European Journal of Integrative Medicine 20 (2018) 73–78

Contents lists available at ScienceDirect

European Journal of Integrative Medicine

journal homepage: www.elsevier.com/locate/eujim

Research paper

Construction of the network of critical paths on Blood-stasis syndrome of hypertension based on mRNA sequencing

Yonghong Lian[a,*], Liguo Chen[b], Lifeng Meng[a], Qinjuan Zhang[a]

[a] First Affiliated Hospital of Guangxi University of Chinese medicine, Nanning, 530023, Guangxi, China
[b] Institute of Integrated Traditional Chinese and Western Medicine, Jinan University, Guangzhou, 510632, Guangdong, China

ARTICLE INFO

Keywords:
Signaling pathway
PPI network
mRNA sequencing
Blood-stasis syndrome
Hypertension

ABSTRACT

Introduction: The objective of this study was to construct a critical network of pathways on Blood-stasis syndrome (BSS) relating to hypertension by using mRNA sequencing and database mining techniques.
Methods: 52 cases of primary hypertension patients were collected and they were further classified into different types of Blood-stasis syndrome groups. Serum samples were collected from different groups and were used to culture Human umbilical vein endothelial cells (HUVECs) so as to establish the cell models of BSS. TRIzol reagent was used to extract total RNA of HUVECs and transcriptome sequencing and analysis was performed based on Solexa Genome Analyzer platform. The differentially expressed genes were compared between the different experimental groups using Office Excel software and the Database for Annotation, Visualization and Integrated Discovery (DAVID) was used to analyze to the functional categories and enriched pathways of the differentially expressed genes. *Interologous Interaction Database (I2D)* was used to construct a protein-protein interaction (PPI) network of the genes in the common pathways of the different HTBS groups. The network was analyzed based on Betweenness centrality, then the hubs of the PPI network were identified as the key genes for Blood-stasis syndrome and linked together to build a critical network of pathways on Blood-stasis syndrome of hypertension.
Results: In contrast with the normal control group (NC) group or the non-blood-stasis syndrome (HTNS) group, a total of 2 378 differentially expressed genes of different types of Blood-stasis syndrome of hypertension (HTBS) groups were selected out. With pathway or gene enrichment analysis using DAVID, 75 key genes in the common pathways with high levels of enrichment were obtained, and were imported into I2D to construct a PPI network. Based on the network analysis results, a critical network of pathways on Blood-stasis syndrome of hypertension were constructed, among which Ubiquitin C (UBC) was the bridge to link the key genes on Hypertension with Blood-stasis Syndrome.
Conclusion: During the process of hypertension, UBC, which plays a key role in maintaining cellular ubiquitin levels, could cause a series of pathological reactions and eventually lead to the occurrence of Blood-stasis syndrome.

1. Introduction

The advent of the post-genomic era has prompted a revolutionary change in the field of biomedical research. The research object has been developing from a single gene or protein to biological network which is composed of a number of biological macromolecules, and the research methods are changing gradually from local or partial research to overall or systematic research, that is, to explore how the biological molecules implement the specific biological function through the dynamic interactions between genes, proteins and other molecules from an overall or systematic perspective [1]. As a result, it has produced a large amount of data, like a volcanic eruption in the post-genomic era. Thus we are facing a challenge which is to research the structure, function, and dynamical mechanism of the interaction networks between biological molecules, through screening, integration, analysis and visualization of the massive data by means of bioinformatics computing tools and means, so as to reveal the relationship between the comprehensive clinical syndrome and the internal network of biological molecules.

In traditional Chinese medicine (TCM), “syndrome” is a comprehensive state depicting a disease which has developed into a certain pathological stage, including the cause, location and nature of the disease as well as the pattern of development. Blood stasis syndrome is

* Corresponding author.
E-mail address: lyuheng@163.com (Y. Lian).

https://doi.org/10.1016/j.eujim.2018.04.004
Received 30 August 2017; Received in revised form 9 April 2018; Accepted 10 April 2018

one of the most common clinical syndromes, which refers to the pathophysiological state of blood stagnation, delayed blood flow, blockade in blood vessel [2]. Lots of researchers have been engaging in elucidating the mechanisms underlying BSS for many decades. However, the primary pathogenic mechanism for BSS remains unclear due to its immense complexity. The research aims to explore the process of formation of Blood-stasis Syndrome of hypertension on the molecular level and find out the drug targets for the treatment of Blood-stasis Syndrome of hypertension. Considering the complexity of BSS, our previous research has established a cell model of blood stasis syndrome in which vascular endothelial cells were incubated with serum of hypertension patients with Blood-stasis syndrome [3]. As we all know, BSS is inevitably related to the pathological changes of blood and vessels. While, endothelium lies between blood and vessels, which provides a structural barrier and also secretes mediators that influence the pathological changes of blood and vessels. Therefore, vascular endothelial injury may be the central process in the formation of BSS. The serum of HT patients with BSS, which contains the pathological information of Blood-stasis syndrome of hypertension, could provide a pathological microenvironment for vascular endothelial cells to some extent. So it is convenient to study BSS using the cell model from the perspective of modern biology.

It must be involved in the signal transduction inside the cell to research the formation of BSS from an overall or systematic perspective. Signal transduction refers to the process which is composed of a series of chemical reactions by which a cell converts one kind of signal or stimulus into another [4]. It is initiated by a stimulus(first messenger),such as light, electricity or chemical molecules, which interact with the cell surface receptors, and then it is transduced to the cell interior through second messengers,which amplify the initial signal, and ultimately to effector molecules, resulting in a cell response to the initial stimulus [5]. Most processes of signal transduction involve ordered sequences of biochemical reactions inside the cell, which constitute signaling pathways. When signaling pathways interact with one another they form a systematic biological molecular network, which allow cellular responses to be coordinated [6]. At each step of the signaling cascade, various controlling factors are involved to regulate cellular actions, responding effectively to cues about their changing internal and external environments [7].

The biological molecular network belongs to the complex network, and centrality analysis provides the methods for us to find out the key points in the network. Centrality is an important indicator in network analysis, which is used to identify the most important vertices within a graph, where the values produced are expected to provide a ranking which identifies the most important nodes [8]. The meanings of “importance” varies by different contexts or purposes, which allows centralities to be classified based on how they measure cohesiveness [9]. The usual methods of centrality analysis are as follows: Degree Centrality, Closeness Centrality, Betweenness Centrality, Flow Centrality and Bottleneck Centrality.

2. Materials and methods

2.1. Case selection and diagnostic criteria

In accordance with the diagnostic criteria for hypertension in “*the Guidelines for Prevention and Treatment of Hypertension in China*” in 2005 [10], 52 cases of primary hypertension patients were collected in the Second Affiliated Hospital of Guangzhou Medical University between December 2011 and June 2012. And they were further classified into five groups: qi deficiency and Blood-stasis (HTBSA), qi stagnation and Blood-stasis (HTBSB), cold-coagulation and Blood-stasis (HTBSC), heat-accumulation and Blood-stasis (HTBSD) and non-blood-stasis syndrome (HTNS) based on the diagnostic criteria for BSS in *Diagnosis and Treatment Guidance for Blood-stasis Syndrome by Integrative Medicine* [11]. 30 healthy volunteers were also selected from the Medical School of Ji’nan University as a normal control group (NC).The research was approved by the local ethics committee and was conducted in accordance with the Declaration of Helsinki. All individuals were asked to give their written informed consent to participate in the research.

2.2. Serum collecting and cell culture

Peripheral venous fasting blood samples were taken from different groups and collected into sterile, capped tubes without an anti-coagulation coating. After self coagulation, the blood samples were centrifuged for 15 min (2 000 r/min, 4 °C). The supernatant was transposed into a sterilized EP tube, inactivated at 56 °C for 30 min and stored in a refrigerator at −20 °C. Therefore, the serum from different groups of individuals were collected and prepared to culture Human umbilical vein endothelial cells (HUVECs). HUVECs (CRL-1730) were purchased from China Center for Type Culture Collection, (Wuhan, Hubei Province, China) and were cultured at 37 °C, 5% CO_2. When the cells were in the logarithmic increasing phase, they were incubated in serum-free F12 K complete medium for 24 h. Then they were cultured with conditioned liquid media containing 90% F12 K, and 10% serum from one of the five HTBS treatment groups for another 24 h. Thus, the cell models of Blood-stasis syndrome were established.

2.3. RNA isolation and library preparation

Then the cells were harvested by incubation in trypsin-EDTA solution, and the TRIzol reagent was used to extract total RNA of HUVECs according to the manufacturer’s protocol. Agarose gels were used to check the purity and degradation of Total RNA and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) was used to evaluate RNA integrity and concentration. An Illumina kit (San Diego, CA, USA) was used to construct the cDNA library for transcriptome sequencing according to the manufacturer’s recommendations. Firstly, mRNA was purified and cleaved into short fragments. Secondly, reverse transcriptase and random primers were used for first strand cDNA synthesis. And then DNA polymerase I and RNase H were used to synthesize the second strand cDNA. Finally, the cDNA fragments were purified, resolved and ligated with sequencing adapters before amplifying with polymerase chain reaction (PCR) to create the cDNA library.

2.4. Transcriptome analysis and gene identification

Transcriptome sequencing and analysis was carried out using Illumina HiSeq 2000 platform in Shanghai Majorbio Bio-Pharm Technology Co.,Ltd. In order to facilitate the analysis, the original image data obtained by Illumina HiSeq 2000 sequencing were transformed into sequence data by Base Calling and stored in a FASTQ format file. Then the quality of the sequence data was evaluated, and high-quality sequences were retained and compared with the human genome sequences. Based on the comparison results, the FPKM value, which means the number of mapped reads per kilobase of exon per million mapped reads, was used to calculate the expression levels of the transcripts. The relative changes of the FPKM values of the Blood-stasis Syndrome of Hypertension (HTBS) groups were compared with the NC group or the HTNS group and the differentially expressed genes (DEG) in each HTBS group were selected. And the common genes in the different HTBS groups were identified as the unique genes for HTBS patients by comparing the differentially expressed genes using Office Excel software (Microsoft, USA).

2.5. Analysis of signaling pathways in Blood-stasis syndromes of hypertension

Signaling pathways are the means by which chemical or physical signals are transmitted through cells as a series of molecular events, which ultimately results in cellular responses. Pathway analysis has