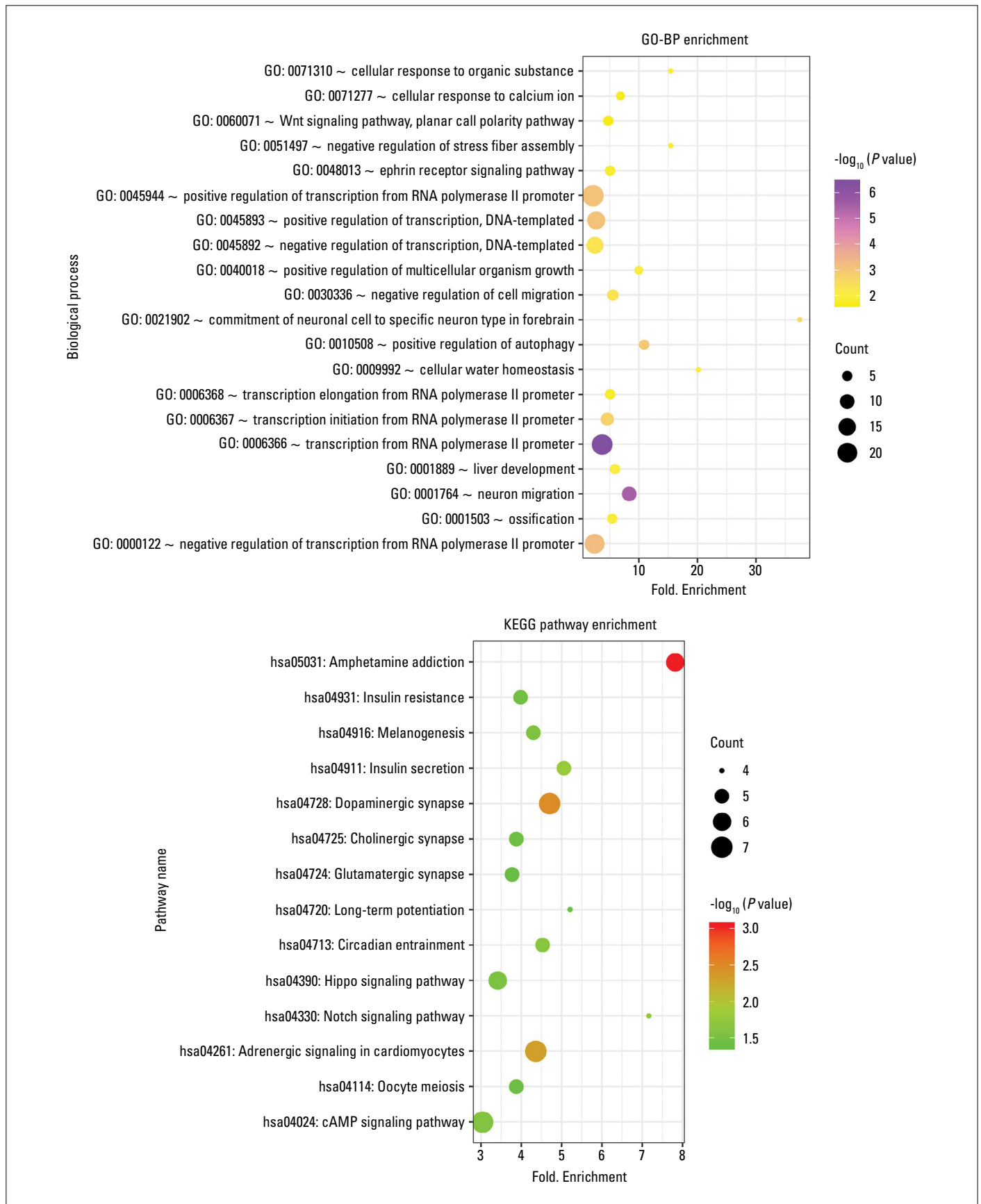
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Figure 3. The top 20 results of functional enrichment analyses for target genes

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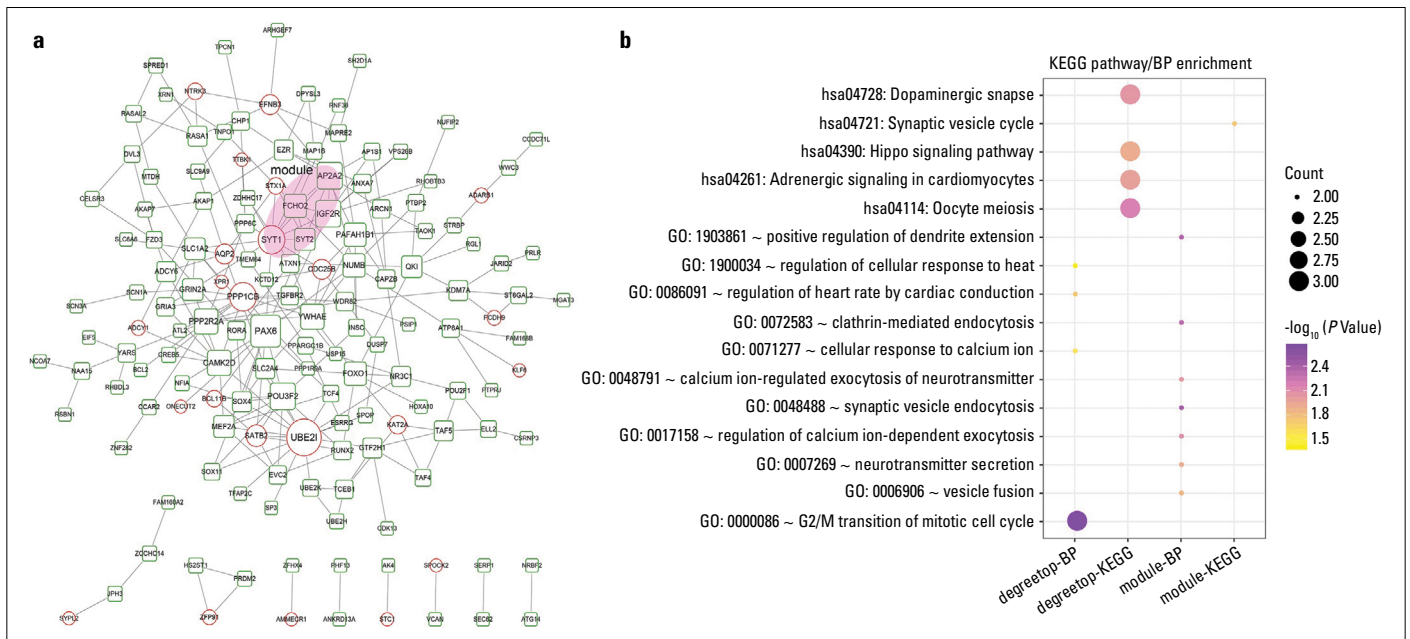DOI:10.14744/AnatolJCardiol.2020.39483

Figure 4. (a) Protein-protein interaction network for target genes; red circle: genes regulated by upregulated miRNA, green circle: genes regulated by downregulated miRNA, higher degree values indicate bigger nodes; (b) KEGG pathways and GO-BP terms for the top 10 genes with higher degrees and the module genes
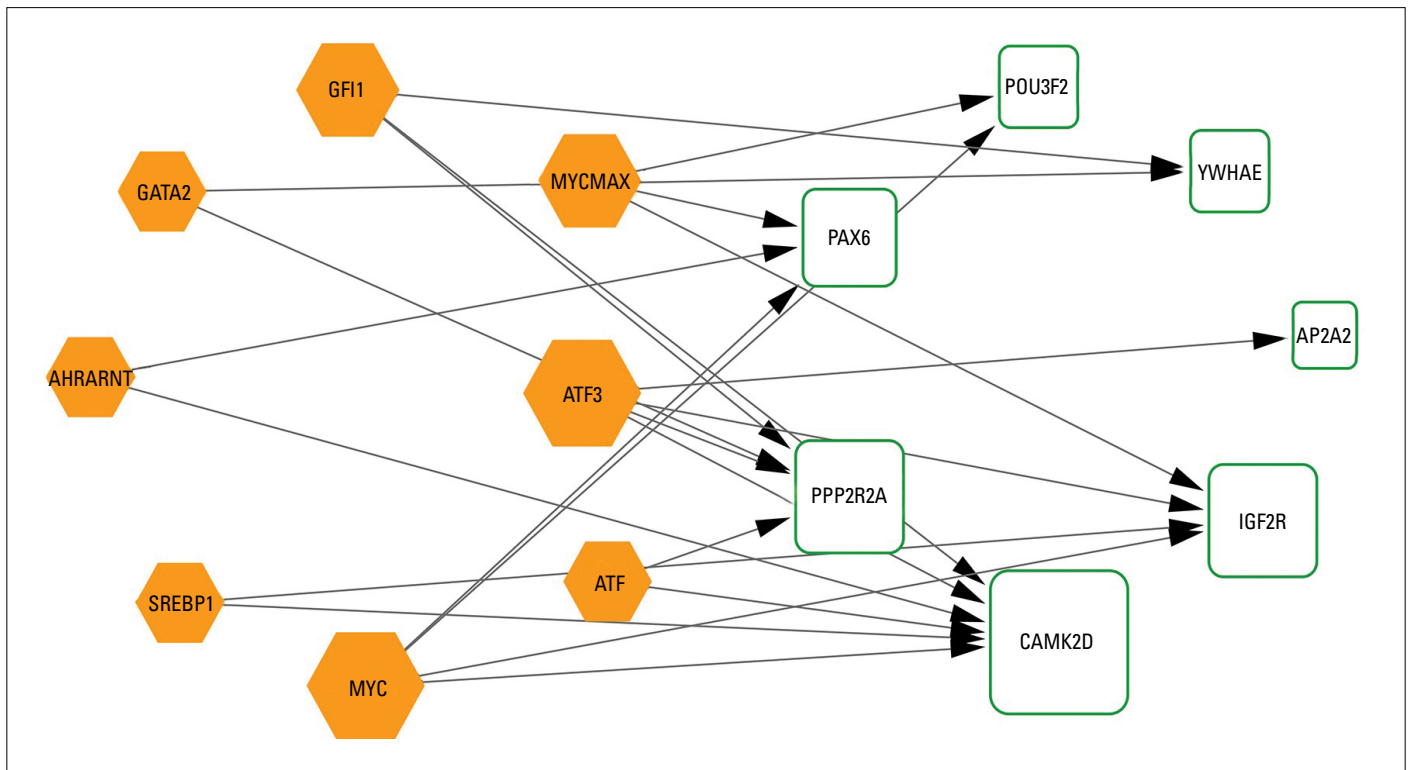

Figure 5. Transcription factors (TFs)-target genes regulatory network. Green squares: genes regulated by downregulated miRNA; Yellow hexagonal: TFs; arrowhead: regulatory direction

the significant roles of apoptosis in the progression of the pro-thrombotic state in AF. *YWHAE* (encoding 14-3-3ε) is required for ventricular morphogenesis (31). Kosaka et al. (32) reported that 14-3-3ε played a significant role in the compaction of car-

diac ventricles by regulating the cell cycle of the cardiomyocyte. This experiment demonstrated that both *in vivo* and *in vitro*, 14-3-3ε played significant roles in cardiac channel activity (33, 34). *AP2A2* is a cardiac target gene of PPAR (35), suggesting the roles

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of *AP2A2* in the heart. Chu et al. (36) indicated that the inhibition of *IGF2R* signaling pathways might be able to prevent the development of pathological cardiac hypertrophy. Co-expression of p53 and *POU4F2* may be significant for controlling pro-apoptotic gene expression in cardiomyocytes after ischemic or hypoxic insults (37). Thus, *CAMK2D, IGF2R, PAX6, POU3F2, YWHAE,* and *AP2A2*, may be crucial in AF or other diseases associated with heart, such as heart failure and arrhythmia. Taken together with our present results, we inferred that *CAMK2D, IGF2R, PAX6, POU3F2, YWHAE,* and *AP2A2* might play essential roles in the development of AF.

Furthermore, in our present study, miR-204-5p targeted *IGF2R, POU3F2,* and *AP2A2*; miR-31-5p targeted *CAMK2D* and *YWHAE*; and miR-223-3p targeted *PAX6*. MiR-204-5p, miR-31-5p, and miR-223-3p had more target genes. Reilly et al. (38) indicated that the upregulation of miR-31 might lead to atrial loss of dystrophin and nNOS in human AF. Yuan et al. (39) suggested that miR-223 might be used in the diagnosis of rheumatic heart disease, complicated with AF. Dai et al. (40) reported that the expression levels of miR-204 could be regarded as the clinical index for the diagnosis of patients with pulmonary arterial hypertension caused by congenital heart disease. Thus, we propose that miR-204-5p, miR-31-5p, and miR-223-3p may be significant miRNAs in the development of AF, and they play significant roles in AF, possibly via their target genes.

It is reported that the Hippo signaling pathway is involved in regulating the survival and proliferation of cardiomyocytes in hearts (41). IP prostanoid receptor activation inhibits cardiomyocyte hypertrophy through cAMP-dependent signaling (42), suggesting the significant role of the cAMP signaling pathway in cardiomyocyte. A previous study has reported that overexpression of cytochrome P450 family 2 subfamily C member 9 (*CYP2C9*), a gene that plays a critical role in the variability of warfarin doses, is associated with an increase in cAMP levels, which suggested that cAMP may be related to the variability in warfarin doses. Given the therapeutic action of warfarin in AF, we speculated that the cAMP signaling pathway might be implicated in AF treatment through *CYP2C9*. In the present study, *CAMK2D* was significantly enriched in adrenergic signaling in cardiomyocytes pathway and cAMP signaling pathway. *YWHAE* was significantly enriched in the Hippo signaling pathway. Thus, we infer that *CAMK2D* plays a role in AF via adrenergic signaling in cardiomyocytes pathway and cAMP signaling pathway affecting cardiomyocyte, and *YWHAE* plays a role in AF via the Hippo signaling pathway affecting cardiomyocyte.

In addition, our present study showed that *CAMK2D, IGF2R, PPP2R2A, PAX6, POU3F2, YWHAE,* and *AP2A2* were targeted by TFs. These TFs included MYC, SREBP1, ATF, ATF3, AHRARNT, MYCMAX, GATA2, and GFI1. Among these TFs, c-Myc is a component of the *VKORC1* promoter transcription factor assembly (43). Notably, *VKORC1* is considered an essential biomarker for its role in contributing to high interindividual variability in warfarin anticoagulant therapy in AF (44). Lee et al. (7) have suggested that the allelic variant of c-Myc would elicit differences in the interaction with *VKORC1*, thereby resulting in an alteration of warfarin sensitivity. GATA2 belongs to the zinc-finger transcription factor family GATA that is involved in the development of blood cells, cardiac development, and cardiomyocyte differentiation (45, 46). It has been reported that the GATA2 transcription factor is involved in the regulation of *CYP2C9*, a vital drug-metabolizing enzyme that metabolizes warfarin (47). However, we did not find any previous studies regarding the association between the development of AF and the other TFs. Hence, further studies are warranted that could explore the roles of these TFs in the development of AF.

Conclusion

In summary, miR-204-5p, miR-31-5p, and miR-223-3p could be significant miRNAs in the development of AF and could prove to be essential biomarkers for its treatment. Moreover, *CAMK2D, IGF2R, PAX6, POU3F2, YWHAE,* and *AP2A2* may be the crucial genes in the development of AF. Furthermore, miR-31-5p→*CAMK2D*→adrenergic signaling in cardiomyocytes pathway-cAMP signaling pathway→cardiomyocytes, and miR-31-5p→*YWHAE*→Hippo signaling pathway→cardiomyocytes could be the two significant molecular mechanisms in the development of AF. Notably, miR-204 was involved in the progression of AF by regulating its target genes *IGF2R, POU3F2,* and *AP2A2*. By contrast, miR-223-3p functioned in AF by targeting PAX6, which is associated with the regulation of apoptosis in AF. However, the lack of experimental verification and a small sample size were the limitations of the current study. Therefore, further studies with large sample sizes and experimental verification are warranted to explore the molecular mechanisms of AF.

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