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Original Article

Exploration of potential biomarkers involved in acute myocardial infarction

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Abstract: Objective: The aim of the current study was to screen potential novel biomarkers involved in AMI. Methods: Microarray data of AMI (GSE66360) was obtained from the Gene Expression Omnibus (GEO) database. Using the limma package, differentially-expressed genes (DEGs) between samples from AMI and healthy controls were screened. Based on the DAVID tool, functional analysis was carried out. Protein-protein interaction (PPI) and transcription factor (TF)-miRNA-target gene regulatory networks were visualized using Cytoscape software. Finally, drug-gene interactions were predicted using the DGIdb database. Results: A total of 339 DEGs between samples from AMI and healthy controls were identified. Upregulated DEGs were mainly enriched in 32 pathways, including osteoclast differentiation, TNF signaling pathways, and transcriptional mis-regulation in cancer. Cytokine-cytokine receptor interaction was the main functional enrichment for downregulated DEGs. IL8, JUN, IL1B, TNF, and FOS were key nodes in the PPI network. In addition, three miRNAs, including has-miR-191, has-miR-101, and has-miR-20, nine TFs, including NFKAPPAB, SRF, IK3, and NFKAPPAB65, and 34 regulatory relationship pairs were integrated. Prediction results revealed that *TNF*, *IL1B*, and *TLR4* might be potential druggable genes for AMI patients. Conclusion: IL8, JUN, IL1B might be novel markers for atherosclerotic plaque instability. MicroRNAs, including miR-191, miR-101, and miR-20, might provide a window for exploration of potential biomarkers for diagnosis and prognosis for AMI patients.

Keywords: Acute myocardial infarction, differentially-expressed genes, miRNAs, functional analysis

Introduction

Although a significant reduction in coronary heart disease has been obtained in recent years [1], acute myocardial infarction (AMI) remains a major cause of death in the general population. It is the most common disease related to Emergency Department crowding each year [2, 3]. Functional testing is the main diagnostic evaluation for AMI. However, because of the disappearance of symptoms of coronary blood flow in some patients, functional testing might lead to missed diagnosis [4]. Thus, it is necessary to explore improved diagnosis methods on other associated circulatory disorders or symptoms, aiming to reduce the risk of the disease.

Occurrence of AMI is usually accompanied with acute thrombo-occlusive disease with the transition from stable atherosclerotic plaque to ruptured plaque. The transition process involves multifactorial disorders, including sheer stress and biochemical factors, such as proinflammatory and vasoactive factors [5, 6]. Recently, most studies have focused on exploring molecular biomarkers associated with AMI diagnosis and treatment, especially serum biomarkers. For example, NLRP3-inflammasome and associated IL-1 beta were demonstrated as prognostic biomarkers of AMI. In vascular lesions, vascular smooth muscle cells (VSMC) have been associated with limit plaque progression and/or plaque stability improvements [7, 8]. A study by Yi et al. suggested that miR-379 might be a novel biomarker for AMI diagnosis, mediated by VSMC [9]. Furthermore, other molecular biomarkers have been put forward, such as urothelial carcinoma-associated 1 [10], miR-208a [11], and pregnancy-associated plasma protein A (PAPP-A) [12]. However, recent biomarkers have been limited in improving diagnosis and clinical therapy of AMI. Identification of novel biomarkers is urgently needed.

Indicating molecular factors suggesting an impending cardiac event, Muse and his colleagues identified a transcriptomic signature of AMI derived from circulating endothelial cells [13]. To explore the potential molecular mechanisms associated with AMI development, microarray data of AMI was downloaded. Differentially-expressed genes (DEGs) between samples from AMI and healthy controls were screened. Functional analysis was further carried out. Aiming to further explore the functional network of DEGs of AMI patients, protein-protein interaction (PPI) and transcription factor (TF)-miRNA-target gene regulatory networks, as well as drug-gene interactions, were predicted.

Material and methods

This study was approved by the Research Ethics Committee at the Shanghai University of Medicine & Health Sciences Affiliated Zhoupu Hospital. All patients provided written informed consent.

Data source

Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/) is a database used to build gene expression data. It is an online resource used to retrieve gene expression data from any species or man-made source [14]. The data expression profile GSE66360 was downloaded from the database. The current study included 49 circulating endothelial cell samples from AMI patients and 50 circulating endothelial cells samples from healthy cohorts. Expression profiles of all samples were analyzed on the platform Affymetrix Human Genome U133 Plus 2.0 Array.

According to the study by Muse et al. [13], inclusion criteria were as follows: Patients aged 18-80 years old of both sexes that presented to one of five San Diego County Medical Centers with a diagnosis of acute myocardial infarction (AMI); Healthy control patients between the ages of 18 and 35, without a history of chronic disease, and diseased control patients (with known but stable cardiovascular disease) between the ages of 18-80 years old; All AMI cases met strict diagnostic criteria, including chest pain symptoms with electrocardiographic (ECG) evidence of ST-segment elevation of at least 0.2 mV in two contiguous precordial leads or 0.1 mV in limb leads, in addition to angiographic evidence of obstructive CAD in the setting of positive cardiac biomarkers.

Data preprocessing and DEGs screening

Original CEL data was obtained from the GEO database. Normalization and background corrections were performed using the R (version 3.4) software package affy (version 1.58.0, http://bioconductor.org/help/search/index.html?q=affy/) [15], including conversion of raw data formats, missing value complements, background correction (MAS method), and data normalization using quantile methods. The probe was annotated with a platform annotation file, removing probes that did not match the gene symbol. If the same gene was mapped by different probes, the average value of the different probes would be defined as the final expression of the gene.

Using the limma package, DEGs from samples between AMI and controls were screened out [16]. The Benjamini-Hochberg method was used to adjust *P*-values. The threshold was defined as $|\log2 (\text{Fold change})| > 1$ and *P*-values < 0.05.

Functional analysis

KEGG pathways and functions of these DEGs [17] were analyzed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) tool. A comprehensive biological information database was included in DAVID. This system can be used to mine biological functions for numerous genes and protein IDs [18] (version 6.8, https://david-d.ncifcrf.gov/). The threshold was designed as count ≥ 2 and *P*-values < 0.05.

Construction of PPI network and module

Search Tool for Retrieval of Interacting Genes (STRING) is an online tool that evaluates PPI networks [19]. Using the STRING (version 10.0, http://www.string-db.org/) database, the PPI of DEGs was analyzed. The input gene was set as a DEG, while the species was set as human beings. PPI score was set as 0.7 to create subsets of high-confidence human PPI networks. The network was visualized by Cytoscape (vesrion 3.2.0, http://www.cytoscape.org/) [20].

CytoNCA (version 2.1.6, http://apps.cytoscape.org/apps/cytonca) was used to analyze the topology properties of the node network. The

Table 1. Primers used in this study

Primer	Primer sequence (5'-3')
IL8-hF	TGGACCCCAAGGAAAACTGG
IL8-hR	TTGCTTGAAGTTTCACTGGCAT
JUN-hF	CCAACTCATGCTAACGCAGC
JUN-hR	CTCTCCGTCGCAACTTGTCA
IL1B-hF	CAGAAGTACCTGAGCTCGCC
IL1B-hR	AGATTCGTAGCTGGATGCCG
GAPDH-hF	TGACAACTTTGGTATCGTGGAAGG
GAPDH-hR	AGGCAGGGATGATGTTCTGGAGAG

parameter was set without weight. Scores of nodes were obtained. The importance of nodes in the PPI network was sequenced by the score [21].

Traditionally, proteins in the same module have the same or similar functions. They act as a module with the same biological role. Thus, the module in the PPI network was explored using MCODE of Cytoscape plugin (degree cutoff = 2, node score cutoff = 0.2, k-core = 2, and max. depth = 100) [22]. The threshold was designed as scores > 5.

TF-miRNA-target regulation forecast

MiRNA prediction was performed using the WebGestalt GAST [21] (http://www.webgestalt.org/option.php) tool. Enrichment analysis of miRNA-target and TF-target of DEGs from modules was performed using the Overrepresentation Enrichment Analysis (ORA) enrichment method. For analysis, species was selected as h-sapiens. The threshold of *P*-values < 0.05 was designed as significant.

Construction of interaction between genes and potential drugs

Drug-gene interactions and gene druggability levels were described in the Drug-Gene Interaction Database (DGIdb, www.dgidb.org) [23]. Based on DGIdb 2.0, the interactions of DEGs of samples from AMI and drugs and gene druggability of DEGs in the modules were analyzed. Moreover, the interaction network was constructed with Cytoscape.

Real-time quantitative polymerase chain reaction (RT-qPCR)

Circulating endothelial cell samples were extracted from patients experiencing AMI (n = 10) and from healthy cohorts (n = 5), according to a previous study [13]. RT-qPCR analysis was performed, detecting expression levels of several key genes. Briefly, total RNA was extracted from circulating endothelial cells (5×10^6) of the two groups using TRIzol® Reagent (Takara, Dalian, China). Next, cDNA was synthesized using PrimeScript™ RT Master Mix (Perfect Real Time) (Takara). Amplification was performed according to the following conditions with an ABI ViiA 7 real-time PCR instrument: 50°C for 3 minutes, 95°C for 3 minutes, 25 cycles of 95°C for 10 seconds, 60°C for 30 seconds, followed by dissociation curve analysis (60°C-95°C: increment 0.5°C for 10 seconds). Primers are shown in **Table 1**.

Statistical analysis

Data are shown as mean ± standard deviation and were analyzed using SPSS 22.0 software and GraphPad prism 5.0 (San Diego, CA). Relative expression levels were normalized to GAPDH and calculated with the $2^{-\Delta Ct}$ method. The overall significance level is set at p = 0.05 or p = 0.01.

Results

Screening of DEGs

As shown in **Figure 1**, the median of expression profile data after standardization was on the same level. There were 339 DEGs, including 281 upregulated genes and 58 downregulated genes, between samples from AMI and healthy controls.

DEGs functional pathways exploration

Figure 2 shows the main KEGG pathway enrichment results. Upregulated DEGs were mainly enriched in 32 pathways, including osteoclast differentiation, TNF signaling pathways, and transcriptional mis-regulation in cancer. Cytokine-cytokine receptor interaction was the only pathway enriched by downregulated DEGs.

Network of PPI and sub-network module construction

PPI networks of DEGs are shown in **Figure 3**. A total of 165 nodes and 454 proteins and protein interaction pairs were obtained. Furthermore, three sub-module networks were calculated based on MCODE of Cytoscape plugin. A

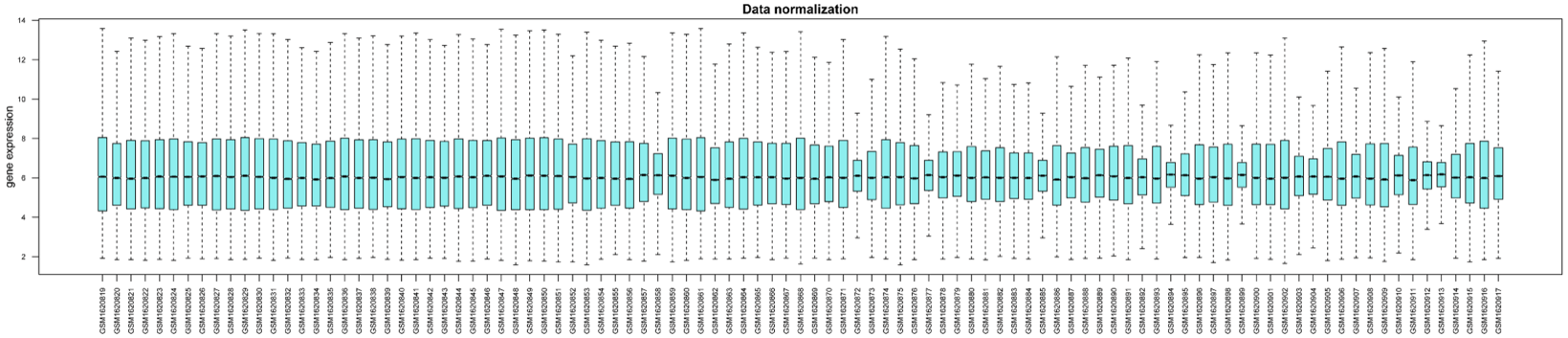

Figure 1. Data normalization boxplot. X axis refers to the log2 of the gene expression; Y axis represents the density.

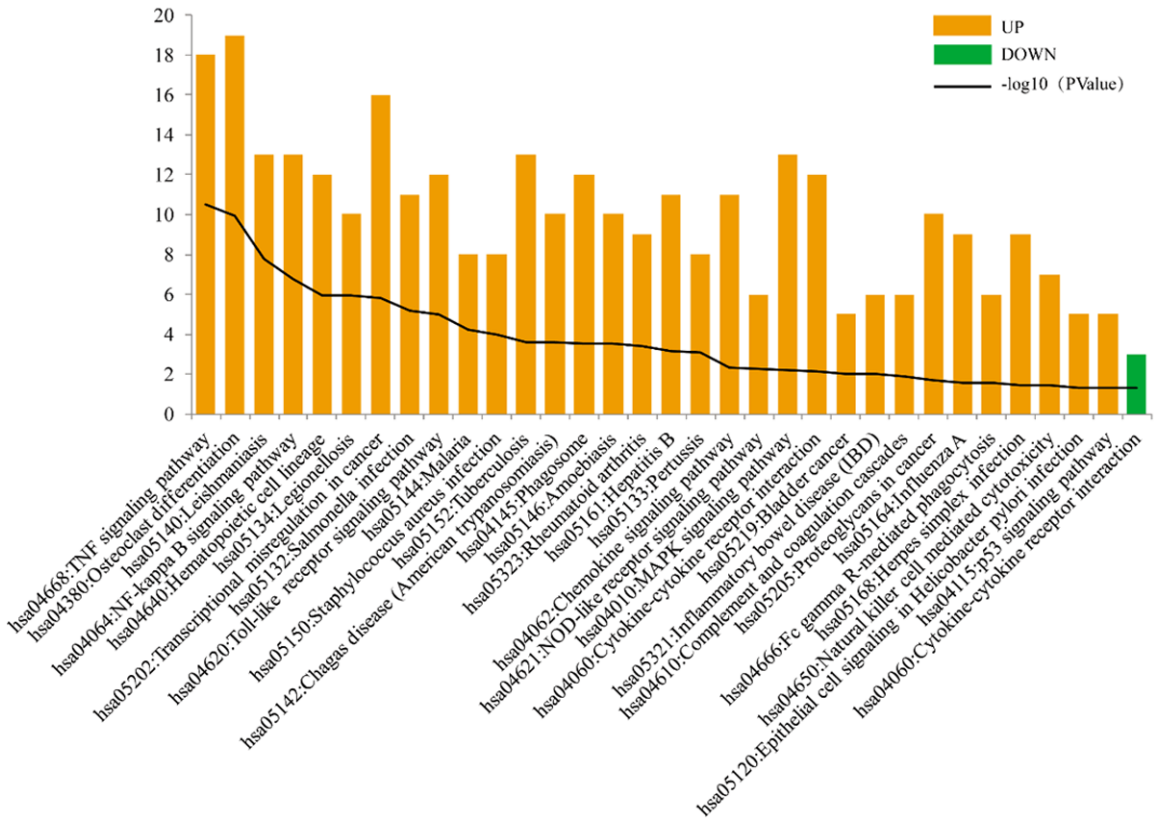

Figure 2. Kyoto Encyclopedia of Genes and Genomes pathway enrichment of differentially-expressed genes (DEGs). Yellow represents upregulated DEGs enrichment, green represents downregulated DEGs enrichment results.

total of 13 nodes and 78 interaction pairs were include in Module A. Module B included 7 nodes and 21 interaction pairs. A total of 9 nodes and 24 interaction pairs were included in Module C. Genes with the top 10 degrees in the PPI network and genes included in the three modules are shown in **Table 2**, including *IL8*, *JUN*, *IL1B*, *TNF*, and *FOS*.

KEGG pathway analysis was further performed on DEGs included in the three modules (**Figure 4**). Genes included in module A were mainly enriched in seven pathways, including chemokine signaling pathways, cytokine-cytokine receptor interaction, and neuroactive ligand-receptor interaction. In total, 26 pathways were involved in genes in module C. The pathways of rheumatoid arthritis and TNF signaling were the main enriched pathways.

Network of TF-miRNA-target gene construction

According to Webgestal prediction, three miRNAs, including hsa-miR-191, hsa-miR-101, and hsa-miR-20, nine TFs, including NFKAPPAB, SRF, IK3, and NFKAPPAB65, and 34 regulatory relationship pairs were integrated, including 13 upregulated genes. As shown in **Figure 5**, Cytoscape was used to construct a TF-miRNA-target network. Of these genes, *EGR1*, *FOS*, and *EDN1* were hub genes, as they had higher degrees than other nodes.

Drug-gene interaction

As shown in **Figure 6**, based on DGIdb predictions of all module genes, this study obtained 51 drug-gene interaction pairs, including 11 upregulated genes, two downregulated regulated genes, and 46 kinds of drugs (etanercept, adalimumab, and infliximab). Regarding these interaction pairs, *TNF*, *IL-1B*, and *TLR4* might be potential druggable genes, as they had higher degrees.

RT-qPCR validation

Expression levels of hub genes *IL8*, *JUN,* and *IL1B* were detected. Results showed that mRNA levels of *IL8*, *JUN,* and *IL1B* were significantly

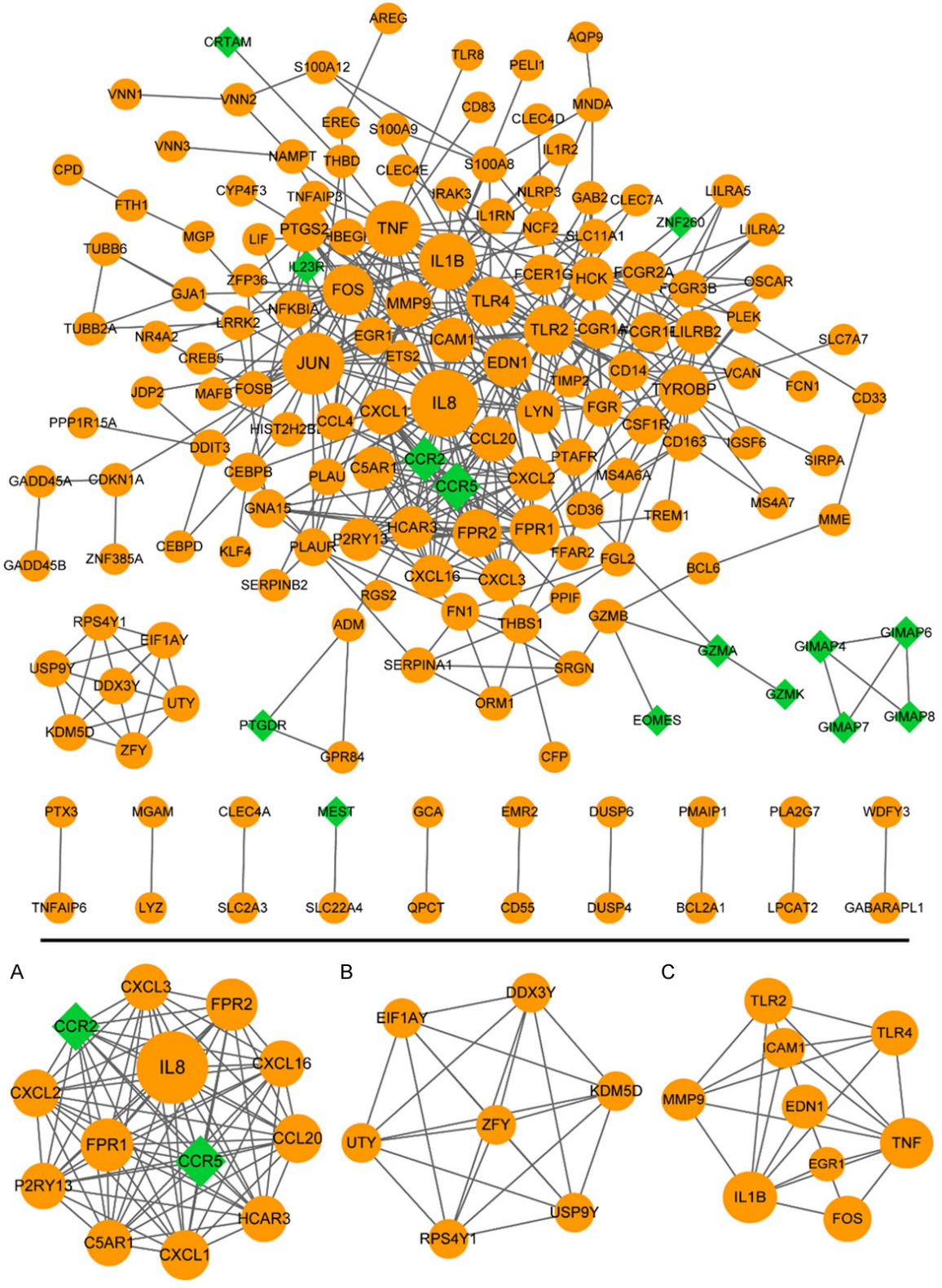

Figure 3. Protein-protein interaction network construction and gene modules screening. Yellow circle represents upregulated genes and green prismatic represents downregulated genes. The node size represents the degree. A huge node refers to a high degree value.

Table 2. Top 10 genes in the protein-protein interaction network of differentially-expressed genes and gene lists in the three modules

Degree top 10			Module A			Module B			Module C		
Nodes	Description	Degree	Nodes	Description	Degree	Nodes	Description	Degree	Nodes	Description	Degree
IL8	UP	35	IL8	UP	35	DDX3Y	UP	6	IL1B	UP	25
JUN	UP	30	FPR1	UP	18	EIF1AY	UP	6	TNF	UP	24
IL1B	UP	25	FPR2	UP	17	KDM5D	UP	6	TLR2	UP	19
TNF	UP	24	CCR5	DOWN	17	ZFY	UP	6	FOS	UP	19
FOS	UP	19	CXCL1	UP	15	UTY	UP	6	TLR4	UP	18
TLR2	UP	19	CCL20	UP	14	USP9Y	UP	6	EDN1	UP	17
TYROBP	UP	19	CCR2	DOWN	14	RPS4Y1	UP	6	MMP9	UP	15
TLR4	UP	18	CXCL2	UP	13				ICAM1	UP	13
FPR1	UP	18	C5AR1	UP	12				EGR1	UP	7
CCR5	DOWN	17	CXCL3	UP	12						
			CXCL16	UP	12						
			HCAR3	UP	12						
			P2RY13	UP	12						

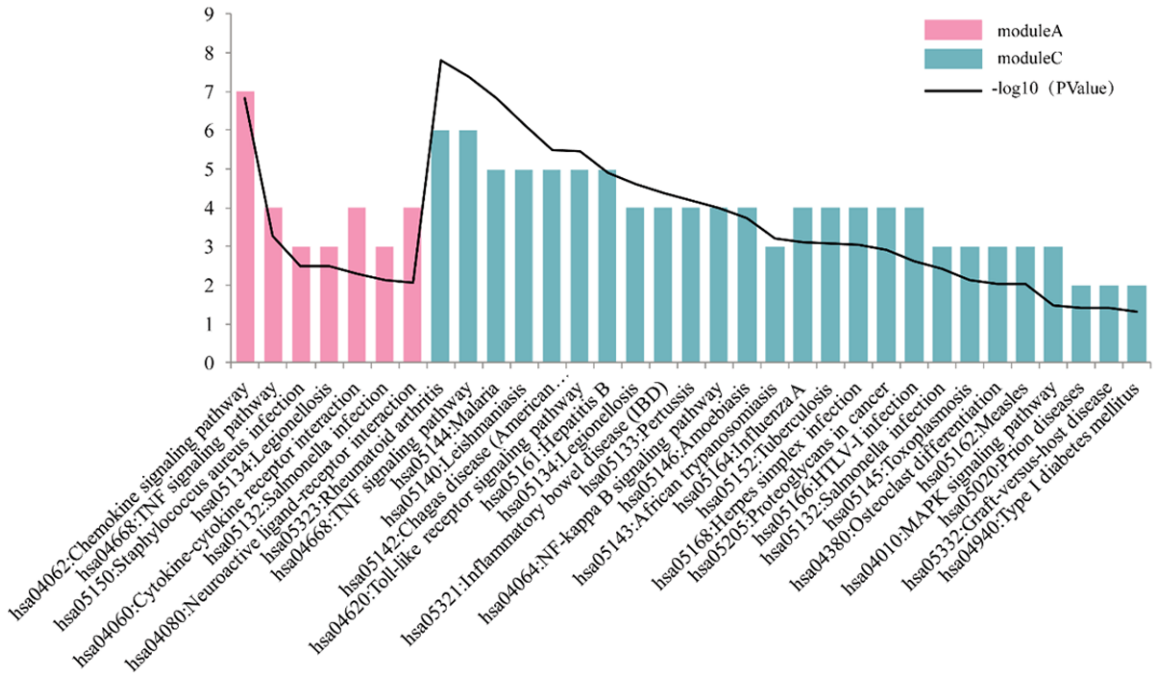

Figure 4. Kyoto Encyclopedia of Genes enrichment (KEGG) results of differentially-expressed genes included in the module. Red cylindrical represents KEGG enrichment of DEGs from module-A; Blue cylindrical represents KEGG enrichment of DEGs from module-C.

upregulated in the AMI group, compared with control samples (**Figure 7**). Results were consistent with analysis results.

Discussion

In the current study, 339 DEGs of AMI and healthy controls were identified. Upregulated DEGs were mainly enriched in 32 pathways, including osteoclast differentiation, TNF signaling pathways, and transcriptional mis-regulation in cancer. Downregulated DEGs were mainly enriched in cytokine-cytokine receptor interactions. IL8, JUN, IL1B, TNF, and FOS were key nodes in the PPI network. In addition, three miRNAs, including has-miR-191, has-miR-101, and has-miR-20, and nine TFs, including NF-KAPPAB, SRF, IK3, and NFKAPPAB65, were key molecules involved in TF-miRNA-target genes network. Finally, prediction results revealed

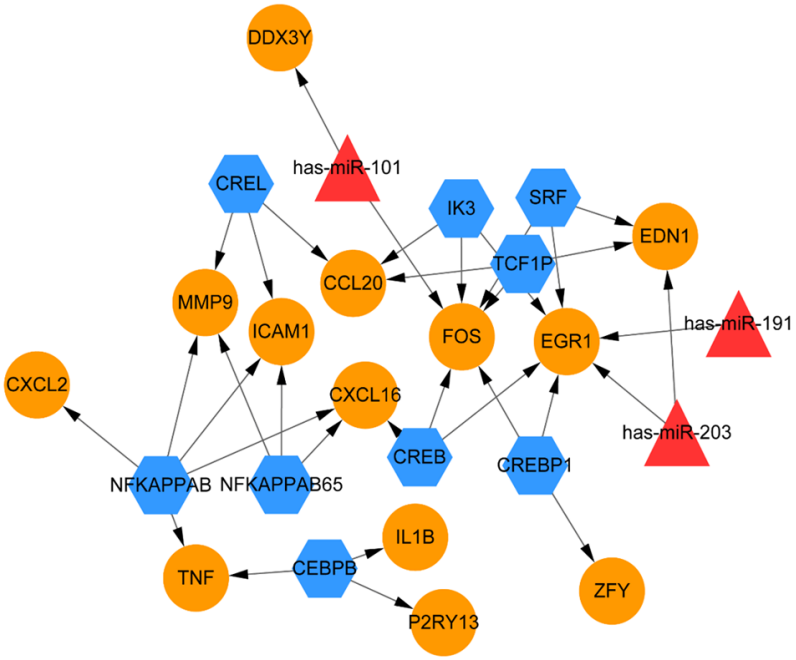

Figure 5. Analysis of the TF-miRNA-target gene regulatory network. The yellow circle represents upregulated differentially-expressed genes, the blue hexagon represents the transcription factor, the red triangle represents miRNAs, and the arrow connecting line indicates the direction of regulation.

that *TNF*, *IL1B*, and *TLR4* might be potential druggable genes for AMI patients.

A study by Muse et al. established the transcriptomic signature of AMI. They demonstrated 11 genes upregulated in AMI, including heparin-binding EGF like growth factor (*HBEGF*), synaptotagmin-like 3 (*SYTL3*), and endothelin 1 (*EDN1*) [13]. These genes were also obtained by the DEGs screening in the current study. Moreover, present data showed that genes, including IL8, JUN, IL1B, TNF, and FOS, were key nodes in the PPI network. Occurrence of AMI is accompanied by acute thrombo-occlusive disease with the transition from stable atherosclerotic plaque to ruptured plaque. The process of atherosclerotic plaque development, progression, and destabilization is accompanied by the inflammatory process. Puz and his colleagues assessed the association between inflammatory molecules TNF, IL-6, and IL-10 in serum levels in patients and stenosis degrees and in ultrasound plaque morphology. Results showed that IL-6 and IL-10 in the serum play important roles in the degree of stenosis and unfavorable changes in atherosclerotic plaque morphology [24]. In accordance with the current study, a previous study also showed that IL-8 plays a key role in the development of atherosclerotic plaque [25]. JUN is another key node in the current microarray data analysis. In a mouse model, c-Jun pathways have been demonstrated as a mediator for IL-6, destabilizing atherosclerotic plaque [26]. Thus, genes such as IL8, JUN, IL1B might be novel markers of atherosclerotic plaque instability.

TNF-alpha has been recognized as a factor that increases susceptibility to heart failure. A progressive chronic inflammatory disorder disease, Prondzinsky and his colleagues put forward that TNF-alpha inducing inflammatory response was associated with clinical outcomes of AMI [27]. Clinical data also showed that susceptibility to AMI was significantly related with genetic polymorphisms in TNF-alpha [28]. Traditionally, NFkappaB is involved in inflammatory response. Current data suggests that NFkappaB was a key TF in the TF-miRNA-target genes network. Dabek and his colleagues demonstrated that destabilization of atherogenic plaque and acute myocardial infarction occurrence was associated with genes involved in NFkappaB signaling pathways [29]. Moreover, TFs, such as SRF, IK3, and NFKAPPAB65, were also evaluated as important factors in the TF-miRNA-target genes network. Although no clinical data has been published concerning these genes, these findings might be useful for future advances in AMI diagnosis.

In recent years, miRNAs have been put forward as novel biomarkers. Many researchers have focused on exploring miRNAs to improve risk stratification, diagnosis, and prognosis of patients with myocardial infarction. For human myocardial injuries in early stages, the potential biomarker roles of many miRNAs have been demonstrated in previous studies, including miRNA-208a [11], miRNA-21 [30], and miRNA-124 [31]. In the current data analysis, several miRNAs, including hsa-miR-191, hsa-miR-101,

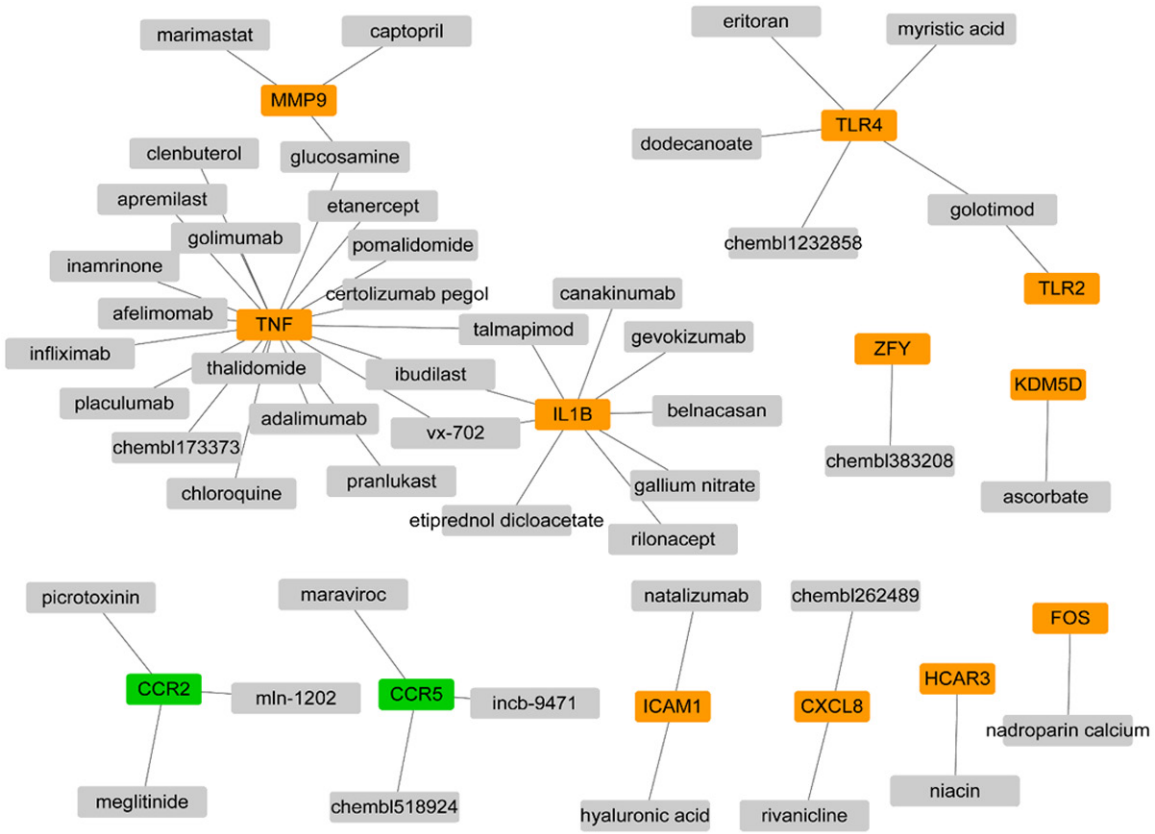

Figure 6. Drug-gene interactions prediction. Yellow square represents upregulated genes, green square represents downregulated genes, and gray square represents the drug.

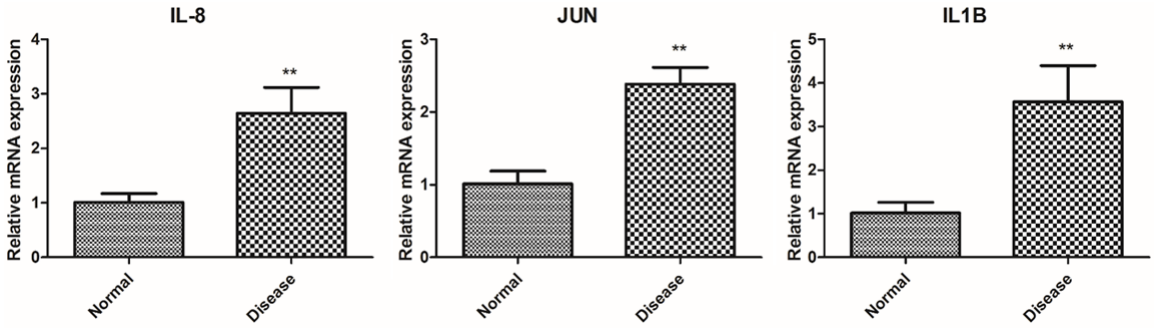

Figure 7. Expression levels of IL8, JUN, and IL1B detected by RT-qPCR. **P < 0.01 compared with controls.

and hsa-miR-20, were defined as important molecules in the development of AMI. Previous studies have shown lower expression levels of miR-191 in AMI [32]. No direct clinical data, supports the regulation roles of miR-101 and miR-20 in AMI. However, previous data has suggested that miR-20 could promote the survival of mesenchymal stem cells exposed to hypoxia [33]. Moreover, miRNA-101 has been widely researched in cancers based on its regulation roles concerning cell proliferation, migration, and angiogenesis [34]. Although no direct roles of miRNA-101 have been found, it cannot be denied that abnormal activation might be related to development of AMI.

In conclusion, 339 DEGs of AMIs and healthy controls were identified. Of these DEGs, *IL8*, *JUN*, and *IL1B* might be novel markers of atherosclerotic plaque instability. MicroRNAs, in-

cluding miR-191, miR-101, and miR-20, might provide a new window, assisting the exploration for potential biomarkers for diagnosis and prognosis for AMI patients.

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Disclosure of conflict of interest

None.

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