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Long non-coding RNA expression profile in permanent atrial fibrillation patients with rheumatic heart disease

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Abstract. – OBJECTIVE: Atrial fibrillation (AF) is the most common type of arrhythmia, especially in rheumatic heart disease (RHD) patients. The differences in structural remodeling and electrical remodeling between the left and right atrium associated with AF in RHD patients are well known, and alterations in the expression profiles of long noncoding RNAs (lncRNAs) in the left atrium have also been investigated. However, the role of lncRNAs in the right atrium (RA) remains largely unknown.

PATIENTS AND METHODS: We identified differentially expressed lncRNAs in RA tissues of RHD patients with AF or a normal sinus rhythm (NSR) using microarray analysis. Then, we performed gene ontology (GO) and KEGG pathway analyses for functional annotation of the deregulated lncRNAs. Finally, we constructed a lncRNA-mRNA co-expression network.

RESULTS: Of the 22,829 human non-coding RNAs analyzed, a total of 1,909 long non-coding RNAs were detected. A total of 182 lncRNAs (117 downregulated and 65 upregulated) were shown to be differentially expressed (fold-change > 1.5) in AF patients compared with NSR patients. Many lncRNAs might be partially involved in an AF-related pathway.

CONCLUSIONS: AF dysregulates the expression of lncRNAs in the RA of RHD patients. These findings may be useful for exploring potential therapeutic treatments for AF in RHD patients.

Key Words:
Long non-coding RNAs, Atrial fibrillation, Microarray.

Introduction

Atrial fibrillation (AF) is the most common type of arrhythmia, which affects 33.5 million individuals worldwide, increasing the risks of ischemic stroke, thromboembolism, congestive heart failure, death, and psychological distress. Furthermore, it impairs the quality of life[1-4], especially in cardiovascular diseases patients who need open-heart surgery[5]. The main mechanisms of AF are atrial electrical remodeling and structural remodeling, which occur and differ in the left atrium (LA) and right atrium (RA)[6,7]. Scholars[6-8] showed that genetic factors play a key role in structural remodeling and electrical remodeling in AF.

Long noncoding RNAs (lncRNAs) are transcripts greater than 200 nucleotides in length with little or no protein coding potential[9]. Evidence has demonstrated that lncRNAs play critical roles in heart development[10] and various heart diseases, including cardiac hypertrophy[11], heart failure[12], and cardiac fibrosis[13]. Recent studies[14,15] have revealed that AF alters the lncRNA expression profiles in peripheral blood samples of cardiac arrhythmia and in the LA of rheumatic heart disease (RHD) patients. However, lncRNA changes in the RA from RHD patients with AF are still unclear. In addition, many investigations[6,7] have reported that the electrical remodeling in the RA plays a critical role in the initiation and maintenance of AF. Our previous researches[7] showed that the distributions of AF-associated small noncoding RNAs (miRNA) in the RA and LA are different and that the same miR-

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NAs in AF are involved in different mechanisms between the RA and LA. Yang et al[5] evaluated the long-term results of two kinds of surgical atrial fibrillation radiofrequency ablations (the biatrial maze and left atrial maze) in concomitant cardiac operations, and the results indicated that the electrical remodeling was different in the LA and RA. Hence, greater knowledge of lncRNA changes in the RA could improve understanding of AF pathogenesis. In the present study, we identified differentially expressed lncRNAs in RA tissues of RHD patients with AF or a normal sinus rhythm (NSR) using microarray analysis. After that, we performed gene ontology (GO) and KEGG pathway analyses for functional annotation of the deregulated lncRNAs. Finally, we constructed the lncRNA-mRNA co-expression network, which could help to understanding the lncRNA network involved in regulating gene expression changes and developing specific therapeutic strategies for AF in RHD.

Patients and Methods

Human Tissue Sampling

The current study conformed to the principles of the Declaration of Helsinki and was approved by the Human Ethics Committee of the First Affiliated Hospital of Sun Yat-Sen University. All patients were informed and gave written consent. Right atrial appendage tissue samples were obtained from patients with rheumatic heart disease, both with a normal sinus rhythm (NSR, n=3) and with atrial fibrillation (AF, n=3). All patients were carefully examined using two-dimensional and Doppler echocardiography and 12-lead electrocardiography. Patients who were allocated to the AF group had permanent atrial fibrillation (documented arrhythmia > 6 months before surgery) with mitral valve stenosis. The exclusion criteria included the following: ischemic cardiomyopathy, myocardial infarction, heart failure, other types of arrhythmias, history of using anti-arrhythmic medications in the past six months, chronic hepatic or renal failure, and diabetes mellitus. Samples were collected as previously described[6].

LncRNA and mRNA Microarray Expression Profiling

Total RNAs were extracted from the six RA tissue samples using TRIzol (Invitrogen, Carlsbad, CA, USA) and were quantified using a Thermo NanoDrop2000 (Thermo Fisher Scientific, Waltham, MA, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) according to the manufacturer's protocol. The RNA was applied to first and second strand cDNA synthesis, and then the double-stranded cDNA was used to generate biotinylated cRNA according to *in vitro* transcription cRNA synthesis. Biotinylated cRNA was then purified by a GeneChip Hybridization Wash and Stain Kit, fragmented and hybridized to a GeneChip® Human Transcriptome Array 2.0 (HTA2.0) (Affymetrix, Santa Clara, CA, USA) according to the affymetrix instructions. The arrays were scanned using a GeneChip Scanner 3000 after washing using a GeneChip Fluidics Station 450. Array images were analyzed and the raw data were extracted using Agilent Feature Extraction software (version 11.0.1.1, Agilent Technologies, Palo Alto, CA, USA). The lncRNAs and mRNA were collected from the authoritative databases including Noncode, GenBank, UCSC Genes, RefSeq, Ensembl, and Broad TUCP. Differentially expressed lncRNAs and mRNAs between AF and NSR were identified by fold change filtering.

Real-Time Quantitative PCR

Real-time quantitative PCR (RT-qPCR) was performed to validate the results of microarray analysis on differentially expressed lncRNAs (SNORD115-22 and BC041938). Relative amounts of target RNA expression were normalized using expression levels of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. Total RNA isolation and high-capacity cDNA reverse transcription were performed as described above. Expression levels of lncRNA SNORD115-22 and lncRNA BC041938 were detected by with a RT-qPCR using TaqMan non-coding RNA assay (lncRNA SNORD115-22: Hs01374454_m1, lncRNA BC041938: Hs03872866_s1) and TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA, USA). RT-qPCR was carried out using an ABI PRISM 7900HT sequence-detection system (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The gene expression levels were calculated with the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

All statistical analyses were performed using IBM SPSS Statistics version 22.0 (IBM Corp., Armonk, NY, USA). All data are presented as the mean ±SEM or proportions. For comparisons, Student's *t*-test was performed. A two-sided p-value < 0.05 was considered statistically significant.

Table I. Clinical characteristics of the AF and NSR patients (n=3, each).

	AF	NSR
Gender (female)	2	3
Age (y)	55.7±4.5	44.3±15.0
BMI (kg/m^2)	25.9±5.4	23.8±1.7
RA (mm)	55.7±7.6	39.7±7.5
LA (mm)	57.3±9.2*	41.7±2.1
LVEDD (mm)	48.3±5.1	49.0±12.8
EF (%)	67.3±5.7	74.7±5.3
NYHA	II(2)/ III(1)	II(1)/ III(2)

BMI: Body mass index; RA: Right atrium; LA: Left atrium; LVEDD: Left ventricular end-diastolic dimension; EF: ejection fraction; NYHA: New York Heart Association.

Results

Clinical Characteristics

Preoperative clinical characteristics were collected from medical records (Table I). Two-dimensional and Doppler echocardiography indicated that the size of the left atrium of AF patients was significantly larger than that of NSR patients, as previously reported[8] while there were no significant differences in terms of gender, age, right atrium diameter, ejection fraction (EF), or NYHA functional classification between the two groups.

Differentially Expressed lncRNAs and mRNAs

LncRNA microarrays with clean data of high quality were compared to the referential genome (hg19). Of the 22,829 human non-coding RNAs analyzed, a total of 1,909 long non-coding RNAs were observed (Figure 1A). A total of 182 lncRNAs were tested to be differentially expressed with $|\log_2^{(\text{fold-change})}| > 1.5$ in AF patients compared with NSR patients (Figure 1B). Among them, 117 and 65 lncRNAs were downregulated and upregulated, respectively. Thirty-three of these lncRNAs

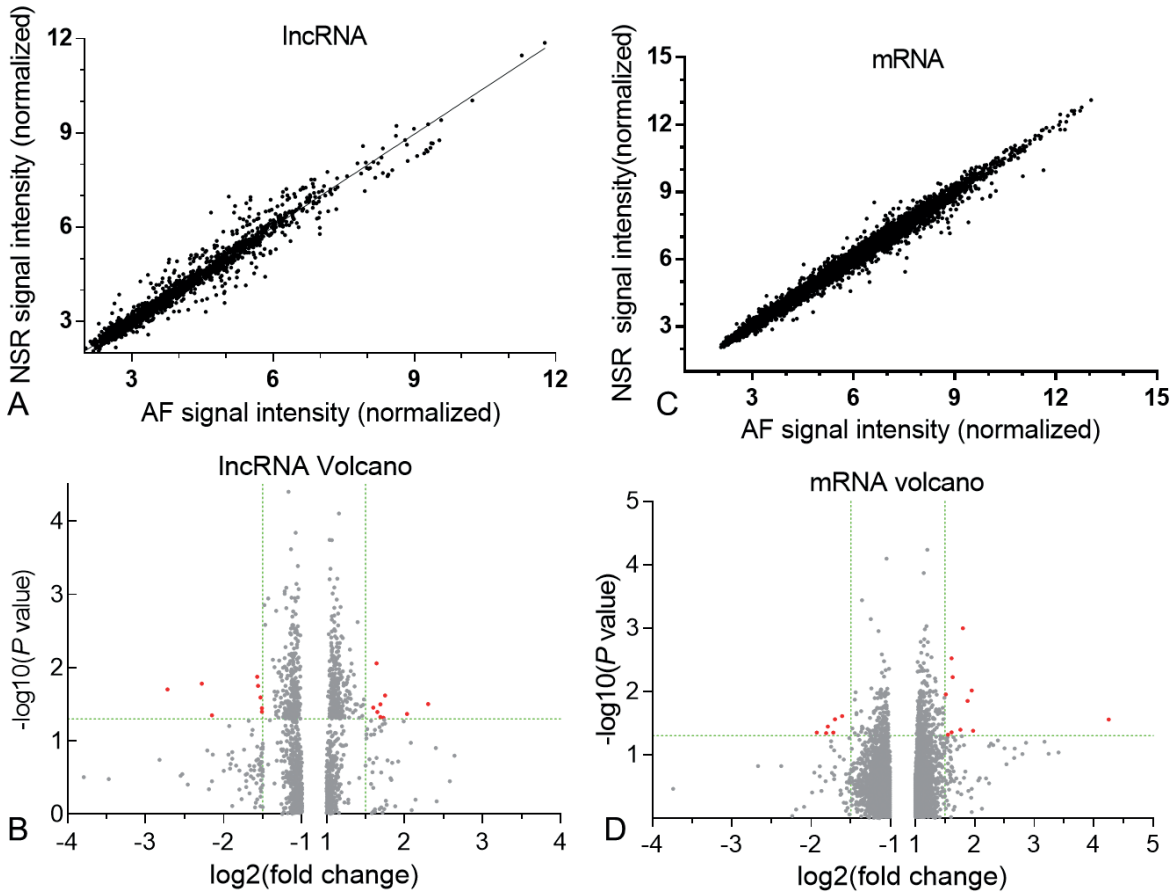

Figure 1. Expression profiles of lncRNAs and mRNAs in AF compared with NSR patients. Scatter-plot of variation in the expression of lncRNAs ***(A)*** and mRNAs ***(C)*** between AF and NSR right atrial samples. The X and Y axes are the bi-weight average signal values (log2 scaled). The Volcano plot of lncRNAs ***(B)*** and mRNA ***(D)*** between AF and NSR groups. The vertical dotted lines delimit 1.5-fold up-and down-regulation. the horizontal dotted line delimits lncRNAs or mRNA corrected p-values < 0.05. X axes are the fold change values (log2 scaled), Y axes are the p-values (log10 scaled).

Table II. Top 10 up/down-regulated lncRNAs in the AF group compared with the NSR group.

lncRNAs name	$log_2^{(fold\text{-}change)}$	*p*-value	Mrna name	$log_2^{(fold\text{-}change)}$	*p*-value
Upregulated*			Upregulated*		
uc010vaf.1	2.3	0.032	ASPN	4.25	0.028
DQ596229	2.3	0.032	CCDC80	1.97	0.042
TCONS_00023347	2.03	0.043	AEBP1	1.95	0.010
DQ589437	1.75	0.024	LTBP2	1.88	0.014
SNORD115-22	1.73	0.049	OMD	1.8	0.001
SNORD115-38	1.69	0.032	DPYSL3	1.76	0.040
SNORD115-32	1.69	0.047	EXT1	1.63	0.006
SNORD115-42	1.65	0.040	FHL2	1.61	0.003
CR608741	1.64	0.009	SMYD2	1.61	0.044
SNORD115-6	1.6	0.035	TNXB	1.55	0.048
Downregulated*			COL14A1	1.51	0.011
uc001eiy.2	-2.72	0.020	Downregulated*		
uc001ejh.1	-2.72	0.020	JAG1	-1.93	0.044
DQ590126	-2.72	0.020	SPHKAP	-1.81	0.045
DQ579288	-2.72	0.020	PDE3A	-1.79	0.036
BC041938	-2.28	0.017	MYBPHL	-1.72	0.045
DQ576791	-2.15	0.045	CHL1	-1.7	0.027
DQ576039	-1.57	0.013	KCNJ5	-1.61	0.024
uc010yty.1	-1.57	0.013			
DQ595787	-1.56	0.018			
TCONS_00005387	-1.53	0.026			
NYHA	II(2)/ III(1)	II(1)/ III(2)			

BMI: Body mass index; RA: Right atrium; LA: Left atrium; LVEDD: Left ventricular end-diastolic dimension; EF: ejection fraction; NYHA: New York Heart Association.

were significantly dysregulated in the AF group compared with the NSR group: 10 lncRNAs were upregulated, while 23 lncRNAs were downregulated ($p < 0.05$). The top ten up/down-regulated lncRNAs in the AF group compared with the NSR group are listed in Table II. Of the 245,349 human mRNAs analyzed, a total of 23,801 mRNAs were detected (Figure 1C, D). Using the same criteria as for the lncRNAs, a total of 132 (47 down and 85 up) mRNAs were shown to be differentially expressed. Among them, 17 mRNAs were significantly dysregulated in the AF group compared with the NSR group: 11 mRNAs were upregulated, while 6 mRNAs were downregulated ($p < 0.05$) (Table II).

Validation of the Microarray Data with RT-qPCR

To verify the data obtained from our microarray analysis, we randomly chose two lncRNAs (BC041938 and SNORD115-22) for quantitative Real-time RT-PCR (RT-qPCR) with 30 human right atrial appendage tissue samples (15 AF samples and 15 NSR samples). The differential expressions of these two lncRNAs were verified as revealed by our microarray results (Figure 2).

Gene Ontology and KEGG Pathway Analysis

The biological function of mRNAs was classified using Gene Ontology (GO) analysis (Figure 3). Differentially expressed transcripts in biological processes were involved in extracellular structure organization, responses to endogenous stimuli, metabolic processes of multicellular organisms, collagen fibril organization, catabolic processes of single-celled organisms, cellular responses to organic substances, multicellular organismal macromolecule metabolic processes, responses to oxygen containing compounds, skeletal system development, and responses to nitrogen compounds. Differentially expressed transcripts in molecular functions were involved in extracellular matrix (ECM) structural constituents, receptor binding, collagen binding, structural molecule activity, glycosaminoglycan binding, protein complex binding, calcium ion binding, integrin binding, and growth factor binding. Differentially expressed transcripts in cellular components were involved in the proteinaceous ECM, ECM, ECM component, basement membrane, collagen trimer, intracellular vesicle, complex of collagen trimers, endoplasmic reticulum parts, and transport vesicles. KEGG pathway enrichment analysis re-

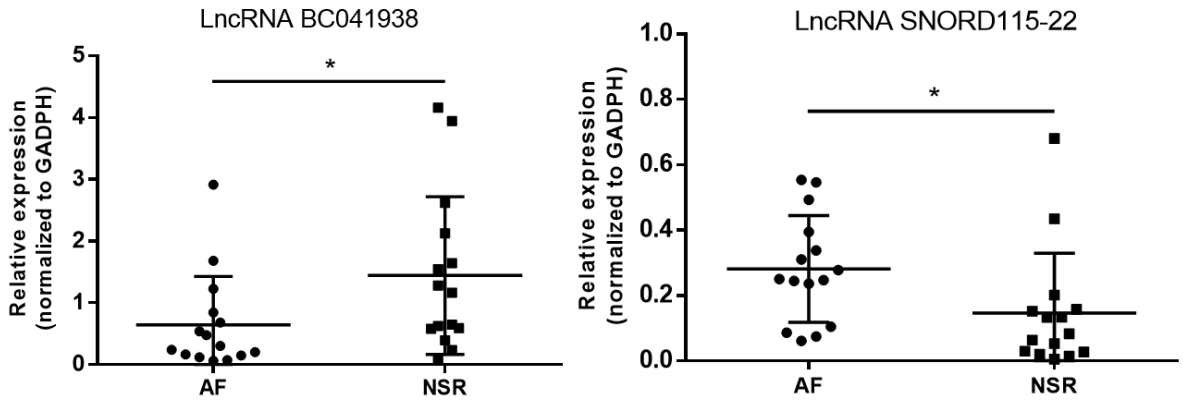

Figure 2. RT-qPCR validation reveals significant lncRNA expression differences in AF samples compared to NSR samples. The expression level of two selected lncRNAs in 15 RHD patients with AF samples compared to 15 patients with NSR. All the differences of five lncRNAs between AF samples and NSR samples were significant.

vealed the top ten potential functional pathways (Figure 3), including ECM receptor interactions, collagen binding, systemic lupus erythematosus, leishmanial infection, asthma, allograft rejection, graft *vs.* host disease, diabetes mellitus, intestinal immune network for IGA production, and autoimmune thyroid disease.

Construction of a Co-Expression Network

LncRNAs play essential roles in AF progression, and regulate target genes primarily through cis- and trans-regulation. The co-expression network of the differentially expressed correlated lncRNAs and putative target mRNAs was constructed by Spearman's correlation coefficients ≥ 0.5 and Pearson's correlation coefficients ≥ 0.5. lncRNAs were ranked according the fold-change (>1.5-fold) and the *p*-value ($p<0.05$). These results indicated that these lncRNAs have roles in human health and disease regulation (Figure 4).

Discussion

AF is the most prevalent heart disease worldwide, causing a tremendous physical and psychological pain in individuals and a heavy burden for countries[1,2]. To date, the effects of pharmacotherapy and radiofrequency ablation for AF are still unsatisfactory, because the mechanisms of AF have not been accurately determined[7]. Therefore, a thorough understanding of the molecular aberrations of AF would contribute to the design and development new therapeutic treatments. Using a high-throughput lncRNA microarray analysis, we identified thousands of differentially expressed mRNAs and differentially expressed lncRNAs in three pairs of RA tissues in AF and NSR patients with RHD. The results of differentially expressed mRNAs demonstrated that some of these might be involved in arrhythmias, such as MYBPHL mRNA. Barefield et al[16] observed that the MYBPHL gene encodes MyBP-HL (myosin-binding protein-H like, a novel myofilament component) and MYBPHL truncations may increase the risk for human arrhythmias. The potential functions of differentially expressed lncRNAs were then assessed through GO enrichment and KEGG pathway analyses and lncRNA-mRNA co-expression analysis, which were principally based on the involvement of lncRNAs' functions with their target gene. The results demonstrated that some of these differentially expressed lncRNAs might be involved in some AF-related pathways, but further investigation is still needed to validate these deductions. Several lncRNAs have recently been identified for their roles in atrial structural remodeling mediated by fibrosis/apoptosis[17-19]. In the current work, the putative functions of lncRNA CR608741 may involve the TGFβ/Smad signaling pathway. The TGFβ/Smad signaling pathway is the most canonical pathway in promoting atrial fibrosis. Qu et al[17] have identified that lncRNA myocardial infarction associated transcript (MIAT) was the first pro-fibrotic lncRNA that controls cardiac fibrosis through upregulation of Furin and TGF-β1 and down-regulation of miR-24. Also, it regulates cardiac function both *in vivo* and *in vitro* models. LncRNA cardiac hypertrophy-associated epigenetic regu-

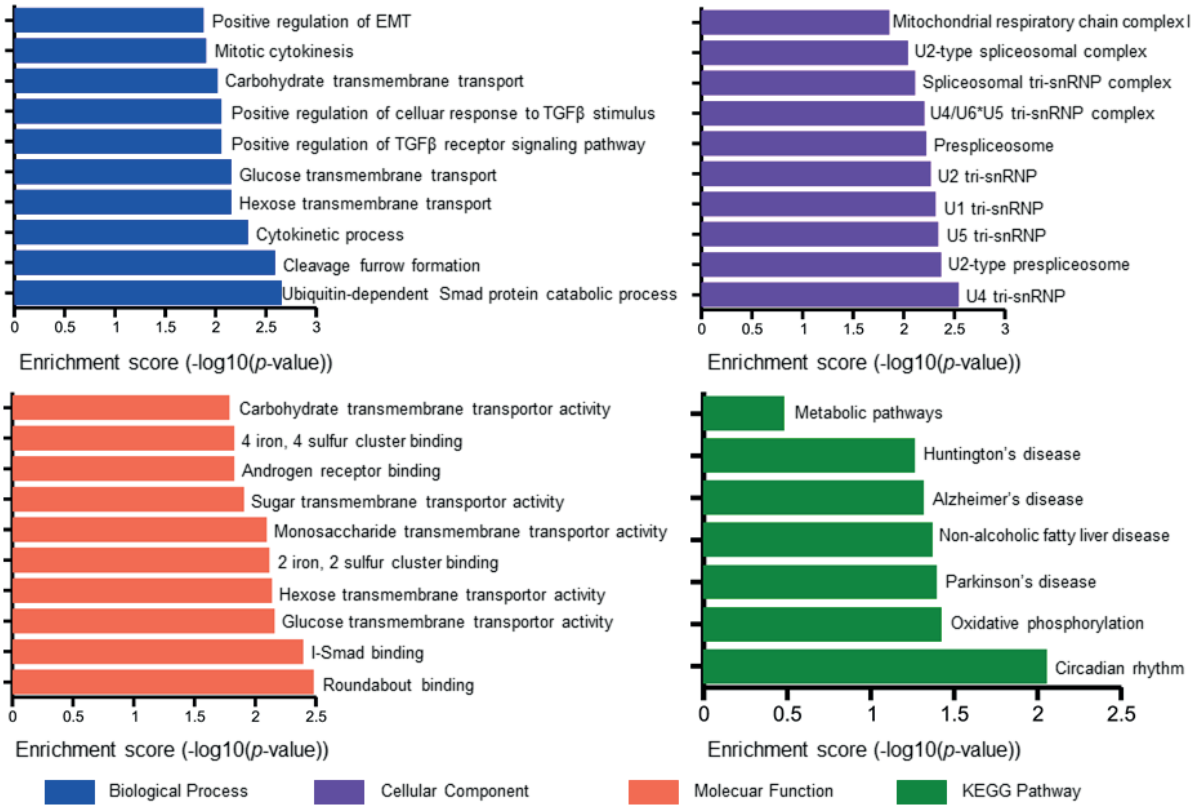

Figure 3. Enrichment analysis of differentially expressed mRNAs. GO-BP: Biological process; GO-MF: Molecular function; GO-CC: Cellular component. The top ten potential functional pathways in KEGG pathway enrichment analysis.

lator (Chaer) knockout significantly attenuated cardiac hypertrophy and fibrosis after transverse aortic constriction surgery[18]. A research indicated that five lncRNAs (n379599, n379519, n384640, n380433 and n410105), which are involved in the TGFβ pathway, regulate the expression and function of extracellular matrix (ECM) synthesis genes and cardiac fibrosis through modulating myofibroblast differentiation in an ischemic cardiomyopathy model[19]. Cardiac apoptosis-related (CARL) lncRNA can act as an endogenous microRNA-539 sponge and suppress mitochondrial fission and apoptosis in cardiomyocytes by relieving miR-539-dependent PHB2 repression[20]. The lncRNA Mhrt was demonstrated to be involved in the regulation of doxorubicin-induced cardiomyocyte apoptosis by the analysis of caspase-3 activity and apoptosis[21]. It is well-established that atrial electrical remodeling, mainly mediated by ion-channel alterations, plays a key role in the initiation and maintenance of AF. Increasing evidence has suggested that electrical remodeling associated lncRNAs are involved in the AF pathogenic mechanism[6]. Li et al[6] showed that the lncRNA TCONS_00075467 in RA tissues was involved in electrical remodeling in a rabbit AF model; then, the silencing of TCONS_00075467 with lentiviruses could shorten the atrial effective refractory period and decrease the L-type calcium current and action potential duration *in vitro*. They also showed that TCONS_00075467 can act as microRNA-328 sponge *in vitro* and *in vivo* and modulate the downstream protein coding gene CACNA1C. Tan et al [22] have demonstrated that the lncRNA HOTAIR is involved in modulation of calcium homeostasis in human cardiomyocytes. They have further confirmed that HOTAIR inhibits the intracellular Ca^{2+} contents via regulation of L-type calcium channels ($Ca_v1.2$). lncRNA LINC00523 may also be involved in regulating intracellular calcium levels[23].

Several reports[24,25] have recently proposed that intrinsic cardiac autonomic neural remodeling plays an important role in the initiation and perpetuation of AF. A new lncRNATCONS_00032546 could shorten the right atrial effective refractory period and increase AF susceptibility by promoting neurogenesis; however, another lncRNA

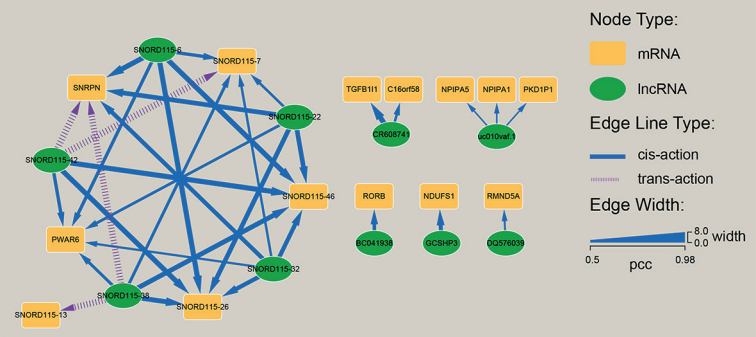

Figure 4. Co-expression networks of lncRNAs and putative target mRNAs.

TCONS_00026102 had the opposite effect and had a positive influence on AF inducibility via complex mechanisms during neural remodeling in a canine AF model.[25]

Conclusions

We showed that AF could dysregulate the expression of lncRNAs in the human right atrium. The potential functions of differentially expressed lncRNAs were assessed through GO enrichment and KEGG pathway analysis and lncRNA-mRNA co-expression analysis. The results demonstrated that some of these differentially expressed lncRNAs might be involved in some AF-related pathways, but further studies are still needed to validate these deductions.

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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