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Expression profiling and ontology analysis of long noncoding RNAs in post-ischemic heart and their implied roles in ischemia/reperfusion injury

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ABSTRACT

Long noncoding RNAs (lncRNAs) play important regulatory roles in cellular physiology. The contributions of lncRNAs to ischemic heart disease remain largely unknown. The aim of this study was to investigate the profile of myocardial lncRNAs and their potential roles at early stage of reperfusion. lncRNAs and mRNAs were profiled by microarray and the expression of some highly-dysregulated lncRNAs was further validated using polymerase chain reaction. Our results revealed that 64 lncRNAs were up-regulated and 87 down-regulated, while 50 mRNAs were up-regulated and 60 down-regulated in infarct region at all reperfusion sampled. Gene ontology analysis indicated that dysregulated transcripts were associated with immune response, spermine catabolic process, taxis, chemotaxis, polyamine catabolic process, spermine metabolic process, chemokine activity and chemokine receptor binding. Target gene-related pathway analysis showed significant changes in cytokine–cytokine receptor interaction, the chemokine signaling pathway and nucleotide oligomerization domain (NOD)-like receptor signaling pathway which have a close relationship with myocardial ischemia/reperfusion injury (MI/RI). Besides, a gene co-expression network was constructed to identify correlated targets of 10 highly-dysregulated lncRNAs. These lncRNAs may play their roles by this network in post-ischemic heart. Such results provide a foundation for understanding the roles and mechanisms of myocardial lncRNAs at early stage of reperfusion.

1. Introduction

Ischemic heart disease (IHD) is the leading cause of death worldwide. An estimated 16.3 million American adults experience these conditions and this number is increasing (Kalanuria et al., 2012). The pathological process leading to IHD (including myocardial infarction, angina pectoris, or both) is very complicated and often accompanied by changes in gene expression (Dabek et al., 2008; Pitts et al., 2008). Data from an increasing number of studies have indicated that noncoding RNAs are associated with important regulatory functions in the heart (Kukreja et al., 2011; Mercer and Mattick, 2013; Small and Olson, 2011). The significance of noncoding RNAs in the regulation of multiple major biological processes impacting development, differentiation and metabolism has brought these neglected molecular players to the forefront of IHD (Ordovas and Smith, 2010).

Recently, long noncoding RNAs (lncRNAs) have become an area of increased research focus. lncRNAs are typically defined as noncoding RNA molecules ranging in length from 200 nt to ~100 kb and lacking the ability for protein-coding (Lee, 2012). They have been shown to exert comprehensive effects on biological processes through a variety of mechanisms and are thought to play roles in the pathophysiology of some diseases such as Alzheimer disease and cancer (Calin et al., 2007; Faghihi et al., 2008). They control protein targeting to genomic loci, epigenetic silencing and serve as scaffolds for multiple proteins (Rinn and Chang, 2012).

Abbreviations: AMI, acute myocardial infarction; BIRC3, baculoviral IAP repeat-containing 3; CXCL1, chemokine (C–X–C motif) ligand 1; CCL9, chemokine (C–C motif) ligand 9; CXCL12, chemokine (C–C motif) ligand 12; EDA, extra domain A; GO, gene ontology; IHD, ischemic heart disease; KEGG, Kyoto Encyclopedia of Genes and Genomes; lncRNA, long noncoding RNA; LAD, left anterior descending artery; MI/RI, myocardial ischemia/reperfusion injury; NOD, nucleotide oligomerization domain; NF-κB, nuclear factor-κB; TNFAIP3, tumor necrosis factor-alpha-inducible protein 3.

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Although lncRNA studies predominate in other fields, such as cancer, the signature of lncRNA expression and possible roles of lncRNAs in the post-ischemic heart have received relatively little attention. In this study, we tested the effects of myocardial ischemia upon lncRNA expression and explore the potential roles of these lncRNAs at early stage of reperfusion.

2. Materials and methods

2.1. Animal

C57Bl/6 mice (males, 4–8 weeks of age) in used in this experiment were obtained from River Animal Experiment Company (Beijing) where they were bred in compliance with the guide to the care and use of laboratory animals draw up by the US National Institutes of Health. The protocol was approved by the Experimental Animal Ethic Committee of Harbin Medical University, China (Animal Experimental Ethical Inspection Protocol No. 2010102). All animals received artificial feed according to the Specific Pathogen Free Animal Criteria, and were allowed free access to food and water.

2.2. Ischemia–reperfusion surgery

The *mice* were anesthetized by using intra-peritoneal injection of pentobarbital sodium with an initial dose of 100 mg/kg. Additional dose was administered during the experiment as needed to maintain the mouse anesthesia, which was indicated by disappearance of their foot withdrawal reflex. Surgical procedures were performed following those described by Palazzo et al. (1998). The body temperature of all subjects was maintained between 36.8 °C and 37 °C via a thermo-regulated surgical table. The trachea was cannulated and the mice were ventilated with a Harvard ventilator before the thoracotomy was performed on the left side in the fourth inter-costal space to remove the pericardium. Then the left anterior descending artery (LAD) at the inferior edge of the left atrium of each mouse was ligatured with an 8–0 prolene suture until myocardial ischemia appeared.

Myocardial ischemia was confirmed by changes in ECG and epicardial cyanosis. After 30 min of ischemia, the occlusion of the left anterior coronary artery was released and reperfusion occurred which was verified by visualizing a marked epicardial hyperemic response. Animals were sacrificed at reperfusion time (2 h after 30 min ischemia). At autopsy, samples collected for RNA analysis from infarct region are confirmed by visual inspection under a dissecting microscope (Leica) by discoloration of the occluded distal myocardium. Sham operated animals underwent the same procedure without occlusion of the LAD.

2.3. Infarct size determination and serum creatine phosphokinase activity

In order to prove the effectiveness of I/R, infarct size of the hearts was tested in this study. The method was performed as follows: After 2 h of reperfusion, the LAD was religated with 7–0 silk suture. Evans Blue dye (1.2 mL of a 2.0% solution, Sigma) was injected through a carotid artery catheter into the coronary circulation to delineate the in vivo area at risk. The heart was rapidly excised and frozen for 20 min, then serially cross sectioned in six, 1 mm thick sections that were then incubated in 1.0% 2,3,5-triphenyltetrazolium chloride (Sigma) for 5 min at 37 °C to demarcate the viable and nonviable myocardium within the area at risk. Slices were weighed and the ischemic region (area at risk) and the infarct region were assessed by a blinded observer using computer-assisted planimetry (NIH Image 1.57).

Besides, serum levels of creatine phosphokinase (CPK) and its myocardium specific isoform (CK-MB) were also measured with the use of commercial kits (Sigma Chemical Co.) for different groups to evaluate model of I/R indirectly (Zingarelli et al., 1998).

2.4. RNA labeling and array hybridization

Kangchen Bio-tech (Shanghai PR China) performed the microarray work in which six samples (3 samples for the reperfusion group and 3 for the sham group) were used for lncRNA microarray analysis. Total RNA from each sample was quantified using the NanoDrop ND-1000 and RNA integrity was assessed using standard denaturing agarose gel electrophoresis. For microarray analysis, the Agilent Array platform was employed. Sample preparation and microarray hybridization were performed based on the manufacturer's standard protocols with minor modifications. Briefly, mRNA was purified from total RNA after removal of rRNA (mRNA-ONLY™ Eukaryotic mRNA Isolation Kit, Epicentre). Then, each sample was amplified and transcribed into fluorescent cRNA along the entire length of the transcripts without 3′ bias utilizing a random priming method. The labeled cRNAs were hybridized onto the mouse lncRNA Array v2.0 (8 × 60 K, Arraystar). After washing the slides, the arrays were scanned by the Agilent Scanner G2505C.

2.5. Data analysis

Agilent Feature Extraction software (version 11.0.1.1) was used to analyze the acquired array images. Quantile normalization and subsequent data processing were performed using the GeneSpring GX v12.0 software package (Agilent Technologies). After quantile normalization of the raw data, lncRNAs and mRNAs with present or marginal ("all targets value") flags in control and reperfusion samples were subjected to further data analysis. Hierarchical clustering was performed to show the distinguishable lncRNA and mRNA expression patterns among samples.

2.6. Gene ontological and pathway analysis

Gene ontology (GO) is used to determine processes or functional categories that were differentially expressed, as described previously (Beissbarth and Speed, 2004). This analysis determined the number of genes in a category present on the array and the number of expression changes that would be part of that category by random chance given the number of differentially expressed genes. To investigate whether genes share a similar biological function, we also searched for overrepresentation in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway. The enriched genes in the KEGG pathway were statistically calculated by hypergeometric distribution. KEGG datasets were downloaded on 2011–12 and contained 211 pathways.

2.7. Gene co-expression network construction

Gene co-expression networks were presented to identify interactions among genes. The method was performed according to Yang's method (Yang et al., 2011). In brief, gene co-expression networks were based on the normalized signal intensity of specific expressed genes. Pearson's correlation co-efficients were used in this study to calculate gene pair correlation based on gene expression. To ensure a visual representation, only the strongest correlations (0.99 or greater) were drawn in these renderings. Each gene corresponds to a node in this network. Two genes connected by an edge indicate a strong correlation. Within the network analysis, a degree is the simplest and most important measure of the centrality of a gene within a network and determines the relative importance. A degree is defined as the number of directly linked neighbors.

2.8. Quantitative real-time polymerase chain reaction

To confirm the expression of lncRNAs by microarray analysis, quantitative real-time was performed for part of them. Briefly, total RNA from cardiac infarcted tissues was extracted using Trizol agent (Invitrogen,