Original Research

Comprehensive analysis of dysregulated genes associated with atherosclerotic plaque destabilization

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Impact statement

Atherosclerotic plaque (AP) destabilization is the fundamental cause of acute cardiovascular events. However, robust gene biomarkers for this pathological process are lacking. In this study, we provided a comprehensive view of the dysregulated genes associated with AP instability. These genes were enriched in biological functions such as extracellular matrix disassembly, collagen catabolic process, response to mechanical stimulus, and PPAR signaling pathway. In addition, several critical pathogenic genes for AP destabilization were obtained, including *ITGAM*, *MMP9*, *PLAUR*, *CCR1*, *CD163*, *CD36*, *ADAM8*, and *IL1RN*. The combination of these genes showed a high performance in identifying individuals with or at increased risk of acute cardiovascular events. The results of our study will improve our knowledge on the molecular mechanisms of AP destabilization and provide novel biomarkers for risk stratification of patients with atherosclerotic cardiovascular disease.

Abstract

Atherosclerotic plaque destabilization is a dominating cause of acute cardiovascular events such as myocardial infarction and stroke. This study aims to identify genetic biomarkers related to atherosclerotic plaque destabilization using bioinformatics. Three transcriptome datasets of human carotid atherosclerotic plaque samples were downloaded from ArrayExpress and Gene Expression Omnibus databases, including E-MATB-2055, E-TABM-190, and GSE120521. With Robust Rank Aggregation analysis, we documented 46 differentially expressed genes between stable and unstable/ruptured plaques. Functional enrichment analysis using DAVID tool demonstrated that these genes were mainly related to biological functions such as extracellular matrix disassembly, collagen catabolic process, response to mechanical stimulus, and PPAR signaling pathway. A protein–protein interaction network for the differentially expressed genes was constructed, and eight pivotal genes (*ITGAM*, *MMP9*, *PLAUR*, *CCR1*, *CD163*, *CD36*, *ADAM8*, and *IL1RN*) were obtained from the network with a connective degree > 5. The expression patterns of these hub differentially expressed genes could be verified in atherosclerotic plaque samples with intraplaque hemorrhage. Using gene set variation analysis, the eight genes were integrated to generate an atherosclerotic plaque destabilization score, which showed a high performance in not only discriminating individuals with myocardial infarction from those with stable coronary illness, but also in predicting future acute cardiovascular events in atherosclerotic patients. In conclusion, the findings of this study will enhance our knowledge on the pathological mechanisms involved in atherosclerotic plaque destabilization, and provide potential gene biomarkers for risk stratification of patients with atherosclerotic cardiovascular disease.

Keywords: Atherosclerotic plaque, bioinformatics, acute cardiovascular events, gene biomarkers

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Introduction

Atherosclerosis, characterized by the initiation and development of fibrofatty lesions within the arterial wall, is a fundamental pathogenesis for cardiovascular disease with increasing morbidity and mortality worldwide.[1] Generally, atherosclerotic plaque (AP) contains a fibrous cap of smooth muscle cells (SMCs) and dense collagens that covers surrounding areas with necrotic fragments, immunoinflammatory infiltrates, cholesterol clefts, and calcium deposits.[2] In very late-staged atherosclerosis, however, the plaques may become unstable as manifested by some key features such as a large necrotic lipid-rich core, a thin fibrous cap, intraplaque neovascularization, and intraplaque hemorrhage (IPH).[3] These histological changes render the plaques more prone to erosion or rupture, which

ultimately leads to arterial thrombosis and thus provokes acute cardiovascular events (CVEs) including myocardial infarction (MI) and stroke.[4] The most commonly used strategy for detecting unstable APs is optical coherence tomography. However, this technique is unable to provide quick risk assessments since it requires invasive and time-consuming coronary angiography.[5] Therefore, there is an urgent need for biomarkers to recognize atherosclerotic patients with plaque destabilization.

In recent years, high-throughput transcriptomes have been increasingly exploited to uncover the pathogenesis of cardiovascular disease. With such approaches, researchers have identified several signaling pathways and functional genes that participate in atherosclerotic disorders. Most of these studies were designed to compare the gene expressions between APs and normal tissues,[6,7] or between early- and advanced-staged APs,[8] while fewer focused on plaque destabilization. For instance, Salem *et al.*[9] measured the microarray data of patients undergoing carotid endarterectomy, and found that chemokine ligand 19 (*CCL19*) was significantly overexpressed in unstable APs. In a RNA-sequencing study by Hung *et al.*,[10] long non-coding RNA *PELATON* was detected highly in unstable carotid APs, possibly linked to key biological processes in plaque progression such as inflammation and macrophage regulation. However, some concerns have been raised on the representativeness and repeatability of an individual microarray or RNA-sequencing study. The small sample sizes and use of different measurement platforms also confounded result interpretation. To address these points, integrated bioinformatics strategies such as robust rank aggregation (RRA) have been developed.[11] But to date, this method has not been applied to analyze AP instability.

In this study, we comprehensively analyzed the transcriptome data of human carotid APs. The differentially expressed genes (DEGs) between stable and unstable/ruptured plaques across three publically accessible datasets were screened out using RRA algorithm. Potential biological processes and pathways of the DEGs were investigated, and a protein–protein interaction (PPI) network was build to harvest hub genes, which were further validated in independent AP samples. Finally, the key DEGs were aggregated to develop a multi-gene biomarker panel for discriminating patients with or at high hazard of acute CVEs. The work flow of our study is summarized in Figure S1.

Materials and methods

Data sources

The transcriptome data of human carotid AP samples were acquired from ArrayExpress (https://www.ebi.ac.uk/arrayexpress/) and Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/) databases, with accession numbers of E-MATB-2055, E-TABM-190, and GSE120521. These *in silico* datasets have compared the transcriptomic profiles between stable and unstable/ruptured plaques, which were produced based on microarray (E-MATB-2055 and E-TABM-190) or RNA-sequencing (GSE120521). The details of the three datasets are shown in Table S1.

Data processing and screening of robust DEGs

The RNA-sequencing data of GSE120521 were converted into TPM values, which denote greater comparability between samples and higher similarity to the transcriptome data profiled by microarrays.[12] The differential expression analyses between stable and unstable plaques in GSE120521 were performed using "limma" package in R. For datasets of E-MATB-2055 and E-TABM-190, the processed data of limma test were accessible and were directly used for subsequent analysis. To obtain robust DEGs, we performed an RRA analysis[11] by integrating the differential expression results of the above three datasets using "RobustRankAggreg" package in R. This approach is computationally useful and statistically stable, which assigned a *P*-value to describe how much better a candidate gene was positioned in the ranked lists than expected by random ordering. A Bonferroni correction was performed for the output *P*-values to reduce false positive results. Genes with $|\text{fold change (FC)}| > 1.5$ and adjusted *P*-value < 0.05 were selected as DEGs between stable and unstable/ruptured APs. The R package "OmicCircos" was utilized to visualize the expression patterns of DEGs from RRA analysis.

Functional annotation

The Database for Annotation, Visualization, and Integrated Discovery (DAVID, https://david.ncifcrf.gov/) is an online tool to uncover the potential biological meaning behind a given gene list.[13] To explore the functional mechanisms related to DEGs, we therefore conducted enrichment analyses of gene ontology (GO)-biological process and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway using DAVID 6.8 program. The results were treated as statistically significant with *P*-value <0.05 and gene count ≥ 3.

PPI network analysis

The Search Tool for the Retrieval of Interacting Genes (STRING, https://string-db.org) is a database that includes comprehensive information of human protein interactions.[14] Here, we uploaded the DEGs list into STRING website to obtain significant protein–protein pairs with combined scores > 0.4. A PPI network was constructed and then displayed using Cytoscape 3.6.0 software. Gene nodes with a connective degree >5 in the network were regarded as hub genes.

Validation of hub DEGs in atherosclerotic lesions

The GSE163154 dataset was downloaded and analyzed to verify the expression patterns of core DEGs between plaque tissues with and without IPH, a critical feature of vulnerable APs.[3] This dataset included 43 human carotid APs, which were measured using the Illumina humanRef-8 v2.0 expression beadchip. The gene levels between IPH

and non-IPH samples were compared using Wilcoxon rank sum test with significance thresholds of $P < 0.05$.

Construction and evaluation of hub gene signature

Since AP destabilization contributed greatly to the development of acute CVEs, we presumed that the combination of hub DEGs may provide a useful biomarker to recognize patients with or at high risk of ischemic disorders such as MI or stroke. To test this, the hub genes were firstly integrated to develop a multi-gene signature using gene set variation analysis (GSVA),[15] which scored individual samples against the hub DEGs set (termed "AP destabilization score" [APDscore]). Receiver operation characteristic (ROC) curve analysis was then performed to appraise the performance of APDscore in discriminating patients with MI from those with chronic coronary artery disease (CAD). The area under the curve (AUC) were measured for datasets including GSE59867 ($n = 157$), GSE123342 ($n = 89$), and GSE62646 ($n = 42$), using "pROC" package in R. In addition, we also examined the predictive ability of APDscore for acute CVEs using the peripheral blood samples from GSE21545 dataset ($n = 97$). Atherosclerotic patients in this cohort were allocated into high- and low-APDscore groups, according to the optimum cut-off values of APDscore generated by R "survminer" package. The difference of CVE-free survival between the two groups was determined using Kaplan–Meier survival analysis and log-rank test, with $P < 0.05$ suggesting the presence of significance. The hazard ratio (HR) for APDscore adjusted by patients' age was calculated using R "survival" package.

Results

Identification and functional annotation of robust DEGs

Robust DEGs between unstable/ruptured and stable plaques were obtained by applying the RRA algorithm across datasets of E-MATB-2055, E-TABM-190, and GSE120521. As a result, we obtained 46 genes that were differentially expressed, of which 28 were upregulated and 18 were downregulated (Table S2). The chromosomal locations and expression trends of the DEGs are exhibited in Figure 1. The list of DEGs was then enriched for biological functions using DAVID web tool. As summarized in Table 1, these genes were predominately associated with GO-biological processes including extracellular matrix disassembly, collagen catabolic process, and response to mechanical stimulus. In addition, KEGG pathway annotation demonstrated that the signaling pathways enriched by the DEGs included PPAR signaling pathway and carbohydrate digestion and absorption.

PPI network analysis

The PPI network of DEGs derived from STRING database contained 36 nodes and 77 edges (Figure 2). The nodes with connective degrees >5 in the network were defined as hub DEGs, including *ITGAM* (degree = 14), *MMP9* (degree = 12), *PLAUR* (degree = 8), *CCR1* (degree = 8), *CD163* (degree = 8), *CD36* (degree = 7), *ADAM8* (degree = 6), and *IL1RN* (degree = 6). These genes were all upregulated in unstable/ruptured as compared to stable AP tissues.

Validation of hub genes

Dataset of GSE163154 was analyzed to validate the expression patterns of hub DEGs, which contained 27 IPH and 16 non-IPH samples. In Figure 3, all the identified hub genes showed a higher expression in plaques with than without IPH (all $P < 0.001$).

Hub gene signature and acute CVEs

The hub DEGs were integrated using GSVA method to generate an APDscore for each peripheral blood sample (Table S3), as described in the "Materials and methods". ROC curve analyses demonstrated that the AUC of APDscore to identify MI patients was 0.837, 0.826, and 0.806, respectively, for datasets of GSE59867 (Figure 4(a)), GSE123342 (Figure 4(b)), and GSE62646 (Figure 4(c)). More importantly, atherosclerotic patients with high APDscore had a poorer CVE-free survival than those with low APDscore (Figure 4(d), log-rank $P < 0.001$) in GSE21545 dataset. The age-adjusted HR for APDscore to predict CVEs was 2.772 (95% confidence interval: 1.025–7.499). These results indicated that the APDscore may be helpful in the risk assessment of atherosclerotic patients.

Discussion

Acute CVEs contribute largely to the unacceptable mortality and disability in patients with atherosclerotic disease,[16] the major cause of which is AP destabilization. Therefore, identification of biomarker related to AP destabilization is important for the management of cardiovascular diseases. In the present work, we documented a total of 46 robust DEGs between stable and unstable/ruptured APs using RRA analysis. These DEGs were mainly enriched in extracellular matrix disassembly, collagen catabolic process, response to mechanical stimulus, and PPAR signaling pathway. PPI network analysis obtained eight hub genes (*ITGAM*, *MMP9*, *PLAUR*, *CCR1*, *CD163*, *CD36*, *ADAM8*, and *IL1RN*), and the expression trends of these genes were further validated in IPH versus non-IPH samples. Finally, we calculated an APDscore using GSVA analysis of the hub DEGs, which could not only discriminate individuals with MI from those with chronic coronary disease, but predict future acute CVEs in atherosclerotic patients.

The robust DEGs were mainly associated with biological functions including extracellular matrix disassembly, collagen catabolic process, response to mechanical stimulus, and PPAR signaling pathway. In addition to cellular components, the extracellular matrix of APs plays a relevant role during atherosclerosis, from its initiation to subsequent progression. Degradation of extracellular matrix components, including elastin, collagen and fibrin, leads a thinning of fibrous cap that makes APs susceptible to disruption and predispose instability.[17] The major mechanical stimuli in atherosclerotic vessels are wall shear stress and plaque structural stress. Local changes in wall shear

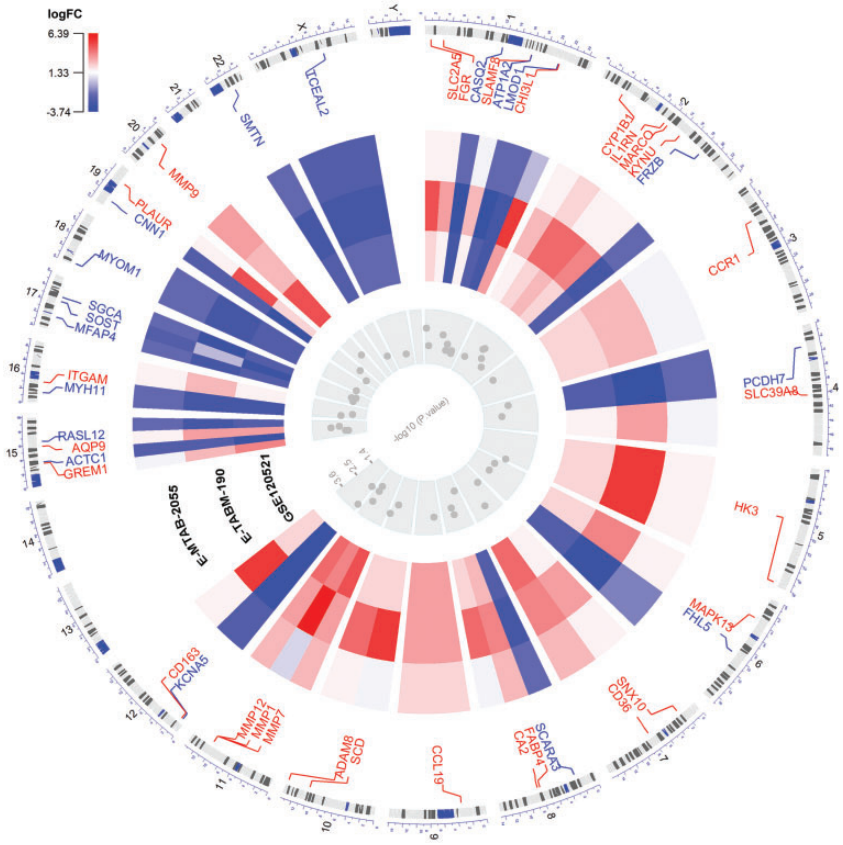

Figure 1. Chromosomal locations and expression patterns of the differentially expressed genes identified by robust rank aggregation analysis of E-MATB-2055 ($n = 48$), E-TABM-190 ($n = 11$), and GSE120521 ($n = 8$). (A color version of this figure is available in the online journal.)
FC: fold change.

Table 1. The significant GO-biological processes and KEGG pathways enriched by DEGs.

ID	Term	*P*-value	Count	Gene symbol
GO-biological processes (top 5)				
GO:0022617	Extracellular matrix disassembly	<0.001	5	*MMP12*, *MMP1*, *ADAM8*, *MMP9*, and *MMP7*
GO:0030574	Collagen catabolic process	<0.001	4	*MMP12*, *MMP7*, *MMP1*, and *MMP9*
GO:0006936	Muscle contraction	0.003	4	*MYOM1*, *SGCA*, *LMOD1*, and *MYH11*
GO:0060048	Cardiac muscle contraction	0.006	3	*ACTC1*, *CASQ2*, and *ATP1A2*
GO:0009612	Response to mechanical stimulus	0.010	3	*CHI3L1*, *KCNA5*, and *SOST*
KEGG pathway				
hsa03320	PPAR signaling pathway	0.001	4	*FABP4*, *MMP1*, *SCD*, and *CD36*
hsa04973	Carbohydrate digestion and absorption	0.008	3	*HK3*, *ATP1A2*, and *SLC2A5*
hsa04976	Bile secretion	0.022	3	*CA2*, *AQP9*, and *ATP1A2*

DEGs: differentially expressed genes; GO: gene ontology; KEGG: Kyoto encyclopedia of genes and genomes; PPAR: peroxisome proliferators-activated receptor.

stress may modify plaque composition towards an unstable phenotype, and the imbalance between plaque structural stress and material strength can promote the rupture of APs.[18] For PPAR signaling, it has been suggested as an important regulator in lipid metabolism, inflammatory response, and atherogenesis.[19] In atherosclerosis mice model, PPARγ agonist rosiglitazone can stabilize the vulnerable APs by improving plaque composition as well as reducing the amount of buried fibrous caps.[20]

In this study, we obtained a total of eight core genes that may function importantly in the course of AP destabilization. Among them, only *MMP9* has been widely studied in vulnerable plaques. The levels of *MMP9* were increased in unstable APs, and its deficiency could inhibit plaque inflammation and prevent plaque instability.[21] *ITGAM*, also known as CD11b, is a biomarker expressed abundantly in inflammatory cells. Blood CD11b(+) dendritic cells and natural killer T cells may mirror the local inflammatory nature in APs.[22] Also, a CD11b-based imaging approach has shown great potential to detect the inflamed APs that is tightly linked to plaque destabilization.[23] Urokinase-type plasminogen activator receptor, a protein encoded by *PLAUR*, was up-regulated in monocyte-derived macrophages and was related to plaque rupture in symptomatic

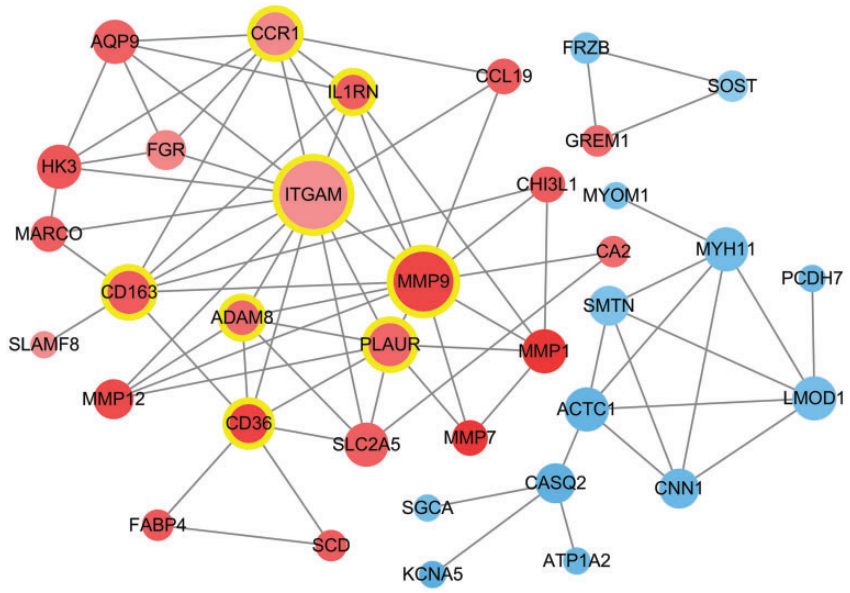

Figure 2. Protein–protein interaction network of the differentially expressed genes. Node color changing from blue to red represents gene expression variations from downregulation to upregulation. Node size reflects the node degree. Node outlined in yellow stands for hub genes. (A color version of this figure is available in the online journal.)

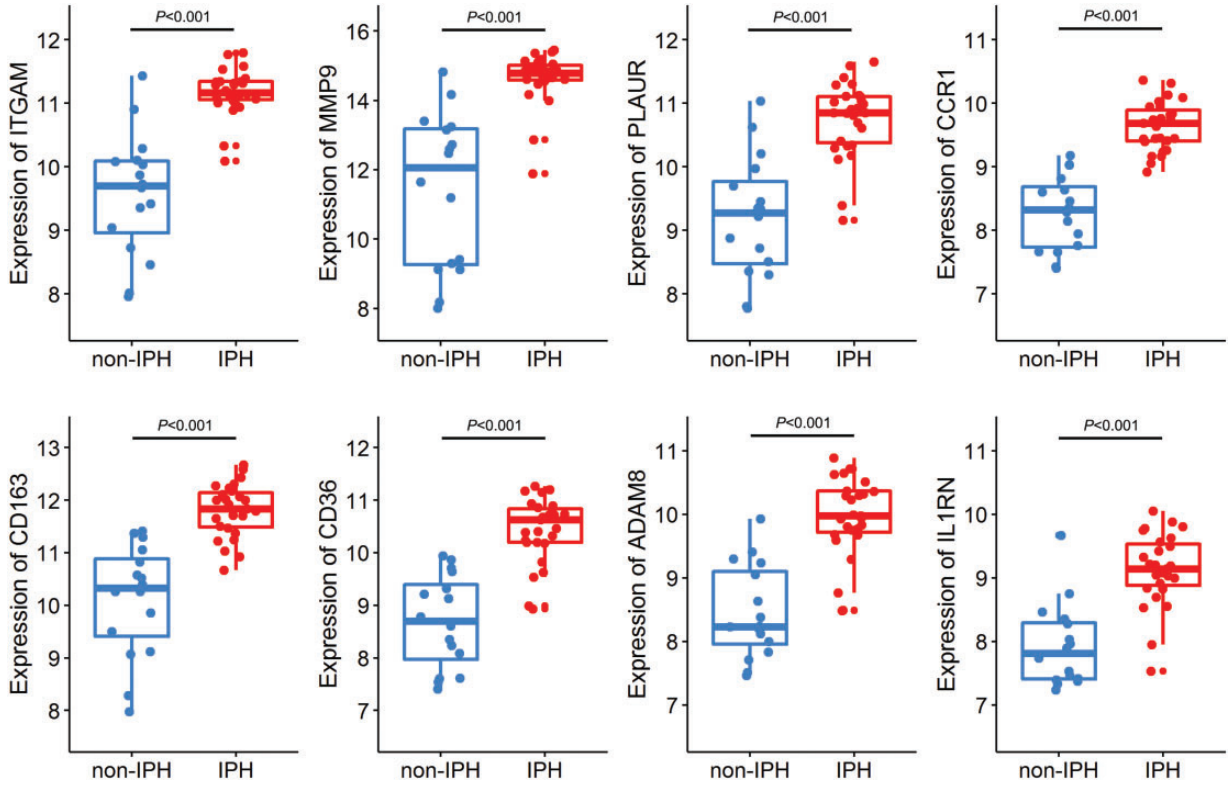

Figure 3. Validation of the expression patterns of key genes in GSE163154 ($n = 43$) dataset. (A color version of this figure is available in the online journal.) IPH: intraplaque hemorrhage.

carotid atherosclerosis.[24] The role *CCR1* in atherosclerosis deterioration is undefined. Braunersreuther *et al*.[25] indicated that *CCR1* deficiency could enhance AP development, while others found no impacts on plaque development or cell composition.[26] For *ADAM8*, its expression has been implicated in human atherosclerosis and MI, but a causal role in plaque progression has not been observed.[27]

In a transcriptome research by Saksi *et al*.,[28] the gene expressions of *CD163*, *CD36*, and *IL1RN* were also higher in symptomatic than in asymptomatic carotid plaques.

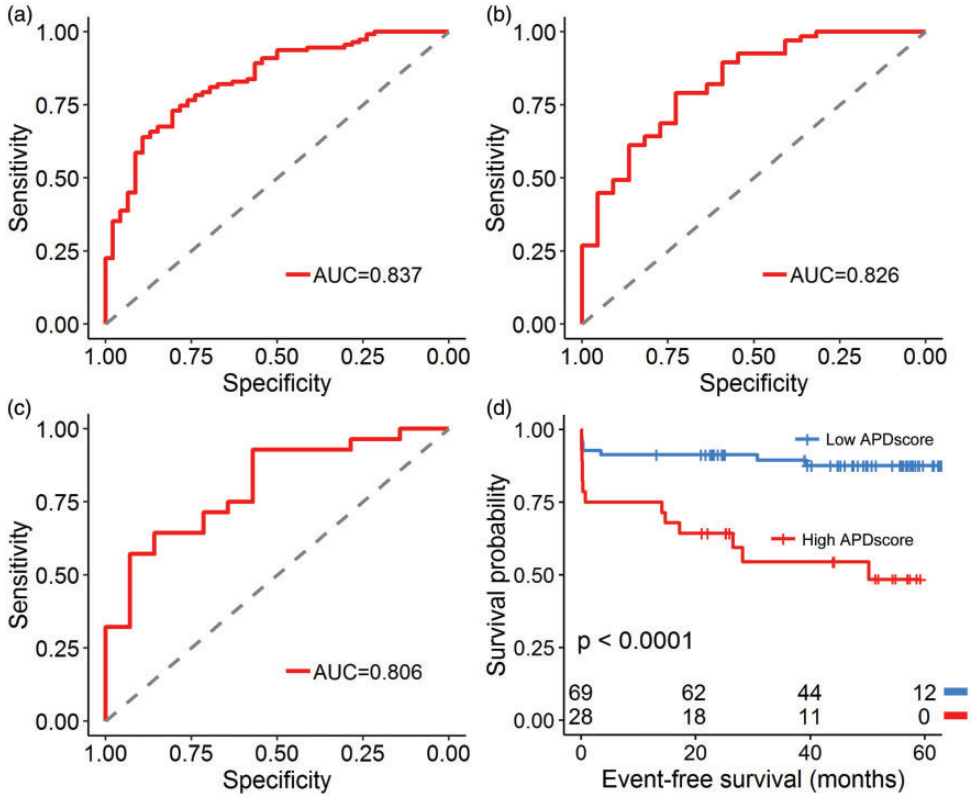

Figure 4. Utility of APDscore in risk stratification of atherosclerotic patients. (a, b, and c) Receiver operation characteristic curves for APDscore to discriminate MI patients in GSE59867 ($n = 157$), GSE123342 ($n = 89$), and GSE62646 ($n = 42$), respectively. (d) Kaplan–Meier survival curve for APDscore to predict acute cardiovascular events in GSE21545 ($n = 97$) dataset. (A color version of this figure is available in the online journal.)
AUC: area under the curve.

Both *CD163* and *CD36* are scavenger receptors highly expressed in monocytes/macrophages. CD163(+) macrophages accelerate intraplaque angiogenesis and inflammation through HIF1α/VEGF-α pathway,[29] and has been linked to a vulnerable plaque phenotype in human atherosclerosis.[30] Cumulative evidence has demonstrated the role of *CD36* in regulating inflammation, lipid metabolism, and function of endothelial cells and SMCs.[31] In atherosclerotic lesions, *CD36* resides in macrophages-rich area of intima and is related to plaque instability.[32] *IL1RN*, an endogenous antagonist of interleukin-1, exerts a critical role in immunoinflammatory response, cell proliferation, and cell damage.[33] Interleukin-1 is a proinflammatory cytokine that expedites atherogenesis, but its inactivation was found to decrease AP stability in advanced atherosclerosis.[34]

Although the biological meanings of most core genes have not been defined in AP destabilization, their expression trends could be validated in an independent dataset. Atherosclerosis is a systematic illness that commonly insults carotid and coronary arteries. These two artery systems are believed to share similar atherosclerotic phenotypes in plaque formation, luminal narrowing, and arterial wall calcification.[35] Thus, the hub genes identified by analyzing carotid APs may be also linked to the acute manifestation of coronary plaque destabilization. To test this hypothesis, we calculated an APDscore based on the gene expressions of the eight hub genes using GSVA method. We found the APDscore had high performances in screening out patients with MI from those with chronic coronary illness. Moreover, the APDscore could predict future acute CVEs in atherosclerotic patients. These results demonstrated that combination of the hub genes may improve the risk stratification of patients with atherosclerosis. Nevertheless, owning to the potential gaps between transcriptomic profiles in blood samples and AP tissues, this result needs additional validations in prospective cohorts.

The strength of our study is that we documented several biological functions and key genes for AP instability using comprehensive bioinformatics methods. However, some limitations should not be ignored. First of all, although the expressions of the hub DEGs were verified using *in silico* data, no cellular or animal experiments were performed to reveal their functional and mechanical implications in AP destabilization. Secondly, some risk factors of AP destabilization cannot be completely balanced between the comparison groups, which may introduce potential bias to our findings.

In summary, by using RRA method we have obtained robust DEGs between stable and unstable/ruptured APs. These genes were mainly implicated in extracellular matrix disassembly, collagen catabolic process, response to mechanical stimulus, and PPAR pathway. The identified eight hub genes may function importantly in AP destabilization, which may be used as biomarkers for risk

assessment of atherosclerotic cardiovascular disease. Future insightful investigations are required to confirm the results of our work.

AUTHORS' CONTRIBUTIONS

QY conceived and designed the study. CQ, YJ and MX conducted the study and contributed to data acquisition, collation and analysis. CQ wrote the manuscript, with key intellectual contents revised by YJ and QY. All authors approved the final submission.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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DATA AVAILABILITY

All transcriptome data used in our study can be downloaded from the publically accessible databases including ArrayExpress and GEO. The details of these data are listed in Table S1.

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SUPPLEMENTAL MATERIAL

Supplementary material for this article is available online.

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