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Original Article

Genome-wide analysis of circular RNA expression profiles in patients with atrial fibrillation

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Abstract: Atrial fibrillation (AF) is one of the most common clinical cardiac arrhythmias. This study was done to screen differentially expressed circular RNAs (circRNAs) in human monocytes from patients with AF and healthy controls using microarray, and preliminarily explore the role of circRNAs in the development of AF. The expression of circRNAs in peripheral blood monocytes of 4 AF patients and 4 healthy donors was detected by chip technology and validated by qRT-PCR. Differentially expressed genes were screened out. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed to identify the function of differentially expressed genes and related pathways. Potential connections between circRNAs and miRNAs were explored by using Cytoscape. 120 differentially expressed circRNAs (FC≥2, P<0.05) were preliminarily screened by circRNA microarray, of which 65 were up-regulated and 55 down-regulated. All of 4 upregulated circRNAs (circRNA_0031, circRNA_1837, circRNA_5901 and circRNA_7571) and 3 out of 4 downregulated circRNAs (circRNA_5801, circRNA_7386 and circRNA_7577) were randomly confirmed by RT-PCR. GO and KEGG analysis suggested that differentially expressed circRNA-related genes are mainly involved in inflammation, immunity, and signaling transduction. CircRNA_7571, circRNA_4648, circRNA_4631 and circRNA_2875 were the first 4 circRNAs with the most binding nodes in the co-expression network. In addition, hsa-miR-328 was the highest positively correlated miRNA in the networks. Our findings demonstrated that there were differentially expressed circRNAs in human monocytes from AF patients. circRNA_7571, circRNA_4648, circRNA_4631 and circRNA_2875 were the first 4 circRNAs with the most binding nodes in the co-expression network. hsa-miR-328 was the largest node that interacted with circRNAs in the co-expression network. circRNAs-hsa-miR-328 network may play a critical role in the pathophysiology and mechanism of AF.

Keywords: circRNAs, atrial fibrillation, chip, expression profile

Introduction

Atrial fibrillation (AF) is one of the most common clinical cardiac arrhythmias. Its incidence is high and increases with age. The age-adjusted prevalence of AF is 0.60% for men and 0.37% for women. Annual incidence of AF is 0.78% for men and 0.40% for women. The lifetime risk of AF in men 40 years and older is 26% for men and 23% for women, while the incidence rate for elderly 60-74 years is as high as 8.0%-11% [1, 2]. At present, the pathogenesis of AF is mainly reflected in atrial structural remodeling, electrical remodeling, inflammation, and genes [3, 4], but the specific pathogenesis has not been clarified. Moreover, there is a lack of new biomarkers with strong specificity for the diagnosis and screening of AF.

With the rapid development of genetic information technology, a class of non-coding RNAs (ncRNAs) that does not encode proteins after transcription and was once considered as "noise" has attracted more attention [5, 6]. Research on ncRNAs associated with AF is currently focused on microRNA (miRNA) and long-chain ncRNA (lncRNA). It has been found that miRNA and lncRNA may play important roles in occurrence and development of AF by regulating the atrial structural remodeling, electrical remodeling, and neural remodeling, and can also be used as biomarkers of AF [7-9]. Circular RNAs (circRNAs) are a family of ncRNAs formed by a special splicing mechanism, which has a closed circular structure and is abundant in eukaryotic transcriptomes. Despite other putative regulatory functions, circRNAs perform as

Table 1. Primer sequences for reverse transcription polymerase chain reaction

Gene name	circbase_id	Primer sequences	Fragment (bp)
GAPDH	-	F: 5'-TCTCTGCTCCTCCCTGTTCTA-3'	177
		R: 5'-ATGAAGGGGTCGTTGATGGC-3'	
circRNA_0031	hsa_circ_0008737	F: 5'-ACUGCCCUAAGUGCUCCUUCUGG-3'	179
		R: 5'-AGAGAAGGGGCCTGAGGGCAGA-3'	
circRNA_1837	-	F: 5'-GCUGGGAUUACAGGCAUGAGCC-3'	192
		R: 5'-GGCTCACGCCTGTAATCCCAGG-3'	
circRNA_5901	hsa_circ_0001240	F: 5'-CAGUGGCCAGAGCCCUGACGUG-3'	159
		R: 5'-TGCTGCCGGGAGCATCGGCCACTG-3'	
circRNA_7571	-	F: 5'-GGUCCAGAGGGCCGTCGT-3'	165
		R: 5'-ATCCCTGTCCATCTCTGGACC-3'	
circRNA_2773	-	F: 5'-GGGGUUCCUGGGGAUGGGAUUU	163
		R: 5'-TCAAAAAGAACCCTAGGAACCCc-3'	
circRNA_5801	hsa_circ_0062426	F: 5'-UGGGUAGAGAAGGAGCUCAGAGGA-3'	181
		R: 5'-CTCTCTGCAGCCCTTTGTCTACCCA-3'	
circRNA_7386	-	F: 5'-UGAGGCCCUUGGGGCACAGUGG-3'	166
		R: 5'-ACACTTAGTGCTTACAAGGGCCTCA-3'	
circRNA_7577	hsa_circ_0006109	F: 5'-UGCCCCACCUGCUGACCACCCUC-3'	166
		R: 5'-CCCGGTGG-CGGCTTGTGGGGCT-3'	

scavengers to capture other RNA molecules. Studies have shown that circRNAs can be used as a miRNA sponge to competitively bind miRNAs, deregulate the regulation of target molecules by these miRNAs, and play an important role in transcription, post-transcription, and translation [10, 11].

Athough AF is associated with a high risk of stroke and death, few studies have performed to explore the role of circRNAs in AF. Whether circRNAs can be used as a diagnostic marker and therapeutic target has not been reported. Thus, in the current study, we evaluated the differential expression profiles of circRNAs in peripheral blood monocytes from AF patients and healthy donors by microarray. Quantitative reverse transcription PCR (qRT-PCR) for differentially expressed circRNAs was subsequently performed to validate the microarray results. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed to identify the functions of differentially expressed genes and related pathways. Furthermore, the potential connections between circRNAs and miRNAs were explored by using Cytoscape.

Materials and methods

Study population and specimen collection

This study included 4 patients with AF diagnosed in the Department of Cardiovascular Medicine of Taizhou People's Hospital in October 2019 (AF group). All of them had paroxysmal atrial fibrillation, and 4 healthy subjects who excluded AF were used as controls (control group). The diagnosis of AF was mainly based on criteria listed in the 2016 ESC Guidelines for the management of atrial fibrillation developed in collaboration with EACTS [12]. Patients with malignant tumor, acute infection, systemic immunity disease, thyroid disease, severe anemia, various organ transplantations and severe heart, lung, liver and renal insufficiency were excluded. About 10 ml of peripheral blood was drawn into ethylene diamine tetra-acetic acid (EDTA) anticoagulant tubes from each participant within 4 hours after admission. Monocytes were purified from PBMCs using Monocyte Isolation Kit II (Miltenyi Biotec, Tokyo, Japan) and frozen for analysis. This study was approved by the Ethics Committee of Taizhou People's Hospital. Written informed consent was obtained from AF patients and controls before entering this experiment.

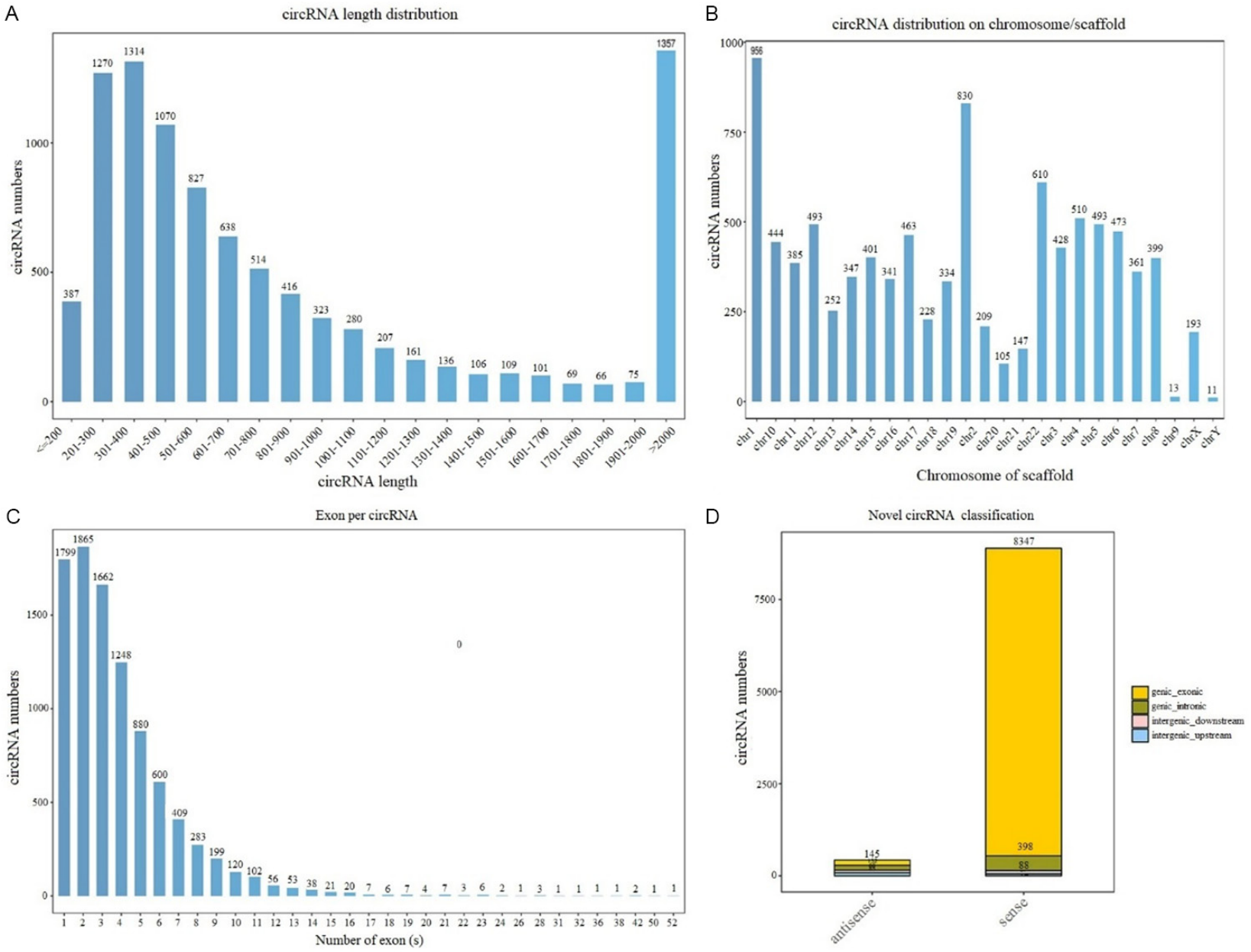

A
circRNA length distribution
circRNA numbers
circRNA length
<=200
201-300
301-400
401-500
501-600
601-700
701-800
801-900
901-1000
1001-1100
1101-1200
1201-1300
1301-1400
1401-1500
1501-1600
1601-1700
1701-1800
1801-1900
1901-2000
>2000
387
1270
1314
1070
827
638
514
416
323
280
207
161
136
106
109
101
69
66
75
1357
B
circRNA distribution on chromosome/scaffold
circRNA numbers
Chromosome of scaffold
chr1
chr10
chr11
chr12
chr13
chr14
chr15
chr16
chr17
chr18
chr19
chr2
chr20
chr21
chr22
chr3
chr4
chr5
chr6
chr7
chr8
chr9
chrX
chrY
956
444
385
493
252
347
401
341
463
228
334
830
209
105
147
610
428
510
493
473
361
399
13
193
11
C
Exon per circRNA
circRNA numbers
Number of exon (s)
1799
1865
1662
1248
880
600
409
283
199
120
102
56
53
38
21
20
7
6
7
4
7
3
6
2
1
3
1
1
1
1
2
1
1
0
D
Novel circRNA classification
circRNA numbers
antisense
sense
145
8347
398
88
genic_exonic
genic_intronic
intergenic_downstream
intergenic_upstream

Figure 1. Characteristics of 9426 circRNAs analyzed by microarray. A. Length distribution of circRNAs. B. Chromosome- or -scaffold distribution of circRNAs. C. Distribution of exon-per-circRNA. D. Category of 9426 circRNAs.

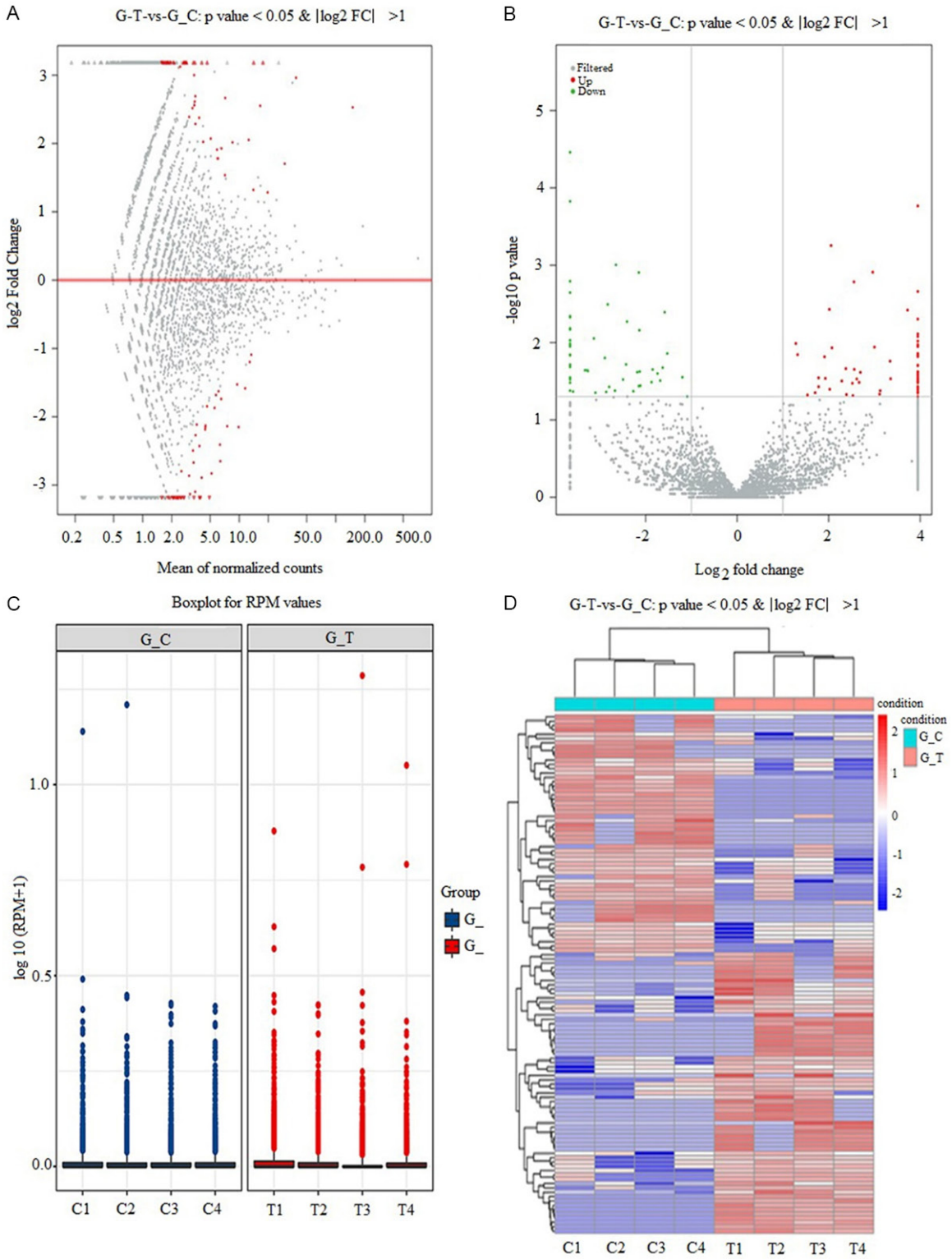


Figure 2. Differentially expressed circRNAs (fold change >2, and P<0.05) between AF group and control group. A. M-A plot for negative binomially distributed simulation data. B. Volcano plots are displayed for visualizing the differential expression of circRNAs. The red and green points in the plot represent the differentially expressed circRNAs

with statistical significance. C. Box plots show the distribution of circRNAs for the two compared samples. The distributions were nearly the same after normalization. D. Hierarchical cluster analysis of all the deregulated circRNAs.

Table 2. Upregulation of circular RNA

circRNA_id	circbase_id	circRNA_Chr	Type	gene	FoldChange	*P*-Value
circRNA_0031	hsa_circ_0008737	Chr1	sense-overlapping	CAMTA1	3.34	0.031
circRNA_0095	-	Chr1	intronic	CAPZB	8.01	0.011
circRNA_0161	-	Chr1	antisense	THEMIS2	4.14	0.001
circRNA_0312	hsa_circ_0004877	Chr1	sense-overlapping	EPS15	4.06	0.011
circRNA_0544	-	Chr1	intergenic		10.15	0.017
circRNA_0685	hsa_circ_0000160	Chr1	sense-overlapping	SUCO	2.49	0.014
circRNA_1166	-	Chr10	intronic	JMJD1C	8.73	0.042
circRNA_1402	-	Chr11	sense-overlapping	IFITM2	5.78	0.049
circRNA_1415	hsa_circ_0000274	Chr11	sense-overlapping	NUP98	5.24	0.047
circRNA_1417	-	Chr11	intronic	NUP98	3.84	0.015
circRNA_1513	hsa_circ_0000302	Chr11	sense-overlapping	SPI1	3.06	0.040
circRNA_1741	hsa_circ_0005589	Chr11	sense-overlapping	ARCN1	4.21	0.012
circRNA_1837	-	Chr12	sense-overlapping	KLRC2	9.3	0.025
circRNA_2116	hsa_circ_0004901	Chr12	sense-overlapping	APAF1	3.88	0.037
circRNA_2294	hsa_circ_0007547	Chr13	sense-overlapping	SKA3	4.18	0.011
circRNA_2371	-	Chr13	sense-overlapping	ELF1	10.23	0.029
circRNA_2482	-	Chr13	sense-overlapping	SLAIN1	3.86	0.020
circRNA_2551	-	Chr14	intergenic		3.8	0.029
circRNA_2616	hsa_circ_0008002	Chr14	sense-overlapping	POLE2	3.24	0.030
circRNA_2681	hsa_circ_0032109	Chr14	sense-overlapping	PPM1A	3.54	0.020
circRNA_3140	hsa_circ_0003916	Chr15	sense-overlapping	PIAS1	5.52	0.002
circRNA_3337	hsa_circ_0000672	Chr16	sense-overlapping	CLEC16A	3.08	0.040
circRNA_3359	hsa_circ_0002771	Chr16	sense-overlapping	PARN	3.64	0.024
circRNA_3421	hsa_circ_0008223	Chr16	sense-overlapping	XPO6	2.91	0.048
circRNA_3448	hsa_circ_0039161	Chr16	sense-overlapping	ITGAX	8.18	0.000
circRNA_4003	hsa_circ_0005347	Chr17	sense-overlapping	BPTF	5.73	0.034
circRNA_4284	hsa_circ_0008699	Chr18	exonic	ZNF516	5.63	0.008
circRNA_4314	hsa_circ_0004891	Chr19	sense-overlapping	CNN2	4.06	0.040
circRNA_4656	hsa_circ_0008847	Chr2	sense-overlapping	MBOAT2	3.76	0.015
circRNA_4657	hsa_circ_0000972	Chr2	sense-overlapping	MBOAT2	2.45	0.010
circRNA_4661	-	Chr2	sense-overlapping	MBOAT2	5.89	0.022
circRNA_4864	hsa_circ_0001006	Chr2	sense-overlapping	RTN4	3.43	0.029
circRNA_4959	-	Chr2	sense-overlapping	DYSF	3.69	0.026
circRNA_5325	-	Chr2	antisense	NOP58	3.21	0.045
circRNA_5335	hsa_circ_0003493	Chr2	sense-overlapping	CARF	3.55	0.026
circRNA_5399	hsa_circ_0058514	Chr2	sense-overlapping	AGFG1	3.89	0.014
circRNA_5664	-	Chr20	intronic	CTSZ	6.47	0.024
circRNA_5691	hsa_circ_0061286	Chr21	sense-overlapping	USP25	3.08	0.045
circRNA_5774	hsa_circ_0008021	Chr21	sense-overlapping	PDXK	13.23	0.004
circRNA_5897	hsa_circ_0008806	Chr22	sense-overlapping	CCDC134	5.19	0.022
circRNA_5901	hsa_circ_0001240	Chr22	exonic	NFAM1	6.34	0.033
circRNA_5988	hsa_circ_0001274	Chr3	sense-overlapping	PLCL2	8.66	0.046
circRNA_6087	hsa_circ_0001289	Chr3	sense-overlapping	SETD2	3.18	0.032
circRNA_6264	hsa_circ_0066959	Chr3	sense-overlapping	HCLS1	3.62	0.028

circRNA_6360	-	Chr3	sense-overlapping	PLOD2	3.69	0.015
circRNA_6574	hsa_circ_0001394	Chr4	exonic	TBC1D14	4.04	0.004
circRNA_6624	-	Chr4	exonic	TLR6	3.43	0.033
circRNA_6644	-	Chr4	sense-overlapping	RBM47	3.13	0.050
circRNA_6903	hsa_circ_0071174	Chr4	sense-overlapping	LRBA	3.18	0.032
circRNA_6955	hsa_circ_0001460	Chr4	sense-overlapping	NEIL3	3.25	0.044
circRNA_6991	-	Chr5	intergenic		5.86	0.002
circRNA_7097	hsa_circ_0072697	Chr5	sense-overlapping	PPWD1	6.69	0.008
circRNA_7571	-	Chr6	sense-overlapping	HLA-A	28.22	0.005
circRNA_7672	hsa_circ_0003700	Chr6	sense-overlapping	FBXO9	6.12	0.030
circRNA_7952	hsa_circ_0004662	Chr6	sense-overlapping	SOD2	5.68	0.011
circRNA_7964	hsa_circ_0078665	Chr6	sense-overlapping	RNASET2	3.43	0.033
circRNA_8132	hsa_circ_0001707	Chr7	intronic	ABCA13	15.44	0.010
circRNA_8233	-	Chr7	sense-overlapping	ANKIB1	3.43	0.037
circRNA_8255	hsa_circ_0007940	Chr7	sense-overlapping	ARPC1B	3.62	0.028
circRNA_8317	hsa_circ_0082096	Chr7	sense-overlapping	ZNF800	4.88	0.031
circRNA_8548	hsa_circ_0006376	Chr8	sense-overlapping	HOOK3	3.31	0.043
circRNA_8895	hsa_circ_0003945	Chr9	sense-overlapping	UBAP2	3.37	0.015
circRNA_9098	hsa_circ_0008192	Chr9	sense-overlapping	PTBP3	4.22	0.014
circRNA_9396	hsa_circ_0001947	ChrX	exonic	AFF2	7.79	0.001
circRNA_9422	hsa_circ_0008297	ChrY	sense-overlapping	DDX3Y	5.27	0.037

Table 3. Downregulation of circRNA

circRNA_id	circbase_id	circRNA_Chr	Type	gene	Fold Change	*P*-Value
circRNA_0259	hsa_circ_0009142	Chr1	sense-overlapping	CAP1	3.41	0.029
circRNA_0323	hsa_circ_0012553	Chr1	sense-overlapping	ZCCHC11	2.88	0.014
circRNA_0831	-	Chr1	sense-overlapping	LYPLAL1	4.38	0.024
circRNA_0835	hsa_circ_0004417	Chr1	sense-overlapping	LYPLAL1	9.69	0.023
circRNA_0947	hsa_circ_0002802	Chr1	sense-overlapping	ZNF124	6.37	0.042
circRNA_0995	hsa_circ_0000211	Chr10	sense-overlapping	SFMBT2	4.55	0.024
circRNA_1111	-	Chr10	sense-overlapping	CCDC7	2.94	0.028
circRNA_1292	-	Chr10	sense-overlapping	EXOSC1	3.23	0.015
circRNA_1335	hsa_circ_0000260	Chr10	sense-overlapping	SMC3	4.44	0.037
circRNA_1450	-	Chr11	sense-overlapping	SERGEF	3.47	0.010
circRNA_1496	-	Chr11	sense-overlapping	PRR5L	3.79	0.011
circRNA_1693	hsa_circ_0006208	Chr11	sense-overlapping	NPAT	7.11	0.003
circRNA_1786	hsa_circ_0002881	Chr12	sense-overlapping	KDM5A	3.08	0.019
circRNA_1787	hsa_circ_0024946	Chr12	sense-overlapping	KDM5A	3.82	0.009
circRNA_1800	-	Chr12	antisense	CACNA1C	5.31	0.005
circRNA_1834	-	Chr12	sense-overlapping	KLRC4-KLRK1	2.95	0.000
circRNA_2370	-	Chr13	exonic	ELF1	3.09	0.021
circRNA_2527	hsa_circ_0004096	Chr13	sense-overlapping	RASA3	4.44	0.001
circRNA_2683	hsa_circ_0032116	Chr14	sense-overlapping	MNAT1	3.67	0.007
circRNA_2773	-	Chr14	intergenic		12.02	0.043
circRNA_2875	-	Chr14	intergenic		3.06	0.030
circRNA_3138	-	Chr15	intronic	PIAS1	4.33	0.036
circRNA_3307	hsa_circ_0007788	Chr16	sense-overlapping	NMRAL1	10.03	0.023
circRNA_3807	-	Chr17	sense-overlapping	CCL3L3	7.42	0.016

circRNA_3830	-	Chr17	sense-overlapping	ERBB2	3.01	0.004
circRNA_4184	-	Chr18	sense-overlapping	RNF138	6.13	0.000
circRNA_4402	-	Chr19	sense-overlapping	ZNF564	3.51	0.014
circRNA_4581	hsa_circ_0003912	Chr19	exonic	DBP	4.63	0.005
circRNA_4624	-	Chr19	sense-overlapping	LILRA1	7.92	0.002
circRNA_4631	-	Chr19	sense-overlapping	KIR2DL1	8.77	0.009
circRNA_4648	-	Chr2	intergenic		4.41	0.007
circRNA_4737	-	Chr2	exonic	GTF3C2	4.23	0.011
circRNA_5440	hsa_circ_0001112	Chr2	sense-overlapping	DGKD	2.13	0.050
circRNA_5625	hsa_circ_0003998	Chr20	sense-overlapping	ARFGEF2	6.95	0.037
circRNA_5801	hsa_circ_0062426	Chr22	sense-overlapping	PPIL2	4.82	0.043
circRNA_5996	-	Chr3	intergenic		4.12	0.021
circRNA_6086	-	Chr3	sense-overlapping	SETD2	4.63	0.005
circRNA_6610	hsa_circ_0069397	Chr4	sense-overlapping	ARAP2	7.28	0.043
circRNA_6775	hsa_circ_0002782	Chr4	sense-overlapping	SLC39A8	5.38	0.019
circRNA_6810	hsa_circ_0007477	Chr4	sense-overlapping	PPA2	5.64	0.030
circRNA_7032	hsa_circ_0072380	Chr5	exonic	ZNF131	4.18	0.009
circRNA_7335	hsa_circ_0006716	Chr5	sense-overlapping	UBE2D2	3.66	0.032
circRNA_7386	-	Chr5	sense-overlapping	SGCD	4.37	0.007
circRNA_7577	hsa_circ_0006109	Chr6	sense-overlapping	C6orf136	2.29	0.028
circRNA_7599	-	Chr6	sense-overlapping	HLA-DRB1	3.16	0.042
circRNA_7797	hsa_circ_0001638	Chr6	sense-overlapping	MFSD4B	3.21	0.031
circRNA_8031	hsa_circ_0005519	Chr7	sense-overlapping	SNX13	8.57	0.045
circRNA_8108	-	Chr7	sense-overlapping	TARP	6.28	0.001
circRNA_8280	hsa_circ_0007395	Chr7	sense-overlapping	KMT2E	12.57	0.033
circRNA_8455	-	Chr8	intronic	ERI1	9.61	0.023
circRNA_8731	hsa_circ_0085438	Chr8	sense-overlapping	TBC1D31	5.03	0.002
circRNA_8841	-	Chr9	sense-overlapping	KIAA2026	3.34	0.025
circRNA_8857	hsa_circ_0008732	Chr9	sense-overlapping	BNC2	3.62	0.022
circRNA_9064	-	Chr9	sense-overlapping	NIPSNAP3A	7.75	0.000
circRNA_9326	hsa_circ_0091175	ChrX	sense-overlapping	BRWD3	3.69	0.020

Main reagents and instruments

Monocyte Isolation Kit II (Miltenyi Biotec, Tokyo, Japan), Trizol reagent (Ambion, USA), RNA purification kit (QIGEN, Germany), PrimeScript RT reagent kit with gDNA Eraser reverse transcription kit (TaKaRa, Japan), quantitative PCR detection kit (TaKaRa, Japan); gene chip detection (Agilent human lncRNA Array V2.0), ND-2000 spectrophotometer (NanoDrop, USA); high-speed refrigerated centrifuge, -80°C refrigerator, and other conventional instruments.

RNA extraction and quality control

Trizol reagent (Ambion, USA) was used to extract the total RNA in monocytes, and QIAGEN Rneasy® Mini Kit (QIGEN, Germany) was used to purify the RNA. The high purity and concentrations of the extracted RNA were tested with NanoDrop nn-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). RNA integrity was tested by using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

circRNAs microarry analysis

Sample labeling and microarray hybridization were performed by Outdo Bio-tech (Shanghai, P.R. China) by the same method as previously described [13]. Thus, double-stranded cDNA was synthesized from the qualified RNA by reverse transcription, and Cy3 fluorescently labeled cRNA was further synthesized. The fluorescence intensity of Cy3 in each sample was scanned by Axon microarray 4000B microarray scanner and the samples with RNA integrity

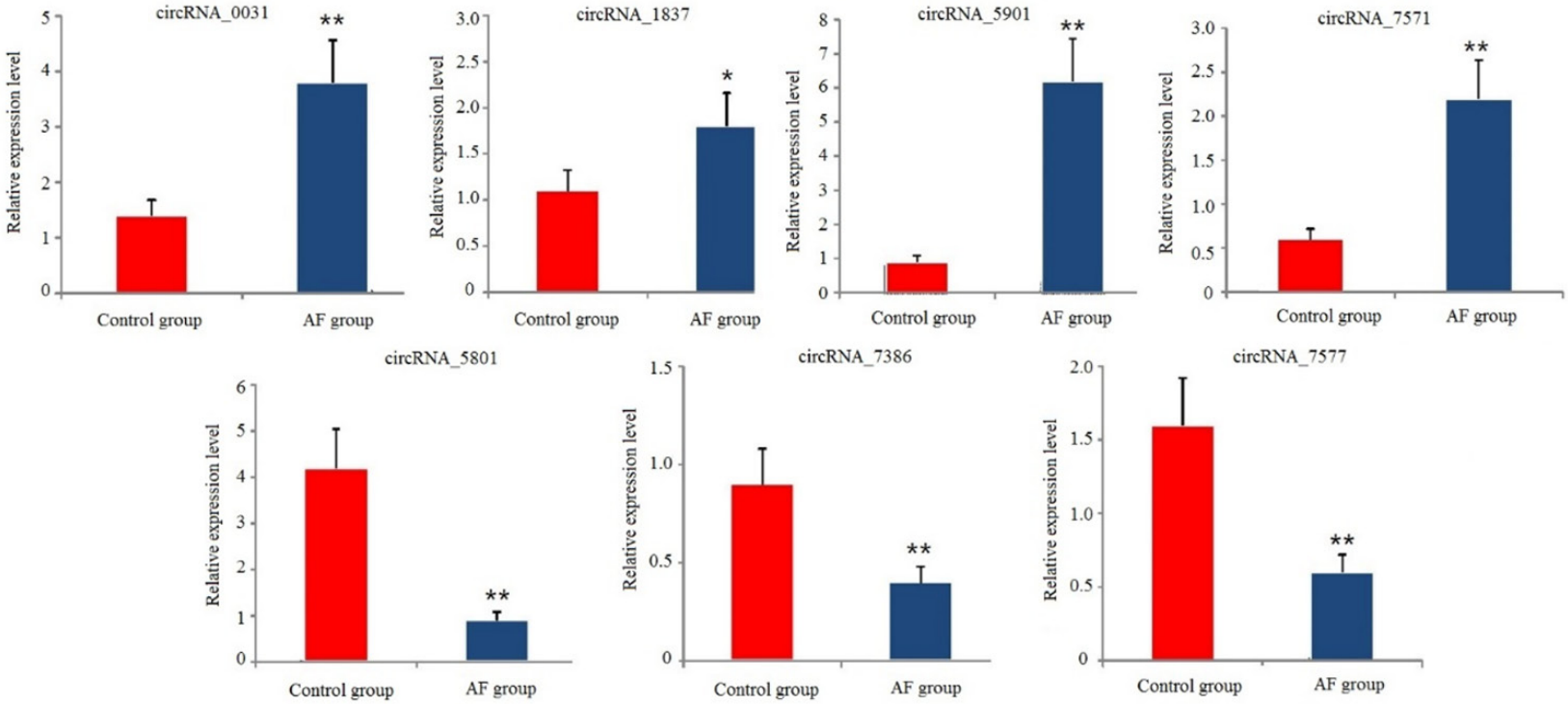
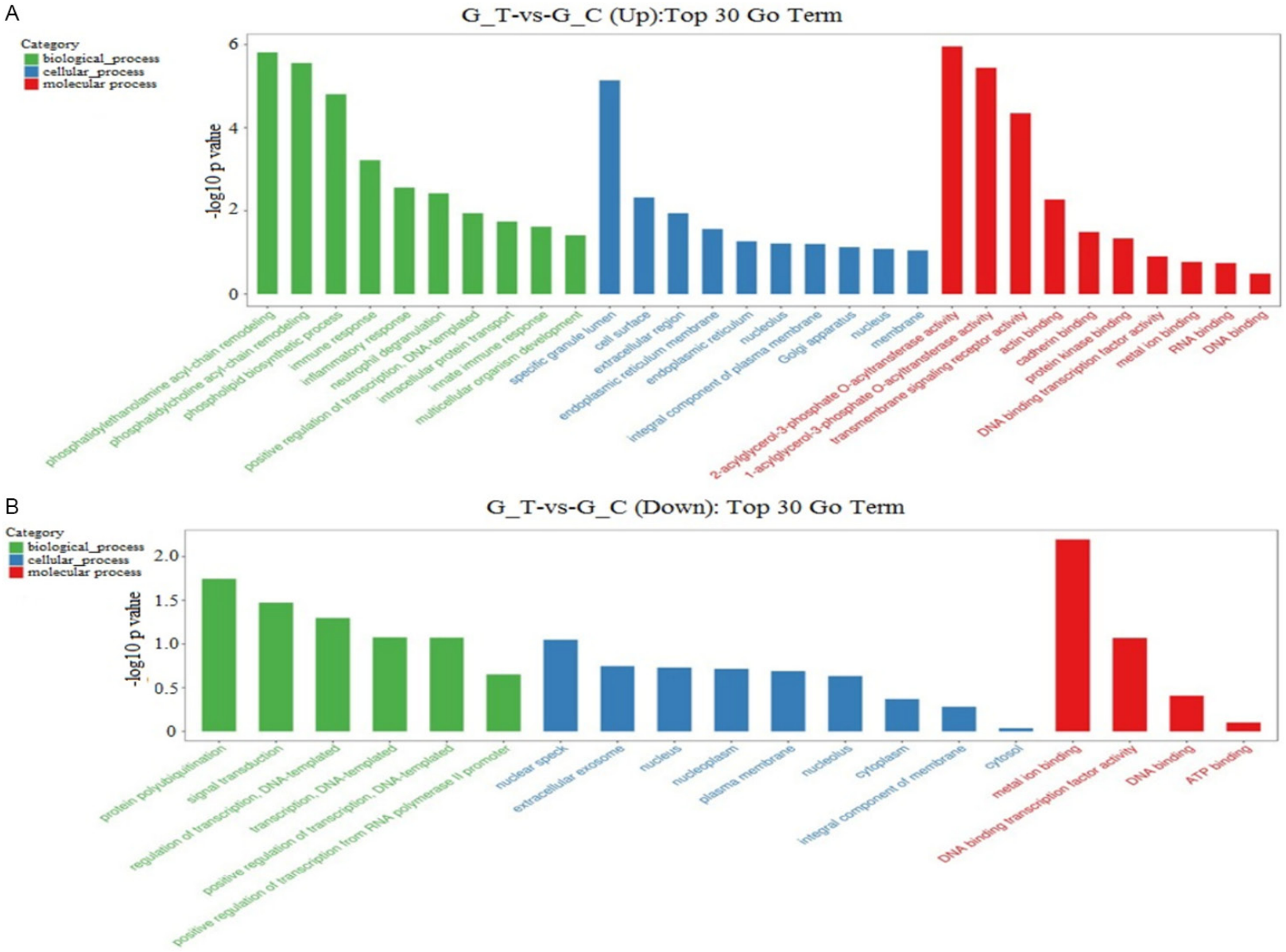


Figure 3. Quantitative reverse transcription-polymerase chain reaction analysis for validation of differentially expressed circRNAs. Compared with control group, *P<0.05 and **P<0.01.

Table 4. GO analysis of dysregulated circRNAs

id	Term	Category	ListHits	*P*-Value	Enrichment_score
GO:0036152	phosphatidylethanolamine acyl-chain remodeling	biological_process	3	1.93E-05	17.4619
GO:0036151	phosphatidylcholine acyl-chain remodeling	biological_process	3	3.45E-05	15.27917
GO:0008654	phospholipid biosynthetic process	biological_process	3	0.000187	10.18611
GO:0032436	positive regulation of proteasomal ubiquitin-dependent protein catabolic process	biological_process	4	0.000739	5.52467
GO:0006955	immune response	biological_process	4	0.000799	5.432593
GO:0010468	regulation of gene expression	biological_process	3	0.001816	5.685271
GO:0006364	rRNA processing	biological_process	3	0.002949	4.989116
GO:0045444	fat cell differentiation	biological_process	3	0.002949	4.989116
GO:0050776	regulation of immune response	biological_process	3	0.004489	4.444848
GO:0006954	inflammatory response	biological_process	4	0.004822	3.621728
GO:0035580	specific granule lumen	cellular_component	3	8.84E-05	12.22333
GO:0009986	cell surface	cellular_component	5	0.001757	3.880423
GO:0005576	extracellular region	cellular_component	6	0.013994	2.339394
GO:0031410	cytoplasmic vesicle	cellular_component	4	0.014215	2.785945
GO:0000151	ubiquitin ligase complex	cellular_component	3	0.02707	2.628674
GO:0005887	integral component of plasma membrane	cellular_component	5	0.062825	1.771498
GO:0005634	nucleus	cellular_component	38	0.064788	1.201621
GO:0005694	chromosome	cellular_component	3	0.071951	1.909896
GO:0005730	nucleolus	cellular_component	8	0.081944	1.509053
GO:0005886	plasma membrane	cellular_component	20	0.111773	1.245056
GO:0047144	2-acylglycerol-3-phosphate O-acyltransferase activity	molecular_function	3	1.39E-05	18.80513
GO:0004888	transmembrane signaling receptor activity	molecular_function	4	3.29E-05	10.5147
GO:0003841	1-acylglycerol-3-phosphate O-acyltransferase activity	molecular_function	3	4.47E-05	14.38039
GO:0001077	transcriptional activator activity, RNA polymerase II proximal promoter sequence-specific DNA binding	molecular_function	4	0.006333	3.39537
GO:0046872	metal ion binding	molecular_function	21	0.011359	1.564229
GO:0004872	receptor activity	molecular_function	3	0.013905	3.216667
GO:0018024	histone-lysine N-methyltransferase activity	molecular_function	3	0.015178	3.134188
GO:0003779	actin binding	molecular_function	5	0.019732	2.341635
GO:0008022	protein C-terminus binding	molecular_function	3	0.036288	2.396732
GO:0003714	transcription corepressor activity	molecular_function	3	0.058097	2.054342

number ≥7 were subjected to the subsequent analysis on the Illumina sequencing platform (HiSeq 2500 or other platform) and 150/125 bp paired-end reads were generated. Junction reads of each sample were counted to evaluate the relative expression of circRNAs in different samples and normalized by DESeq software. The fold-change between different samples was calculated. The statistical significance was calculated by t test. circRNAs with fold-change >2 and P<0.05 were regarded as significant differential expression.


A
G_T-vs-G_C (Up):Top 30 Go Term
Category
biological_process
cellular_process
molecular process
-log10 p value
0
2
4
6
phosphatidylethanolamine acyl-chain remodeling
phosphatidylcholine acyl-chain remodeling
phospholipid biosynthetic process
immune response
inflammatory response
neutrophil degranulation
positive regulation of transcription, DNA-templated
intracellular protein transport
innate immune response
multicellular organism development
specific granule lumen
cell surface
extracellular region
endoplasmic reticulum membrane
endoplasmic reticulum
nucleolus
integral component of plasma membrane
Golgi apparatus
nucleus
membrane
2-acylglycerol-3-phosphate O-acyltransferase activity
1-acylglycerol-3-phosphate O-acyltransferase activity
transmembrane signaling receptor activity
actin binding
cadherin binding
protein kinase binding
DNA binding transcription factor activity
metal ion binding
RNA binding
DNA binding
B
G_T-vs-G_C (Down): Top 30 Go Term
Category
biological_process
cellular_process
molecular process
-log10 p value
0
0.5
1.0
1.5
2.0
protein polyubiquitination
signal transduction
regulation of transcription, DNA-templated
transcription, DNA-templated
positive regulation of transcription, DNA-templated
positive regulation of transcription from RNA polymerase II promoter
nuclear speck
extracellular exosome
nucleus
nucleoplasm
plasma membrane
nucleolus
cytoplasm
integral component of membrane
cytosol
metal ion binding
DNA binding transcription factor activity
DNA binding
ATP binding

C

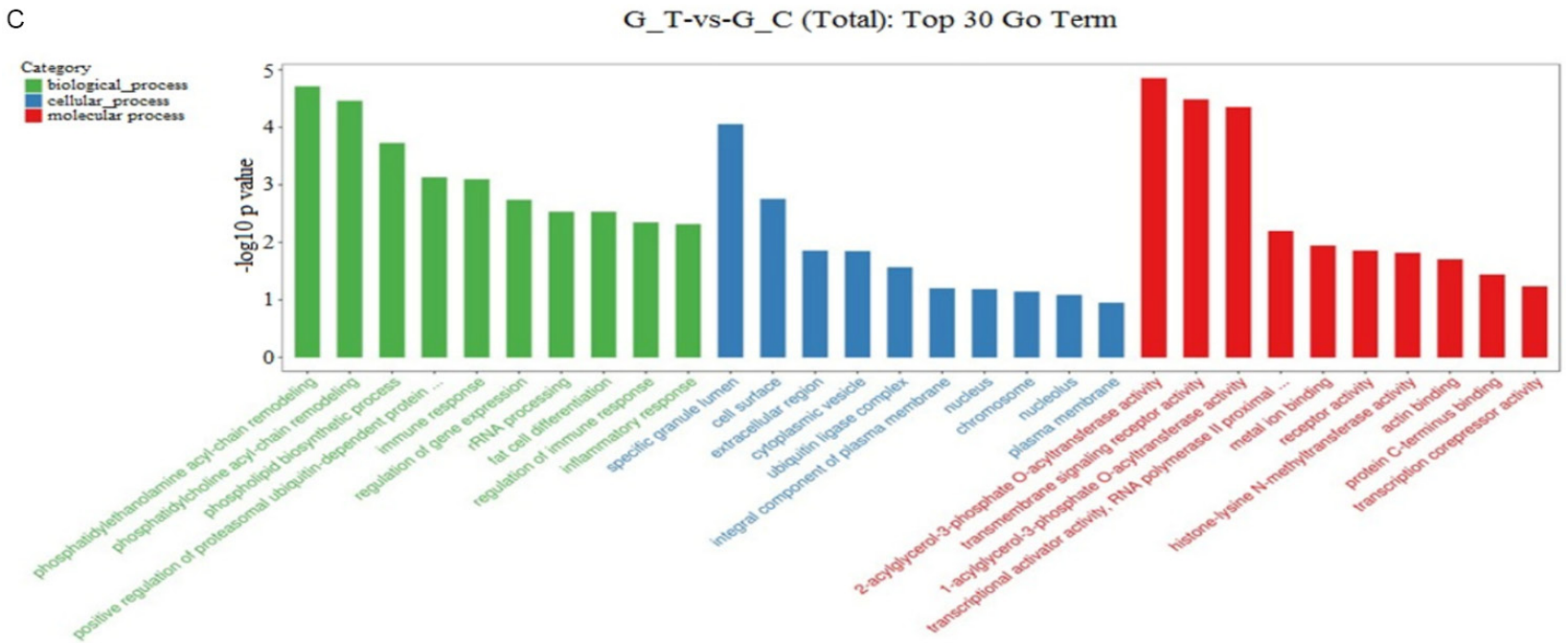
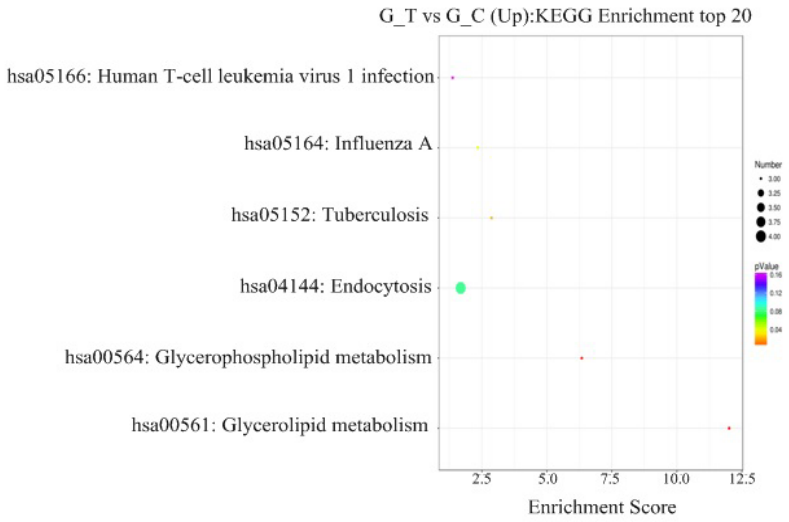
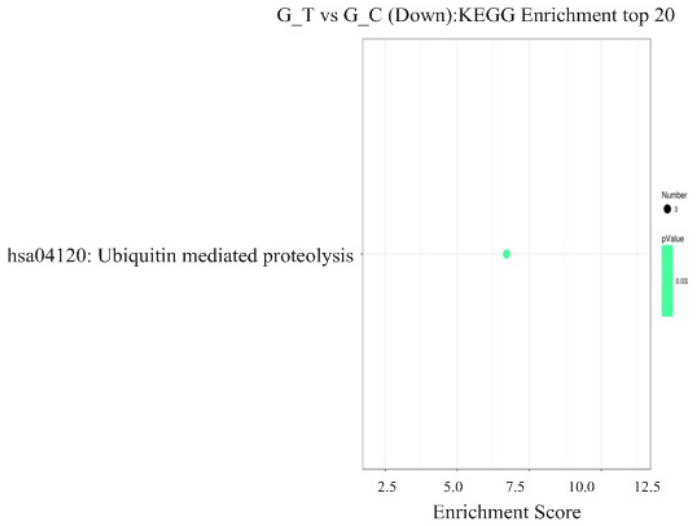


Figure 4. The results of Gene Ontology analysis. A. The top 10 neighbor coding genes of GO enrichment correspond to the upregulated circRNAs. B. The top 10 neighbor coding genes of GO enrichment correspond to the downregulated circRNAs. C. The top 10 neighbor coding genes of GO enrichment correspond to the total differentially expressed circRNAs.


G_T vs G_C (Up):KEGG Enrichment top 20
hsa05166: Human T-cell leukemia virus 1 infection
hsa05164: Influenza A
hsa05152: Tuberculosis
hsa04144: Endocytosis
hsa00564: Glycerophospholipid metabolism
hsa00561: Glycerolipid metabolism
2.5
5.0
7.5
10.0
12.5
Enrichment Score
Number
pValue


G_T vs G_C (Down):KEGG Enrichment top 20
hsa04120: Ubiquitin mediated proteolysis
2.5
5.0
7.5
10.0
12.5
Enrichment Score
Number
pValue

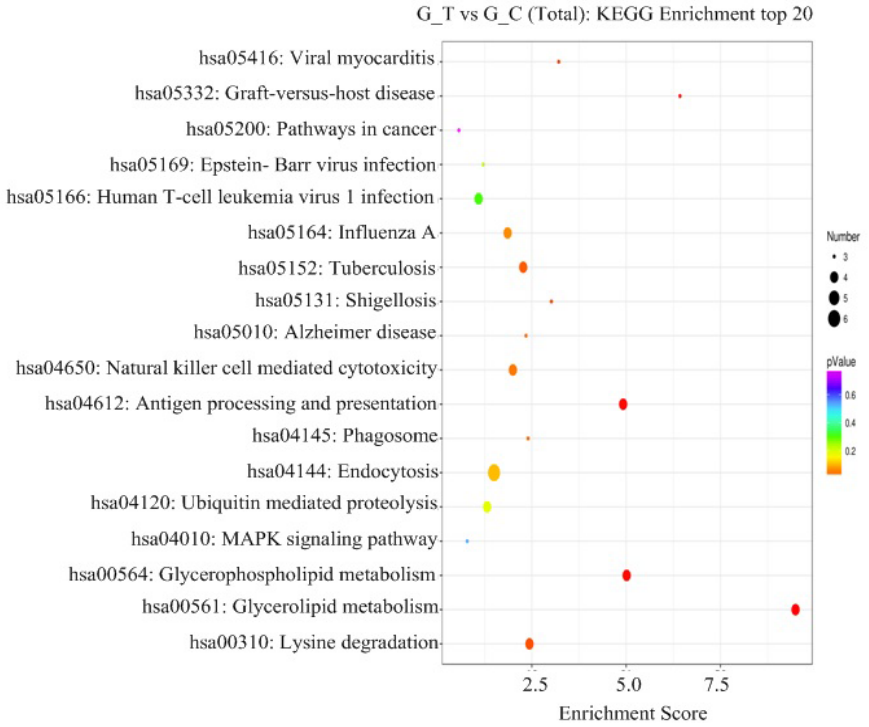

G_T vs G_C (Total): KEGG Enrichment top 20
hsa05416: Viral myocarditis
hsa05332: Graft-versus-host disease
hsa05200: Pathways in cancer
hsa05169: Epstein- Barr virus infection
hsa05166: Human T-cell leukemia virus 1 infection
hsa05164: Influenza A
hsa05152: Tuberculosis
hsa05131: Shigellosis
hsa05010: Alzheimer disease
hsa04650: Natural killer cell mediated cytotoxicity
hsa04612: Antigen processing and presentation
hsa04145: Phagosome
hsa04144: Endocytosis
hsa04120: Ubiquitin mediated proteolysis
hsa04010: MAPK signaling pathway
hsa00564: Glycerophospholipid metabolism
hsa00561: Glycerolipid metabolism
hsa00310: Lysine degradation
2.5
5.0
7.5
Enrichment Score
Number
pValue

Figure 5. Bubble map of top 20 pathway terms in KEGG enrichment analysis of dysregulated circRNAs. A. The top 20 pathway terms of the upregulated circRNAs. B. The top 20 pathway terms of the downregulated circRNAs. C. Top 20 pathway terms of the total differentially expressed circRNAs.

qRT-PCR validation of differentially expressed circRNAs

In order to confirm the results of microarray analysis, four upregulated circRNAs (circRNA_0031, circRNA_1837, circRNA_5901 and circRNA_7571) and four downregulated circRNAs (circRNA_2773, circRNA_5801, circRNA_7386 and circRNA_7577) were selected randomly for validation by qRT-PCR. Simply, 1 μl of cDNAs was added to 12.5 μl of SYBR-Green Gene Expression Master Mix (Applied Biosystems, Inc.), 10.5 μl of DEPC-treated water, and 0.5 μl of reverse and forward primers. The gene expression level of target circRNAs was normalized to the housekeeping gene GAPDH (Sangon Biotech, Shanghai, China) and calculated using the ($2^{-\Delta\Delta Ct}$) method. The primer sequences for RT-PCR are shown in **Table 1**.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis

GO analysis of differentially expressed circRNAs, including the domains of biological processes, cellular components, and molecular function were further analyzed with the Database for Annotation, Visualization, and Integrated Discovery (DAVID) Bioinformatics Resource v6.8. KEGG analysis of differentially expressed circRNAs was performed to find the pathways they participated in by the KEGG Ontology-Based Annotation System (KOBAS) 2.0.

Construction of circRNA-microRNA networks

Functional sponging activity of circRNAs over miRNAs was analyzed by the prediction of miRNA target binding sites over the sequences of differentially expressed circRNAs. Enrichment results of total differentially expressed circRNAs were sorted by *p* value, and the potential connections between circRNAs and miRNAs were further explored by using Cytoscape 3.4.0 (http://cytoscape.org/).

Results

Analysis of differentially expressed circRNA

In total, 9426 circRNAs were detected by microarray, and the relevant characteristics are shown in **Figure 1**. There were 120 differentially expressed circRNAs between AF patients and controls (fold-change >2 and P<0.05) (**Figure 2**). Among these, 65 circRNAs were up-regulated (**Table 2**) and circRNAs 55 were down-regulated (**Table 3**).

qRT-PCR validation of differentially expressed circRNAs

Four upregulated circRNAs (circRNA_0031, circRNA_1837, circRNA_5901 and circRNA_7571) and four downregulated circRNAs (circRNA_2773, circRNA_5801, circRNA_7386 and circRNA_7577) were selected randomly for qRT-PCR validation to confirm the microarray results. As a result, all of 4 upregulated circRNAs (P<0.05 or P<0.01 for circRNA_0031, circRNA_1837, circRNA_5901 and circRNA_7571, respectively) and 3 out of 4 downregulated circRNAs (P<0.05 or P<0.01 for circRNA_5801, circRNA_7386 and circRNA_7577, respectively) showed a significantly different expression (**Figure 3**), which was consistent with microarray results.

GO and KEGG analysis of dysregulated circRNAs

To explore the functions of dysregulated circRNAs in AF patients compared with normal controls, GO gene enrichment analysis and KEGG pathway analysis were underwent by using DAVID and KOBAS. GO analysis indicated that the largest enriched biologic processes include phosphatidylethanolamine acyl chain remodeling, phosphatidylcholine acyl chain remodeling and phospholipid biosynthesis. The largest enriched cell composition includes special particles, cell surfaces, and extracellular areas. The largest enriched molecular functions include: 2-acyl glycerol-3-phosphate acyltransferase activity, transmembrane signal receptor activity, 1-acyl glycerol-3-phosphate acyltransferase activity (**Table 4**; **Figure 4**). The bubble map of top 20 pathway terms in KEGG enrichment analysis is shown in **Figure 5**. Differentially expressed circRNAs and the comparison of all genes at KEGG Level 2 (including cell growth and death, transcription and development) are shown in **Figure 6**. According to

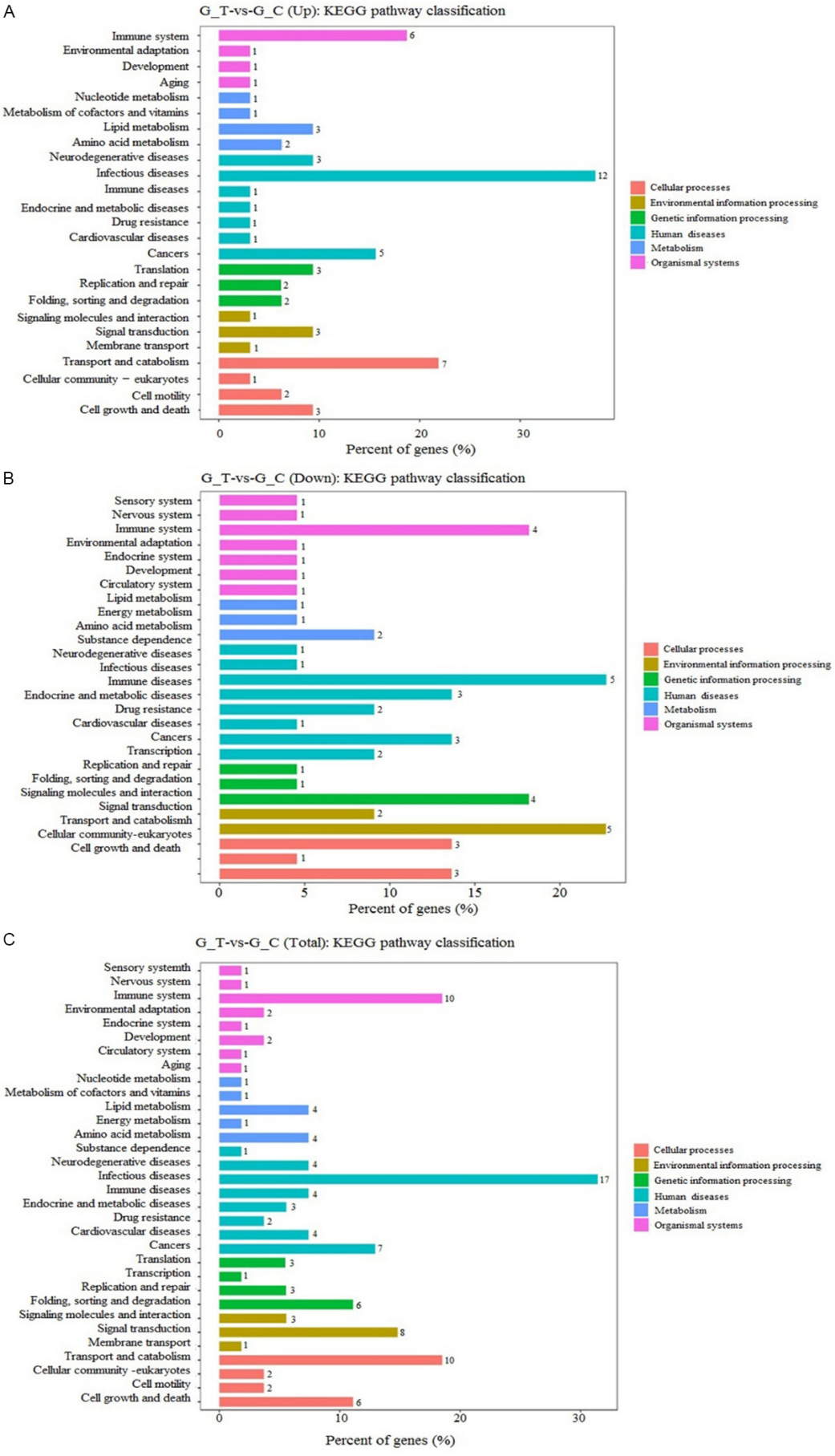

A
G_T-vs-G_C (Up): KEGG pathway classification
Immune system
Environmental adaptation
Development
Aging
Nucleotide metabolism
Metabolism of cofactors and vitamins
Lipid metabolism
Amino acid metabolism
Neurodegenerative diseases
Infectious diseases
Immune diseases
Endocrine and metabolic diseases
Drug resistance
Cardiovascular diseases
Cancers
Translation
Replication and repair
Folding, sorting and degradation
Signaling molecules and interaction
Signal transduction
Membrane transport
Transport and catabolism
Cellular community − eukaryotes
Cell motility
Cell growth and death
6
1
1
1
1
1
3
2
3
12
1
1
1
1
5
3
2
2
1
3
1
7
1
2
3
0
10
20
30
Percent of genes (%)
Cellular processes
Environmental information processing
Genetic information processing
Human diseases
Metabolism
Organismal systems
B
G_T-vs-G_C (Down): KEGG pathway classification
Sensory system
Nervous system
Immune system
Environmental adaptation
Endocrine system
Development
Circulatory system
Lipid metabolism
Energy metabolism
Amino acid metabolism
Substance dependence
Neurodegenerative diseases
Infectious diseases
Immune diseases
Endocrine and metabolic diseases
Drug resistance
Cardiovascular diseases
Cancers
Transcription
Replication and repair
Folding, sorting and degradation
Signaling molecules and interaction
Signal transduction
Transport and catabolismh
Cellular community-eukaryotes
Cell growth and death
1
1
4
1
1
1
1
1
1
2
1
1
5
3
2
1
3
2
1
1
4
2
5
3
1
3
0
5
10
15
20
Percent of genes (%)
Cellular processes
Environmental information processing
Genetic information processing
Human diseases
Metabolism
Organismal systems
C
G_T-vs-G_C (Total): KEGG pathway classification
Sensory systemth
Nervous system
Immune system
Environmental adaptation
Endocrine system
Development
Circulatory system
Aging
Nucleotide metabolism
Metabolism of cofactors and vitamins
Lipid metabolism
Energy metabolism
Amino acid metabolism
Substance dependence
Neurodegenerative diseases
Infectious diseases
Immune diseases
Endocrine and metabolic diseases
Drug resistance
Cardiovascular diseases
Cancers
Translation
Transcription
Replication and repair
Folding, sorting and degradation
Signaling molecules and interaction
Signal transduction
Membrane transport
Transport and catabolism
Cellular community -eukaryotes
Cell motility
Cell growth and death
1
1
10
2
1
2
1
1
1
1
4
1
4
1
4
17
4
3
2
4
7
3
1
3
6
3
8
1
10
2
2
6
0
10
20
30
Percent of genes (%)
Cellular processes
Environmental information processing
Genetic information processing
Human diseases
Metabolism
Organismal systems

Figure 6. Results of KEGG enrichment analysis. A. The top 30 neighbor coding genes of KEGG enrichment correspond to the upregulated circRNAs. B. The top 30 neighbor coding genes of KEGG enrichment correspond to the downregulated circRNAs. C. The top 30 neighbor coding genes of KEGG enrichment correspond to the total differentially expressed circRNAs.

KEGG results, differentially expressed circRNA-related signaling pathways are mainly involved in inflammation and the immune system.

Construction of circRNA-miRNA co-expression network

According to the significantly altered circRNAs, the miRNA binding sites for each circRNA were explored by using Cytoscape and the significance of shared miRNAs for each circRNA-miRNA pair was estimated to construct the circRNA-miRNA co-expression network. Finally, the top 300 networks of circRNA-miRNA were extracted and drawn according to the *p*-value of the enrichment results of the total dysregulated circRNAs (**Figure 7**). As shown in **Figure 7**, circRNA_7571, circRNA_4648, circRNA_4631, and circRNA_2875 are the first four circRNAS with the most binding nodes in the co-expression network, which suggests that the four circRNAs may play important roles in AF, and could be regarded as key circRNAs. In addition, hsa-miR-328 was the largest node that interacted with circRNAs in the co-expression network, which suggested that circRNA-hsa-miR-123p may play a key role in the pathogenesis of AF.

Discussion

circRNA has received attention in the field of non-coding RNAs in recent years, and was first discovered in RNA viruses. circRNA is different from linear RNA in that it does not have a 5' end cap and a 3' end tail structure. It has a covalent closed loop structure and is not easily degraded by exonuclease, so it can stably exist in tissue or body fluids. In addition, the circRNA sequence is highly conserved, with a certain timing and tissue specificity [14], which suggests that circRNA may be highly conserved among different species and play an important role in the occurrence and development of disease. It also has potential as a biomarker of disease.

AF is the arrhythmia with the highest clinical incidence, which can induce heart failure, stroke, peripheral vascular embolism and other fatal cardiovascular and cerebrovascular diseases. So far, the pathogenesis of AF is still unclear, and specific biochemical diagnostic markers are also lacking [15, 16]. In recent years, the important role of ncRNAs in ontogenetic development and the occurrence and development of various diseases has become increasingly apparent. More and more studies have shown that ncRNAs are closely related to the occurrence and maintenance of AF [17, 18]. Further studies of circRNAs in AF patients can help clarify the pathogenesis of AF and find more stable AF markers. However, the relationship between circRNAs and AF is still unclear.

In this study, microarray was used to screen the differentially expressed circRNAs in peripheral blood monocytes between 4 AF patients and 4 healthy individuals. The results showed that 9426 circRNAs were abnormally expressed in AF patients, and 120 circRNAs were differentially expressed (fold-change >2 and $P<0.05$) compared with the healthy control group, of which 65 circRNAs were up-regulated and 55 circRNAs were down-regulated, indicating that these differentially expressed circRNAs may play an important role in the pathogenesis of AF. All of 4 upregulated circRNAs (circRNA_0031, circRNA_1837, circRNA_5901 and circRNA_7571) and 3 out of 4 downregulated circRNAs (circRNA_5801, circRNA_7386 and circRNA_7577) selected randomly for qRT-PCR validation showed a significantly different expression, which was consistent with microarray results. In order to further understand the roles of differentially expressed circRNAs in AF, the bioinformatics analysis of genes involved in the differentially expressed circRNAs was conducted. GO analysis of differentially expressed circRNAs suggests that the largest enriched biological process was mainly involved in phosphatidylethanolamine acyl chain remodeling, phosphatidylcholine acyl chain remodeling, and phospholipid biosynthesis. The largest enriched cell composition was mainly involved in special particles, cell surfaces, and extracellular areas. The largest enriched molecular functions were mainly involved in 2-acyl glycerol-3-phosphate acyltransferase activity, transmembrane signal

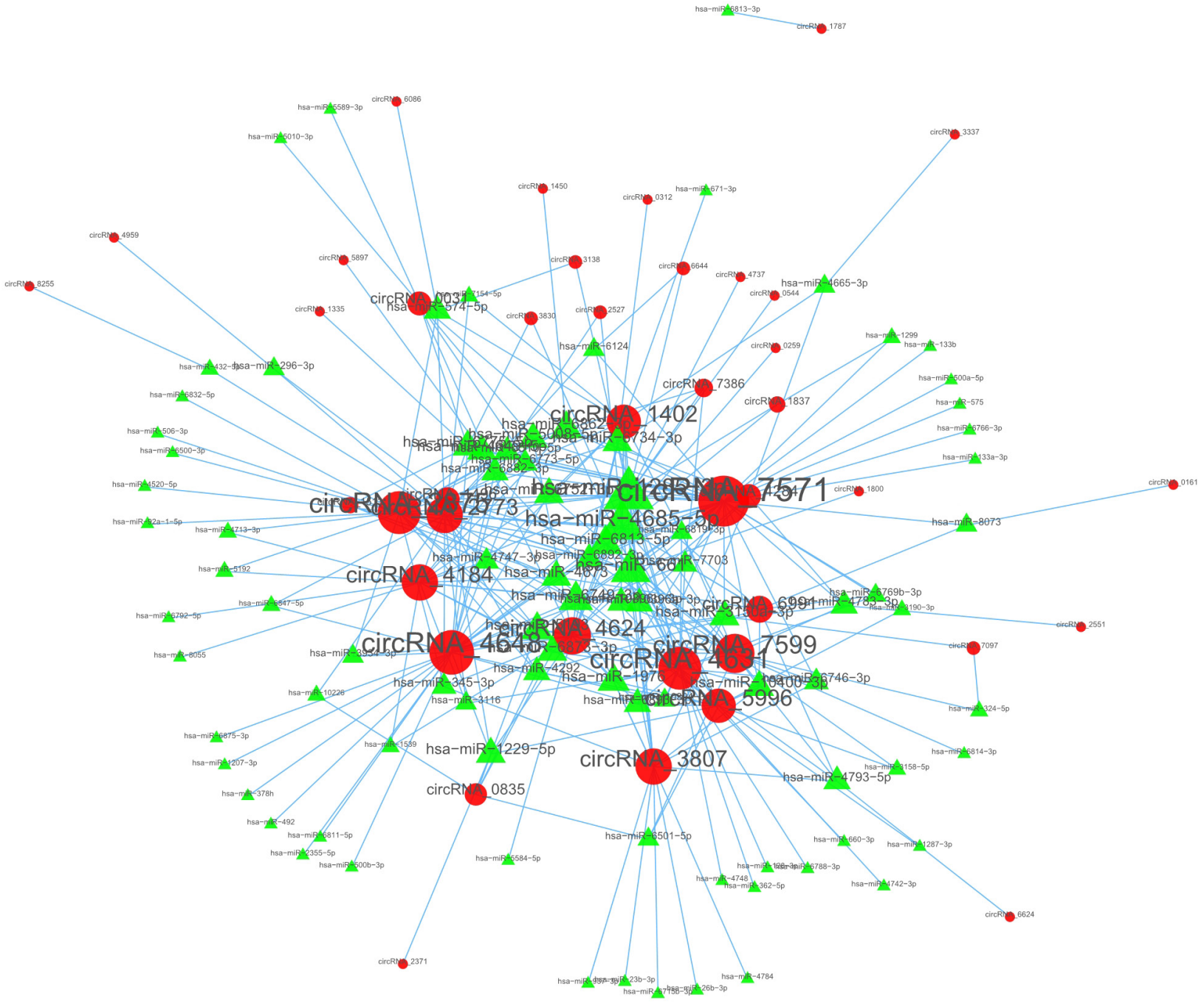

hsa-miR-6813-3p
circRNA_1787
circRNA_6086
hsa-miR-6589-3p
hsa-miR-6010-3p
circRNA_3337
circRNA_1450
circRNA_0312
hsa-miR-671-3p
circRNA_4959
circRNA_5897
circRNA_3138
circRNA_6644
circRNA_4737
hsa-miR-4665-3p
circRNA_8255
circRNA_0031
hsa-miR-574-5p
circRNA_0544
circRNA_1335
circRNA_3830
circRNA_2527
hsa-miR-6124
circRNA_0259
hsa-miR-1299
hsa-miR-133b
hsa-miR-296-3p
circRNA_7386
circRNA_1837
hsa-miR-500a-5p
hsa-miR-575
hsa-miR-6832-5p
circRNA_1402
hsa-miR-506-3p
hsa-miR-6766-3p
hsa-miR-6500-3p
hsa-miR-133a-3p
hsa-miR-6773-5p
hsa-miR-6832-3p
circRNA_7571
circRNA_1800
circRNA_0161
hsa-miR-4520-5p
circRNA_2073
hsa-miR-4685-5p
hsa-miR-8073
hsa-miR-92a-1-5p
hsa-miR-4713-3p
hsa-miR-6813-5p
hsa-miR-4747-3p
hsa-miR-7703
hsa-miR-5192
circRNA_4184
hsa-miR-4673
hsa-miR-6769b-3p
hsa-miR-6847-5p
circRNA_6991
hsa-miR-4783-3p
hsa-miR-6792-5p
hsa-miR-3190-3p
circRNA_2551
circRNA_4624
circRNA_4648
circRNA_7599
circRNA_7097
hsa-miR-8055
hsa-miR-6873-3p
circRNA_4631
hsa-miR-4292
hsa-miR-345-3p
hsa-miR-6746-3p
hsa-miR-10226
hsa-miR-3116
circRNA_5996
hsa-miR-324-5p
hsa-miR-6875-3p
hsa-miR-1229-5p
hsa-miR-6814-3p
hsa-miR-1207-3p
circRNA_3807
hsa-miR-3158-5p
hsa-miR-4793-5p
hsa-miR-378h
circRNA_0835
hsa-miR-492
hsa-miR-6811-5p
hsa-miR-6501-5p
hsa-miR-660-3p
hsa-miR-1287-3p
hsa-miR-2355-5p
hsa-miR-6584-5p
hsa-miR-500b-3p
hsa-miR-6788-3p
hsa-miR-4748
hsa-miR-362-5p
hsa-miR-4742-3p
circRNA_6624
circRNA_2371
hsa-miR-4784
hsa-miR-23b-3p
hsa-miR-26b-3p

Figure 7. circRNA-miRNA coexpression network explored by using Cytoscape. The size of each node represents functional connectivity of each circRNA. The network consists of 37 circRNAs and 90 miRNAs. The red node represents circRNA and the green node represents miRNA. circRNA_7571, circRNA_4648, circRNA_4631 and circRNA_2875 were the four largest nodes in the network. hsa-miR-328 was the highest positive correlated miRNA in the networks.

receptor activity and 1-acyl glycerol-3-phosphate acyltransferase activity, etc. According to KEGG results, differentially expressed circRNAs-related signaling pathways were mainly involved in inflammation and the immune system. circRNA_7571, circRNA_4648, circRNA_4631 and circRNA_2875 were the first four circRNAs with the most combined nodes in the circRNAs-miRNA co-expression network. In addition, hsa-miR-328 was the largest node that interacts with circRNAs in the circRNAs-miRNA co-expression network. This suggests that circRNA-hsa-miR-328 may play a key role in the pathogenesis of AF. It has been reported that hsa-miR-328 plays a role in the proliferation and collagen production of atrial fibroblasts and is involved in the formation and maintenance of AF [19, 20], which is consistent with our findings.

At present, studies on the circRNAs of AF are just beginning; further experiments are needed to confirm these circRNAs and related pathways and biologic processes discovered. In this study, for the first time, the relationship between circRNAs and AF was explored. Specific circRNAs were found to be expressed in AF peripheral blood monocytes, which not only provided a reliable standard basis for the diagnosis of AF at the gene level, but also provided a new research direction for studying the pathogenesis and prognosis of AF at the molecular level. However, the numbers of samples in this study were small; large-sample clinical validation of RT-PCR would be required and further analysis of differentially expressed circRNAs in peripheral monocytes had not been further explored in the present study. Further analysis and related experimental studies will further clarify the role of these circRNAs in the occurrence and development of AF.

Acknowledgements

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Disclosure of conflict of interest

None.

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