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Research paper

Global transcriptomic study of atherosclerosis development in rats

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ABSTRACT

Atherosclerosis is a chronic disease of the arterial wall and a leading cause of death worldwide. Though the pathophysiology of atherosclerotic lesion formation has been studied, we still lack evidence of the global changes in the artery during atherosclerosis. In this report, we induced atherosclerosis in rats and conducted GeneChip analysis on carotid arteries with or without plaque formation. We found that molecular pathways underlying plaque formation in atherosclerosis were related to immune response, angiogenesis, cell proliferation, apoptosis and hypoxic microenvironments, suggesting that the pathophysiology of atherosclerosis is varied. In addition, we showed that three lncRNAs, GAS5, SNHG6 and Zfas1, were significantly increased in the plaque of atherosclerosis patients compared to normal people. A complex interaction of mRNA and lncRNA was identified in atherosclerosis. Our results provide a global transcriptomic network of atherosclerosis development in rats and possible targets that could lead to new clinical applications in the future.

1. Introduction

Atherosclerosis, the principal cause of heart attack, stroke and gangrene of the extremities, accounts for up to 50% of deaths in Western countries (Weber and Noels, 2011). It is generally recognized that atherosclerosis results from an excessive inflammatory response to damage to the endothelium and smooth muscle of the artery wall, followed by plaque formation and thrombosis (Libby et al., 2011). The genesis of atherosclerotic lesions is complex because of the large number of growth factors, cytokines, regulatory molecules and cell types that participate in this process (Koenen and Weber, 2010). Current treatments for atherosclerosis are mainly based on drugs that lower the plasma cholesterol concentration and blood pressure (Steffens et al., 2005). Yet, mortality associated with atherosclerosis is still high. Uncovering novel molecular pathways such as microRNAs and long non-coding RNAs (lncRNAs) responsible for atherosclerosis development may provide valuable prognostic biomarkers and potential therapeutic targets.

The transcriptome is the entire set of RNA molecules in a cell, including mRNA, rRNA, tRNA, microRNA and lncRNA. Whole-transcriptome analyses have revolutionized our understanding of the cause and progression of many diseases such as cancer (Ponting et al., 2009). Previously, mRNA was primarily studied, but current research has suggested that non-coding RNA such as microRNA and lncRNA also play important roles in normal and disease development (Shi et al., 2013a). In atherosclerosis studies, whole transcriptome analyses are rare and only a few microRNAs and lncRNAs were found to be involved in the progress of this disease. Understanding the whole transcriptome associated with plaque formation would provide opportunities to develop new diagnostic and therapeutic strategies to induce the regression of lesions and to possibly prevent their formation. In this study, we used a rat atherosclerosis model to characterize the global transcriptome during atherosclerosis development, including mRNA and lncRNA, and analyzed the associated gene ontology (GO) and pathways. The results from this study increase our understanding of atherosclerosis development and may also provide a rationale for future clinical studies in atherosclerosis diagnosis and therapy.

2. Materials and methods

2.1. Experimental model of atherosclerosis in rats

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The animal experiments were approved by the Committee on Laboratory Animal Research of Shanghai Jiao Tong University, China, and conducted according to the guidelines of the Laboratory Animal Center of Shanghai Jiao Tong

Abbreviations: FDR, False Discovery Rate; GO, Gene Ontology; SD, Sprague Dawley; CTA, Computed Tomography Angiography; MRA, Magnetic Resonance Angiography; DSA, Digital Subtraction Angiography; lnc-RNA, Long non-coding RNA; CXCL, Chemokine ligang.

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University School of Medicine. Six- to eight-week-old male Sprague-Dawley (SD) rats were purchased from the Shanghai Slac Animal Center (Shanghai, China). The experimental model of atherosclerosis in rats was established according to the literature (Sasaki et al., 1994). Briefly, the rats were fed a high-fat diet for 8 weeks to establish the hyperlipidemia model. Afterwards, the left common, external and internal carotid artery was exposed and temporarily clipped. A 2F embolectomy balloon catheter (Forgaty, USA) was inserted into the left common carotid artery through a V-shaped incision on the external carotid artery. The balloon was inflated with saline solution to distend the common carotid artery at a pressure of 6 kPa and was then drawn into the external carotid artery 3 times. Upon removal of the catheter, the external carotid artery was ligated, and the wounds were closed. Rats were anesthetized, and a nasal cannula was used to supply air. Breathing rate and rhythm were monitored every 15 min during the surgery, and the animals did not display any pain. Post-operative animals were returned to their cages lying prone in a quiet environment. While the rats recovered, breathing rate and rhythm were monitored every hour for 6 h. Two rats died because of improper anesthesia. Four weeks after the injury, the mice were sacrificed after overdoses of chloral hydrate and carotid arteries were excised for analysis.

2.2. Measurements of total cholesterol, high-density lipoprotein and low-density lipoprotein

Total cholesterol, high-density lipoprotein and low-density lipoprotein were measured in rats using a Biochemical analyzer (AU5800, Beckman Coulter, USA) before and after the 8 weeks on a high-fat diet. The body weight of the rats in each group was collected and analyzed at week 12.

2.3. Patient data

Carotid artery tissues were collected from eight patients who had severe ICA stenosis through CEA procedures at the Department of Neurosurgery, Renji Hospital, Shanghai Jiao Tong University School of Medicine with informed consent and institutional review board approval. All patients obtained a confirmed diagnosis of ICA stenosis of >70% through CTA, MRA, or DSA. As the excised part was longer than the length of the lesion, we took the ends of the carotid artery plaque as the comparable normal control.

2.4. Reverse transcription-PCR (RT-PCR) analysis

Cellular RNA was isolated by Tri-Reagent (Molecular Research Center) according to the manufacturer's instructions. DNA was removed from the samples using DNase treatment (DNA-free kit, Ambion Applied Biosystems), and cDNA was synthesized from the purified RNA using Moloney murine leukemia virus reverse transcription kit (Promega, Madison, WI, USA). *GAPDH* primer sets were used to produce a normalization control. We used the following primer sequences:

GAS5-F: 5′-ACACAGGCATTAGACAGAA-3′;
GAS5-R: 5′-CCAGGAGCAGAACCATTA-3′;
ZFAS1-F: 5′-AGTTGCAGTCAGGCTTCATAC-3′;
ZFAS1-R: 5′-TCGTCAGGAGATCGAAGGTTG-3′;
SNHG6-F: 5′-AAGAGCCGTTAGTCATGCCG-3′;
SNHG6-R: 5′-ATGCCGCGTGATCCTAGTAGT-3′;
GAPDH-F: 5′-GTCTCCTCTGACTTCAACAGCG-3′;
GAPDH-R: 5′-ACCACCCTGTTGCTGTAGCCAA-3′.

Real-time RT-PCR was carried out in triplicate with the SYBR Green PCR Master Mix (Applied Biosystems, CA, USA) and a 7900HT Fast Real-Time PCR machine (Applied Biosystems, CA, USA).

2.5. Microarray hybridization and data mining

Total RNA from the left or right carotid artery of atherosclerosis rats was amplified and labeled with biotin according to the standard Affymetrix® protocol. The fragmented and biotinylated cDNA was then subjected to hybridization with the Affymetrix GeneChip Rat Gene 2.0 ST Array (Affymetrix, Santa Clara, CA). Chip analyses were repeated three times. DAVID Bioinformatics Resources analyses of biological themes were performed to explore the underlying themes of those statistically significant, differentially-expressed genes in terms of biological relevance, *e.g.*, functional relevance as identified by Gene Ontology (GO) enrichment analysis and pathway enrichment analysis. The Benjamini-Hochberg derived step-up procedure of False Discovery Rate (FDR) was applied to account for multiple hypotheses testing to assess the significance of the biological theme enrichments.

2.6. Statistical analysis

We built a large lncRNA-mRNA network to identify the interactions between genes and lncRNA (Pujana et al., 2007). More focused lncRNA-mRNA networks were built according to the normalized signal intensity of specific genes and lncRNA. For each pair of gene-lncRNA, gene-gene or lncRNA-lncRNA, we calculated the Pearson correlation and choose the significant correlation pairs with which to construct the network (Prieto et al., 2008). In a network analysis, degree centrality is the simplest and most important measure of the relative importance of a gene or lncRNA based on its centrality within a network. Degree centrality is defined as the number of links incident upon a node (Barabasi and Oltvai, 2004). Statistical analysis was performed using the Student's *t*-test. A P value <0.05 was considered statistically significant.

3. Results

Fig. 1A is a schematic model showing the experimental procedures of atherosclerosis induction in rats. First, male SD rats were fed a high-fat diet for 8 weeks to establish the hyperlipidemia model. The left carotid artery was then injured by surgical procedures. Four weeks post-injury, the left carotid arteries of the rats showed clinical features of atherosclerosis.

Next, body weight, total cholesterol, high-density lipoprotein and low-density lipoprotein were measured in atherosclerotic rats and control rats. The results showed that atherosclerotic rats had a higher body weight (583.3 ± 23.5 g versus 424.7 ± 11.6 g) and a higher total cholesterol level (3.0 ± 0.09 versus 2.3 ± 0.14 mg/dL) compared to control rats (Fig. 1B). High-density lipoprotein is considered to be an anti-atherosclerotic factor that promotes removal of cholesterol in peripheral tissues (including the arterial wall), thereby preventing the occurrence of atherosclerosis (Kosmas et al., 2014). We showed that high-density lipoprotein was lower in atherosclerotic rats compared to control rats (0.6 ± 0.01 versus 1.1 ± 0.2 mg/dL) (Fig. 1B). However, low-density lipoprotein is considered a pro-atherosclerotic factor, whose deposits in the brain and blood vessels gradually form atherosclerotic plaques (Kosmas et al., 2014). As expected, low-density lipoprotein was higher in atherosclerotic rats compared to control rats (1.7 ± 0.04 versus 0.7 ± 0.03 mg/dL) (Fig. 1B). Arteries in the AS model group had a significant amount of atheromatous plaques as shown by H&E staining (Fig. 1C). These data suggest a successful induction of atherosclerosis in rats.

To better understand changes in the transcriptome in atherosclerosis, we used the left carotid artery as the experimental group and the right carotid artery as the control group. The Affymetrix GeneChip Rat Gene 2.0 ST Array was used to screen and compare the transcriptome patterns between the two groups. The Affymetrix GeneChip Rat Array can simultaneously detect >27,000 transcripts including mRNA and long non-coding RNA (lncRNA). We found that a total of 1886 genes were differentially expressed in the left carotid artery with 1005 being