Characterization of circRNA-associated ceRNA networks in patients with nonvalvular persistent atrial fibrillation

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Abstract. Circular RNAs (circRNAs) are non-coding RNAs forming closed-loop structures, and their aberrant expression may lead to disease. However, the potential network of circRNA-associated competing endogenous RNA (ceRNA) involved in nonvalvular persistent atrial fibrillation (NPAF) has not been previously reported. In the present study, four left atrial appendages (LAA) of patients with NPAF and four normal LAAs were examined via RNA sequencing, and their potential functions were investigated via bioinformatics analysis. The circRNA-enriched genes were analyzed using Gene Ontology (GO) categories, while the enrichment of circRNAs was detected via the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. A total of 296 significantly dysregulated circRNA transcripts were obtained, with 238 upregulated and 58 downregulated. A number of circRNAs were further confirmed using reverse transcription-quantitative polymerase chain reaction analysis. Furthermore, the more comprehensive circRNA-associated ceRNA networks were examined in patients with NPAF. GO categories and KEGG annotation analysis of circRNAs revealed that the circRNA-associated ceRNA networks were likely to influence AF though alterations in calcium and cardiac muscle contraction. The circRNA-associated ceRNA networks revealed that dysregulated circRNAs in NPAF may be involved in regulating hsa-microRNA (miR)-208b and hsa-miR-21. To the best of our knowledge, this study presents the circRNA-associated ceRNA networks in NPAF for the first time, which may have potential implications for the pathogenesis of AF. This study reveals a potential perspective from which to investigate circRNAs in circRNA-associated ceRNA networks (hsa_circRNA002085, hsa_circRNA001321) in NPAF, and provides a potential biomarker for AF.

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Abbreviations: circRNA, circular RNA; ceRNA, competing endogenous RNA; NPAF, nonvalvular persistent atrial fibrillation; LAA, left atrial appendages; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; AF, atrial fibrillation; FC, fold-change; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; TGF-β1, transforming growth factor-β1

Key words: NPAF, RNA-seq, circRNAs, ceRNA, pathway

Introduction

Atrial fibrillation (AF) is the most common type of cardiac arrhythmia in humans, characterized by irregular and rapid electrical activity of the atria (1,2). In 2010, AF affected ~33.5 million people, causing ~5 million new cases each year worldwide (3,4). AF causes 130,000 mortalities/year in the USA (5) and affects 2-3% of the European population (4,6,7). The number of patients with AF is predicted to increase rapidly in the coming decades and to gradually reduce quality of life (8,9). In Asia, the number of patients with AF and AF-associated stroke is estimated to reach 72 million and 2.9 million by 2050, respectively (3,10). Warfarin is commonly applied to treat nonvalvular persistent atrial fibrillation (NPAF) and research on NPAF has been extensive (11,12), although the current treatment options remain inadequate. Thus, it is necessary to identify a novel biomarker for the prediction, diagnosis and treatment of NPAF.

Circular RNAs (circRNAs), non-coding RNAs identified in 1991 (13), regulate gene expression and act as microRNA (miRNA) 'sponges' by competing with endogenous RNA (ceRNA) networks to suppress specific miRNA activity (14,15). circRNAs are associated with numerous diseases and may serve an important role in diagnosis or pathogenesis (16,17). Previous

studies have demonstrated that circRNA-miRNA-mRNA networks are likely to be involved in certain diseases (18,19). However, there is little research regarding the functions of circRNAs in AF, particularly NPAF. The present study aimed to identify a novel biomarker for diagnosis of AF.

Materials and methods

Ethics approval statement. Written informed consent was obtained from patients prior to collection of left atrial appendages (LAAs), which were abandoned due to surgical techniques. The present study was conducted in accordance with the Declaration of Helsinki, and all experimental procedures were approved by the Ethics Committee of Shanghai East Hospital (approval no. 040-2017) as per relevant guidelines and regulations (clinical research registration no. ChiCTR-RRC-17014230).

Clinical specimens. A total of four male patients with NPAF without valvular disease as an AF group, and four healthy male organ donors as a control group, were recruited between January 2016 and December 2017. AF specimens were collected from the LAAs of patients with NPAF during LAA excision along with surgical atrial fibrillation ablation. Normal LAAs were collected from healthy donors. Excised LAAs were stored in RNA later in -20˚C prior to RNA extraction. Characteristics of the four patients are listed in Table I.

RNA extraction and qualification. Total RNA was isolated using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol, and dissolved in RNase-free water. RNA purity was assessed using a Nanodrop ND-2000 device (Thermo Fisher Scientific, Inc., Wilmington, DE, USA) with an absorbance (A)260/A280 of 1.8-2.0, and integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). The RNA integrity values of the RNAs obtained from the eight LAA specimens were 7.9, 7.7, 7.1, 7.3, 6.8, 7.2, 8.1 and 7.5, respectively.

RNA sequencing. Sequencing libraries were prepared as recommended by the VAHTS™ Total RNA-seq (H/M/R) Library Prep kit (Illumina, Inc., San Diego, CA, USA). Ribosomal RNA was removed using target-specific probes, RNase H and DNA polymerase I (Finnzyme; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and subsequently fragmented into pieces. Using reverse transcriptase and random primers, the RNA fragments were copied to the first strand of cDNA: 1 cycle of 25˚C for 10 min; and 1 cycle of 50˚C for 15 min and 85˚C for 5 min, and the second strand was synthesized using DNA polymerase I, RNase H and dNTPs (dUTP, dATP, dGTP and dCTP; Invitrogen; Thermo Fisher Scientific, Inc.). Subsequently, single 'A' bases were added to the fragments of cDNA and then the adapter was ligated. To select the appropriate fragment size for sequencing, the library fragments were selected with VAHTSTM DNA Clean Beads (Vazyme Biotech Co., Nanjing, China). The second strand of cDNA was digested using an UDG (uracil-N-glycosylase) enzyme (Thermo Fisher Scientific, Inc.). Following cluster generation, 150-bp paired-end reads were produced by sequencing the libraries on the Illumina, Inc. Hiseq X10 platform.

Differential expression analysis. The expression levels of circRNAs were measured by RNA sequencing and expressed as 'transcripts per kilobase million'. Differentially-expressed circRNAs were analyzed using DESeq2 based on the negative binomial distribution test, and the thresholds were $P<0.05$ and fold-change (FC) >2. P-values were calculated using a statistical algorithm (20).

Validation of circRNAs by reverse transcription-quantitative PCR (RT-qPCR). A number of differential circRNAs were randomly selected to test the accuracy of the RNA sequencing data by RT-qPCR. Total RNA was reverse transcribed into cDNA using PrimeScript™ RT Reagent kit (Takara Bio, Inc., Shiga, Japan) according to standard procedures. A total reaction volume of 20 μl, including 1,000 ng RNA, 4 μl 5X PrimeScript RT Master Mix and RNase Free dH_2O. The thermocycling conditions were as follows: 37˚C for 15 min, then 10 sec at 85˚C. RT-qPCR was performed with a SYBR Green kit (Promega Corporation, Madison, WI, USA) in the QuantStudio™ 6 Flex system (Applied Biosystems; Thermo Fisher Scientific, Inc.), The reactions were incubated in 384-well plates at 50˚C for 2 min, and 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min, followed by a dissociation curve. The internal reference was 18s rRNA (Applied Biosystems; Thermo Fisher Scientific, Inc.). Relative expression was quantified using the $2^{-\Delta\Delta Cq}$ method (21). The primers are listed in Table II.

Gene ontology (GO) and kyoto encyclopedia of genes and genomes (KEGG) enrichment analysis. The differential or target genes of circRNAs were estimated and determined using GO enrichment analysis at the perl module (GO: TermFinder, http://search.cpan.org/dist/GO-TermFinder/) The KEGG enrichment of target genes of differential circRNAs was tested using R functions (22) (P hyper and Q value). The significantly-enriched GO terms and KEGG pathways met the criterion of corrected $P<0.05$.

circRNA-ceRNA interaction prediction. The circRNA-miRNA interactions were predicted using TargetScan 6.0 (http://www.targetscan.org/vert_60/), which identifies miRNA targets and determines whether or not a given target is conserved across a given set of species. The sequences of circRNAs were predicted as miRNA binding seed sequence sites using potential miRNA response elements.

Statistical analysis. Statistical analyses was conducted using SPSS 19.0 software (IBM Corp., Armonk, NY, USA). When comparing two groups of profile differences, the fold-change (i.e. the ratio of the group averages) between the groups for each circRNA was computed. The differential expression of circRNAs was assessed using a Student's unpaired t-test. Error bars in the figures represent the means ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Overview of RNA-seq. No significant between-group difference was identified in the expression levels of 15,777 circRNAs. In total, 296 differential circRNAs were identified with FC>2 and $P<0.05$. Among them, 238 were

Table I. Demographic characteristics of patients.

No.	Age, years	Sex	NYHA	INR	Coronary artery	Disease complications	Duration of AF, years	Drug treatments
1	64	Male	II-III	3.38	Negative	Type II diabetes	2	Warfarin, furosemide, spironolactone and Cordarone
2	76	Male	II-III	0.92	Negative	Negative	3	Warfarin, Cordarone, spironolactone, furosemide and Betaloc
3	69	Male	II-III	2.03	Negative	Negative	4	Warfarin, Betaloc, furosemide, spironolactone and Cordarone
4	60	Male	II-III	2.00	Negative	Negative	2	Warfarin, furosemide and spironolactone

NYHA, New York Heart Association; INR, International Normalized Ratio; AF, atrial fibrillation.

Table II. The list of circRNAs qRT-PCR primers between NPAF and controls.

CircRNA	Forward primer	Reverse primer
hsa_circRNA011430	CGAGCAGTACCCCACAATGG	TCTGTTGGCATGCTGCTGAA
hsa_circRNA015317	ATTAGGCAGACTCTTCAAAACGC	AAACCCCCACCCACAAAGCA
hsa_circRNA016587	TAGGCACAGCTCCTCCAGAT	TGTGAGATGCTTCACTGCATTC
hsa_circRNA003585	GCTGCCCAATGATCTGCTTG	CCCTGCTTGCAGCTGTAGAAT
hsa_circRNA015019	GGAGCCAAAGCCTAATCCGC	CTGCTGCCAAGGCATACTCA
hsa_circRNA003126	ATGGACTGGCGGATCAAGGA	TGGCCACCAGTCACAAGGTA

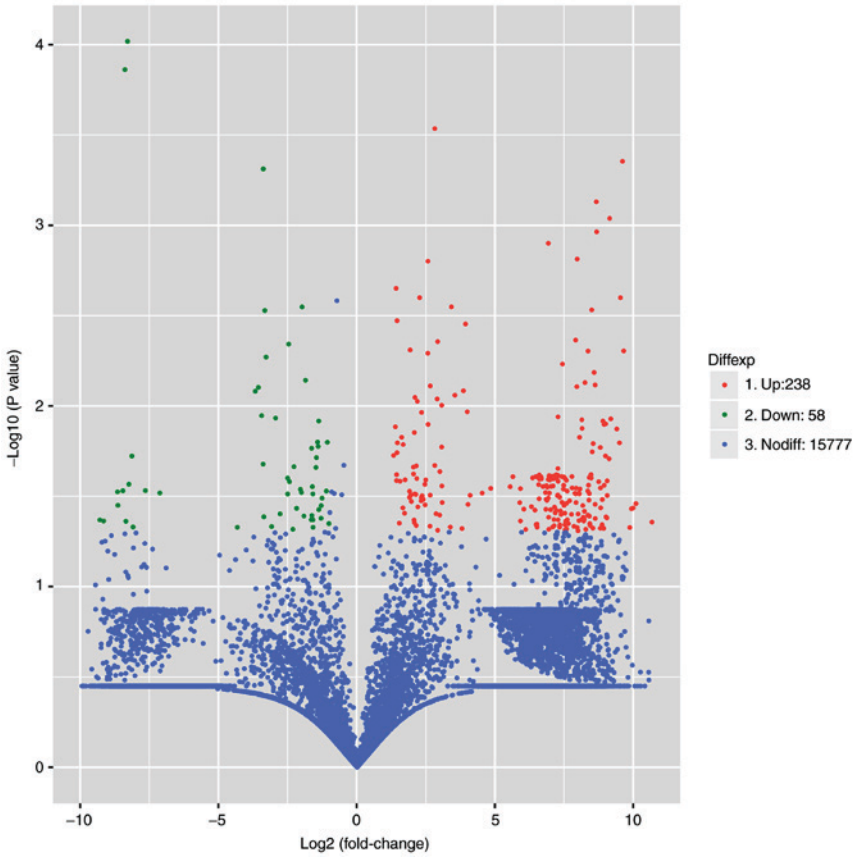

Figure 1. Volcano plot of circRNAs between the NPAF and control groups illustrates the distribution and number of circRNAs. Red and green represents upregulated and downregulated circRNAs, respectively, with fold-change >2 and P<0.05. Blue represents unaltered circRNAs. NPAF, nonvalvular persistent atrial fibrillation; circRNA, circular RNA.

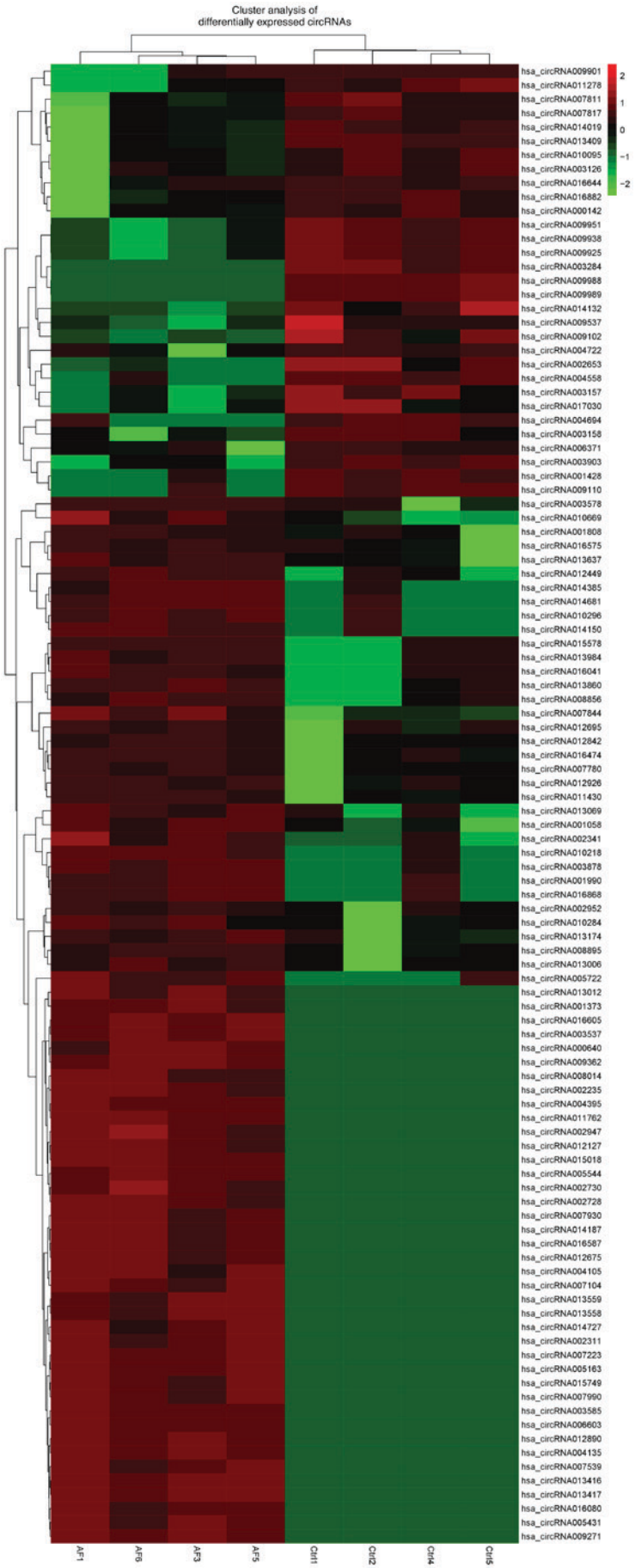

Figure 2. Heatmap of differentially-expressed circRNAs between the NPAF and control groups. The expression levels of circRNAs are represented by colored bars. The red and green color represents upregulated and downregulated circRNAs, respectively. Higher intensity of color indicates a greater degree of up- or downregulation. AF, NPAF; Ctrl, control. NPAF, nonvalvular persistent atrial fibrillation; circRNA, circular RNA.

upregulated and 58 were downregulated in NPAF tissues compared with the controls (Figs. 1 and 2). The upregulated circRNAs (data not shown; available at https://1drv.ms/w/s!Al7sl_MjqbmWgQDki8ZjwTQd2j0H) with 'FC >2' and downregulated circRNAs (data not shown; available at https://1drv.ms/w/s!Al7sl_MjqbmWgQEKE-v8hCjzTevu) are listed. A number of the circRNAs were used in the subsequent analysis.

Validation of circRNAs by RT-qPCR. A total of six dysregulated circRNAs were randomly selected as representatives to validate the RNA sequencing data by RT-qPCR, and the primers are listed in Table II. All circRNAs were well-validated by qPCR, and the directions of the changes were consistent with the RNA-seq data (Fig. 3). The RT-qPCR data suggested the RNA-seq-identified circRNAs are reliable and require further research.

Functional enrichment analysis: GO and KEGG pathway analysis. The differential circRNAs were annotated with GO, and were identified to be involved in the following functions: Cell component, biological process and molecular function (Fig. 4). GO analysis revealed that a number of functional pathways were enriched. Some of the top 10 terms were as follows: 'Voltage-gated calcium channel activity is involved in AV node cell action potential' in cell component GO (no. 0086056); 'cardiac muscle contraction is regulated by calcium ion signaling' in bioprocess GO (no. 0010882); 'release of sequestered calcium ion into cytosol is regulated by sarcoplasmic reticulum' in bioprocess GO (no. 0010880); and 'regulation of cardiac muscle cell action potential is involved in regulation of contraction' in bioprocess GO (no. 0098909), which were the most closely associated with AF. These criteria from the GO analysis were also applied to the KEGG enrichment analysis. The top 20 KEGG pathway analysis demonstrated that the most significant pathways involved in AF were 'arrhythmogenic right ventricular cardiomyopathy' (no. ko05412) and 'cardiac muscle contraction' (no. ko04260; Fig. 5).

Construction of circRNA-ceRNA interaction network. To analyze the interaction between differential circRNAs and ceRNAs, a ceRNA network in NPAF was investigated using RNA sequencing data. To examine which circRNAs were vital for NPAF progression, nine differential circRNAs were selected from those with calcium-associated parental genes. Complete sequences of hsa_circRNA-011785, -001321, -003878, -002085, -003884, -003876, -007410, -007411 and -004558 are listed in Table III. Details are provided in Table IV. A representative network of circRNAs and miRNAs is presented in Fig. 6. The interactions between hsa_circRNA002085 and hsa-miRNA (miR)-21, and between hsa_circRNA001321 and hsa-miR-1, suggest the possibility of an NPAF regulatory mechanism.

Discussion

In total, 296 differential circRNAs were identified between NPAF tissues and the controls (FC>2; P<0.05), including 238 upregulated circRNAs and 58 downregulated circRNAs. To validate the RNA sequencing data, six representative dysregulated circRNAs (hsa_circRNA-011430, -015317, -016587, -003585, -015019 and -003126) were selected. The RT-qPCR results suggested that the RNA sequencing-identified circRNAs are reliable and merit further research. The significantly dysregulated circRNAs in patients with NPAF may serve a regulatory role in the mechanism of AF progression.

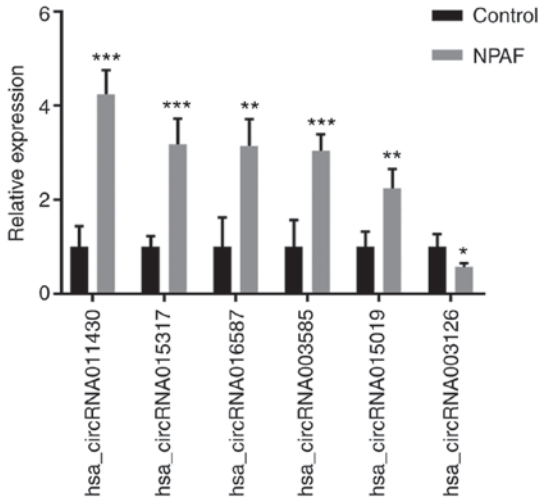

Figure 3. Validation of differentially expressed circRNAs between the NPAF and control groups by reverse transcription-quantitative polymerase chain reaction. A total of six circRNAs were validated and 18s was used as the internal control. The validation rate of the six circRNAs was approaching 100%, demonstrating that the circRNA sequencing data were reliable. *P<0.05, **P<0.01, ***P<0.001 vs. control. NPAF, nonvalvular persistent atrial fibrillation; circRNA, circular RNA.

Unlike linear RNAs, circRNAs are covalently linked to form closed-loop structures without 5' caps or 3' tails (18,23-25). In circRNAs, a downstream splice donor is joined by 'back-splicing' to the upstream splice acceptor (17,26,27). circRNAs are associated with, and serve important roles in the diagnosis and pathogenesis of, numerous diseases (16,17), including colorectal cancer (28), breast cancer (29) and gastric cancer (30). Furthermore, circRNAs are able to upregulate the expression levels of fibrosis-associated genes in cardiac fibroblasts (31). Other studies have reported that a circRNA-ceRNA network may be present in certain diseases (18,19). However, the circRNAs and circRNA-ceRNA network leading to AF remain to be elucidated.

AF remains a common cause of stroke, heart failure and cardiovascular mortality worldwide. AF may occur idiopathically, which is associated with familial inherent specific genetic mutations (32). Certain mechanisms leading to AF are primarily associated with the remodeling of ion channel functions, including those of K^+ channels, and cellular Ca^{2+} handling and release (33-36). Other mechanisms of AF include mutations and the abnormal expression of genes encoding cardiac ion channels, including potassium voltage-gated channel subfamily Q member 1, potassium voltage-gated channel subfamily E regulatory subunit 2, potassium voltage-gated channel subfamily J member 2 (KCNJ2) and sarcoplasmic/endoplasmic reticulum calcium ATPase 2A (SERCA2) (36).

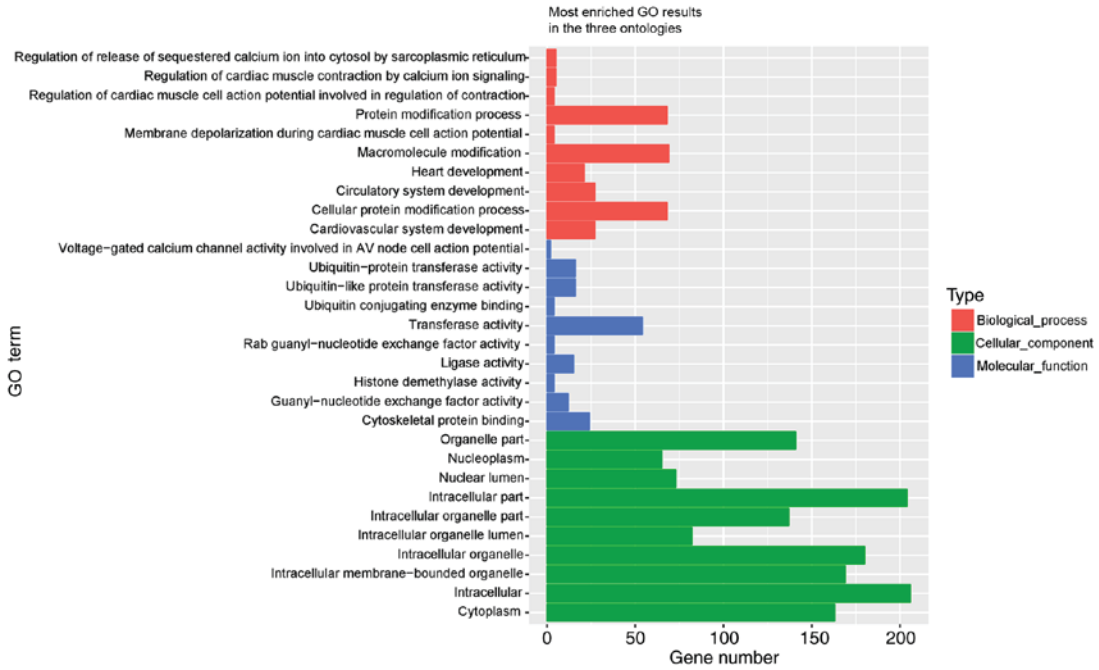

Figure 4. GO annotations and enrichment analysis of the differentially expressed circRNAs between the NPAF and control groups. The differentially expressed circRNAs between the NPAF and control groups were annotated with respect to three components (cellular component, biological process and molecular function). Q<0.05. GO, gene ontology; NPAF, nonvalvular persistent atrial fibrillation; circRNA, circular RNA.

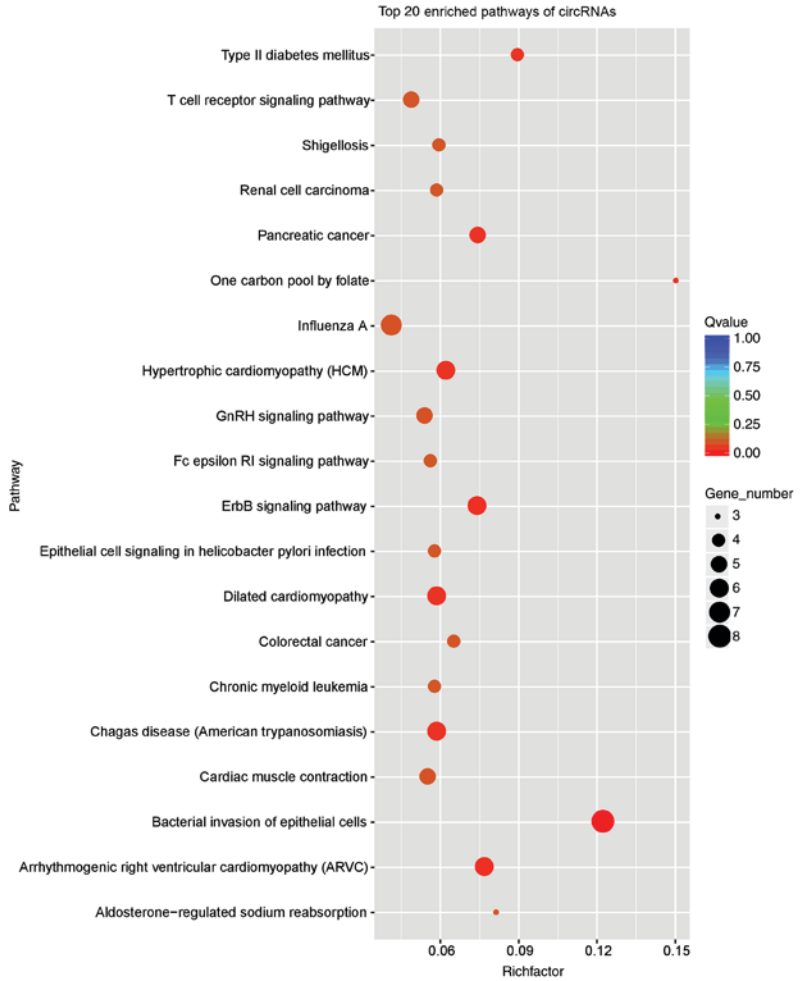

Figure 5. Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis of the differentially expressed circRNAs between the nonvalvular persistent atrial fibrillation and control groups. The number of genes is represented by the size of the circle, and the Q-value is represented by the color. circRNA, circular RNA.

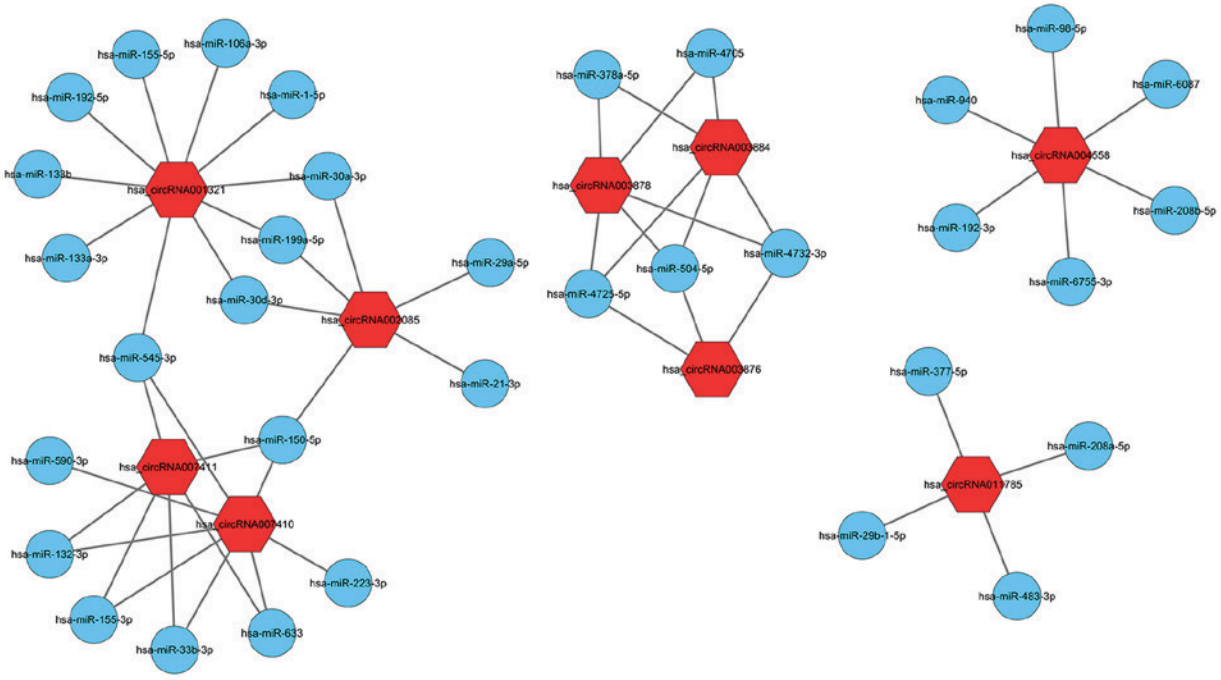

Figure 6. cirRNA-miRNA networks between the NPAF and control groups. Red and blue represents circRNAs and miRNAs, respectively. miRNA/miR, microRNA; circRNA, circular RNA.

For instance, calcium gene expression was identified to be abnormal in AF (37), and was hypothesized to contribute to the propensity for structural remodeling in AF. The calcium signaling pathway is essential in the electrical remodeling of AF and may induce the recurrence of AF (37). Abnormalities in intracellular Ca^{2+} handling are crucially involved in AF-initiated focal activity and perpetuation through rapidly firing foci and reentry (37).

GO analysis performed using differential circRNAs between the NPAF and control groups demonstrated that a number of functional pathways were enriched. The primary cell component was GO (no. 0086056) 'voltage-gated calcium channel activity is involved in AV node cell action potential'. The main bioprocesses were GO (no. 0010882) 'cardiac muscle contraction is regulated by calcium ion signaling', GO (no. 0010880) 'release of sequestered calcium ion into cytosol is regulated by sarcoplasmic reticulum' and GO (no. 0098909) 'regulation of cardiac muscle cell action potential is involved in regulation of contraction'. The specific genomes were 'CACNB2, CACNA1C' (GO:0086056); 'SLC8A1, ATP2A2, CACNA1C, ANK2, RYR2' (GO:0010882); 'DHRS7C, CACNA1C, SLC8A1, RYR2, ANK2' (GO:0010880); 'CACNA1C, ATP2A2, RYR2, ANK2' (GO:0098909). These genes are primarily involved in Ca^{2+} channels and may be the most closely associated with AF. A total of two of the most significant top 20 KEGG pathways were 'arrhythmogenic right ventricular cardiomyopathy' (no. ko05412) and 'cardiac muscle contraction' (no. ko04260), which were also associated with AF. Differential expression of circRNAs may be affected by AF and atrial remodeling. The present study identified a number of differential circRNAs in AF.

The circRNA-ceRNA interactions were constructed to examine which circRNAs serve important roles in NPAF progression. A total of nine differential circRNAs (hsa_circRNA-011785, -001321, -003878, -002085, -003884, -003876, -007410, -007411 and -004558) from calcium-associated parental genes were selected. miRNAs serve multiple roles in atrial fibrillation, including regulating electrical remodeling by targeting the genes involved in different ion channels, and regulating structural remodeling in cardiac tissues by increasing cardiac fibrosis or apoptosis. Different miRNAs have been demonstrated to be upregulated or downregulated in patients with AF (21), and may target different genes to regulate cardiac function. Different miRNAs may target single genes and serve similar roles, including repressing I_{K1} (potassium current) by targeting KCNJ2 via miR-1 and miR-26 (22,38). In the present study, certain circRNAs were also dysregulated in AF, which might indicate an association. The interactions between hsa_circRNA004558 and miR-208b, between hsa_circRNA002085 and hsa-miR-21, and between hsa_circRNA001321 and hsa-miR-1 may suggest a possible regulatory mechanism in AF. For instance, miR-208b was demonstrated to be upregulated in patients with AF and an ovine model. Furthermore, a high miR-208b level was demonstrated to increase MYH7 expression and alter the subcellular localization of connexin43 (39). miR-208b has been reported to reduce the expression levels of CaV1.2 and SERCA2, which further reduce L-type Ca^{2+} current density and sarcoplasmic reticulum Ca^{2+} load/release, respectively (39). These alterations are hallmarks of atrial remodeling during AF (40). miR-21 is reportedly associated with atrial fibrosis regulation in AF, which inhibits the proliferation of cardiac fibroblasts by inactivating the transforming growth factor (TGF)-β1/mothers against decapentaplegic homolog (Smad)2 signaling pathway (41).

miR-21-3p may regulate sepsis-associated cardiac dysfunction and the development of cardiac hypertrophy. When miR-21-3p is inhibited, such diseases may be treated via a protective strategy (42,43). The upregulated inward

Table III. Complete sequence of differentially expressed circRNAs between the NPAF and control groups.

circ_ID	Sequence
hsa_circRNA011785	AAAATGTAGGCCGGTTAATCACTCCTGCCAAAAAGCTTGAAGATACAATACGTCTTGCTGAACTAGTCATTGAAGTTCTTCAGCAAAATGAG GAGCACCACGCAGAGCCACATGTTGATAAAGGAGAAGCCTTTGCGTGGTGGTCAGATTTAATGGTGGAGCATGCGGAGACGTTCCTGTCAC TCTTTGCAGTAGACATGGATGCAGCCTTAGAGGTGCAACCTCCAGACACATGGGACAGTTTTCCACTATTTCAGCTGCTGAATGATTTTCTCC GTACTGACTATAATTTGTGCAATGGAAAATTTCACAAACACCTGCAAGACCTGTTTGCCCCACTTGTTGTTAGATATGTGGATCTGATGGAGTC CTCAATTGCACAATCCATTCACAGGGGCTTTGAGCGGGAGTCATGGGAACCAGTCAAGAGTTTAACCAGTAACCTACCCAATGTGAACCTAC CCAATGTGAACCTTCCCAAAGTACCAAATCTACCAGTTAACATCCCTCTAGGCATCCCACAAATGCCTACTTTTTCGGCACCGTCATGGATGG CTGCTATATATGATGCGGATAATGGGTCAGGCACCTCAGAAGATCTGTTTTGGAAACTTGACGCCCTTCAGACCTTCATTCGGGACCTGCACT GGCCTGAAGAAGAGTTTGGAAAGCACCTGGAACAACGGCTGAAGTTGATGGCAAGTGACATGATCGAATCTTGTGTCAAAAGAACCAGGA TTGCATTTGAAGTTAAGCTGCAAAAAACCAGTCGATCAACAGATTTTCGAGTCCCACAGTCAATATGCACCATGTTTAATGTTATGGTTGATG CCAAAGCTCAATCAACAAAACTTTGCAGCATGGAAATGGGCCAAGAGCATCAATACCATTCAAAAATAGACGAACTAATTGAAGAAACTGTT AAAGAAATGATAACACTCTTGGTTGCAAAGTTCGTTACTATCTTGGAAGGAGTGCTGGCAAAATTATCCAGATATGACGAAGGGACTTTGTTT TCTTCTTTTCTGTCATTTACCGTGAAGGCAGCTTCCAAATATGTGGATGTACCTAAACCCGGGATGGACGTGGCCGACGCCTACGTGACTTTCG TCCGCCATTCTCAGGATGTCCTGCGTGATAAGGTCAATGAGGAGATGTACATAGAAAGGTTATTTGAT
hsa_circRNA001321	GAGGAATTAGAAGGTCTTCATCTATGTCTTATGTTGATGGCTTCATAGGGACATGGCCCAAAGAGAAAAGATCATCAGTGCATGGCGTATCAT TTGATATTTCTTTTGATAAAGAAGATAGTGTACAGAGATCCACTCCAAACCGAGGAATCACTCGTTCTATTAGTAATGAAGGACTTACTCTGAA CAACAGTCATGTATCTAAACACATTAGGAAAAATTTGTCCTTCAAGCCAATAAATGGAGAAGAGGAAGCAGAGAGCATTGAAGAAGAACTTA ATATAGATTCTCACAGTGACCTCAAATCTTGTGTGCCCCTTAACACAAATGAACTAAATTCTAATGAGAATATTCATTACAAGCTTCCAAATGGA GCTTTACAAAATAGAATACTTCTTGACGAGTTTGGCAATCAGATCGAGACACCAAGCATTGAAGAAGCATTACAAATAATTCATGATACTGAA AAATCTCCTCATACACCTCAGCCAGACCAAATTGCTAATGGCTTCTTTCTTCATAGTCAAGAAATGAGTATCTTAAATTCAAATATCAAGTTAAA TCAATCTAGTCCTGATAATGTAACTGATACGAAAGGTGCCTTGAGTCCCATAACTGACAATACTGAAGTAGACACTGGAATTCACGTTCCTTC AGAAGATATTCCTGAAACTATGGACGAAGATTCTTCGTTGAGAGATTATACTGTAAGCTTGGACTCTGACATGGATGATGCATCTAAATTTCTTC AGGATTATGATATTCGAACTGGCAACACCAGGGAAGCTTTGAGTCCTTGTCCAAGTACTGTAAGTACCAAGTCTCAGCCAGGCAGCAGTGCTT CTTCTAGTTCTGGAGTTAAAATGACCAGCTTTGCTGAACAAAAATTCAGGAAACTGAATCATACCGATGGAAAAAGTAGTGGAAGCAGTTCT CAAAAAACTACACCAGAAGGCTCTGAACTTAATATTCCTCATGTGGTTGCTTGGGCACAAATTCCAGAAGAAACAGGGCTTCCACAGGGACG GGACACTACCCAGCTGTTGGCCTCTGAAATGGTGCATCTTAGGATGAAACTAGAAGAAAAGAGGCGTGCTATAGAAGCCCAGAAAAAGAAA ATGGAAGCTGCTTTTACCAAACAGAGACAGAAAATGGGAAGGACAGCATTCCTTACTGTAGTGAAAAAGAAAGGGGATGGGATATCTCCTCT ACGAGAGGAAGCGGCGGGTGCAGAAGATGAGAAAGTATATACTGATCGAGCAAAAGAAAAGGAATCACAAAAAACTGATGGACAAAGGAG CAAGTCACTGGCAGATATAAAAGAGAGCATGGAGAATCCTCAAGCCAAATGGCTAAAGTCTCCAACTACACCTATTGATCCTGAGAAGCAGT GGAACCTGGCAAGCCCCTCAGAAGAAACTTTAAATGAAGGAGAGATTTTAGAATATACCAAATCCATTGAAAAGTTAAATTCATCCCTGCATT TTCTACAACAAGAAATGCAACGCTTGTCACTTCAGCAGGAGATGTTAATGCAGATGAGAGAGCAACAATCTTGGGTGATTTCACCTCCACAA CCCTCTCCACAGAAACAGATTCGAGATTTTAAGCCTTCTAAGCAGGCAGGCCTGTCATCAGCCATTGCACCATTCTCCTCAGACTCCCCTCGT CCTACTCACCCATCTCCACAGTCTTCTAACAGGAAAAGTGCATCTTTTTCTGTTAAAAGTCAAAGGACTCCTAGGCCAAATGAGTTAAAAATA ACACCTTTGAATCGAACCTTGACACCTCCTCGGTCTGTGGATAGCCTTCCTCGGTTAAGGAGGTTTTCACCAAGTCAAGTTCCTATTCAAACT AGGTCATTTGTATGTTTTGGGGATGATGGAGAACCTCAGTTAAAGGAATCCAAACCTAAAGAGGAAGTTAAAAAGGAGGAATTGGAATCCAA AGGGACTTTGGAACAGCGTGGACATAATCCAGAAGAAAAGGAAATCAAACCTTTTGAGTCAACAGTCTCTGAAGTCCTATCACTGCCTGTCA CAGAGACTGTATGTCTGACACCAAATGAGGACCAATTGAATCAACCCACAGAACCCCCTCCTAAACCCGTTTTCCCACCCACTGCTCCAAAA

Table III. Continued.

circ_ID	Sequence
	AATGTTAATCTGATTGAAGTTTCCCTCTCAGATTTGAAACCCCCTGAAAAGGCTGATGTACCTGTTGAAAAATATGATGGAGAAAGTGATAAA
	GAACAATTTGATGATGACCAGAAAGTATGCTGTGGATTCTTTTTTAAGGATGATCAAAAAGCAGAAAATGATATGGCAATGAAACGGGCAGCT
	TTGTTGGAGAAAAGATTAAGAAGGGAAAAGGAAACTCAGCTCCGGAAACAACAGTTGGAAGCAGAAATGGAGCATAAGAAGGAGGAAACAAGG
	CGTAAAACTGAGGAAGAACGTCAGAAGAAAGAAGATGAGAGAGCACGCAGAGAATTTATTAGGCAAGAATATATGAGGCGGAAACAACTGA
	AATTCTAATGGAAGATATGGATACAGTAATTAAACCCCGTCCTCAAGTAGTAAAACAAAAAAAACAGCGACCAAAATCTATTCACAGAGATCA
	TAGAATCCCCCAAAACACCAATAAAGGGTCCTCCAG
hsa_circRNA003878	GTCAGGATCGTGTGATTGTAAACTAGTCAGGTCAGGGTCGTGTGATTGTAAACTAGTCAGGTCAGGGTCGTGTGATTGTAAACTAGTCAG
hsa_circRNA002085	TGCTGGGATTACAGGCGTGACCATGGCACCGAGAACAATTTTTTGAAATTTAAGTTTTGGTGTTAAAGGTTTGGGTCAAGTTAAGCAGAGCTT
	GGGCTTGACACATATGTACACACGTGTGTGTGGGTGCGATGTATGTATTCTGCAACTGGCTATGACACCAATGCCCAGATGTCAAGTGGGAAA
	TTTTCCAAGTTCTTGACAGCATCCAAGCATATATACTTTAGGCAGATACTTATATTCTTTGGTCTGGCAATGAGTGAGGAGAATAATAGATGAGA
	CCTTTGGAAACGATGAAAATGATCATTACAGACCCATCCACACTTGATAGTTGGAGCAGTTGTGCCTGGAGGTGGATTGGGAGAACTTCTCTTTC
	GAGCTTTCTGAAGTTTTCCTTTAAATCAGTTGAGCTATTGATATAAAAATTGTCTCAGAGTCCTAATATGCTTATTATACATTTTGTAG
	TTGGTTATAGCTCCAAGTGACTCAGTCACCAGAAAAGTCACTTGAAAGTTGGTGTATCTCTACAAAACCTTGGTGAACATTTAAATCACCTTT
	TTAAATATTACACTCTTCATGGAACTTTACTTTTAGTAATGTCAAACATCTGCCTACAAAATGGAATGTTATTTTACATGTATACTAACTTGT
	TCTTCAAAATGCAGCACCTCCTTTCACTATACAGACGGTCCTTGACATACACTGGTTCAACTTAAGATTGTGCAAGTTTATTACAAATTTTTC
	AGGACATAACCTTATCATAAGTCAAGGTCTGAACTTTACGATGGTTTGACGTCAGACTTTTTACTGGGTCTGTCCACGTGTTAAGTACATTTT
	GAGTTACCATATTTTCTATTTATGATGGGTTTAGTGAAGTGTAACTCTATCATAAGTTGGGGAGCATCTGTGTTGCCAAAACCACTGGAGGGC
	TACAATATGGCTGGAGGGAATGGAAGAATTTTTCTGGTATATAGAAAGAAGTGAGGCTGACATTTGCAGGCAGAACTGCTGCTTTATCGCAAG
	GTGACATAGTCTTTTTCCACTTTAAGAGCCTAGTAGCAGTCCCTAGAAATCACTGAAATCATCTTTGAGATATATAAGAGTGTAAGGTTTATA
	TAGGAATCCCTGCAACTTCACACATTTGAGAAGGAACAGGATAGGCCTCCCTTCCTCTGTGGATTCAGGGGCTCCGTAATTATCAAGGGAAGG
	TATAAGTTTCACATAATACCTACAGGGCCTTTCATTTTCATTTCATGCATACTATTTTACTTAATTTTGAGCATCTTAGAATTGCTTGCTTCA
	AGTTATAAGTAGATAGTTTTTGAAATTAATTTGCTTCCTAAAGCAATTCATTTATTGTGCTCAGATTTTGCTCACTTGAAAGTCCTATATTCT
	GTGTAGAACTTGGAATTCTATATAAACAGAACAATGGTTTGAATTGCAAAAAAAGCCCATGATCAGTATCTTAGTTTGCCAATGGCTGGGCAT
	GGTGGCTCATGCCTGTCATCCCAACA
hsa_circRNA003884	GTGTGATTGTAAACTAGTAAGGTCAGGATCGTGTGATTGTAAAGTAGTCAGGTCAGGATCGTGTGATTGTAAACTAGTCAGGTCAGGGTC
hsa_circRNA003876	GTCAGGGTCGTGTGA TTGTAAACTAGTCAG
hsa_circRNA007410	GCAGAGGAAAATATTGACTTATTAGATGATGGCTCTAATTCTTTTGCAACTGACTTGTCATCAGGAACTATTAACCACAAGAAATACATCAAG
	TTTTCTAAAACAATAGAGAAGGAAATTTCACCGGAAATTAGGAGTTTGAGCCCAGAATATAAAAAAATATTTGAAACATCAATAATCTTTTGT
	GGAGAAGAAAAGTCCTCTGATTTTTCAGGAGAAAAAAAAGTTGGGAGAAAGAGTTTACAAGTACAACAGCACAGTAAAAGAACTGAGATTATC
	CCTCCTTTTCTGAAGCTGTCAAAGGAGAAGGTGACAAGGAAAGAAAACTCTTTATGCAAGTTGCCGAATCAGTACAGCGTTCACAAGACTTCA
	TCACCTCTTTGTACATCTTCTGCAATTACTCGGGAAAAGGAAATGCTGTCTAACCTCTACATGACATTATATGATGAAGTAACCCATGGATAT
	TTACACTCAAAAGAATTAAGTGCACTTCATAAAGCCTGTAAAATTTTTAGTAAAATTCGAAGTGGTAAGATTTATGTGAATGATCTTCCAGTG
	ATCCTTTGCATCTTGAGAATTTCTATAAGTGATTTAGAAATGCGACAGGCACTAAAGACTGTTGATATTGATGCATTCCAGGATGCCTTGAAG
	ATTTTCTGTAGGATAAAAGGTGGTCGAGTTTCAACTGATGACGTGTTTGCTGTTTTGGATAGCATGGGTATCCCTATAAACCGTGAAATTTTA
	GAAGAAGTGACAAAACATACCTATATTGACA

Table III. Continued.

circ_ID	Sequence
hsa_circRNA007411	AGGAAAATATTGACTTATTAGATGATGGCTCTAATTCTTTTGCAACTGACTTGTCATCAGGAACTATTAACCACAAGAAATACATCAAGTTTT CTAAAACAATAGAGAAGGAAATTTCACCGGAAATTAGGAGTTTGAGCCCAGAATATAAAAAAATATTTGAAACATCAATAATCTTTTGTGGAG AAGAAAAGTCCTCTGATTTTTCAGGAGAAAAAAAAGTTGGGAGAAAGAGTTTACAAGTACAACAGCACAGTAAAAGAACTGAGATTATCCCTC CTTTTCTGAAGCTGTCAAAGGAGAAGGTGACAAGGAAAGAAAACTCTTTATGCAAGTTGCCGAATCAGTACAGCGTTCACAAGACTTCATCAC CTCTTTGTACATCTTCTGCAATTACTCGGGAAAAGGAAATGCTGTCTAACCTCTACATGACATTATATGATGAAGTAACCCATGGATATTTAC ACTCAAAAGAATTAAGTGCACTTCATAAAGCCTGTAAAATTTTTAGTAAAATTCGAAGTGGTAAGATTTATGTGAATGATCTTCCAGTGATCC TTTGCATCTTGAGAATTTCTATAAGTGATTTAGAAATGCGACAGGCACTAAAGACTGTTGATATTGATGCATTCCAGGATGCCTTGAAGATTT TCTGTAGGATAAAAGGTGGTCGAGTTTCAACTGATGACGTGTTTGCTGTTTTGGATAGCATGGGTATCCCTATAAACCGTGAAATTTTAGAAG AAGTGACAAAACATACCTATATTGACAGTGA
hsa_circRNA004558	ACCAATCTGACCTTCGTTGGCTGCGTGGGCATGCTGGATCCTCCGAGAATCGAGGTGGCCTCCTCCGTGAAGCTGTGCCGGCAAGCAGGCATC CGGGTCATCATGATCACTGGGGACAACAAGGGCACTGCTGTGGCCATCTGTCGCCGCATCGGCATCTTCGGGCAGGATGAGGACGTGACGTCA AAAGCTTTCACAGGCCGGGAGTTTGATGAACTCAACCCCTCCGCCCAGCGAGACGCCTGCCTGAACGCCCGCTGTTTTGCTCGAGTTGAACCC TCCCACAAGTCTAAAATCGTAGAATTTCTTCAGTCTTTTGATGAGATTACAGCTATGACTGGCGATGGCGTGAACGATGCTCCTGCTCTGAAG AAAGCCGAGATTGGCATTGCTATGGGCTCTGGCACTGCGGTGGCTAAAACCGCCTCTGAGATGGTCCTGGCGGATGACAACTTCTCCACCATT GTGGCTGCCGTTGAGGAGGGGCGGGCAATCTACAACAACATGAAACAGTTCATCCGCTACCTCATCTCGTCCAACGTCGGGGAAGTTGTCTG

circRNA, circular RNA.

Table IV. Parental genes corresponding to the differentially expressed circRNAs.

A, upregulated circRNAs

circ_ID	P-value	FC	Chr	Start	End	Str	Loci	Gene list	Description
hsa_circRNA003878	0.003525	15.10051	chr12	2255950	2256039	-	Exon	CACNA1C	Calcium voltage-gated channel subunit α1C
hsa_circRNA003884	0.027306	94.9909	chr12	2256001	2256090	-	Exon	CACNA1C	Calcium voltage-gated channel subunit α1C
hsa_circRNA003876	0.033396	116.6174	chr12	2255950	2255979	-	Exon	CACNA1C	Calcium voltage-gated channel subunit α1C
hsa_circRNA007411	0.042442	295.9099	chr17	47335200	47361525	+	Exon	EFCAB13	EF-hand calcium binding domain 13
hsa_circRNA007410	0.041746	522.6585	chr17	47335196	47361521	+	Exon	EFCAB13	EF-hand calcium binding domain 13
hsa_circRNA002085	0.025361	101.3261	chr10	18508039	18509552	-	Exon	CACNB2	Calcium voltage-gated channel auxiliary subunit β2
hsa_circRNA011785	0.026138	2.935789	chr3	62438104	62499268	-	Exon	CADPS	Ca^{2+} dependent secretion activator
hsa_circRNA001321	0.012686	5.92702	chr1	200847640	200853495	+	Exon	CAMSAP2	Calmodulin regulated spectrin associated protein family member 2

B, downregulated circRNAs

circ_ID	P-value	FC	Chr	Start	End	Str	Loci	GeneList	Description
hsa_circRNA004558	0.007921	0.08415	chr12	110340659	110342448	+	Exon	ATP2A2	ATPase sarcoplasmic/endoplasmic reticulum Ca^{2+} transporting 2

circRNA, circular RNA; FC, fold-change; Chr, chromosome; Str, strand.

rectifier currents (I_{K1}) associated with the electrical remodeling of AF are required to maintain AF. Additionally, miR-1 expression is downregulated in patients with AF, which may increase the levels of inwardly rectifying potassium channel (Kir)2.1 subunits and I_{K1} (44,45). miR-4732-3p is targeted by three circRNAs (hsa_circRNA-003876, -003878 and -003884), and represses TGF-β signaling by targeting Smad2 and Smad4, and promoting cell proliferation (46). The TGF-β and Smad signaling pathways serve essential roles in atrial fibrosis.

The results of the present study provide a potential novel insight into the molecular mechanisms and therapeutic implications of AF. The circRNA-ceRNA interactions identified may act as biosignatures for AF, and provide evidence for identifying a novel agent for the diagnosis and gene-targeted therapy of AF.

The current study has a few limitations. Firstly, though the effects of clinical patient characteristics (e.g. comorbidities, duration of AF and use of drugs) on the analysis of the RNA network were considered during patient selection, it is not possible to disregard these effects. Secondly, the circRNA data for NPAF and normal tissues were obtained from RNA sequencing, although further validation is required, including through the use of knockdown and overexpression experiments on target circRNAs and miRNAs.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

CG and YLu conceived and designed the experiments. YZ, XK and JL performed the experiments. XM, YLi, DL and LW analyzed the data. YZ, CG and YLu wrote the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Written informed consent from patients was obtained prior to collection of the left atrial appendages, which were abandoned due to the surgical technique. The study was approved by the ethics committees (no. 040-2017) in accordance with the relevant guidelines and regulations (clinical research registration no. ChiCTR-RRC-17014230). The patients provided written informed consent.

Patient consent for publication

The patients provided written informed consent for publication.

Competing interests

The authors declare that they have no competing interests.

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