Using Time-course Data to Identify Stress- and LDL-induced MicroRNA Target Genes for Vascular Smooth Muscle Cells

Chien-Hung Huang, Jin-Shuei Ciou, Shun-Tsung Chen, Ke-Rung Tzeng, Jeffrey J. P. Tsai, Nilubon Kurubanjerdjit, Ka-Lok Ng*, *Member, IAENG*

***Abstract*— A majority of the wall of blood vessels is composed of the smooth muscle cell. It is known that abnormal proliferation of the vascular smooth muscle cell (VSMC) is a major cause of restenosis and atherosclerosis. Cardiovascular diseases (CVDs) are the number one cause of death globally. MicroRNAs (miRNAs) play an important role in the regulation of VSMC phenotype that may play crucial roles in causing CVDs.**

In this study, we employ in silico analysis to examine how the VSMC response when subjected separately to mechanical stress and oxidized form of low-density lipoprotein (ox-LDL) cholesterol using time-course microarray data. We found a group of common differentially expressed genes (DEGs) that are involved in these two conditions. Gene set enrichment analysis suggested that the enriched biological processes are cell-cycle-related processes. This group of common DEGs are putative candidates that might have a synergistic effect on VSMC proliferation. MiRNA-regulated DEGs are identified.

A publicly available web-based platform was constructed to record the results of the identified DEGs involved in the two conditions and their upstream miRNA regulators can be accessed too.

***Index Terms*—cardiovascular disease, low-density lipoprotein cholesterol, mechanical stress, time-course microarray, vascular smooth muscle cell, microRNAs**

I. Introduction

A majority of the wall of blood vessels is composed of the smooth muscle cell. Abnormal proliferation of vascular smooth muscle cell (VSMC) is a major cause of restenosis (vessel renarrowing), and atherosclerosis. There are many studies suggest that vascular injury triggers VSMC dedifferentiation which result in VSMC changes from a contractile to a synthetic phenotype [1]. This type of transition between different phenotypes is referred to as phenotypic modulation. Contractile VSMCs are elongated, spindle-shaped cells, whereas synthetic VSMCs are less elongated and have a cobblestone morphology [2]. Synthetic response is the major cause of restenosis and atherosclerosis (AT) following the development of neointimal hyperplasia [3].

Cardiovascular diseases (CVDs) is a series of diseases related to the circulatory system, such as coronary heart attacks, arrhythmia, and cerebrovascular diseases (strokes) etc. These diseases originated in vascular lesions, such as abnormal vascular tone and vasomotor etc., often have a similar cause and disease process, and they are the leading causes of death in most of the developed countries. Therefore, how to improve the diagnosis, treatment and prevention of CVDs is an urgent and important issue.

The vascular function and structure abnormalities will lead to its morphological and molecular changes in blood vessels, cause a series of responses, such as endothelial damage, inflammatory cascades initiate, migration and phenotype changes in VSMC, as well as extracellular matrix (ECM) imbalances [1] [4].

The vessel wall is an initiative and integrative organ, which owns three main cell types, including endothelial cells (ECs) lining the tunica intima, VSMC in the tunica media and fibroblasts within the adventitia, may through producing locally active substances to self-modulate the structure and function of the blood vessel for responding to various stimuli. The hypertrophy and proliferation of VSMC contribute to the formation of AT, hypertension, and restenosis [4-6].

Various local or systemic risk factors may initiate AT by inducing endothelial dysfunction and vascular injury. It is known that upon the vascular wall exposed to high pressure (such as mechanical stretch) or oxidized form of low-density lipoprotein (ox-LDL) cholesterol, both will lead to differential gene expression, then promote the growth and migration of VSMC, result in severe vascular lesions and sequelae which caused CVDs [5-10].

Mechanical force is a particularly important modulator in circulatory systems. Once mechanical forces (such as pressure-induced mechanical stretch) exert injurious forces on the vessel wall, where mechanical forces are largely disturbed, followed by the modulation of gene expression is observed in VSMC. As a result of up- and down-regulation

The work of Chien-Hung Huang is supported by the grant MOST 104-2221-E-150-039, and National Formosa University, under the grant of EN103B-B1005. The works of Ka-Lok Ng, Jin-Shuei Ciou and Jeffrey J.P. Tsai are supported by the grant of MOST 102-2632-E-468-001-MY3. The work of Ka-Lok Ng is also supported by the grants: MOST 104-2221-E-468-012 and 103-asia-06.

Chien-Hung Huang is with the Department of Computer Science and Information Engineering, National Formosa University, Taiwan 63205 (Phone: +886-56315588; e-mail: chhuang@nfu.edu.tw). Jin-Shuei Ciou, Shun-Tsung Chen, Ke-Rung Tzeng and Jeffrey J.P. Tsai are with the Department of Biomedical Informatics, Asia University, Taiwan 41354. (e-mail: zxrwater@gmail.com, stc100218003@gmail.com, ncrazj@hotmail.com, jjptsai@gmail.com)

Nilubon Kurubanjerdjit is with the School of Information Technology, Mae Fah Luang University, Chiang Rai, Thailand 57100 (e-mail: sendtoopal@gmail.com).

*Corresponding author, Ka-Lok Ng is with the Department of Bioinformatics and Medical Engineering, Asia University, Taiwan 41354. Department of Medical Research, China Medical University Hospital, China Medical University, Tai-chung 40402, Taiwan (e-mail: ppiddi@gmail.com).

of specific genes, key cellular processes may be modulated, such as cell proliferation, apoptosis, cell migration and the synthesis, degradation and reorganization of the ECM. These differential expressions of genes will encode relevant factors to counter the effects of mechanical forces exerted on the vessel wall and minimize its notable complications. Namely, mechanical forces may directly perturb or alter the manner of the genes in the cell, thereby initiating signalling pathways usually used by growth factors [4-7, 11].

On the other hand, many studies support that LDL cholesterol plays a central role in the pathogenesis of AT, and the ox-LDL activates both cell-mediated and immune responses, that perpetuate the chronic inflammatory reactions characteristic of AT, and also has been shown to induce VSMC from the contractile state transform to the migratory and proliferative state [11-15].

LDL is a major risk factor in AT development and formation. The lowering LDL cholesterol may reduce or prevent the occurrence of CVDs [16-18]. VSMC exposed to atherogenic stimuli, such as ox-LDL, express high levels of a variety of lipid-binding membrane receptors for cholesterol uptake. When the binding occurs it may result in the accumulation of high levels of cholesterol and cholesteryl esters [14, 19, 20].

LDL is oxidated into the ox-LDL form, which will become more toxic and take part in inflammation response to contribute the plaque formation and development by multiple mechanisms, including promoting endothelial cell dysfunction, forming macrophage foam cell, and modulating VSMC phenotype state. Clearly, the ox-LDL effect is mediated by the transcriptional induction of proinflammatory cytokines and growth factors, initiated multiple signal transduction pathways that induced effector genes of cell proliferation, migration and ECM formation. Finally, these transcriptional alterations result in abnormal cell growth and apoptosis, and phenotypic alteration in VSMC [15].

Time-course gene expression experiments have been widely used for studying a wide range of biological processes, due to their capability to capture the dynamical behavior of the systems. To analyze the differential expression of genes of VSMC separately subjected to mechanical stress and ox-LDL at the molecular level, we proposed to make use of the time course microarray experiment for identifying significantly abnormal expression gene (namely differentially expressed genes, DEGs), further understanding of the commonality among these two conditions, i.e. mechanical stress and ox-LDL.

In this work, DEGs are identified first. Then, we conduct gene set enrichment analysis to highlight the most relevant biological process terms associated with a given gene list.

MicroRNAs (miRNAs) have been known for about 20 years ago. They play crucial roles in many biological processes, such as cell development, differentiation, signaling, metabolism, and stress responses. In a previous work, we had investigated the molecular mechanisms of plant miRNAs in host-pathogen interaction (HPI) [21]. It was found that the miRNA-targeted genes involved in response to hormone and hormone mediated signaling pathways, that could play an essential role in HPI.

The first experimental report on the role of miRNA in VSMC was not discovered until 2007 [22]. Recent studies have indicated that a number of specific miRNAs, i.e. miR-21 [22], miR-143/145 [23] and miR-221/222 [24], are involved in regulation of VSMC. The above-mentioned works strongly suggested that miRNAs play an important role in the regulation of VSMC phenotype. Hence, in this study we extended our previously published work [25] by including the miRNA-regulated DEGs information and investigated their biomedical consequences.

II. METHODS

A. Datasets

To examine the correlation between stress and LDL contribute to the response of VSMC, we make use of the time course microarray experiment. For the stress-induced condition, E-MEXP-569 was downloaded from ArrayExpress database [26]. The experiment compared the gene expression profiles of the VSMC in response to a cyclical mechanical strain over a time course of 0, 2, 4 and 24 hours. Each sample consisted of two replicates prepared from human aortic VSMC purchased from Cambrex Bioscience.

For the ox-LDL condition, GSE13139 was downloaded from Gene Expression Omnibus [27]. This experiment measured the genes expression changes activated by OxLDL binding to LOX-1 of VSMCs at several different time course of 0, 2, 6, 12 and 24 hours. Each time point measurement was repeated twice to examine the temporal patterns of the gene expression in response to ox-LDL. Each sample consisted of three replicates prepared from HAECT cells.

It is known that defects in miRNAs can possibly induce diseases, such as cancer formation [28]. The importance of this hypothesis can be understood in terms of the regulation relationships between miRNA and their target genes. For instance, if the upstream miRNA is defective, its effect could be amplified downstream. In this study, the miRNA targeted gene data are obtained from miRTarBase (version 4.5) [29] and TarBase (version 5) [30].

B. Differentially Expressed Genes Identification

Many statistical methods are available for microarray data analysis. The use of false discovery rate (FDR) is a common approach to discover significant genes [31]. Another approach is to use ANOVA to investigate the impact of microarray gene expression values within a single factor [32]. Among the many statistical methods, Significance Analysis of Microarray (SAM) [33-34]. Empirical Bayes Analysis of Microarrays (EBAM) [35], and empirical Bayes statistics (eBayes) [36] are three commonly employed approaches to identify DEGs.

SAM is a statistical method for identifying DEGs by comparing two or more groups of samples. It uses repeated permutations of the data to estimate FDR based on observed versus expected score, which is obtained from randomized data. A gene, which has an observed score that deviates significantly from the expected score, is considered as a DEG.

EBAM performs one and two class analyses using either a modified t-statistic or standardized Wilcoxon rank statistic, and a multiclass analysis using a modified F-statistic.

Moreover, this function provides an EBAM procedure for categorical data such as SNP data and the possibility of employing a user-written score function.

The EBAYES algorithm computes moderated t-statistics, moderated F-statistics, and log-odds of differential expression by empirical Bayes shrinkage of the standard errors towards a common value.

In a previous study [37] our study suggested that, EBAYES, SAM, and EBAM, achieve a similar level of cancer gene prediction accuracy, i.e. around 20%, therefore, EBAYES is adopted in the present analysis.

The publicly available microarray data analysis package Bioconductor [36, 38]. was adopted in the present study. In particular, we used the EBAYES algorithm, an intrinsic function of the limma package, to identify DEGs, assuming a FDR less than 1% [31]..

C. Gene Set Enrichment Analysis

Functional annotation of the DEGs is given by implementing the Database for Annotation, Visualization and Integrated Discovery, DAVID [39]. DAVID accepts batch annotation and conducts GO term enrichment analysis to highlight the most relevant GO terms associated with a given gene list.

III. RESULTS

To determine the DEGs, we made use of the Linear Models for Microarray Data (*limma*) package, which is available from *Bioconductor*. Details are described in the following section.

A. Differentially expressed gene identification

In this study, Robust Multi-array Average (RMA) was used for gene expression normalization. After that, a model matrix (use the function, model.matrix) was created with rows and columns denote the replicates and the time points respectively. Then, we seeked a linear model to describe each gene using the lmFit function provided by the limma package [28, 29]. [40-41].

DEGs are determined by first constructing the contrast matrix (use the function, cont.matrix), which make pair-wise comparisons between the two replicates. EBAYES analysis was subsequently conducted on the previous results, and the DEGs were selected by setting a FDR threshold of 0.01.

A total of 473 stress-induced DEGs and 8217 LDL-induced DEGs were obtained. There are 289 common DEGs (268 probe IDs, some of the probe IDs have multiple gene symobols) involved in both conditions, in which at least 15 genes are relevant to VSMC phenotypic modification. The results of the identified DEGs involve in the two conditions can be accessed at http://ppi.bioinfo.asia.edu.tw/vsmc/, which provide several important genetic information, such as, the chromosomal locations, GenBank, cytoband and pathway information.

B. The results of gene set enrichment analysis

A total of 268 common DEGs (probe IDs) were submitted to DAVID for clustering, thus, enriched biological processes (BPs) related gene groups were obtained.

Details of the top two clusters (with enrichment scores (ES) 8.12 and 3.48 respectively) enriched BPs information are presented in Table 1. The top five found enriched BPs are cell-cycle-related processes, such as, M phase, regulation of mitotic cell cycle, cytoskeleton organization and spindle organization. For more details about the results of enriched analysis, see our web site information.

Many studies have noted that haemodynamic factors and oxidized low-density lipoprotein regulating VSMC; for instance, (i) with increasing LDL concentration, shear stress increases LDL uptake by human ECs when co-cultured with VSMC [42], and (ii) trigger many cell-cycle-related molecules [43-44]. In particular, the results of Ref. 43 indicated that ox-LDL and mechanical stretch have a synergistic effect on VSMC proliferation, which is induced through a stimulation of nuclear protein import via HSP60 and an activation of the MAPK pathway [43]..

TABLE I
THE RESULTS OF GENE SET ENRICHMENT ANALYSIS

Enrichment Score: 8.12	**Count**	**p-value**	**Benjamini**
cell cycle	43	7.66E-14	1.23E-10
cell cycle process	32	1.60E-10	1.28E-07
cell cycle phase	27	3.18E-10	1.70E-07
M phase	24	4.57E-10	1.83E-07
mitotic cell cycle	24	4.44E-09	1.19E-06
nuclear division	17	1.36E-07	3.11E-05
mitosis	17	1.36E-07	3.11E-05
M phase of mitotic cell cycle	17	1.74E-07	3.49E-05
organelle fission	17	2.35E-07	4.19E-05
cell division	15	1.07E-04	1.32E-02
Enrichment Score: 3.48	**Count**	**p-value**	**Benjamini**
cytoskeleton organization	19	6.91E-05	1.00E-02
microtubule cytoskeleton organization	10	2.99E-04	2.50E-02
spindle organization	6	4.74E-04	3.12E-02
microtubule-based process	12	1.21E-03	5.71E-02

C. The results of miRNA-regulated DEGs

Among the common DEGs, 53 of the DEGs do not have upstream miRNA regulators. For the rest of the interaction, one can classify the miRNA-regulated DEGs in three types of modules, i.e. one- to-one, one-to-many, and many-to-many. A total of 185 miRNA-regulated clusters are found. Figure 1 shows a many-to-one module between several miRNAs and their target gene, MAT2A.

We have established a web-based platform to provide the following information: (i) stress-induced DEGs, (ii) ox-LDL induced DEGs, (iii) the results of gene set enrichment analysis for the common set of DEGs based on BP annotation, and (iv) the upstream miRNAs for the common set of DEGs. The database is freely accessible at http://ppi.bioinfo.asia.edu.tw/vsmc/.

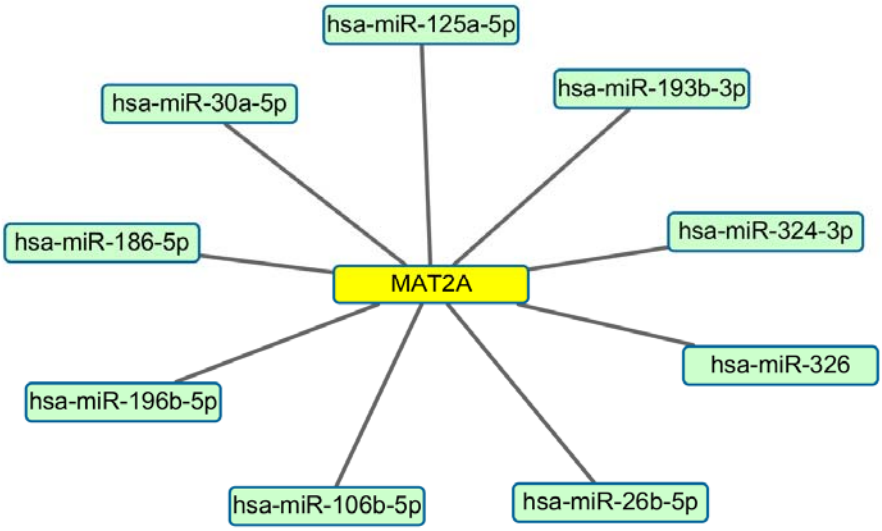

Figure 1. The many-to-one miRNA-regulated module for the DEG, MAT2A.

IV. Conclusions

To examine how VSMC in response to mechanical stress and ox-LDL, we employed time-course microarray experiment to identify the DEGs. Our results have suggested that mechanical stress or ox-LDL may induce a group of common DEGs. Analysis indicated that this group of DEGs could be clustered, in which cell-cycle-related events are the major enriched BPs. Furthermore, this group of common DEGs may be worth for further study, because they could have a synergistic effect on VSMC proliferation.

Time series data are of great interests both for application and scientific research. They have been widely used for studying a wide range of problems, such as (i) applying motif discovery (repeated subseries in a single time series data) and anomaly detection (unusual subseries) in pattern recognition [45], (ii) forecasting of wheat imports [46], and (iii) forecast the enrollments and stock index [47]. In the present work, we have demonstrated the usefulness of employing time series data in biomedical applications.

MiRNAs are emerging as key components in gene regulatory pathways in CVD studies. As miRNAs regulate the expression of their target genes, they play critical roles in a variety of cellular processes, such as phenotypic modification and stress response. However, their precise functional roles are still largely unknown in the context of gene regulatory networks. Also, miRNAs can target multiple genes and thereby the biological functions of a single miRNA can be diverse. It is critical to identify miRNA-directed target genes to investigate the biological functions of miRNAs [21].

There are some tasks are undergoing to examine how VSMC react in response to mechanical stress. A three-phase study is proposed to examine this problem. The gene association network for VSMC can be inferred by using Gaussian graphical model. Graph theory and cluster analysis is employed to analyze the gene association network [48].

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