

Interferon regulatory factor 3 is a potential biomarker to predict the process of atherosclerosis

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Abstract. Cardiovascular diseases is one of the major public health and medical concerns globally. Atherosclerosis is a condition where plaque build-up narrows the blood vessels, ultimately preventing sufficient blood flow to the heart. In current research, the role played by the human innate immune system in the development of atherosclerosis is a topic of interest. Although the specific relations require further studies, macrophages have been identified to play a key role in the progression of atherosclerosis due to their ability to mediate inflammation. Nevertheless, the mechanism that how macrophages cause inflammatory responses in atherosclerotic process and what is the risk factor that can describe the inflammation in clinic still remains unclear. This paper reviews the mechanism of macrophages to trigger inflammation and their relationships to atherosclerosis. In this study, Interferon Regulatory Factor 3 (IRF3) and its-related cytokine interleukin 1 β was found to be upregulated in patients with acute coronary disease. Afterwards, the over-expressed IRF3 level and its RNA transcription was further confirmed in atherosclerotic ApoE^{-/-} mice. Our results indicated that IRF3 was up-regulated during atherosclerotic process, which might be a potential biomarker of atherosclerosis and its-related coronary disease in clinic.

1. Introduction & Background

Cardiovascular disease has consistently been the leading cause of mortality globally, followed by cancers and respiratory diseases [1]. Because cardiovascular diseases is considered chronic and may cause irreversible damage, related prevention and treatment have been a major focus in healthcare. Among cardiovascular diseases, the most common forms are Coronary Artery Disease, Peripheral Artery Disease, and Carotid Artery Disease. The commonality among them is that arteries are unable to supply blood to and from the heart as it narrows and hardens due to plaque build-up, a condition known as atherosclerosis [2]. Risk factors to atherosclerosis are both genetic and lifestyle-based [3]. From a gene perspective, a person is more at risk for atherosclerosis if he or she has a family history of heart disease. From a life style perspective, unhealthy diet, lack of exercise, and alcohol and tobacco use are all considered negative health determinants of atherosclerosis. It is worth noting that atherosclerosis and other heart conditions often come hand in hand with other chronic conditions, such as hypertension, diabetes, and hypercholesterolemia [4]. As a result, prevention and treatment of atherosclerosis is often related to and complicated one's blood pressure, blood sugar, and cholesterol. The mechanism of atherosclerosis is highly related to the human innate immune system and usually begins when there is damage in the artery and plaque is formed to repair the damage. As plaque continues to grow, it reaches a point of rupture. Once rupture, the human innate immune system recruits

specialized molecules and cells to the damaged site and forms blood clot. As a result, the artery is narrowed and blood flow is decreased.

The innate immune system serves as the human body's natural defense against foreign pathogens. In contrast to adaptive immunity, innate immunity produces a much quicker, efficient response when a foreign pathogen is detected [5]. In order to carry out its tasks, the innate immune system relies on different types of cells, such as phagocytic cells, lymphoid cells, and natural killer cells. Among them all, phagocytic cells circulate around and are capable of engulfing and ultimately destroying a particle, a process known as phagocytosis [6]. When an innate immune response is triggered, phagocytic cells locate may be deployed to detect and destroy the pathogen and its associated debris. Phagocytosis is often accompanied or followed by inflammation which is carried out by inflammatory cells and cytokines. While inflammation is beneficial when the human body is under foreign pathogen invasion, prolong inflammation is damaging and may be life-threatening.

Among the phagocytic cells involved with the innate immune system, macrophages play a crucial role in the rupture repair and immune response maintenance of damaged arteries [7-8]. Macrophages are a type of large phagocytic cells that can be recruited by the innate immune system to participate in repair. In response to environmental cues, macrophages can appear as either the classic, pro-inflammatory M1 phenotype or the alternative/anti-inflammatory/ M2 phenotype [9]. Due to their plasticity between subtypes and remarkable ability

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to detect pathogenic signals, macrophages can have a diverse range of roles in development and repair. Generally speaking, macrophages and other immune cells are released when the body is under attack by a foreign pathogen. To fight off the pathogen, immune cells incite inflammatory responses. In the case of atherosclerosis, macrophages can maintain and worsen the situation because it can secrete proinflammatory factors, such as cytokines and chemokines, in response to toll-like receptor ligands, interferons, and other pathogen-associated molecular complexes[10-11]. Interferons are particularly important during viral infections and are activated by viral products. The key functions of various interferons include cell-to-cell communication under pathogen invasion, triggering immune protection by immune cells, and preventing viruses from growing and dividing[12]. Mechanically, interferons begin by binding to the receptors on cell-surface, thereby activating the intracellular JAK-STAT pathway. Once the JAK-STAT compound moves within the cell nucleus, it can then turn on the transcription of IFN-stimulated genes (ISGs), including interferon regulatory factors (IRFs). Finally, the ISGs then proceeds to respond to pathogens by establishing pro-inflammatory responses[13]. During the immune response, an alternative pathway may be used to activate IRFs, known as the *st*ING pathway. Upon binding of STING and cyclic GMP-AMP synthase (cages), interferon regulatory factor 3 (IRF3) is induced, leading to the activation of proinflammatory Type I interferons and macrophages [14]. Nevertheless, whether IRF3 and its-related signaling could be served as biomarkers to indicate the atherosclerosis in clinic is still unclear.

2. Materials and Methods

2.1 Data Source and Bioinformatical Analysis

Coronary atherosclerosis mRNA expression data of GEO, database gene were downloaded from Gene Expression Omnibus (GEO) (<http://www.ncbi.nlm.nih.gov/geo/>). GEO dataset GSE202625 GPL23934 Ion Torrent S5 (Homo sapiens) with 27 of coronary artery disease patients and 25 of healthy volunteers was included for further analysis. Gene differential expression analysis were performed using R/Bioconductor edgeR.

2.2 qPCR

Total RNA was extracted from atherosclerotic plaques of ApoE^{-/-} mice by using Trizol reagent and reverse-transcribed into cDNA with PrimeScript RT Master Mix. The process of qRT-PCR amplification was performed using the Step One Plus Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) with FastStart Universal SYBR Green Master. All the experiments mentioned here were performed according to relevant manufacturer's instructions. The formula $2^{-\Delta\Delta Ct}$ (Ct means the cycle threshold) was used to normalize the relative expression of mRNA.

2.3 Enzyme-linked Immunosorbent Assay

Whole blood was collected after ApoE^{-/-} mice were fed with a western diet for 8 weeks, followed by the serum isolated by a low-speed centrifugation at 4 oC temperature. Afterwards, Serum IL-1 β , IL-18, IFN- γ and IRF3 levels were determined by enzyme-linked immunosorbent assay (ELISA) kit.

2.4 Statistical Analysis

Every experiment has been performed for at least 3 times. The comparison between two groups was conducted by independent Student's t-test. SPSS 21.0 software were used to perform all the analyses. All data were expressed as mean \pm SD. Differences were considered statistically significant at P < 0.05.

3. Results

After analyzing the differences in the gene expression of between the CAD patients and no-CAD volunteers. Over 200 of significant DEGs (Figure 1A, $|\log_2$ FoldChange>1.0, p <0.05). Among them, the gene expression of IRF3 and its-related factor IL-1 β and IL-18 were found to be significantly upregulated in the blood (Figure 1B-E).

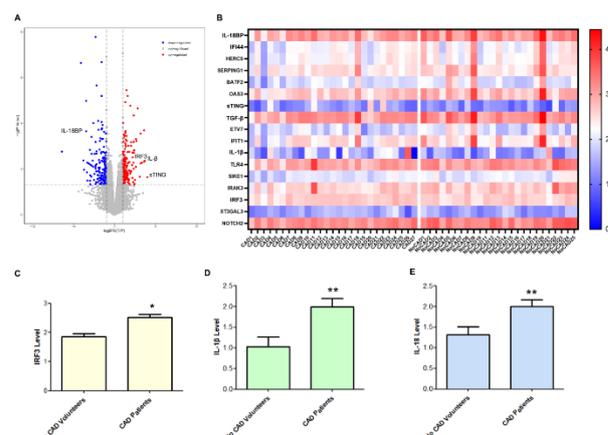


Figure 1. RNA sequencing of blood collected from CAD patients and no CAD volunteers. * P < 0.05 and ** P < 0.01 v.s. no CAD volunteers.

Furthermore, to confirm whether IRF3 was the biomarker to describe the process of atherosclerosis, the atherosclerotic ApoE^{-/-} mice were established by 8-week western diet. Afterwards, the diseased aortic arch was collected and the mRNA transcriptional level of IRF3 and its related pathway genes was evaluated by qPCR. As was shown in figure 2A, the mRNA level of IRF3 was remarkably restricted in ApoE^{-/-}, with the transcriptional level of IL-1 β , IL-18 and IFN- γ (Figure 2B-D) increased, when compared to those of the wide type mice.

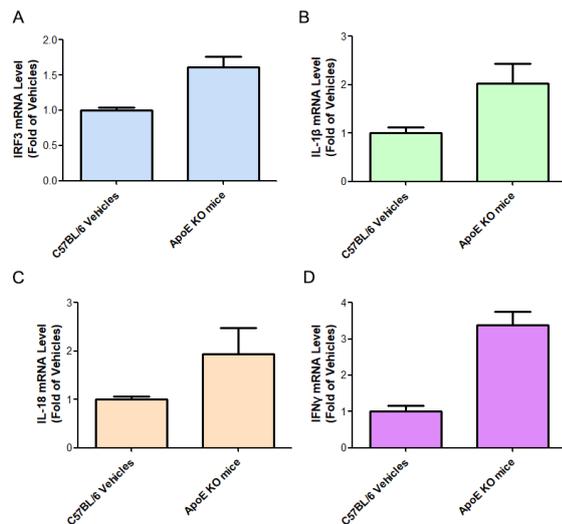


Figure 2. qPCR analysis of IRF3 (A), IL-1β (B), IL-18 (C) and IFN-γ (D) in ApoE^{-/-} mice.

The level of IRF3, IL-1β, IL-18 and IFN-γ were further measured in serum of ApoE^{-/-} mice. As a result, level of those pro-inflammatory cytokines were all remarkably upregulated in atherosclerotic mice (Figure 3). Therefore, our results indicated that IRF3 might be a biomarker to be able to determine the process of atherosclerosis in clinic.

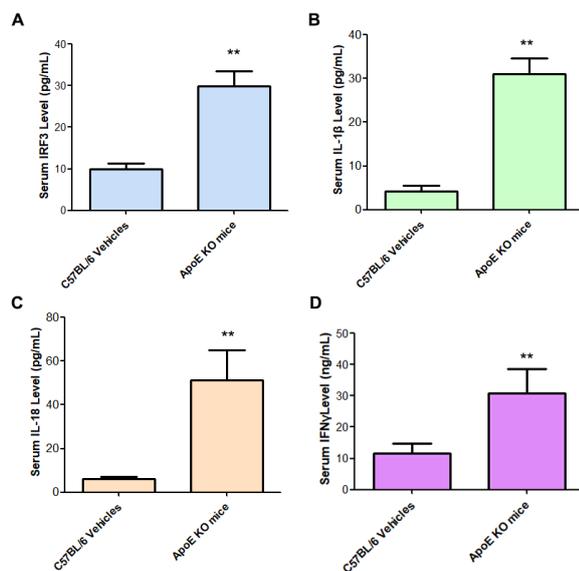


Figure 3. ELISA analysis of inflammation-related cytokines IRF3 (A), IL-1β (B), IL-18 (C) and IFN-γ (D) in ApoE^{-/-} mice. ** P < 0.01 v.s. C57BL/6 vehicles.

4. Discussion and Conclusions

The incidence of various cardiovascular diseases is accelerating exponentially with the world civilization industrialized [15] (PMID: 36094529). In the year 2022, approximate 18 million patients with cardiovascular diseases occurred and around 30% of them died due to the disease process [16] (PMID: 35042750). Although a series of efforts like coronal artery angiography, intravascular ultrasound and optical coherence tomography have been carried out to benefit the early

metaphase diagnosis of the cardiovascular disease. Lack of biomarkers still resulted in the poor diagnostic rate of the disease at their early stage. Thus, further investigation against the potential biomarker of cardiovascular disease is urgently required.

IRFs are a family of proteins that are transcription factors for a wide range of genes associated with innate immune and adaptive immune responses. Among them, IRF3 has been proved to be constitutively expressed in healthy cells and acts as a key player in rapid innate responses regulation [17] (PMID: 35127372). Recently, IRF3 has been demonstrated to be a risk factor that is associated with poor outcome of myocardial infarction (MI), the late stage of cardiovascular disease. It has been well documented that during MI ischemia, macrophages accumulated at heart vasculature, and followed by the cGAS/sTIRG/IRF3 axis activation, which finally resulted in cardiac inflammatory damage [18] (PMID: 29106401). However, whether IRF3 could be a potential biomarker of early stage of cardiovascular disease need further evaluation.

In our study, we focus on the role of IRF3 in the early stage of cardiovascular disease. We found that the mRNA transcription of IRF3 and sTIRG was upregulated in cells from peripheral blood of CAD patients, with inflammatory cytokine IL-1β and IL-18 over-expressed, indicating that IRF3 might be related to the process of cardiovascular disease.

Although the role of IRF3 was preliminarily evaluated by analysing the online RNA expression data of GEO, ApoE^{-/-} mice were still established to confirm the prediction role of IRF3 in early cardiovascular disease. Our results showed that the serum IRF3 level together with its transcription activity in diseased aortic arch was up-regulated, while the inflammatory cytokines IL-1β, IFN-γ and IL-18 also increased, indicating that increased IRF3 level might be related to the activated inflammation in atherosclerotic aortic arch, which highlight the prediction role of IRF3 in cardiovascular disease.

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