

Circulating circRNA expression profile and its potential role in late recurrence of paroxysmal atrial fibrillation post catheter ablation

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ABSTRACT

BACKGROUND Catheter-based pulmonary vein isolation (PVI) is an effective and well-established intervention for symptomatic paroxysmal atrial fibrillation (PAF). Nevertheless, late recurrences of atrial fibrillation (LRAF) occurring during 3 to 12 months are common, and the underlying mechanisms remain elusive. Circular RNAs (circRNAs) in atrial tissue have been linked to the pathophysiological mechanisms and progression of PAF in a few studies. However, their expression patterns in peripheral blood and regulatory function in LRAF are not clear.

METHODS In the present study, the expression profile of circulating circRNAs in three paired nonvalvular PAF patients with or without LRAF was investigated by high-throughput sequencing and validated by quantitative real-time polymerase chain reaction (qRT-PCR). Bioinformatics analyses, including Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, and circRNA/miRNA regulatory network, were performed to predict the functions and potential regulatory roles of differentially expressed (DE) circRNAs.

RESULTS A total of 12,834 circRNAs, comprising 5,491 down-regulated and 7,343 up-regulated circRNAs, were found to be DE in blood samples from the two groups in peripheral blood between LRAF and non-recurrence control individuals. The most enriched GO categories in terms of molecular function, biological process, and cellular component features were catalytic activity, cellular metabolic process, and intracellular part, respectively. The KEGG enrichment study revealed that the most important metabolic process controlled by DE circRNAs is endocytosis. In the circRNA/microRNAs interaction network, four up-regulated circRNAs (*hsa_circ_0002665*, *hsa_circ_0001953*, *hsa_circ_0003831*, and *hsa_circ_0040533*) and one down-regulated circRNA (*hsa_circ_0041103*) were predicted to play potential regulatory roles in the pathogenesis of LRAF.

CONCLUSIONS This investigation discovered the expression pattern of circulating circRNAs that is indicative of PAF late recurrence, which may serve as risk markers or therapeutic targets for LRAF after PVI.

Paroxysmal atrial fibrillation (PAF) is one of the most prevalent cardiac arrhythmias in adults, which can develop into persistent atrial fibrillation after repeated episodes.^[1] It has been demonstrated that a larger PAF burden is linked to a higher risk of ischemic stroke.^[2] As a result, it is thought that maintaining stable sinus rhythm (SR) is

the optimal goal for treating symptomatic PAF. Current clinical guidelines suggest catheter-based pulmonary vein isolation (PVI) as a first-line treatment for symptomatic or drug-resistant PAF^[3,4] because it is effective in maintaining SR.^[5,6] Arrhythmia recurrence following PVI is unfortunately common, occurring in 20%–60% of cases, and necessitates recur-

rent ablation procedures or resumption of antiarrhythmic medication therapy.^[7,8]

Atrial fibrillation (AF) recurrences can be divided into two categories: early recurrence and late recurrence. Any recurrence within 3 months period post-CA is considered an early recurrence of AF (ERAF). Half of the ERAFs occur within the first 14 days after ablation,^[9] which is considered to be related to post-operative atrial tissue scarring, edema, inflammation, or increased sympathetic excitability, and 60% of ERAFs may disappear on their own as the above reactions decrease.^[10,11] A late recurrence of atrial fibrillation (LRAF) is one that occurs more than 3 months following the procedure.^[12] Previous research revealed that the main causes of LRAF might be inadequate transmural injury from radiofrequency energy or the reconnection of previously isolated pulmonary veins.^[13] However, the molecular mechanism of LRAF is not yet fully understood.

Non-coding RNAs (ncRNA), which include microRNA (miRNA), long non-coding RNA (lncRNA), and circular RNA (circRNA), are RNAs transcribed from the genome but not translated into protein. An increasing corpus of research has indicated that ncRNAs play a role in the pathophysiology processes of AF, such as aberrant calcium handling, structural or electrical remodeling, and dysregulation of neurohormones.^[14] Among them, circRNAs may be excellent candidates for diagnostic biomarkers and therapeutic targets for AF recurrence because they are endogenous, highly conserved, stable in mammalian cells, and prevalent in disease states.^[15-17] The expression of circRNAs in the atrial tissue has been implicated to be closely connected with AF,^[18] no matter in PAF or persistent AF patients,^[19] with valvular heart diseases^[20,21] or non-valvular heart diseases.^[22] Although the results are variable and unsuitable for use as clinical diagnostics, these little investigations have provided the groundwork for understanding the molecular causes of AF. We are aware of only one publication that examined the association between LRAF and left atrial appendage circRNAs following surgical ablation in individuals with valvular AF.^[23] Few studies have examined how circRNA expression patterns in peripheral blood and their roles in PAF late recurrence post-ablation. By using RNA sequencing (RNA-seq), we were able to identify the differentially expressed (DE) circRNAs in the peripheral blood of patients with LRAF and

non-recurrence PAF in this investigation. The purpose of the following step was to estimate the function and molecular pathways of dysregulated circRNAs using bioinformatic studies such as Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, and circRNAs/miRNA interaction network. Our findings offered fresh understandings of the functions of circRNA in LRAF and suggested potential biomarkers or therapy targets.

METHODS

Study Subjects

This study has been registered in the Chinese Clinical Trials Registry (ChiCTR2100049230). The Ethics Committee of the Chinese PLA General Hospital authorized all study protocols (approval ID: S2021-096-01), which were carried out in conformity with the Declaration of Helsinki. Written informed consent was obtained from all patients before participation. Before enrolling in this study, six non-valvular PAF patients who had evident symptoms or were intolerant to anti-arrhythmic drug treatment had routed CA-based PVI in our hospital from October 2020 to February 2021. Nonvalvular PAF is defined as the occurrence of intermittent AF episodes lasting fewer than seven days in individuals without signs of artificial heart valves and moderate to severe mitral stenosis. Following surgery, dabigatran (Pradaxa, 150 mg po bid) was prescribed for three months for all patients. To identify AF recurrence, we did 12-lead ECG or 24-h ambulatory ECG at every three-month outpatient visit following the procedure. In addition, if the patient has any possible symptoms of arrhythmia such as palpitations or chest tightness, we recommend him undergo a 12-lead or 24-h ambulatory ECG in a timely manner. The development of AF, atrial flutter, or atrial tachycardia (atrial tachycardia duration 30 s) more than 3 months following RFCA is typically referred to as an LRAF. In the present study, three of the participants had PAF recurrence (without taking OAT) between 3 to 12 months after ablation (LRAF group), while the other three had no recurrence episode at least a year after procedure (Control group). None of them had severe liver or kidney disease, autoimmune disease, a malignant tumor, valvular heart disease, structural heart disease other than left ventri-



cular hypertrophy, left atrial enlargement (left atrial diameter > 50 mm), or any systemic inflammatory disease. Each patient underwent a standard clinical evaluation that included physical examination, serial 12-lead ECG examination, 24-h Holter monitoring of the ECG, echocardiogram, and laboratory tests. The clinical characteristics of these patients were listed in Table 1.

Blood Sample Collection and Storage

Each patient had 4 cc of peripheral blood samples taken into a PAXgene Blood RNA Tube (Qiagen, Valencia, CA, USA) after fasting for 8 to 12 hours. The samples were then processed by spending two hours at ambient temperature, eight hours in a 4 °C refrigerator, more than 24 h in a -20 °C refrigerator, and lastly eight hours in a -80 °C refrigerator for storage prior to RNA extraction.

Total RNA Extraction and Quality Control

The three pairs of blood samples had their total RNA extracted in accordance with the manual for the PAXgene Blood RNA Kit (QIAGEN, Germany). A NanoDrop ND-2000 ultra-micro spectrophotometer (Thermo Scientific, Wilmington, DE, USA) was used to measure the RNA's concentration and purity. Standard denaturing agarose gel electrophoresis was used to check the integrity of the RNA.

CircRNA Sequencing

The RNA sample preparation and circRNAs high-throughput sequencing (circRNA-seq) were per-

formed by the Beijing Genomics Institute (BGI, Shenzhen, China) in accordance with their established protocols (<https://www.genomics.cn/>). Briefly, total RNA was used in accordance with the manufacturer's recommendations to deplete the rRNAs using the NEBNext rRNA Depletion Kit (New England Biolabs, Inc., Massachusetts, USA). Utilizing the NEBNext® Ultra™ II Directional RNA Library Prep Kit (New England Biolabs, Inc., Massachusetts, USA), RNA libraries were created. Last but not least, 150 bp paired-end reads were used for library sequencing on an Illumina Hiseq 4000 sequencer.

CircRNA-seq Data Analysis

By deleting reads with poor quality, splice contamination, and a high concentration of unknown base N, the raw data produced by sequencing was filtered. Following this, the reference genome was compared to the filtered data, and the circRNAs identifier (CIRI) and find_circ programs both predicted circRNAs. CircRNAs were quantified, and a differential expression analysis was carried out after merging the outcomes from the two software packages. The number of total reads was used to standardize and log₂ transform the raw junction reads for all samples. A fold change (FC) threshold of 2 and a *P*-value of 0.001 were used to find circRNAs that were differentially expressed (DE).

Functional Analysis of DE CircRNAs

GO and KEGG pathway enrichment analyses

Table 1 Clinical characteristics of the study patients.

	LRAF1	LRAF2	LRAF3	Control1	Control2	Control3
Sex	Male	Female	Male	Male	Female	Female
Age, yrs	64	55	40	66	76	65
Duration of PAF, months	48	104	48	26	240	36
LVEF, %	55	57	57	65	62	63
Coronary artery Disease	+	-	+	+	+	+
Hypertension	+	+	-	+	+	+
Diabetes	-	+	-	-	+	-
Dyslipidemia	-	+	-	-	+	+
Smoking	+	-	+	+	-	-
Drinking	-	-	+	+	-	-
OAT	-	-	-	-	-	-

LRAF: late recurrences of atrial fibrillation; LVEF: left ventricular ejection fraction; OAT: oral anticoagulant therapy; PAF: paroxysmal atrial fibrillation.



were carried out using the R package clusterProfiler in order to predict the functional annotation of DE circRNA host genes in LRAF.

Validation of Candidate CircRNAs Using qRT-PCR

According to the manufacturer's instructions, RNA reverse transcription was carried out using the Prime-Script RT kit (Takara Bio, Nijmegen, Japan) to create complementary DNA. SYBR-Green PCR Mix (Takara, Tokyo, Japan) in the ViiA 7 Real-time PCR system (Applied Biosystems Inc., Foster City, CA, USA) was used to find the expression of circRNAs. Table 2 depicts the PCR primer sequences. The $2^{-\Delta\Delta CT}$ approach was used to calculate the levels of circRNA expression that were normalized to the house-keeping gene β -actin. Each sample underwent three parallel studies.

Prediction of the CircRNA/miRNA Interaction Network

To establish the circRNA/miRNA interaction network, we used Arraystar's miRNA target prediction software, which is based on miRanda (<http://www.microrna.org>) and TargetScan (<http://www.targetscan.org>), to search for miRNA response elements (MREs) on the aforementioned five validated DE circRNAs. To further visualize the interactions, Cytoscape software was employed.

Statistical Analysis

Statistical analyses were conducted using SPSS software (version 21.0, SPSS, IL, USA) and Graph-Pad Prism version 8.0 (GraphPad Software, CA, USA). Significant difference between the control and the LRAF groups was compared using Student's *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

CircRNA Expression Profiling in LRAF Patients

We used high-throughput sequencing to find the dysregulated circRNAs and analyze the circRNA expression profiles in LRAF patients. A Venn diagram analysis revealed that 15,961 DE circRNAs, including 4,942 circRNAs that overlapped the two groups, were discovered in LRAF patients and non-recurrence controls (Figure 1A). 3,127 circRNAs out of these were excluded from the analysis because they had a differential FC < 2 or *q* value > 0.001. Thus, a total of 12,834 DE circRNAs, comprising 5,491 down-regulated and 7,343 up-regulated ones, were included in the following analysis. These circRNAs distribute across all the human chromosomes (Figure 1B), whose average lengths are around 2,000 nucleotides (Figure 1C). After normalization, the DE circRNA distributions were essentially the same, as seen in the box plot (Figure 1D). Exon-type

Table 2 Primers designed for qRT-PCR validation of candidate circRNAs.

Gene name	Forward and reverse primer (5' to 3')	Product length (bp)
hsa_circ_0002665	F: GAGGTAACCTCGCTATCTGGA-3 R: CTTGCCATTCACTGACATTA	145
hsa_circ_0001953	F: GCAGTCATAGATGCCAGCGG R: TGGCTCTGTGGCTGCAATT	149
hsa_circ_0003831	F: TTAATCAGTGACAACGCAGC R: ATTTGAGAAGTGCCATCACC	102
hsa_circ_0040533	F: GCCTGACCTCCTGGTTACCA R: GGCGAGAGATCTTTCGGTGA	160
hsa_circ_0041103	F: GGCAGAAGGCCTCTCTCCTG R: GCCCCACTGGTGAGACTGAA	112
β -actin	F: GAGAAAATCTGGCACCACACC R: GGATAGCACAGCCTGGATAGCAA	177

has: Homo sapiens; qRT-PCR: quantitative real-time polymerase chain reaction; F: forward; R: reverse.

circRNAs make up 69% of the up-regulated and 64% of the down-regulated circRNAs among these dysregulated circRNAs. Exon, intron, and intergenic circRNA counts were shown in Figure 1E, and the number of circRNAs from circBASE and non-circBASE in each sample was shown in Figure 1F. The scatter plot displays the differences in DE circRNA expression between the two groups (Figure 1G). Significantly aberrant circRNAs are shown in the volcano plot for LRAF (Figure 1H). A distinct

expression profile of circRNAs between the LRAF and the control group can be found using hierarchical clustering analysis (Figure 1I). Table 3 lists the top 30 circRNAs that are quite up- and down-regulated.

Bioinformatics Analysis of the DE CircRNAs

GO and KEGG pathways analysis was used to investigate the molecular functional roles of the DE circRNA-target gene. The top 30 significantly en-

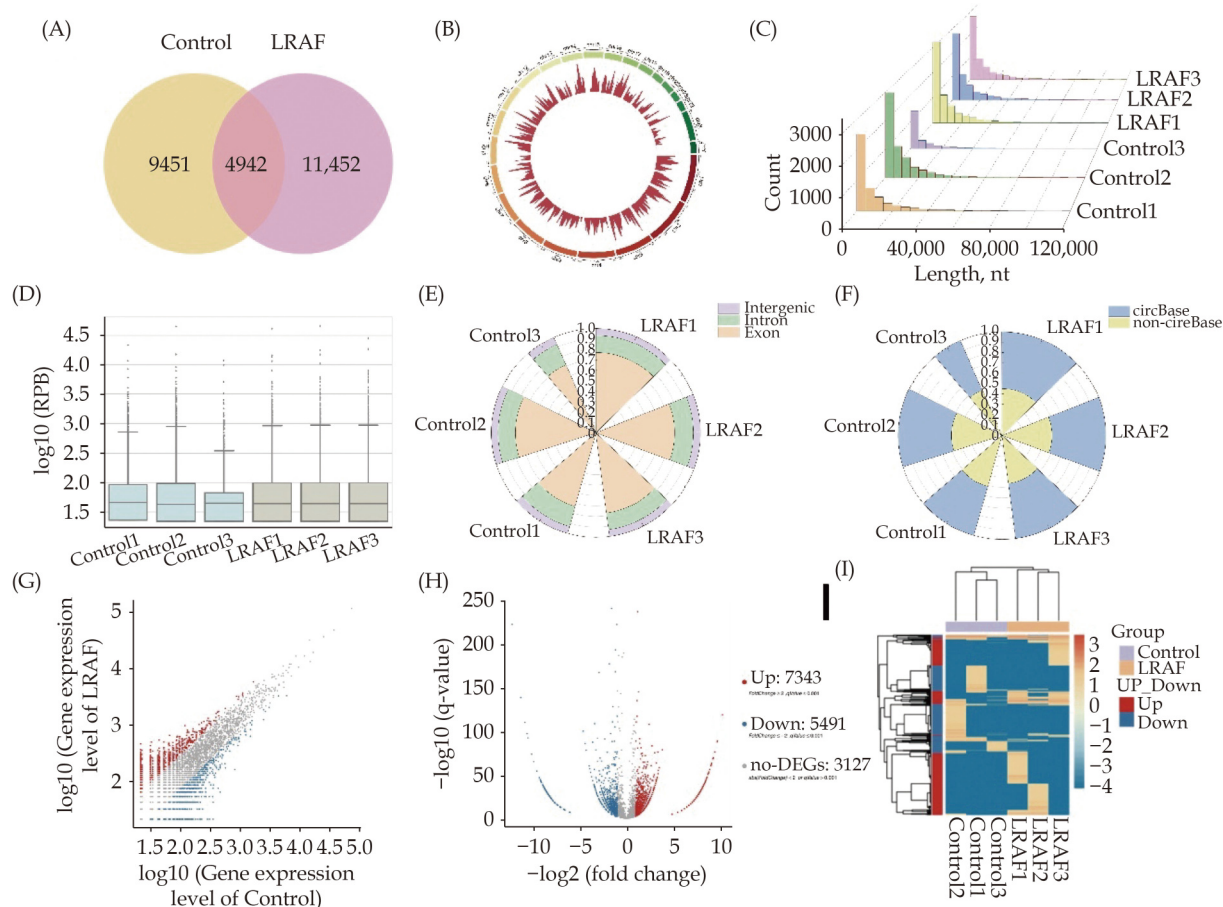


Figure 1 Expression profiles of circRNAs between LRAF and control group. (A): Venn diagram analysis presenting the number of circRNAs found in two groups. (B): Distribution of DE circRNAs on the chromosomes in two groups. The chromosome is shown by the outside circle, and the change in circRNA number on the chromosome is represented by the inner circle. (C): The distribution of all circRNAs' lengths. (D): The circRNAs distributions between samples are displayed in the box plot. (E): Statistical chart illustrating the distribution and classification of circRNAs in each sample. The number of circRNAs from exon, intron, and intergenic circRNA in each sample is depicted in the figure. (F): Statistical graph of the circRNAs number annotated or unannotated on circBase for each sample. (G): Scatter-plot expression of differential circRNAs in two groups. The logarithmic values of circRNA expression in the LRAF or control group are shown on the Y or X axis, respectively. (H): Volcano plot of DE circRNAs in LRAF. Up- and down-expressed circRNAs are represented by the red and blue points, respectively. The Y-axis displays the $-\log_{10}$ transformed significance values, while the X-axis displays the $\log_2$ transformed difference multiplier values. (I): Hierarchical clustering analysis of DE circRNAs. Each column indicates one patient blood sample, and each row represents a single circRNA. The expression levels of circRNAs were represented by a color scale. The red and blue color indicate up- and down-expressed circRNA, respectively. The representative DE circRNAs were clustered with $-\log_{10}$ values. LRAF: Late recurrence of atrial fibrillation; DE: differentially expressed.



Table 3 Top 30 up-regulated and down-regulated circRNAs in LRAF patients.

CircRNA ID	Genomic Length	log ₂ FC ^a	Regulation	P-value	Chromosome	Gene name	CircRNA type
chr5:1073748 1272395	198647	10.2600	Up	3.01E-122	chr5	10723:SLC12A7 7015:TERT	Exon
hsa_circ_0006677	14221	9.7256	Up	8.73E-92	chr1	WDR78	Exon
hsa_circ_0005921	7779	9.7053	Up	9.10E-91	chr7	-	Intergenic
hsa_circ_0001621	10637	9.4538	Up	3.51E-79	chr6	CASP8AP2	Exon
chr9:107513237 107521452	8215	9.2941	Up	1.43E-72	chr9	NIPSNAP3A	Exon
hsa_circ_0001760	26105	9.2662	Up	1.81E-71	chr7	MGAM	Intronic
hsa_circ_0001730	461	9.1181	Up	6.91E-66	chr7	EPHB4	Exon
hsa_circ_0056248	18642	9.0794	Up	1.69E-64	chr2	PTPN4	Exon
hsa_circ_0000182	21671	9.0397	Up	4.20E-63	chr1	FLVCR1	Exon
hsa_circ_0004117	17869	9.0397	Up	4.20E-63	chr10	C10orf76	Exon
chrY:22669237 22683186	13949	8.9135	Up	7.45E-59	chrY	-	Intergenic
hsa_circ_0001061	17785	8.8228	Up	5.71E-56	chr2	CCDC138	Exon
chr1:207820661 207830696	10035	8.7796	Up	1.21E-54	chr1	CR1L	Intronic
hsa_circ_0003959	2359	8.7305	Up	3.57E-53	chr2	GPR75-ASB3	Exon
chr17:76388557 76394432	5875	8.6271	Up	3.39E-50	chr17	PGS1	Exon
chr15:64984326 64984444	118	8.6271	Up	3.39E-50	chr15	OAZ2	Intronic
chr3:148300643 148310052	9409	8.5775	Up	7.94E-49	chr3	-	Intergenic
hsa_circ_0079619	18202	8.5724	Up	1.09E-48	chr7	MPP6	Exon
hsa_circ_0004581	5939	8.5157	Up	3.62E-47	chr3	USP4	Exon
chr9:3484973 3490345	5372	8.5156	Up	3.62E-47	chr9	RFX3	Intronic
hsa_circ_0006435	1231	8.5156	Up	3.62E-47	chr9	UGCG	Intronic
chr1:168034828 168037701	2873	8.5104	Up	4.99E-47	chr1	DCAF6	Exon
hsa_circ_0007907	572	8.5104	Up	4.99E-47	chrY	ZFY	Exon
hsa_circ_0029708	25305	8.4620	Up	9.02E-46	chr13	ZDHHHC20	Exon
hsa_circ_0091291	7544	8.4620	Up	9.02E-46	chrX	NUP62CL	Exon
chr10:46158028 46159290	1262	8.4565	Up	1.25E-45	chr10	ZFAND4	Exon
hsa_circ_0002290	8506	8.4565	Up	1.25E-45	chr11	RSF1	Exon
chr17:67270083 67280213	10130	8.4565	Up	1.25E-45	chr17	ABCA5	Intronic
chr10:17775834 17818169	42335	8.4565	Up	1.25E-45	chr10	TMEM236	Intergenic
hsa_circ_0002423	438	8.4565	Up	1.25E-45	chr5	-	Intergenic
chr16:223510 227461	3951	-12.2145	Down	4.81E-226	chr16	3040:HBA2 3039:HBA1	Exon
chr7:141755358 141780745	25387	-11.3035	Down	6.03E-142	chr7	MGAM	Intronic
chr7:141754554 141778808	24254	-10.8701	Down	2.28E-113	chr7	MGAM	Intronic
chr16:77204726 77225530	20804	-10.6377	Down	2.14E-100	chr16	MON1B	Intergenic
hsa_circ_0000693	41634	-10.5424	Down	1.69E-95	chr16	3687:ITGAX 3681:ITGAD	Exon
hsa_circ_0068641	697	-10.1254	Down	1.27E-76	chr3	TM4SF19	Exon
chr19:54781743 54843496	61753	-9.9043	Down	4.06E-68	chr19	LILRB2	Intergenic
chr19:2292149 2292516	367	-9.5711	Down	5.57E-57	chr19	LINGO3	Intronic
chr17:78305806 78306431	625	-9.5711	Down	5.57E-57	chr17	RNF213	Exon
hsa_circ_0052174	1376	-9.3103	Down	1.68E-49	chr19	RDH13	Exon
chr7:33644477 33682530	38053	-9.2262	Down	2.68E-47	chr7	BBS9	Intergenic
hsa_circ_0007552	992	-9.2190	Down	4.10E-47	chr12	RILPL1	Exon
chr7:102755507 102769239	13732	-9.1673	Down	8.23E-46	chr7	NAPEPLD	Exon
chr10:98469324 98469740	416	-9.1598	Down	1.27E-45	chr10	PIK3AP1	Exon

Continued

CircRNA ID	Genomic Length	log ₂ FC ^a	Regulation	P-value	Chromosome	Gene name	CircRNA type
hsa_circ_0040528	13393	-9.1446	Down	3.00E-45	chr16	WDR59	Exon
hsa_circ_0004405	7253	-9.1293	Down	7.13E-45	chr5	FAM169A	Exon
chr3:186681573 186692262	10689	-9.1059	Down	2.62E-44	chr3	ST6GAL1	Intronic
hsa_circ_0057623	12026	-9.0902	Down	6.27E-44	chr2	ANKRD44	Exon
hsa_circ_0048466	531	-9.0822	Down	9.70E-44	chr19	NFIC	Exon
hsa_circ_0006699	2646	-9.0742	Down	1.50E-43	chr19	PRR12	Exon
chr3:196050614 196054462	3848	-8.9574	Down	7.28E-41	chr3	TM4SF19	Exon
chr7:139818913 139838990	20077	-8.9222	Down	4.36E-40	chr7	KDM7A	Exon
hsa_circ_0002958	632	-8.9133	Down	6.83E-40	chr6	FARS2	Exon
hsa_circ_0029926	5743	-8.9043	Down	1.07E-39	chr13	FRY	Exon
hsa_circ_0011240	16731	-8.8953	Down	1.68E-39	chr1	PUM1	Exon
chr1:205585605 205597002	11397	-8.8770	Down	4.15E-39	chr1	ELK4	Intronic
chr17:60087911 60088594	683	-8.8770	Down	4.15E-39	chr17	MED13	Exon
chr12:112670808 112673588	2780	-8.8492	Down	1.62E-38	chr12	HECTD4	Exon
hsa_circ_0044171	1090	-8.8398	Down	2.55E-38	chr17	MAP3K14	Exon
hsa_circ_0033016	47666	-8.8303	Down	4.02E-38	chr14	ITPK1	Exon

LRAF: late recurrence of atrial fibrillation; has: Homo sapiens; ^aFC: fold change.

riched GO terms for biological process (BP), molecular function (MF), and cellular component (CC) were displayed in Figure 2. The intracellular component, catalytic activity, and cellular metabolic process, in that order, were the most enriched CC, MF, and BP activities.

According to KEGG analysis, endocytosis, Fc gamma R-mediated phagocytosis, inositol phosphate metabolism, ubiquitin-mediated proteolysis, and protein processing in the endoplasmic reticulum are the key pathways connected to host genes that are enriched. The top 30 significantly enriched pathways were shown in Figure 3.

Validation of the Candidate CircRNAs by qRT-PCR

In order to further verify the accuracy of circRNA-seq and prove the existence of DE circRNAs, we reviewed the pertinent literature and chose 5 of the most DE circRNAs (FC > 3, P-value < 0.05, high expression value of original signal) for subsequent qRT-PCR verification. While the four up-regulated circRNAs (hsa_circ_0002665, hsa_circ_0001953, hsa_circ_0003831, hsa_circ_0040533) were shown to be considerably higher in the LRAF group (Figure 4 A-D) compared to the control group, the expression level of hsa_circ_0041103 was significantly

lower (Figure 4E), which was consistent with the original circRNA-seq results (Figure 4F).

Construction of the CircRNA/miRNA Interaction Network

This paper offers annotated data of the five validated circRNAs binding to miRNAs, creating an interaction network between circRNAs and miRNAs. The five DE circRNAs are expected to interact with a total of 190 miRNAs in this network (Figure 5). Notably, the following miRNAs are interacting with several circRNAs in the network: hsa-miR-1468-3p, hsa-miR-6736-3p, hsa-miR-3194-3p, hsa-miR-5580-5p, hsa-miR-4518, hsa-miR-103a-3p, hsa-miR-107 and hsa-miR-16-5p.

DISCUSSION

To the best of our knowledge, this is the only study to explore the circRNA expression profile in peripheral blood in Chinese PAF patients with LRAF following CA-based PVI. The results of this work indicate that numerous DE circRNAs characteristic of PAF late recurrence exist in peripheral blood which could be useful indicators for risk assessment or therapeutic targets for LRAF after PVI.

The current circRNA-seq results came from three



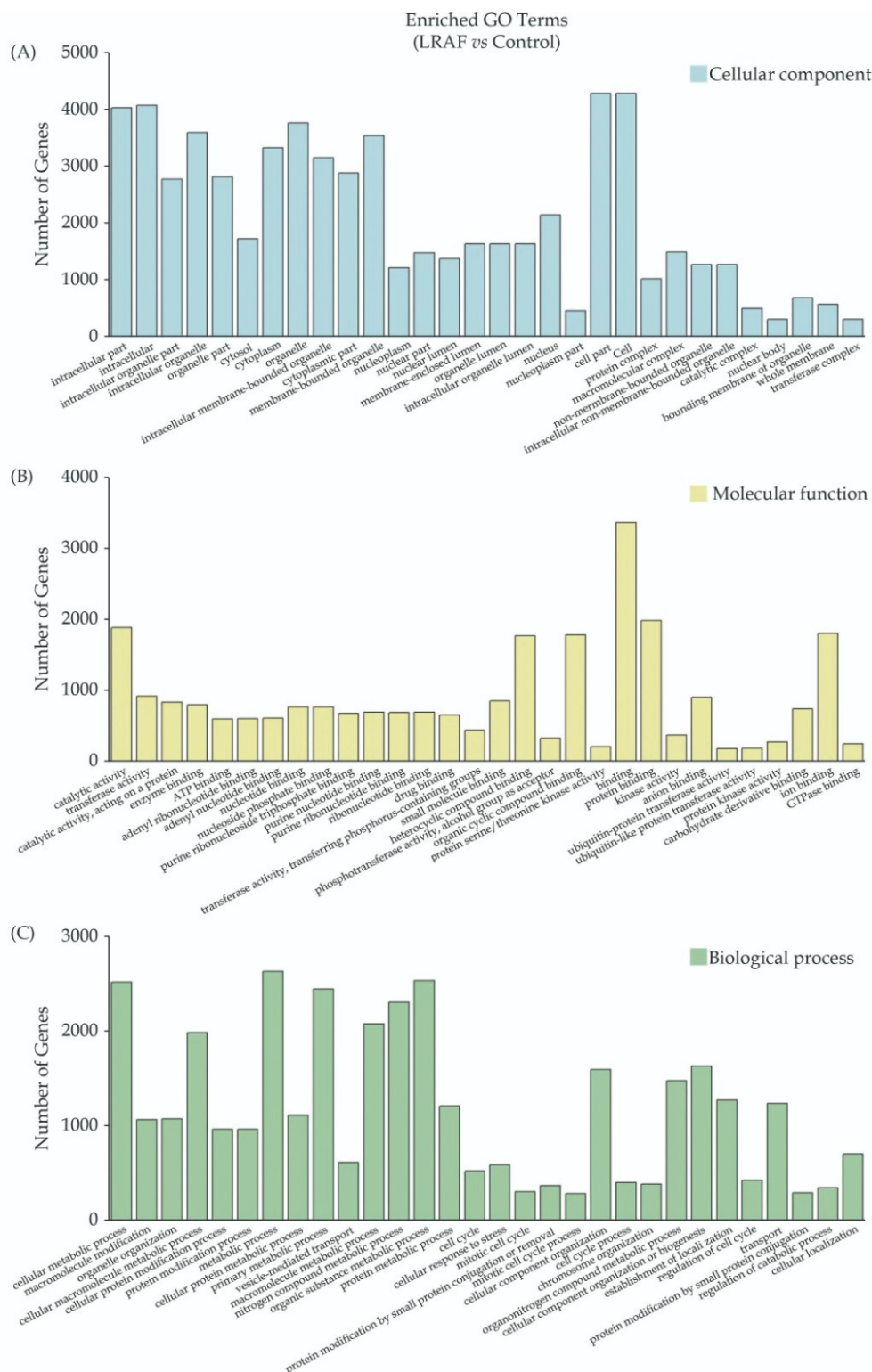


Figure 2 GO enrichment analysis of the DE circRNAs parental genes. (A): Cellular Component; (B): Molecular Function; and (C) Biological Process. X-axis represents the top 30 GO terms corresponding to differential circRNAs, and Y-axis represents number of genes (*P* value). LRAF: late recurrence of atrial fibrillation; GO: Gene Ontology; DE: differentially expressed.

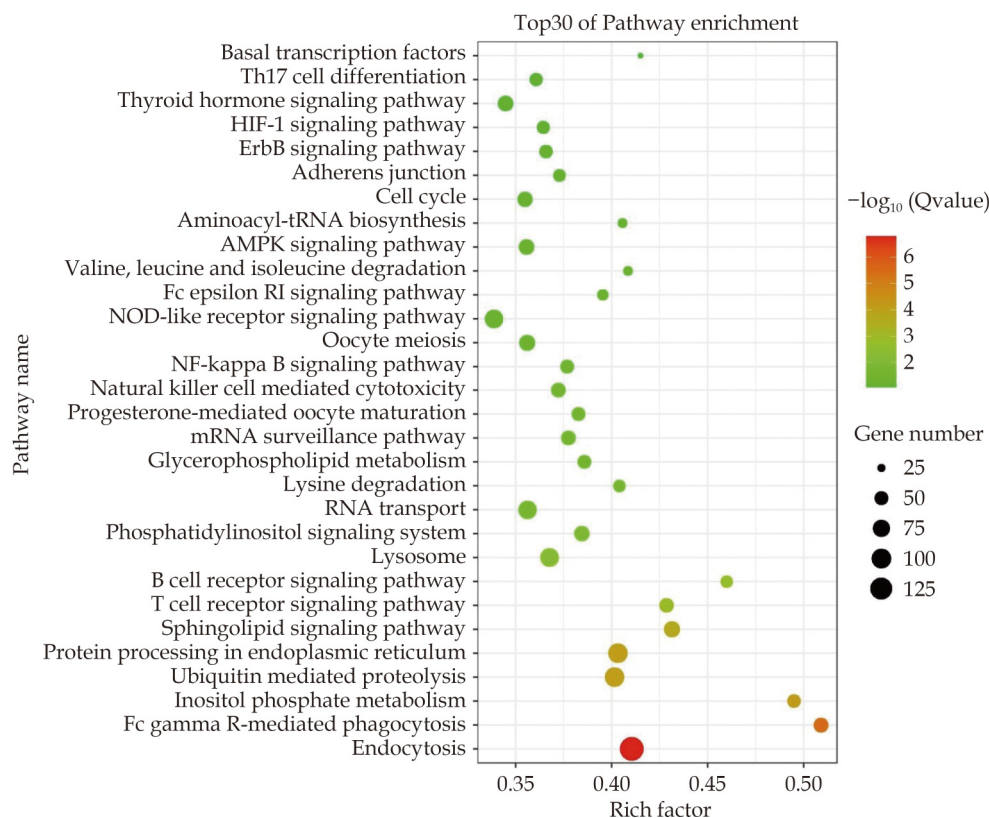


Figure 3 Bubble map of KEGG pathway enrichment analysis of the DE circRNAs parental genes. X-axis represents the rich factor value and Y-axis represents the pathway name. Size and color of each dot represents the number of enriched host genes corresponding to DE circRNAs in the pathway and $-\log_{10}(q\text{-value})$, respectively. DE: differentially expressed; KEGG: Kyoto Encyclopedia of Genes and Genomes.

paired non-valvular PAF patients who had or didn't have LRAF. To reduce the impact of catheter procedure factors on LRAF, all six patients received the CA-based PVI over a short period of time from the same skilled electrophysiologist. In comparison to the control group, LRAF patients had a total of 12,834 DE circRNAs, with 5,491 down-regulated and 7,343 up-regulated circRNAs. DE circRNAs can be found on every chromosome. More than 60% of these circRNAs were classified as exon types.

Then, using GO enrichment and KEGG pathway analysis, we looked into the probable involvement of the distinct circRNAs in LRAF post-CA. Intracellular components, catalytic activity, and cellular metabolic processes are the most abundant GO activities in terms of CC, MF, and BP, respectively. These findings revealed possible differences in the cellular and molecular components of circulating blood, as well as their function and metabolism, in patients with LRAF compared to non-relapse controls. Endocytosis, Fc gamma R-mediated phagocytosis, in-

ositol phosphate metabolism, ubiquitin-mediated proteolysis, and protein processing in the endoplasmic reticulum are among the significantly enriched pathways identified by KEGG analysis. The findings suggest that LRAF is caused by dysregulated circRNAs through a cascade of molecular and signal alterations.

We used qRT-PCR to verify five circRNAs with different multiples of > 3 and statistical differences, and the results were consistent with the sequencing results: has_circ_0002665, has_circ_0003831, has_circ_0001953, and has_circ_00040533 expression were up-regulated, while has_circ_0041103 expression was down-regulated. The DE circRNAs listed above are all AF-associated circRNAs that were recently found. Among these, has_circ_0041103 regulates FOXK1 expression via sponging miRNA-107, which has been implicated in increasing bladder tumor cell proliferation, migration, and infiltration.^[24] As a member of the FOX family, FOXK1 regulates signal transduction, cell cycle progression, and metabolism.^[25]



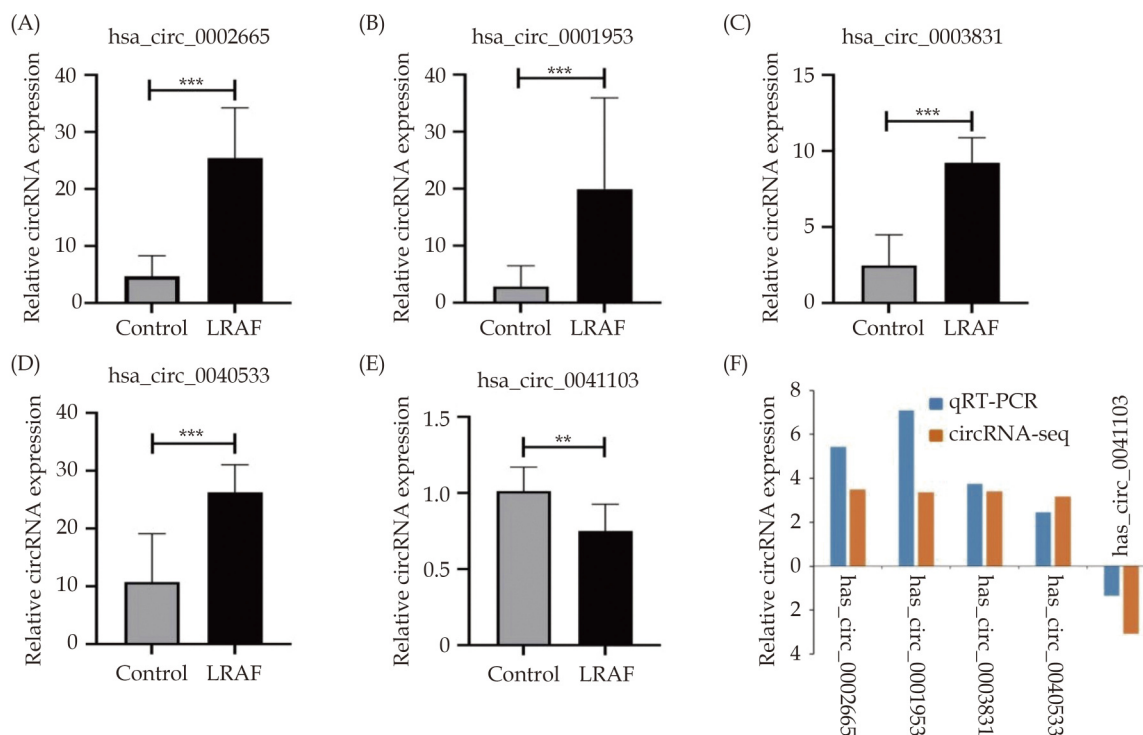


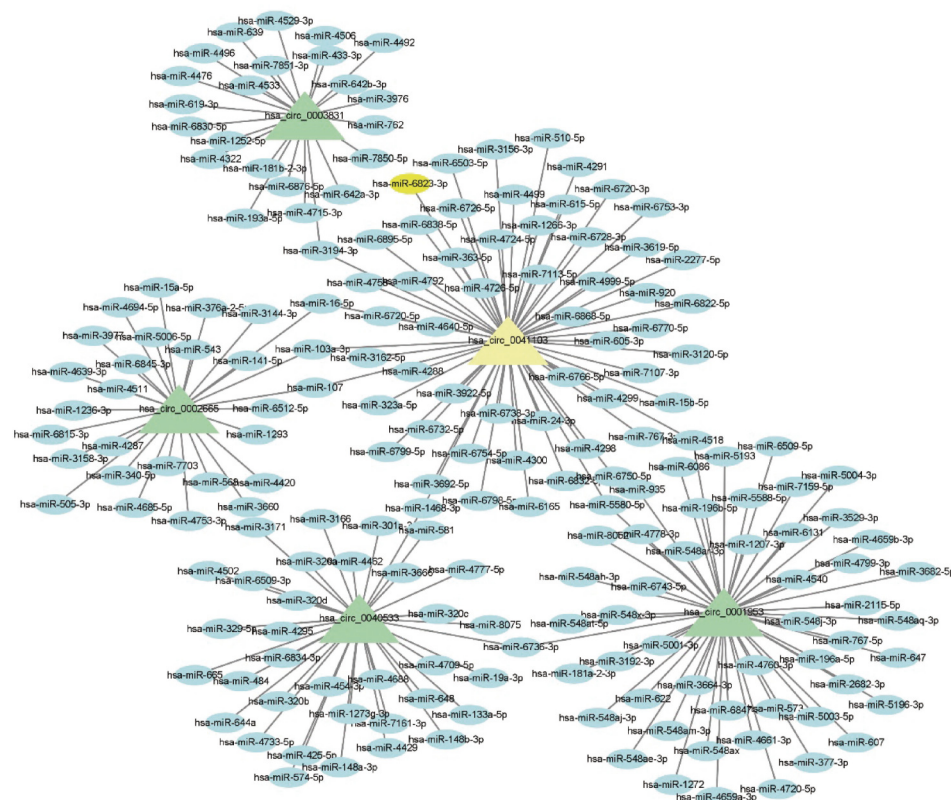
Figure 4 Validation of DE circRNAs by qRT-PCR. (A): hsa_circ_0002665; (B): hsa_circ_0003831; (C): hsa_circ_0001953; (D): hsa_circ_0040533; (E): hsa_circ_0041103; and (F): the relative expression levels of circRNAs detected by qRT-PCR and circRNA-seq. X-axis represents the DE circRNAs, while Y-axis represents the relative expression level of circRNA in LRAF compared to the control group. Blue and orange column represents qRT-PCR and circRNA-seq results, respectively. ** $P < 0.01$; *** $P < 0.001$ vs control group. circRNAs: circular RNAs; circRNA-seq: circRNAs high-throughput sequencing; DE: differentially expressed; LRAF: late recurrence of atrial fibrillation; qRT-PCR: quantitative real-time polymerase chain reaction.

FOKK1 was discovered to play a role in regulating epithelial-mesenchymal transition and interstitial lung fibrosis by Wang, *et al.*^[26] Whether FOKK1 also participates in the regulation of atrial interstitial fibrosis, and whether hsa_circ_0041103 contributes to the atrial remodeling via miRNA-107/FOKK1 axis need to be explored.

Another DE hsa_circ_0002665 is implicated in the regulation of gene transcription factor 25 (TCF25), which has been linked to arrhythmogenic cardiomyopathy pathogenesis.^[27] TCF25 functions as a transcription factor that inhibits serum response factor (SRF), a protein that has been linked to muscle development and cell proliferation. In mice, overexpression of SRF causes cardiomyopathy and cardiac hypertrophy.^[28] It's also worth checking whether hsa_circ_0002665 regulates the TCF25 gene to contribute to the mechanism of LRAF or atrial cardiomyopathy following PVI.

Several investigations have found that distinct miRNAs have a role in the pathophysiology of AF, suggesting that they could be used as therapeutic

targets. Using high-throughput sequencing, Lu, *et al.* examined the miRNA expression profile of atrial fibrillation patients and discovered that miR-328, miR-145, miR-222, miR-1, miR-162, miR-432, and miR-493b were down-regulated, whereas miR-634, miR-664, miR-9, miR-152, miR-19, miR-454, miR-146, and miR-374a were up-regulated in the blood samples of atrial fibrillation patients.^[29] Mun, *et al.* found that the expression of miR-103a, miR-320d, miR-107, miR-486, and miR-let-7b in peripheral blood exosomes in AF patients was considerably up-regulated.^[30] CircRNAs contain abundant miRNAs binding sites, which serve as miRNAs sponges in cells and then tune gene expression by trapping target miRNAs. In our study, the DE hsa_circ_0002665 in LRAF patients is predicted to be the ceRNA for miR-103a and miR-107, while hsa_circ_0040533 is predicted to be the ceRNA for miR-320d, which has been reported to play an important regulatory role in AF-related pathophysiology in the studies mentioned above. However, the exact mechanism by which these DE circRNAs contribute to the regulating of LRAF is yet to be determined.



In the present study, we took peripheral blood as a sample, rather than tissue or blood from the left atrium during electrophysiological procedure, which has been repeatedly used in previous AF studies.^[18-21] Although the DE circRNAs in tissue or blood from the left atrium might more specifically reflect the local biological response and possible mechanisms of LRAF, the samples extraction from left atrium right after transeptal puncture is an intrusive and technically difficult process, which need further ethical committee permission. It is simpler to identify and ease clinical screening of biomarkers since the expression of DE circRNAs in peripheral blood has a certain association with the expression in atrial blood. Our studies explored the expression pattern of circRNAs in peripheral blood and their role in LRAF, and it might offer fresh understanding of how circRNAs work in LRAF and suggests possible convenient biomarkers.

Several limitations should be mentioned. First,

despite the fact that the same skilled operator performed the CA-based PVI on all of the enrolled patients, the results may have been affected by the relatively small sample size. Second, the difference between the actual PAF duration and the ECG-based PAF length (such as a silent episode of PAF) may have an impact on the ablation success rate. Third, because this study only involved non-valvular PAF patients, patients with persistent atrial fibrillation or valvular disease cannot extrapolate its findings. Fourth, some of our selected PAF patients had a wide range of AF durations, which could have impacted the data's impartiality. However, it is crucial to note that the length of PAF history does not always indicate the magnitude of AF burden, which has recently been identified as an even more important factor that impacts the prognosis and efficiency of therapy for AF. Unfortunately, there are presently no recognized methods for measuring the burden of AF, which is also one of the future research topics for AF. As a result, we will expand our sample size to allow for stratification of patients according to their

PAF duration and conduct further analyses to assess the potential impact of this factor on the outcomes of interest. Furthermore, the potential for these DE circRNAs to serve as biomarkers for PAF relapse post-PVI should be investigated in a larger, multicenter investigation in the future to enhance the findings.

Conclusions

Taken together, our circRNA-seq findings revealed that a large quantity of DE circRNAs in peripheral blood may be linked to PAF late recurrence post PVI. Additionally, the networks of interactions between five novel, DE circRNAs and microRNAs may point to fresh understandings of the regulatory processes underlying LRAF and serve as a starting point for in-depth research.

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