Expression profile of circulating lncRNAs in patients with atrial fibrillation

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Abstract

Background: Atrial fibrillation (AF) is one of the most common cardiac arrhythmias. However, the exact pathophysiology and mechanisms about AF remains unclear. Accumulating studies have demonstrated that long non-coding RNAs (lncRNAs) play a vital role in the regulation of almost all biological processes. However, relationships between AF and lncRNAs are still unknown. Methods: The peripheral blood monocytes of a total of 20 patients with AF and 20 healthy subjects were collected for gene chip technology to detect differentially expressed lncRNAs. Reverse transcription polymerase chain reaction (RT-PCR) was applied for further verification. 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. Results: There were 19 lncRNAs differentially expressed (FC ≥ 2, p <0.05), of which 6 were up-regulated and 13 were down-regulated. Two of three upregulated lncRNAs (P=0.014 and 0.006 for HNRNPU-AS1and LINC00861, respectively) and two of three downregulated lncRNAs (P=0.028 and 0.032 for RP11-443B7.3 and CTD-2616J11.14, respectively) were randomly confirmed by RT-PCR. And showed a significantly different expression with the RNA-seq results. GO analysis showed that differentially expressed genes enriched in differentially expressed transcripts in biological process were mainly involved in metabolic process, catabolic process and biosynthetic process. Differentially expressed transcripts in cellular component were mainly involved in nuclear lumen, organelle lumen and cytoplasm et al. Differentially expressed transcripts in molecular function were mainly involved in protein binding, RNA binding and molecular function et al. KEGG enrichment pathway analysis showed that some of the enrichment pathways associated with differentially expressed lncRNAs include Calcium signaling pathway, NF-kappa B signaling pathway, cytokine-cytokine receptor interaction and Toll-like receptor signaling pathway, etc. HNRNPU-AS1 was the highest positive correlated lncRNA in the networks. Conclusions: The expression of lncRNA in peripheral blood of AF patients is different from that of normal people. The physiological functions of these differentially expressed lncRNAs may be related to the pathogenesis of AF, which provide experimental basis and new therapeutic target for prognosis and treatment of patients with AF. HNRNPU-AS1 may play a important role in the pathophysiology and mechanisms of AF.

Background

Atrial fibrillation (AF) is one of the most common cardiac arrhythmias. There is a positive relation between the prevalence of AF and age, with a prevalence of 0.5% among individuals aged 50 to 59 years and up to 8.8% among individuals aged 80 to 89 years [1]. As reported, AF is associated with increased global public health risk such as stroke, heart failure and cardiovascular mortality [2, 3]. Over the last 20 years, the major mechanisms involved in AF have been illustrated as electrical and structural remodeling, reentry and ectopic firing, inflammation and oxidative stress [4, 5]. However, the exact underlying pathophysiology and mechanisms about AF remains unclear.

Long non-coding RNAs (lncRNAs) are non-protein-coding RNAs longer than 200 nucleotides in length and and seldom encode proteins [6]. Accumulating studies have demonstrated that lncRNAs play a vital role

in the regulation of almost all biological processes, including development, differentiation, metabolism. Moreover, lots of lncRNAs have been functionally elucidate in regulating a great number of genes involved in cancers, myocardial infarction and heart failure and may be potential novel biomarkers for diseases diagnosis and prognosis, as well as therapeutic targets [7-9]. However, relationships between AF and lncRNAs are still unknown. Recently, we have performed a microarray analyses on human left atrial appendage in AF patients and indicated that there are differentially expressed lncRNAs in AF, which may contribute to the pathogenesis of AF [10].

As a type of white blood cell present in the peripheral circulation, monocytes have been reported to closely linked to outcomes in patients with cardiovascular disease and the high numbers of monocytes are associated with increased risk of recurrent myocardial infarction, hospitalization and cardiac death [11, 12]. Yet, the role of monocytes in determining outcomes amongst AF patients is unknown, the lncRNAs expression of peripheral circulating monocytes in AF has not been investigated. Thus in the present study, in order to determine whether there is a dysregulation of lncRNAs in monocytes of AF patients compared with healthy people, lncRNA microarray is used and real-time quantitative reverse transcription PCR (qRT-PCR) for specific differentially expressed lncRNAs is used subsequently to validate the microarray results. We think that these data could help highlight the exact underlying pathophysiology and mechanisms about AF and provide new therapeutic strategies in AF patients.

Methods

Study population

In this study, 20 patients with AF and 20 matched control subjects were recruited from our hospital. Written informed consent was obtained from AF patients and controls before entering this experiment and the study protocol was approved by the Ethics Committee of Taizhou People's Hospital. The diagnosis of AF was mainly based on the criteria listed in 2016 ESC Guidelines for the management of atrial fibrillation [13]. Information about baseline characteristics was collected from the medical records by a special doctor and provided in Table 1.

Monocytes collection, RNA preparation and construction of the lncRNA microarray

About 8 ml of blood samples from each subject was drawn into ethylene diamine tetra-acetic acid (EDTA) - anticoagulant tubes, then diluted 1:1 with PBS, separated with Ficoll's density gradient centrifugation and subsequent Magnetic Cell Sorting. Total RNAs were isolated from the isolated monocytes using Trizol (Invitrogen, USA) and were quantified using a NanoDrop spectrophotometer (IMPLEN, CA, USA). A total amount of 3 μg RNA per sample was used as input material for the RNA sample preparations, products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system. Quantile normalization and subsequent data processing were performed using the GeneSpring GX v11.5.1 software package (Agilent, USA). Differentially expressed lncRNAs between the two groups were identified by Fold Change filtering. The threshold set for upregulated and downregulated lncRNAs was more than two-fold.

Validation by reverse transcription polymerase phain reaction (RT-PCR)

To verify the microarray data, we randomly selected 6 differentially expressed lncRNAs and detected their expression changes by RT-PCR. A total of 3μg RNA was used for reverse transcription. Detection of the amplified cDNA was performed with the rotor gene Q series (Qiagen, USA). GeneAmp PCR System 7500 (Applied Biosystems, USA) was used for RT-PCR. Data are presented as fold changes of these genes relative to the expression of GAPDH as a housekeeping gene. The results were analyzed according to the $2^{-\triangle\triangle Ct}$ method [14]. Primers used for RT-PCR are listed in Table 2.

Gene Ontology (GO) and KEGG enrichment analysis

Gene Ontology (GO) enrichment analysis of differentially expressed genes or lncRNA target genes were implemented by the GOseq R package, in which gene length bias was corrected. GO terms with corrected P value less than 0.05 were considered significantly enriched by differential expressed genes. KEGG is a database resource for understanding high-level functions and utilities of the biological system, such as the cell, the organism and the ecosystem, from molecular-level information, especially large-scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies (http://www.genome.jp/kegg/). We used KOBAS software to test the statistical enrichment of differential expression genes or lncRNA target genes in KEGG pathways.

Construction of lncRNA-mRNA regulatory co-expression network

To evaluate the interaction between the differentially expressed lncRNAs and their target mRNAs, co-expression network was constructed on the basis of the correlation between the differentially expressed lncRNAs and mRNAs (Pearson correlation coefficient, the absolute value >0.95; P-value < 0.05) using the Cytoscape 3.7.0 software (http://cytoscape.org/).

Statistical analysis

SPSS software 22.0 was used for statistical analyses. All data were presented as the mean ±standard deviation (SD). Comparisons between the two groups were performed using Student's t-test. Statistical significance was determined at $p < 0.05$.

Results

Analysis of differentially expressed lncRNAs

In total, 675 lncRNAs were analyzed by microarray. A total of 19 lncRNAs was calculated as differentially expressed between AF group and control group (fold change >2, and $p < 0.05$). In which, 6 lncRNAs were upregulated and 13 lncRNAs were downregulated (Figure 1, Table 3).

Microarray validation by RT-PCR

To validate the microarray results, six differentially expressed lncRNAs were randomly selected to to verify their expression level by qPCR, including 3 up-regulated lncRNAs (HNRNPU-AS1, AC005786.7 and LINC00861) and 3 down-regulated circRNAs (RP11-443B7.3, CTD-2616J11.14 and CTD-2616J11.3). As a result, 2 out of 3 upregulated lncRNAs (P = 0.014 and 0.006 for HNRNPU-AS1and LINC00861, respectively) and 2 out of the three downregulated lncRNAs (P = 0.028 and 0.032 for RP11-443B7.3 and CTD-2616J11.14, respectively) showed a significantly different expression with the RNA-seq results (Figure 2).

GO and KEGG pathway analysis of differentially expressed lncRNAs

Differentially expressed target genes of the lncRNAs were further analyzed to GO analysis, which included biological process, cell component and molecular function three categories.The top 20 significant GO terms of each subgroup are shown in Figure 3. As shown in Figure 3, differentially expressed transcripts in biological process were mainly involved in metabolic process, catabolic process and biosynthetic process. Differentially expressed transcripts in cellular component were mainly involved in nuclear lumen, organelle lumen and cytoplasm et al. Differentially expressed transcripts in molecular function were mainly involved in protein binding, RNA binding and molecular function et al. Next, KEGG pathway enrichment analysis for the differentially expressed lncRNAs was performed to identify pathways and further study biological function. We performed the critical pathways with low P-values ($P < 0.05$) and used-lg p-value to describe the significance level of the pathway enrichment. The top 20 pathway terms of KEGG analysis are shown in Figure 4. Briefly, most enriched pathways were related to the Calcium signaling pathway, NF-kappa B signaling pathway, cytokine-cytokine receptor interaction and Toll-like receptor signaling pathway.

Construction of lncRNA-mRNA regulatory co-expression network

According to the differential expression of lncRNAs, the potential co-expression network between lncRNAs and mRNAs was constructed by using Cytoscape. As shown in Figure 5, HNRNPU-AS1 was the highest positive correlated lncRNA in the networks, which suggests that HNRNPU-AS1 may play a important role in the pathophysiology and mechanisms of AF.

Discussion

As one of the most common cardiac arrhythmias, AF is responsible for the risk of ischemic stroke and further results in significant morbidity, mortality and poor quality of life. Various evidence suggest that AF presence affects an estimated 33.5 million individuals in the global world with an overall prevalence of 1-2% in the general population and an increases with age up to 20% in octogenarians [3,15]. However, the efficacy of presently available therapeutic approaches is limited. Thus, elucidating the mechanisms underlying AF onset and progression is helpful for AF therapeutic innovation. Our previous study has demonstrated that there was differentially expressed lncRNAs in atrial tissues from AF patients and dysregulated lncRNAs may play regulatory roles in the mechanism of AF [10]. To our knowledge, there are very few studies revealing lncRNA expression profiles of monocytes from AF population. Ferthermore,

compared with atrial tissues, monocytes are easilier obtained and their RNA quality meets the standard of RNA sequencing (RNAseq).

In the current study, as an outstanding technology for disease excavation, RNAseq was applied to identify differentially expressed lncRNAs of monocytes between AF patients and healthy people. We identified 19 differentially expressed lncRNAs of monocytes between AF patients and healthy people, among which 6 lncRNAs were down-regulated and 13 lncRNAs were up-regulated. Compared with the differentially expressed lncRNAs in human left atrial appendage of AF patients , the numbers of differentially expressed lncRNAs in circulating peripheral blood were significantly lower.

To further identify whether the differentially expressed lncRNAs are associated with AF, 3 up-regulated lncRNAs (HNRNPU-AS1, AC005786.7 and LINC00861) and 3 down-regulated circRNAs (RP11-443B7.3, CTD-2616J11.14 and CTD-2616J11.3) were randomly selected for qT-PCR validation. The results were in accordance with the differential expression observed in RNAseq.

GO enrichment analysis showed that the differently expressed lncRNAs were mainly associated with metabolic process, catabolic process and biosynthetic process in biological process , nuclear lumen, organelle lumen and cytoplasm in cellular component, protein binding, RNA binding and molecular function in molecular function. Furthermore, enriched pathways demonstrated that most enriched pathways were related to the Calcium signaling pathway, NF-kappa B signaling pathway, cytokine-cytokine receptor interaction and Toll-like receptor signaling pathway. Accumulating evidence illustrates that atrial tachyarrhythmia causes atrial hypertrophy by activation of the Calcium signaling pathway, which thereby contributes to structural remodeling of human atria [16]. Reports showed that NF-kappa B signaling pathway and Toll-like receptor signaling pathway are closely associated with apoptosis, immunity, inflammation, and oxidative stress [17,18]. All these demonstrated that these signaling pathways might be involved in the initiation and development of AF and differentially expressed lncRNAs may participate in the pathogenesis of AF.

In the current study, co-expression network analysis between lncRNA and mRNA was constructed to predict the key lncRNAs that related to AF by Cytoscape 3.01.HNRNPU-AS1, which was shown to have the highest correlated degree, may play a important role in the co-expression network. The results indicate that the regulation of HNRNPU-AS1 may affect the onset, progression, and maintenance of AF through regulating the expression of their corresponding mRNAs.

In summary, Our results illustrated a profile of lncRNAs differentially expressed in monocytes of AF patients and differentially expressed lncRNAs may play core roles in the mechanism of AF. Although the mechanisms of the discovered lncRNAs in AF remain to be elucidated, we hope our novel discovery will lead to more studies that will determine its function.

Declarations

Acknowledgments

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Disclosure of conflict of interest

None.

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Tables

Table1 Clinical characteristics of AF patients and matched controls

Parameters	AF patients (n-20)	Controls (n=20)	P-value
Age (years)	64.15±6.65	63.65±5.99	>0.05
Male/female	10/10	12/8	>0.05
Body mass index (kg/m^2)	23.96 ± 0.98	23.38 ± 0.62	>0.05
smoking	2	2	>0.05
Alcohol abuse	0	0	>0.05
NYHA class I/II/III/IV	20/0/0/0	20/0/0/0	>0.05
SBP (mm Hg)	130.75 ± 15.38	129.35 ± 14.57	>0.05
DBP (mm Hg)	73.45 ± 8.54	72.40 ± 8.21	>0.05
LAD (cm)	4.06 ± 0.42	3.24 ± 0.25	<0.05
LVEF (%)	54.92 ± 5.21	56.37 ± 6.28	>0.05

AF atrial fibrillation, SBP systolic blood pressure, DBP diastolic blood pressure, NYHA New York Heart Association, LAD left atrium diameter, LVEF left ventricular ejection fraction

Table 2 The primer sequences for qRT-PCR

Gene_id	Gene_name	Primer sequences	Length
ENSG00000188206.6	HNRNPU-AS1	F:5′-GGAAGCTGTACACTGGAGGT-3′ R:5′-GCGCTAGCACACTGCAATTA-3′	182
ENSG00000267436.1	AC005786.7	F:5′-CAGCAGAGTCCACCAAGC-3′ R:5′-TGAGCTCAGTCCAGTTCACC-3′	168
ENSG00000245164.6	LINC00861	F:5′-GCCATTCTTCAAGGACTTCACA-3′ R:5′-CAGCTCCAATTTCCAATTCTGC-3′	112
ENSG00000258082.1	RP11-443B7.3	F:5′-TCACTAGTGTGCCGTCTGAA-3′ R:5′-GTCGGAACACAGAACACCTG-3′	154
ENSG00000268889.1	CTD-2616J11.14	F:5′-AGCAACTATCTTGGCAACATCCT-3′ R:5′-AACAACCCTACTTAACGAAACCC-3′	105
ENSG00000254760.1	CTD-2616J11.3	F:5′-TTGGAGAATGCCGTTGAGATG-3′ R:5′-GCAAAGAGTAGGGTCCTGTGGT-3′	113
GAPDH-F		F:5′-CATGAGAAGTATGACAACAGCCT-3′	177
GAPDH-R		R:5′-AGTCCTTCCACGATACCAAAGT-3′	

Table 3 Differentially expressed lncRNAs

Differentially expressed	Gene_id	Gene_name	AF_FPKM	C_FPKM	Foldchange	pvalue
Upregulated	ENSG00000188206.6	HNRNPU-AS1	8.68	0.72	12.06	0.015
	ENSG00000247982.6	LINC00926	25.85	12.33	2.08	0.043
	ENSG00000245164.6	LINC00861	23.56	3.183	7.21	0.003
	ENSG00000267436.1	AC005786.7	1.91	0.11	18.63	0.002
	ENSG00000271895.2	RP4-635E18.8	9.84	0.23	42.7	0.001
	XLOC_099061	-	2.05	0.18	3.48	0.023
Downregulated	ENSG00000230470.1	GS1-115G20.1	1.23	3.43	2.78	0.033
	ENSG00000241163.7	LINC00877	2.22	4.65	2.11	0.035
	ENSG00000239794		3.72	8.68	2.34	0.032
	ENSG00000175611.11	LINC00476	20.42	49.47	2.47	0.039
	ENSG00000254760.1	CTD-2616J11.3	2.05	6.61	3.31	0.013
	ENSG00000255441.1	CTD-2616J11.2	2.46	7.51	3.12	0.026
	ENSG00000258082.1	RP11-443B7.3	0.74	2.63	3.77	0.002
	ENSG00000261222.2	CTD-2006K23.1	2.12	5.66	2.66	0.002
	ENSG00000268889.1	CTD-2616J11.14	2.02	6.55	3.28	0.012
	ENSG00000271614.1	LINC00936	2.72	8.18	3.02	0.002
	ENSG00000279491.1	RP11-810P12.7	2.11	4.33	2.06	0.042
	XLOC_060065	-	0.86	2.73	3.41	0.046
	XLOC_109634	-	0.22	2.12	9.64	0.001

Figures

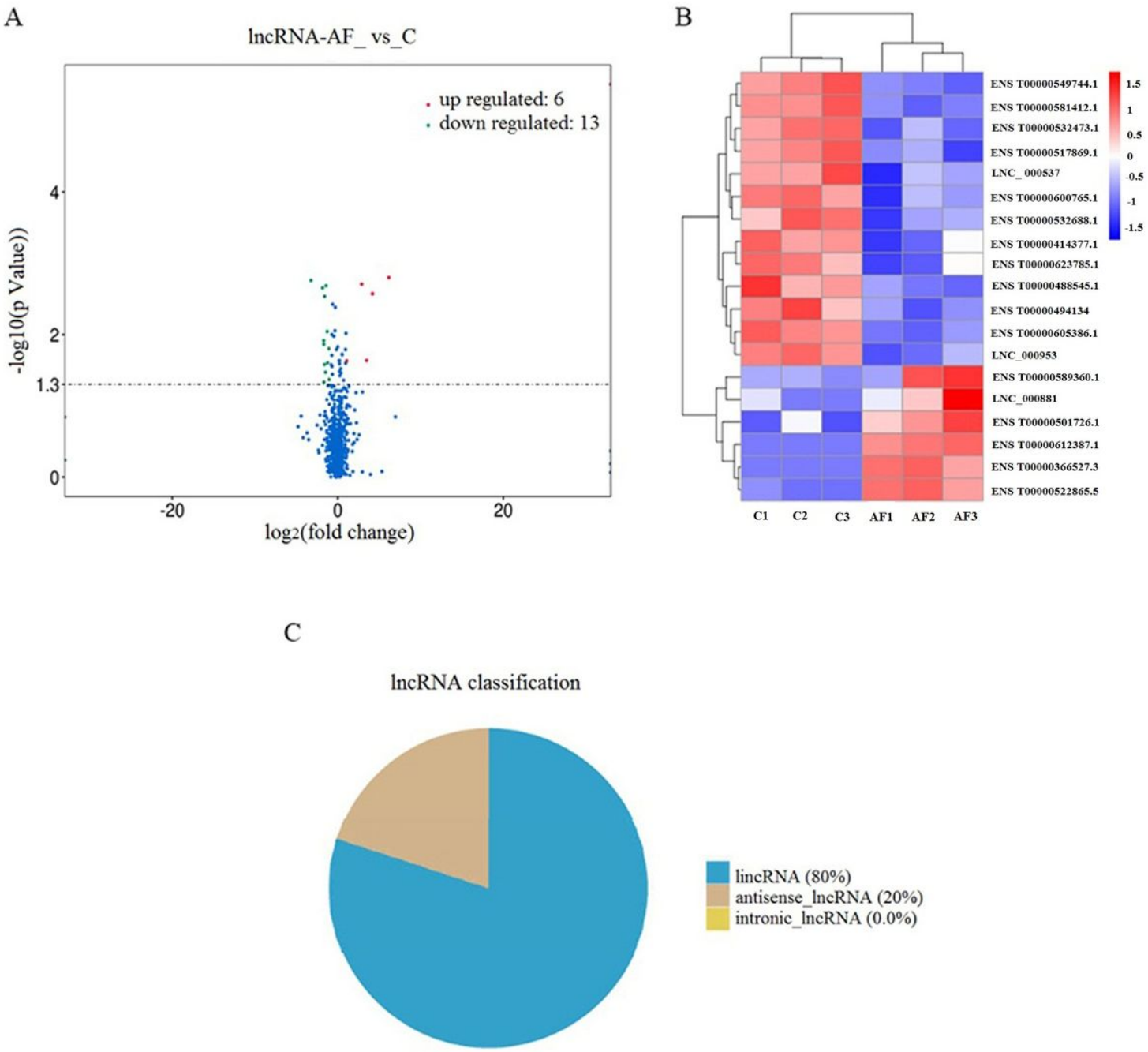

Figure 1

LncRNA microarry expression data between AF group and control group. (A) Volcano plot of lncRNAs between AF group and controls. The horizontal dashed line delimit 2.0-fold up- and down-regulation. Red plots represent up-regulated lncRNAS and green plots represent down-regulated lncRNAs with >2.0 fold-

changge and corrected P value <0.05. (B) Cluster analysis of differentially expressed lncRNAs of AF patients and healthy controls. In the color scheme, red indicates higher expressiom, and green indicates lower expression. (C) Category of differentially expressed lncRNAs.

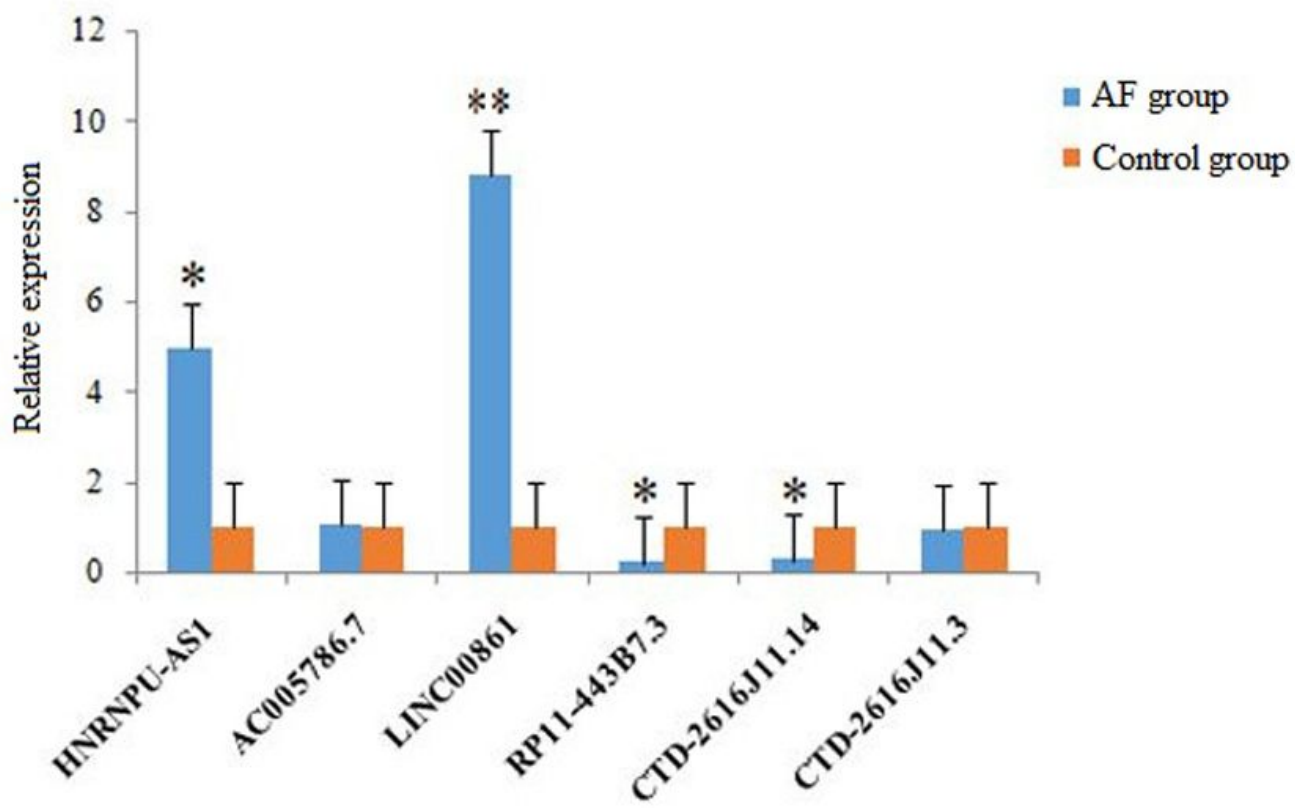

Figure 2

LncRNAs expression validated by real-time RT-PCR. Each RT-PCR assay was performed for three times. *P < 0.05, **P<0.01, compared with control group.

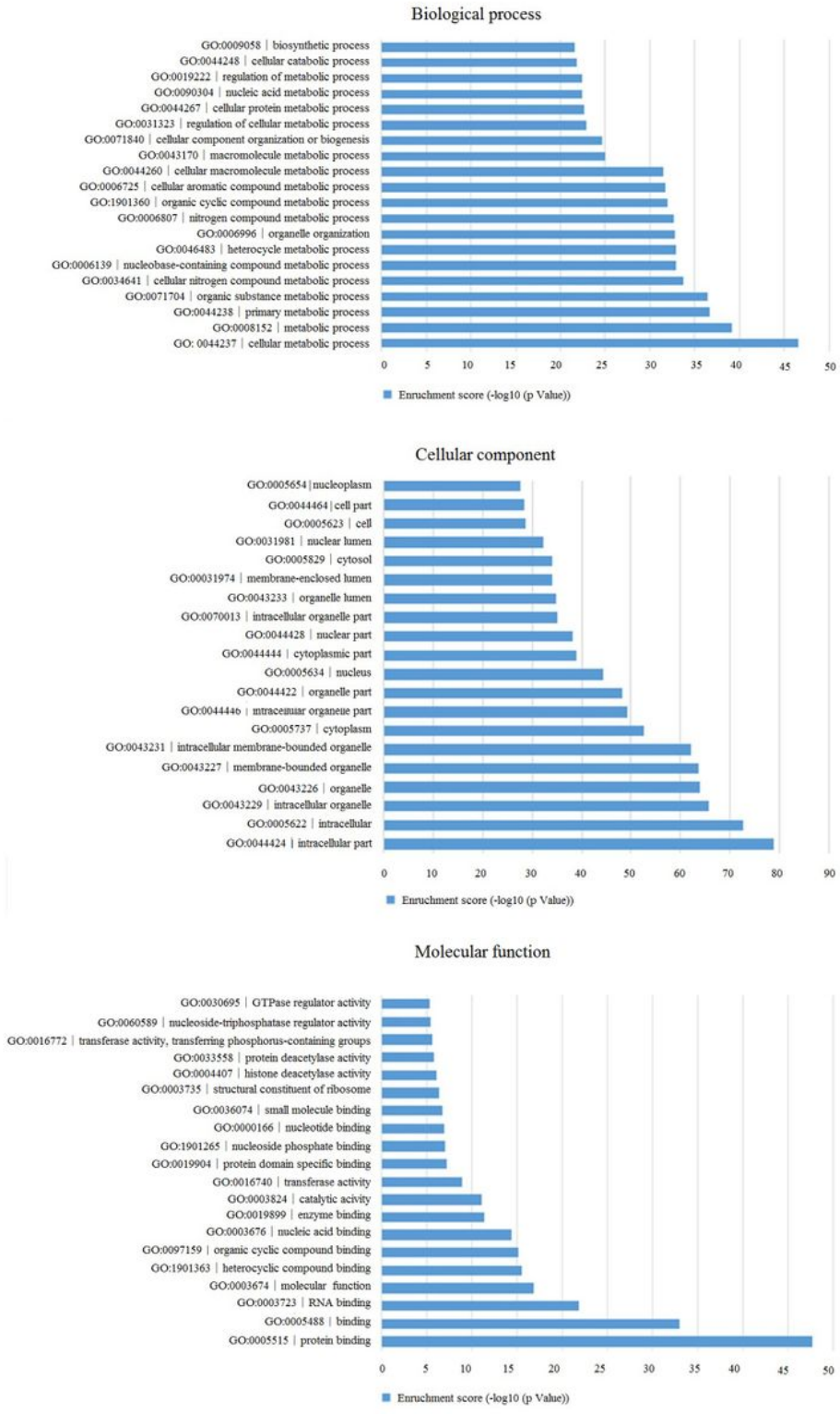

Figure 3

The top 20 results of GO analysis (biological processs, cellular component and molecular function)

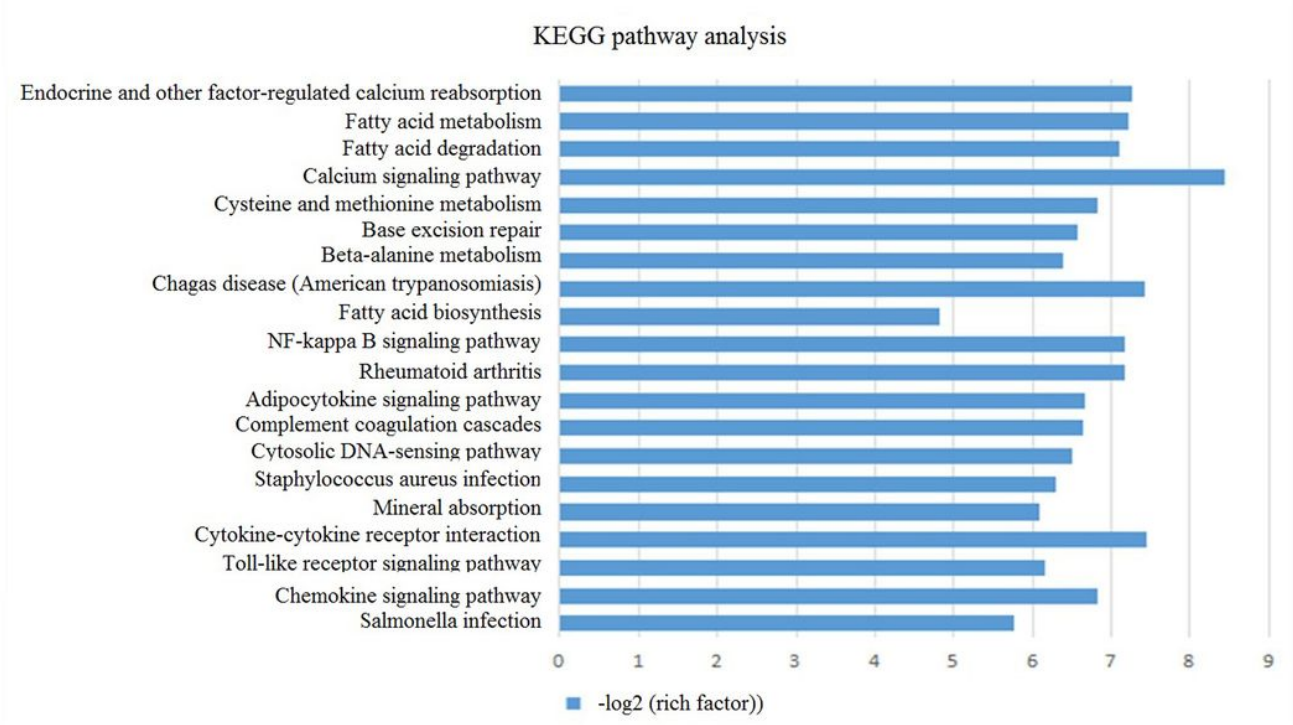

Figure 4

The top 20 neighbor coding genes of KEGG enrichment corresponds to the differentially expressedlncRNAs

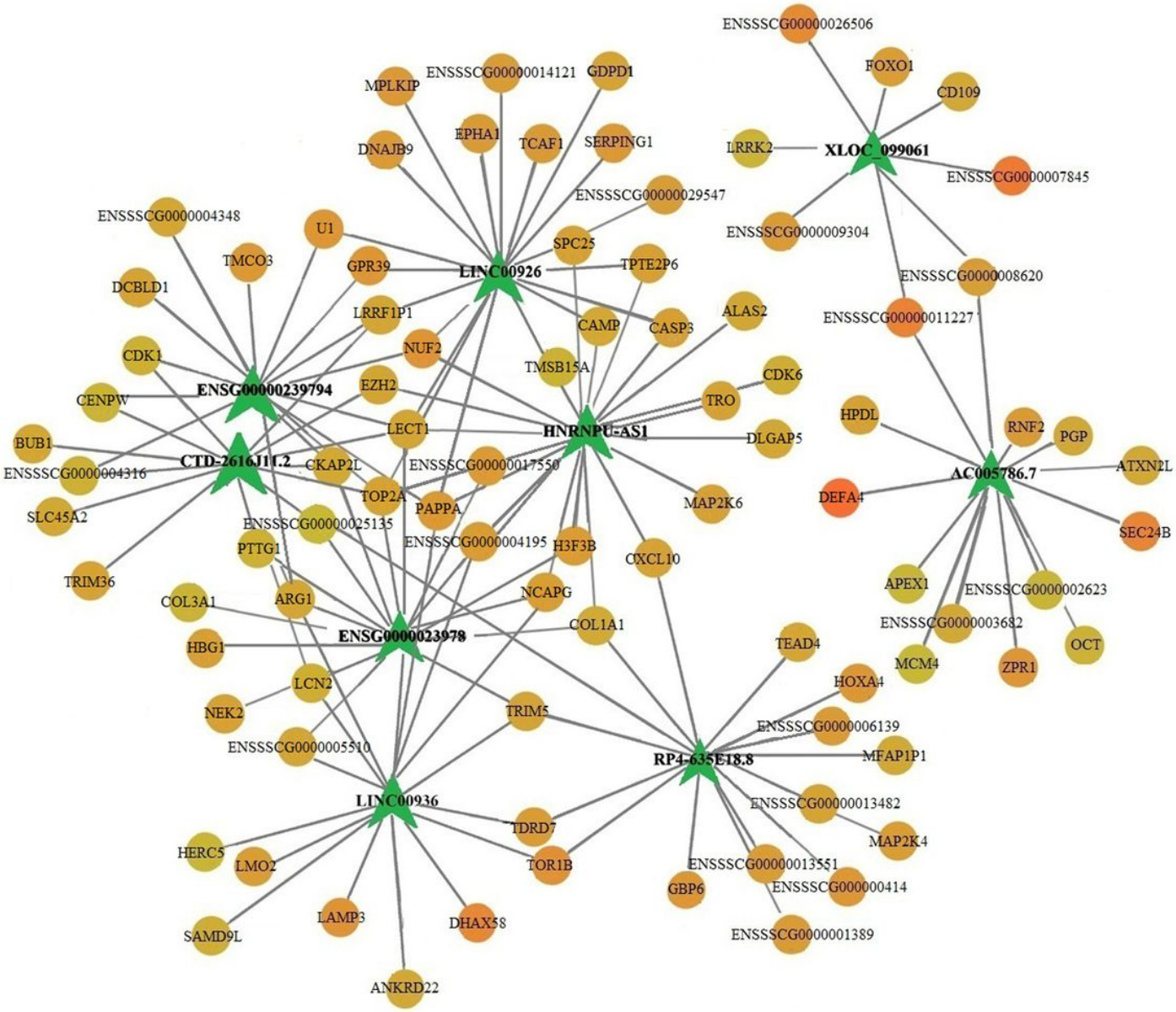

Figure 5

LncRNA-mRNA co-expression network explored by using Cytoscape. The size of each node represent functional connectivity of each lncRNA. The green node represents lncRNA and the yellow node represents mRNA.