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Expression profile of long non-coding RNAs in rat models of OSA-induced cardiovascular disease: new insight into pathogenesis

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Abstract

Purpose Long non-coding RNAs (lncRNAs) are a recently identified class of regulatory molecules involved in the regulation of numerous biological processes, but their functions in a rat model of chronic intermittent hypoxia (CIH) remain largely unknown. Therefore, for further investigation, we aimed to explore lncRNA expression profiles and reveal their potential functional roles in rat models of CIH.

Methods We used a well-established CIH rat model and conducted lncRNA microarray experiments on the heart samples of rats with CIH and under normoxia control. Differentially expressed lncRNAs and mRNAs were identified via fold-change filtering and verified by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Bioinformatics analyses were applied to reveal the potential roles of key lncRNAs. Co-expression analysis was conducted to determine the transcriptional regulatory relationship of lncRNAs and mRNAs between the two groups.

Results Our data indicated that 157 lncRNAs and 319 mRNAs were upregulated, while 132 lncRNAs and 428 mRNAs were downregulated in the rat model of CIH compared with sham control. Pathway analyses showed that 31 pathways involved in upregulated transcripts and 28 pathways involved in downregulated transcripts. Co-expression networks were also constructed to explore the potential roles of differentially expressed lncRNAs on mRNAs. LncRNAs, namely, XR_596701, XR_344474, XR_600374, ENSRNOT00000065561, XR_590196, and XR_597099, were validated by the use of qRT-PCR.

Conclusions The present study first revealed lncRNAs expression profiles in a rat model of CIH, providing new insight into the pathogenesis of obstructive sleep apnea-induced cardiovascular disease.

Keywords Long non-coding RNA · Obstructive sleep apnea · Cardiovascular disease · Expression profiles

Introduction

Obstructive sleep apnea (OSA) is a sleep breathing disorder characterized by partial or complete collapse of the upper airway during sleep, which affects about 5% of the adult population. OSA patients usually have comorbidities of systemic or pulmonary hypertension, diabetes, or obesity [1]. Numerous studies show that as for cardiovascular diseases, such as ischemic heart disease, hypertension, and congestive heart failure, OSA is identified as an independent risk factor [2–4]. The pathogenesis includes sympathetic activation, endothelial dysfunction, oxidative stress, and inflammation [5–8]. The development of cardiovascular disease generally requires an exposure to sleep apnea for several years. However, even a healthy OSA patient can exhibit a subtle modification in vascular remodeling, such as atherosclerosis change [9, 10]. Therefore, studying the specific pathophysiology of OSA-induced cardiovascular disease and the exact underlying mechanisms is critical.

Recently, widespread attention has been focused on lncRNAs, a novel class of non-coding RNAs, which are over 200 nucleotides in length. As known, lncRNAs are nearly pervasively transcribed in mammalian genomes [11]. Accumulating evidence indicates lncRNAs participate in

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various regulatory processes, where they may potentially serve as signals, decoys, guides, and scaffolds [12, 13]. LncRNAs could affect numerous cellular processes, for instance, cell growth, differentiation, and apoptosis [14]. LncRNAs may also play critical role in both normal development and disease [15]. Previous work has demonstrated that a set of lncRNAs are differentially expressed in lots of human diseases such as cardiovascular disease, cancer, and psychiatric disease [16]. For example, lncRNA SNHG1 negatively regulates miR-145a-5p in nasopharyngeal carcinoma, which contributes to an enhancement of NUAK1 and promotes cancer cell metastasis and invasion [17]. However, no study reports lncRNA expression and its function in a rat model of OSA. And the features of lncRNA expression profile and their potential functions in OSA are still unknown.

In the present study, we conducted lncRNA microarray analysis of left ventricular tissues from CIH rodent model. Bioinformatic analyses were further used to predict the potential biological functions of important lncRNAs. Overall, these findings may provide a strong foundation for further research on the roles of lncRNAs in OSA-induced cardiovascular disease.

Materials and methods

Animal model of CIH and left ventricular samples

The experiments were performed on male Sprague-Dawley rats (280–330 g), which were obtained from the animal experiment center of Fujian Medical University. Rats were divided into two groups: the normoxia group (21% O_2, 24 h per day, 8 weeks, $n = 3$) and CIH group (6%O_2 60 s, 21% O_2 alternating 60 s, 8 h per day, 8 weeks, $n = 3$). The rat model of CIH was validated by MC Lai et al. [18]. During CIH exposure, animals were placed in commercial hypoxic chambers connected to a supply of N_2 and O_2 gas. The chamber was periodically filled with N_2 and O_2 manipulated by computer-controlled timed solenoid valves to regulate the degree and time of hypoxia. Also, the chamber was equipped with O_2, CO_2, humidity, and temperature sensors to monitor the O_2 concentration. Each cycle of CIH lasted for 120 s. The process was composed of filling the chamber with N_2 for the first 60 s and filling it again with O_2 for another 60 s. The gas flow rate was adjusted to make a 6% nadir of O_2 during every cycle and then followed by restoration of O_2 to 21%. The rats had free access to water and chow with constant temperature, humidity, and light:dark cycle (light from 07:00 to 19:00). Rats were euthanized 8 weeks after being exposed to the respective treatment and left ventricular tissues were obtained. With the approval of Fujian Medical University, we performed all experimental procedures.

Hematoxylin and eosin staining

In order to investigate the injury of myocardium after CIH, we fixed the ventricular tissue in 4% formaldehyde and paraffin embedded. Next, we used hematoxylin and eosin to stain all the 5-μm-thick sections to further histological analysis. Finally, for observing the histological changes, the heart tissues were then placed under an optical microscope at × 400 magnification.

RNA extraction and purification

TRIzol reagent (Invitrogen, CA, USA) was used to isolate total RNA which was from the 3 CIH specimens and the paired normoxia tissues. Then, we used a NanoDrop ND-1000 spectrophotometer (OD 260 nm, NanoDrop, Wilmington, DE, USA) to quantify the total RNA. Meanwhile, RNA integrity was estimated by using standard denaturing agarose gel electrophoresis. At last, the ratio of absorbance at 260 to 280 nm (A260/A280) was used to assess the purity of RNA.

LncRNA and mRNA microarray

Arraystar Rat LncRNA Microarray V2.0 has a total of 13,611 lncRNAs collected from all major public databases and repositories, such as Refseq, Ensembl, lncRNAdb, and scientific publications, and a total of 24,626 protein coding mRNAs. They were employed to analyze all differentially expressed lncRNA and mRNA in CIH animal models.

RNA labeling and Array hybridization

In this study, according to the Agilent One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technology) with minor modifications, sample labeling and array hybridization were fulfilled. In brief, mRNA was purified following removal of rRNA (mRNA-ONLY™ Eukaryotic mRNA Isolation Kit, Epicenter). Each sample was then amplified and transcribed into fluorescent cRNA, which was along the entire length of the transcripts without 3′ bias making use of a random priming method (Arraystar Flash RNA Labeling Kit, Arraystar). RNeasy Mini Kit (Qiagen) was used to purify the labeled cRNAs. We applied NanoDrop ND-1000 to measure the concentration and activity of the labeled cRNAs (pmol Cy3/μg cRNA). A total of 1 μg of each labeled cRNA was

Table 1 Primers used for qRT-PCR

Primer name	Sequence (5′-3′)	Tm (°C)	Lengths (bp)
β-actin	F: 5′-CGAGTACAACCTTCTTGCAGC-3′ R: 5′-ACCCATACCCACCATCACAC-3′	60	202
XR_596701	F: 5′-CCAGTTTACAGAGATTCCTCAGG-3′ R: 5′-GCCAAGTGACATGAACAAAGTG-3′	60	163
XR_344474	F: 5′-TCTCAGCTCTGTCTGCACCA-3′ R:5′ TCTCCTGTCCTCGTCTCCAT 3′	60	117
ENSRNOT00000065561	F: 5′-TTTACTTGCTCCTTCCCCTTCT-3′ R: 5′-TGAGCGTACACCAGCACTTC-3′	60	101
XR_600374	F: 5′-CCATGTGAAGCAGAGTGACAGA-3′ R: 5′-CTGTTGTAATGCTCCAAAGGCT-3′	60	123
XR_590196	F:5′-GCCTCCTCAACTGTTCCATAA-3′ R: 5′-CATACTTTCCTAGTGCCTCCTGT-3′	60	202
XR_597099	F: 5′-TCTGCTCAGCCAGCTCCTAA-3′ R: 5′-CCAAGCTGTCTCTCAAGTGCT-3′	60	296

fragmented via the addition of 5 μl 10× blocking agent and 1 μl of 25× fragmentation buffer. Next, we then heated the mixture at 60 °C for 30 min. Furthermore, we diluted the labeled cRNA by adding 25 μl 2× GE hybridization buffer. Subsequently, 50 μl of hybridization solution was dispensed into the gasket slide, and then it was assembled to the LncRNA expression microarray slide. The slides were incubated for 17 h at 65 °C in an Agilent Hybridization Oven. To the end, the hybridized arrays were washed, fixed, and scanned with the use of the Agilent DNA Microarray Scanner (part no G2505C). The analysis was finished by KangChen Bio-tech, Shanghai, China.

Computational analysis

We applied the Agilent Feature Extraction software (version 11.0.1.1) to analyze all the acquired array images. We used the GeneSpring GX v12.1 software package (Agilent Technologies) to perform the analysis of quantile normalization and subsequent data. Then, for further data analysis, we chose all the lncRNAs and mRNAs which at least 3 out of 6 samples have flags in present or marginal ("all targets value"). We identified all the differentially expressed lncRNAs and mRNAs between the two groups through fold-change filtering and *P* value/FDR filtering. Both GO analysis and pathway analysis were used to illuminate the functions of these differentially expressed mRNAs. Hierarchical clustering and combined analysis were also carried out by using in-house scripts. Furthermore, a coding–non-coding gene co-expression network (CNC) was drawn, which was done by using 9 mRNAs and all differentially expressed lncRNAs. To illustrate the CNC network, Cytoscape (v2.8.2) with Pearson coefficient ($|r|$) > 0.97) was used.

qRT-PCR

According to the manufacturer's instructions, total RNA was extracted from rat hearts samples with the use of TRIzol reagent (Invitrogen) and converted into cDNA via the use of the SuperScript™ III RT kit (Invitrogen). The expression levels of

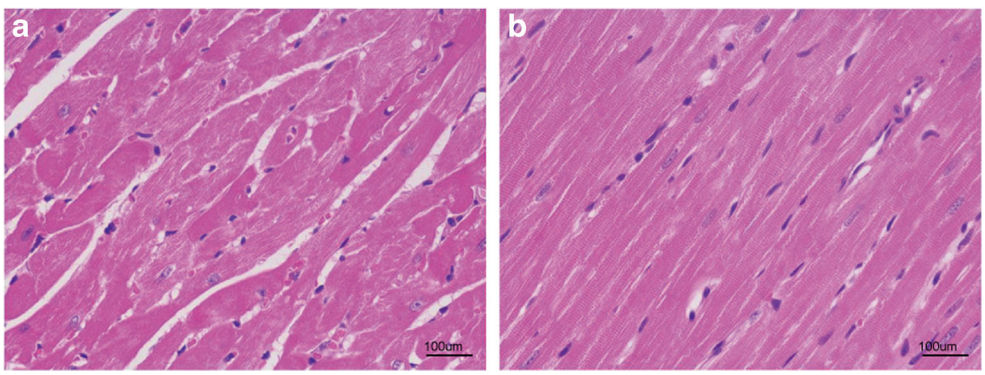
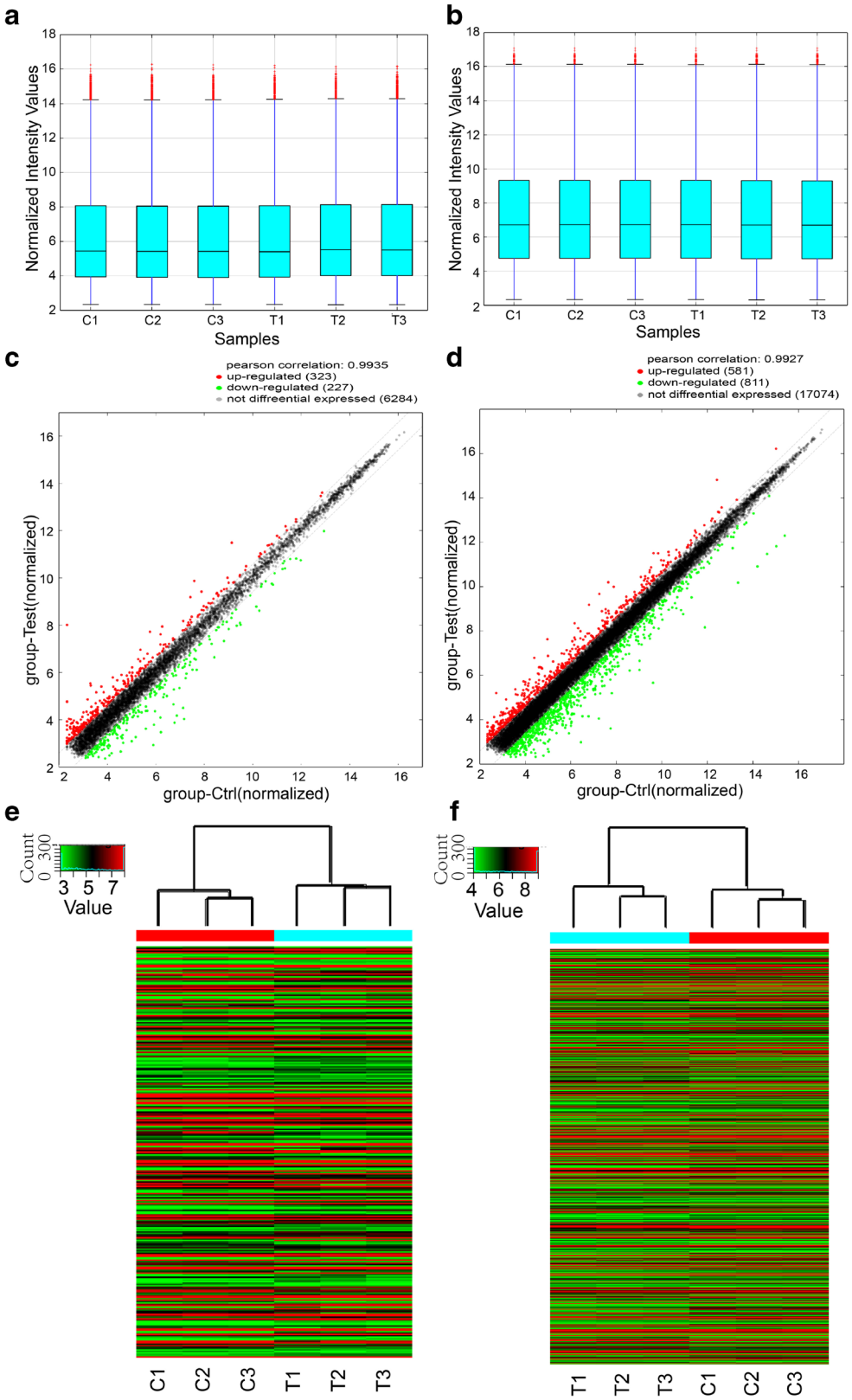


Fig. 1 Effect of CIH on the histopathology of myocardium. **a** Cardiac histology of normoxia rats with normal architecture. **b** Cardiac histology of CIH rats with abnormal myocardial architecture (magnification × 400)



6 selected lncRNAs were verified by qRT-PCR using SYBR Green assays (Arraystar). The above 6 selected lncRNAs were normalized to β-actin, and the detection was independently repeated three times. The $2^{-\Delta\Delta CT}$ method was performed to obtain relative fold changes of the expression of each lncRNA. The primers were listed in Table 1.

◀ **Fig. 2** Different expression profiles of lncRNA and mRNA between CIH and control samples. **a**, **b** Box plots of lncRNA and mRNA showed the distributions of intensities from all samples. **c**, **d** Scatter plots of lncRNA and mRNA. The values of *X* and *Y* axes indicated the averaged normalized signal values of each group (log2 scaled). Green lines are fold-change lines. Middle line indicates a fold change of 1 or no difference between CIH and control samples. LncRNAs beyond the range of green line have more than 1.5-fold difference between CIH and control samples. **e**, **f** Heat map and hierarchical clustering. Fold changes ≥ 1.5 and $P < 0.05$ were taken as the cut-off criteria for deregulation of lncRNAs. Red color indicates relatively up-regulation, and green color indicates relatively downregulation

Statistical analysis

The expression levels of lncRNAs and mRNAs between the CIH and control group were calculated via unpaired *t* test. To perform GO and pathway analysis, Fisher's exact test was employed to evaluate significance of GO terms or pathway identifiers' enrichment. Pearson coefficient was used to analyze the CNC network construction. $P < 0.05$ was conducted to demonstrate a statistically significant difference.

Results

Cardiac histopathological changes

To assess whether CIH damage the cardiac structure, an analysis of ventricular histopathological changes was performed via the use of both hematoxylin and eosin. After

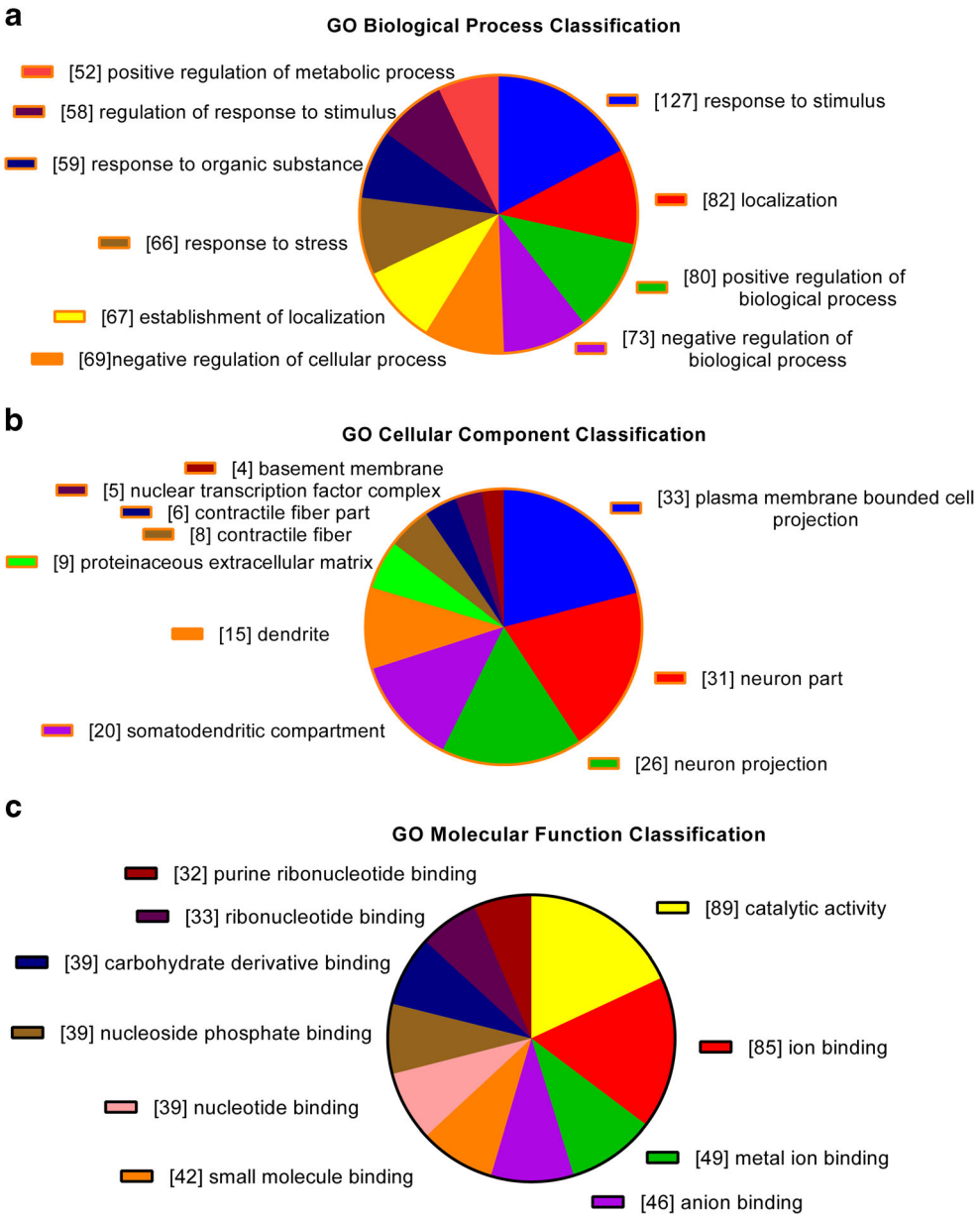


Fig. 3 GO analysis. **a**–**c** GO terms for the upregulated mRNAs: **a** biological process; **b** cellular component; **c** molecular function. **d**–**f** GO terms for the downregulated mRNAs: **d** biological process; **e** cellular component; **f** molecular function

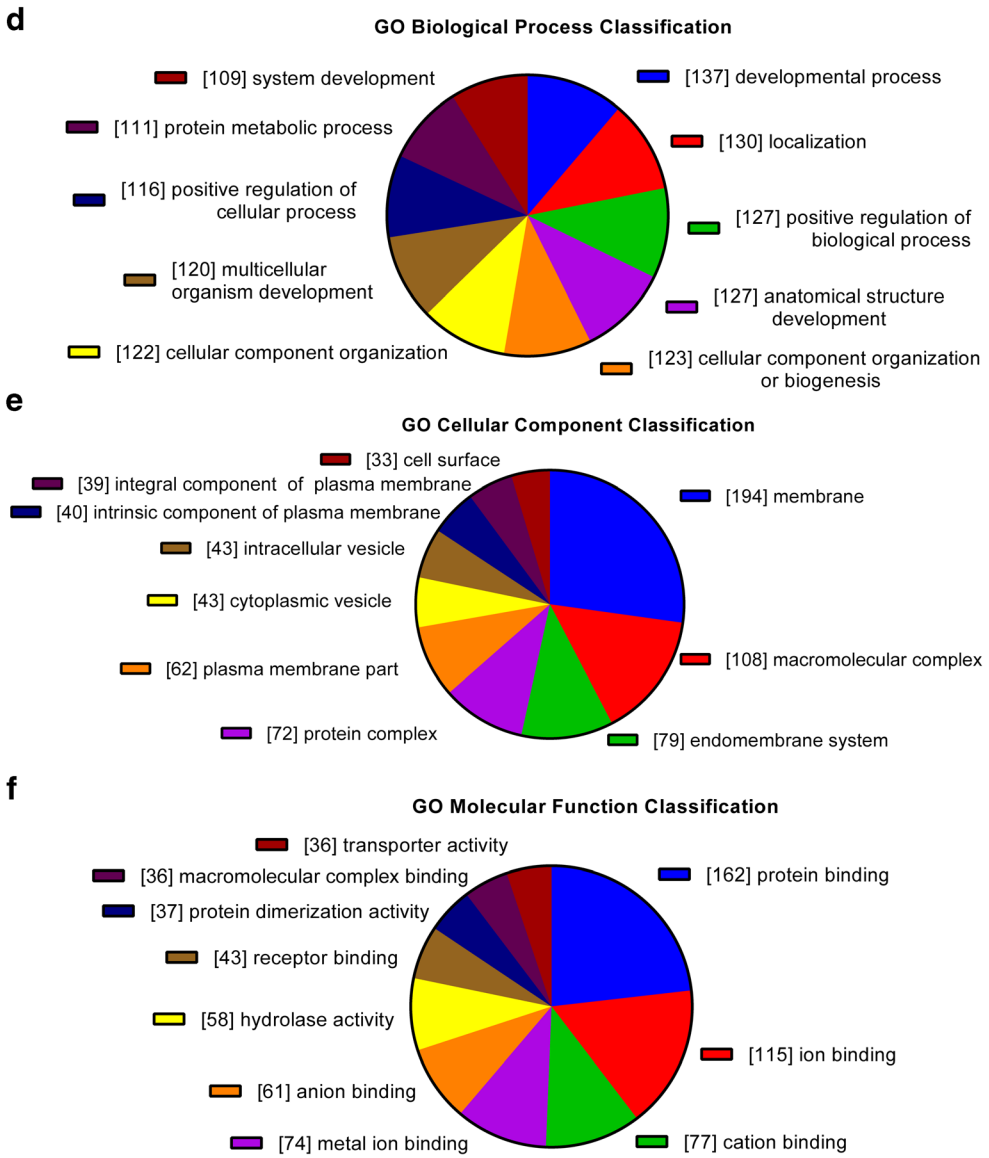


Fig. 3 continued.

viewing × 400 magnified images, the left ventricular myocardium showed no significant changes in the control group (Fig. 1a). The abnormal myocardial architecture, cardiomyocyte atrophy, and the distortion of cell nucleus were shown obviously in the CIH group (Fig. 1b).

Differentially expressed lncRNAs and mRNAs

To make sure the quality of the microarray data, we utilized box plot and scatter plot. As showed in Fig. 2a, b, we used box plot to compare the distributions of the intensities. In addition, scatter view was employed to assess the variation of lncRNA and mRNA expression between the 2 groups (Fig. 2c, d). At last, we made use of hierarchical clustering to show distinguishable lncRNA and mRNA expression profiles (Fig. 2e, f). Compared with the control group, 289 lncRNAs were significantly differentially expressed (fold change ≥ 1.5, $P < 0.05$). Among these differentially expressed lncRNAs, a total number of 157 lncRNAs were upregulated, while 132 lncRNAs were downregulated. Similarly, 747 mRNAs were differentially expressed (fold change ≥ 1.5, $P < 0.05$) between the two groups. Among these differentially expressed mRNAs, 319 mRNAs were upregulated, while 428 mRNAs were downregulated.

GO analysis

GO analysis was used to determine genes and describe gene product enrichment, which involved in classification of biological process (BP), cellular component (CC), and molecular function (MF). We found that the most highly GO classifications for the upregulated transcripts were rhythmic process (Fig. 3a; BP), contractile fiber (Fig. 3b; CC), and MAP kinase

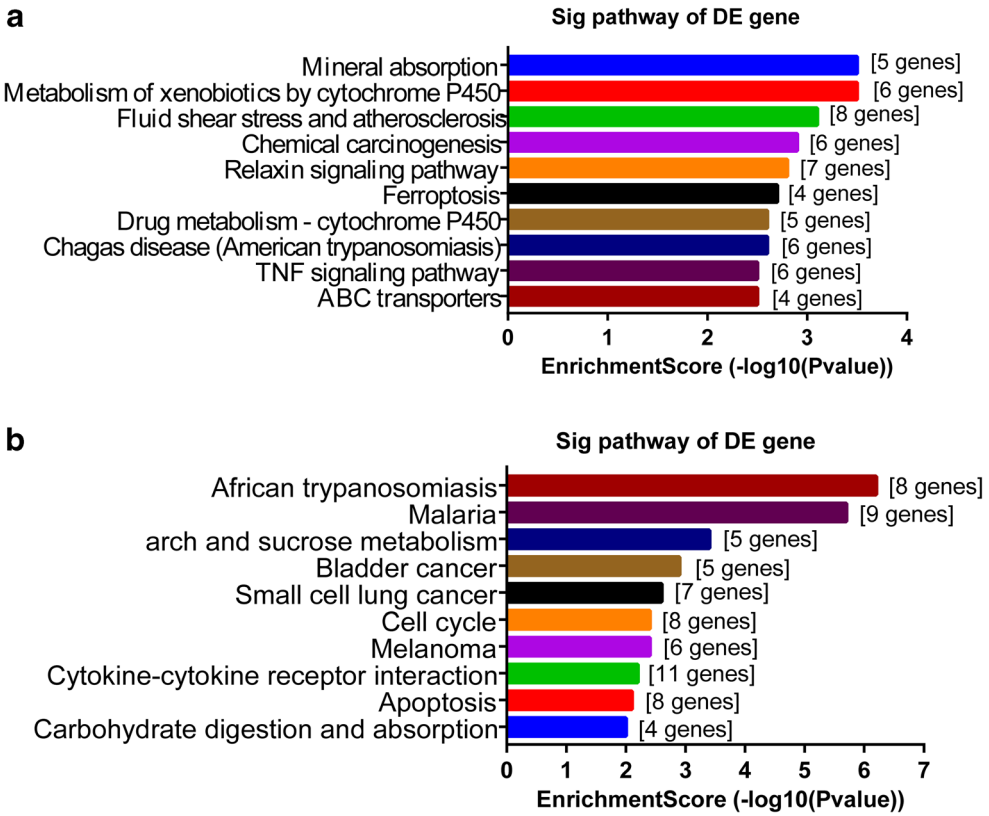


Fig. 4 Top 10 significant pathways associated with upregulated and downregulated genes are listed

tyrosine/serine/threonine phosphatase activity (Fig. 3c; MF). The most highly GO terms for the downregulated transcripts were oxygen transport (Fig. 3d, BP), hemoglobin complex (Fig. 3e, CC), and oxygen carrier activity (Fig. 3f, M).

Pathway analysis

According to the latest KEGG database, we found that 31 pathways enriched in the upregulated genes (Fig. 4a). The most enriched network was "Mineral absorption-Rattus norvegicus (rat)" with 5 genes annotated with this term. Meanwhile, 28 pathways enriched in the downregulated transcripts (Fig. 4b). However, the most enriched network was "African trypanosomiasis-Rattus norvegicus (rat)" with 8 transcripts annotated with this term. Among these pathways, Li S et al. reported that the gene category "apoptosis" had been implicated in the promotion of OSA-induced progressive heart failure [19].

Construction of CNC network

Nine important coding genes, including Nfkb1, Dusp1, Mapk12, Fos, vascular endothelial growth factor A (Vegfa), BCL2 binding component 3(Bbc3), cyclin B1(Ccnb1), tubulin alpha 3B(Tuba3b), and the cyclin-dependent kinase inhibitor 1A(Cdkn1a), were selected to construct a CNC network. In this CNC network construction, a single lncRNA could have a correlation with many mRNAs, and vice versa. For example, the lncRNA XR_591224 is correlated with Nfkb1, Fos, and Mapk12. These genes primarily participate in cell cycle, cytokine-cytokine receptor interaction, apoptosis, fluid shear stress and atherosclerosis, and TNF signaling pathway. These results indicate that the lncRNAs in the network likely perform similar functions and thus might play a key role in regulating the development of OSA-related cardiovascular disease. Figure 5 shows the results of the CNC analysis of 9 coding genes.

qRT-PCR

To validate the reliability of our microarray results, three upregulated lncRNAs (XR_596701, XR_344474, and ENSRNOT00000065561) and three downregulated lncRNAs (XR_600374, XR_590196, and XR_597099) were randomly selected to perform qRT-PCR verification in same RNA samples used for the microarray analyses. To our interesting, the qRT-PCR results were in agreement with the microarray findings, which demonstrated the same trends of up- or downregulation for each differentially expressed lncRNA (Fig. 6).

Discussion

Increasing evidences have revealed that lncRNAs play crucial roles in multiple cardiovascular diseases [20]. However, their

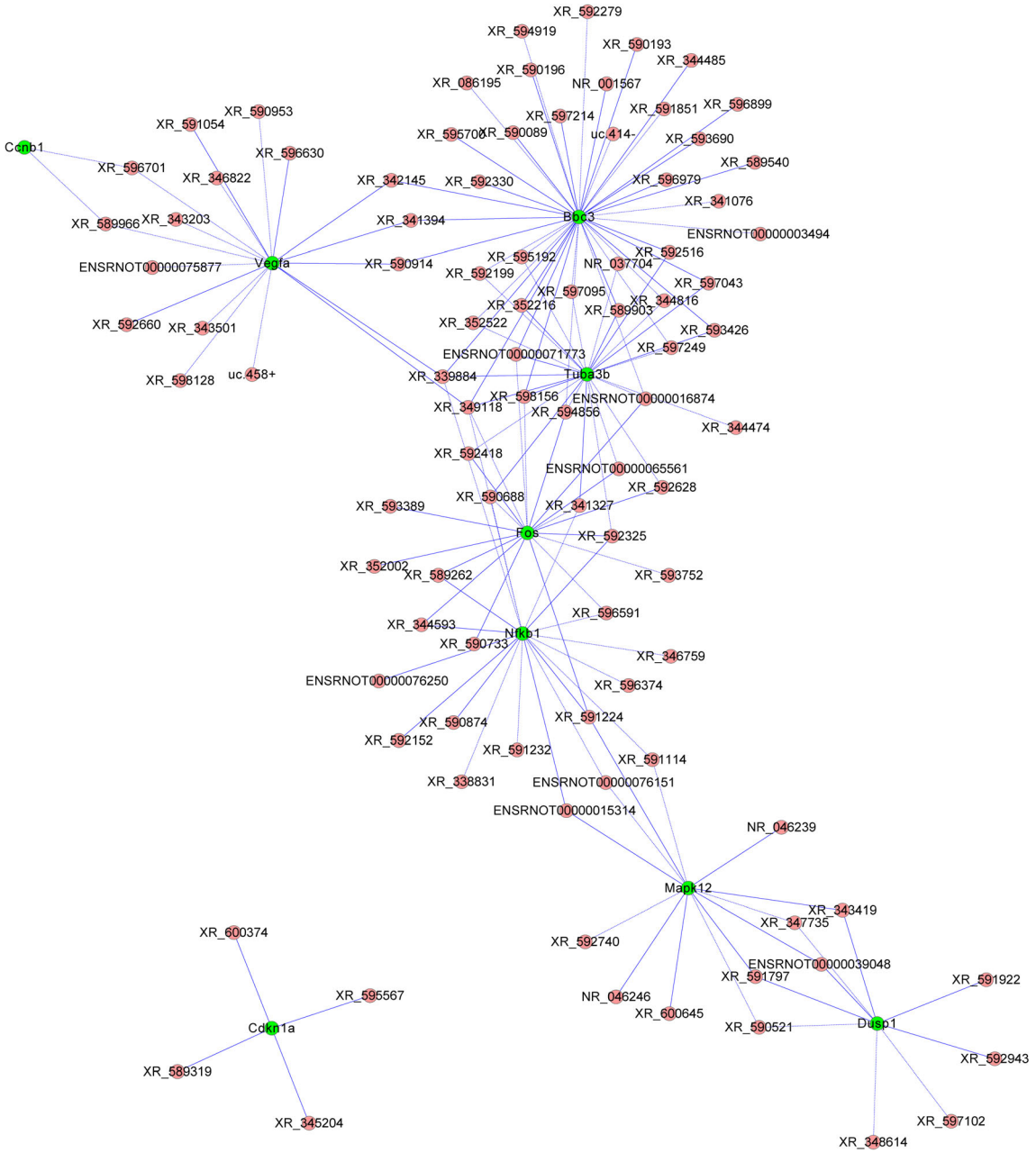


Fig. 5 CNC analysis of 9 differentially expressed mRNAs. The green nodes represent mRNAs, pink nodes represent lncRNAs. Solid line indicates positive correlation, and dotted line represents negative correlation

relative expression levels in OSA-related cardiovascular diseases remain unclear. In this article, we investigated the expression levels of lncRNAs and mRNAs in 3 different pairs of heart tissue via the use of microarray chips, which includes 13,611 lncRNAs and 24,626 mRNAs. Two hundred eighty-nine dysregulated lncRNAs were identified. Most of the dysregulated lncRNAs are novel lncRNAs, whose functions have not yet been elucidated. To validate the reliability of the microarray finding, we randomly chose six lncRNAs and determined their expression levels via the use of qRT-PCR. Bioinformatics analyses, including GO analysis, pathway, and CNC analysis, were then conducted to predict their functional roles.

In view of multiple reasons, the probabilities to perform human research on the relation between OSA and cardiovascular diseases are still at a preliminary stage. First, OSA-induced cardiovascular diseases patients often display with confounding risk factors such as obesity, diabetes mellitus, and smoking. Second, OSA patients often demand several years to initiate cardiovascular diseases diagnosed clinically. Third, it is neither easy nor to ethically sound to conduct more invasive techniques in these patients. All these risk factors limit the investigation of causal interaction between OSA and cardiovascular diseases. To cope with this disadvantage, OSA animal models have yet been explored [21] and used to investigate the pathogenesis of

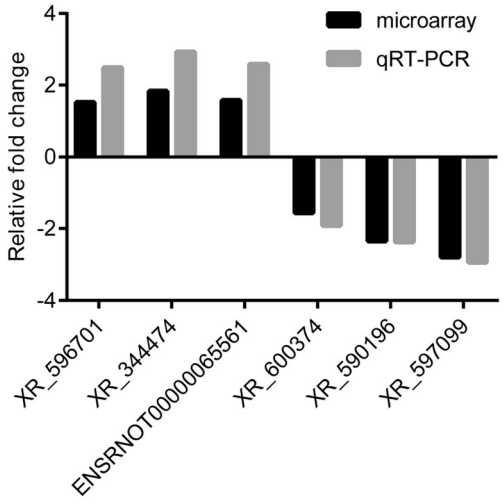


Fig. 6 Relative fold changes of lncRNAs by microarray and qRT-PCR. The upwards represents upregulation, while downwards represents downregulation

OSA-related complications [22]. Therefore, we used CIH mice model instead of human samples in this study.

To better understand lncRNA profiles in OSA-related cardiovascular diseases, targets of lncRNA were predicted. In addition, bioinformatics analysis, such as GO and pathway analysis, was performed. Among these pathways, the "apoptosis" pathway was demonstrated to be linked to the promotion of OSA-induced cardiovascular disease [19, 23].

In cardiovascular research, lncRNAs play crucial roles in atherosclerosis, heart failure, coronary artery disease, and myocardial infarction [24]. Animal studies support the idea that the enhanced atherosclerosis known to occur in OSA is owed to the increase of lipid peroxidation. In accordance with this hypothesis, direct evidence has been acquired that CIH can result in the development of atherosclerotic lesions. Exposure to CIH for 12 weeks was prone to the formation of atherosclerotic plaques, but a combined with high-fat diet was in need for that effect to happen [25]. In the present study, the fluid shear stress and atherosclerosis pathway displayed associations with the OSA-induced cardiovascular disease.

The inflammatory response, together with oxidative responses, is the main risk factor for OSA-induced cardiovascular disease. In accordance with these results, GO analysis indicated that the genes were enriched for the GO terms which were relevant to immune response, response to stress, and response to lipid. It is noteworthy that the response to lipid terms enriched in both the upregulated and downregulated genes in our study. Meanwhile, pathway analysis also showed that inflammation-associated pathways such as apoptosis, TNF signaling pathway, fluid shear stress, and atherosclerosis were involved in the present study. It was reported that tumor necrosis factor-α is a vital pro-inflammatory cytokine markers in OSA and stable coronary heart disease patients [24].

Finally, a CNC network was constructed to reveal the correlation between lncRNAs and dysregulated mRNAs. To our knowledge, lncRNAs influence transcription of proximal or distal genes via cis- and trans-acting mechanisms [25, 26]. As shown in the CNC network, all the nine genes are regulated by lncRNAs via the way of cis- and trans-regulation. The malfunction of regulating this CNC network was likely to be a vital step for the pathogenesis of OSA-induced cardiovascular disease. Further research is demanded to investigate the functions of all differentially expressed lncRNAs.

However, there are some disadvantages that should be taken into account in this research. First, exposure to CIH during daylight is not enough to imitate real OSA condition. Second, owing to the lack of hypercapnic stimulus, the CIH animal model is not the same as that in human. Third, lncRNAs and mRNAs validated by qRT-PCR should be further verified on cell level. Therefore, the above factors should be also considered when delineating result.

Conclusions

Taken together, to the best of our knowledge, the study first revealed that OSA altered lncRNA expression profiles in the rat heart. These findings could aid in advancing our foundation of knowledge on the pathogenesis of OSA-induced cardiovascular disease.

Author contributors All authors directly participated in the study and have approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable guidelines for the care and use of animals were followed.

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