

RUNX1 and CCL3 in Diabetes Mellitus-Related Coronary Artery Disease: A Bioinformatics Analysis

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Background: Cardiovascular complications are a major cause of death and disability in patients with diabetes mellitus, but how such complications arise is unclear.

Methods: Weighted gene correlation network analysis (WGCNA) was performed on gene expression profiles from healthy controls, individuals with diabetes mellitus, and individuals with diabetes mellitus-associated coronary artery disease (DMCAD). Phenotypically related module genes were analyzed for enrichment in Gene Ontology (GO) terms and Kyoto Gene and Genome Encyclopedia (KEGG) pathways. Predicted biological functions were validated using gene set enrichment analysis (GSEA) and ClueGo analysis. Based on the TRRUST v2 database and hypergeometric tests, a global network was built to identify transcription factors (TFs) and downstream target genes potentially involved in DMCAD.

Results: WGCNA identified three modules associated with progression from diabetes mellitus to DMCAD. The module genes were significantly involved in biological processes related to interferon and viral infection, while GSEA of DMCAD samples suggested involvement in viral myocarditis, chemokine signaling and phagosomes. RUNX1 was identified as a potential TF regulating these module genes. Analysis of the global regulatory network of TFs and their targets suggested that CCL3 may be a key regulator in DMCAD.

Conclusion: We found bioinformatic evidence that CCL3 may be a key regulator and RUNX1 a key TF in DMCAD.

Keywords: diabetes mellitus, coronary artery disease, transcription factors

Introduction

Diabetes mellitus is a common metabolic disease that can lead to macro- and microvascular complications, such as coronary artery disease.¹ Individuals with diabetes mellitus are at higher risk of coronary artery disease than the general population, and they are nearly twice as likely to be hospitalized for myocardial infarction or to die from cardiovascular problems as those without diabetes.² Coronary angiography has become the standard diagnostic method for coronary artery disease (CAD) and can improve the early detection of clinical disease.³ However, coronary angiography is expensive and invasive, so there is a need for better diagnostic methods, such as finding a new biomarker.

In the previous studies, the key genes of diabetes mellitus-related coronary heart disease⁴ and ischemic stroke related to diabetes were acquired.⁵ In that work, a classifier that can diagnose coronary artery disease was developed,⁶ but it is not applicable to diabetes mellitus-associated coronary artery disease (DMCAD). How DMCAD arises is poorly understood, although it seems to involve the ubiquitin-proteasome system,⁷ inflammatory factors (fibrinogen, C-reactive protein, galectin-3) and metabolic factors such as lipoproteins.⁸⁻¹⁰ We wondered whether the transcription factor (TF) RUNX1 might also be involved. RUNX1 is involved in vertebrate hematopoiesis, and it is one of the genes most frequently mutated in hematological malignancies.¹¹ There is also growing evidence that RUNX1 plays a role in non-hematopoietic tissues of various epithelial origin.^{12,13} Consistent with the present study, RUNX1 may also contribute to atherosclerotic plaque formation,¹⁴ and the downregulation of RUNX1 in diabetes mellitus leads to defects in macrophage maturation.¹⁵

We also wondered whether chemokines might be involved in DMCAD. These factors recruit leukocytes to sites of injury and activate proliferation of vascular smooth muscle cells, neovascularization and platelets.^{16,17} CLL3 is two-sided in that it not only has anticancer properties but also promotes cancer development. CC chemokines, including CLL3, are important in the initiation of immune responses.¹⁸ They induce the recruitment of dendritic cells, neutrophils, monocytes, macrophages, NK cells and T cells to sites of inflammation.¹⁸ In tumors, tumor-associated macrophages¹⁹ and MSCs²⁰ secrete CLL3, which promotes tumor growth.²¹ CCL3 causes migration and invasion of cancer cells via CCR5.²² In particular, CC chemokine ligand (CCL) 3 has been detected in human atherosclerotic lesions.²³

Here we performed gene expression profiling of healthy individuals, individuals with diabetes mellitus, and those with DMCAD. Our analyses implicate RUNX1 in the progression from diabetes mellitus to DMCAD, and they implicate CCL3 as a key regulator of DMCAD pathology.

Materials and Methods

Data Collection and Processing

Gene expression profiles were downloaded from the Gene Expression Omnibus (GEO; <https://www.ncbi.nlm.nih.gov/>).²⁴ The GSE90074 dataset, based on the GPL6480 platform,²⁵ contains gene expression profiles from mononuclear cells in whole blood from 55 patients with coronary artery disease, 17 with diabetes mellitus, 38 with DMCAD and 33 healthy controls. For the present study, we excluded the data from patients with coronary artery disease alone. The *normalizeBetweenArrays* function in the “limma” package²⁶ was used to normalize gene expression profiles. When the same gene was detected using multiple probes, the expression level was defined as the average across all probes. Probes were excluded if they hybridized to multiple genes. The workflow of the present study is shown in Figure 1.

Weighted Correlation Network Analysis (WGCNA)

Using the “WGCNA” package²⁷ in R, we examined the correlations between genes in GSE90074 and phenotype, which was categorized as normal (1), diabetes mellitus (2) or DMCAD (3). We focused on genes that strongly correlated with phenotype and were associated with a significant p value. Candidate power (1 to 20) was used to test average

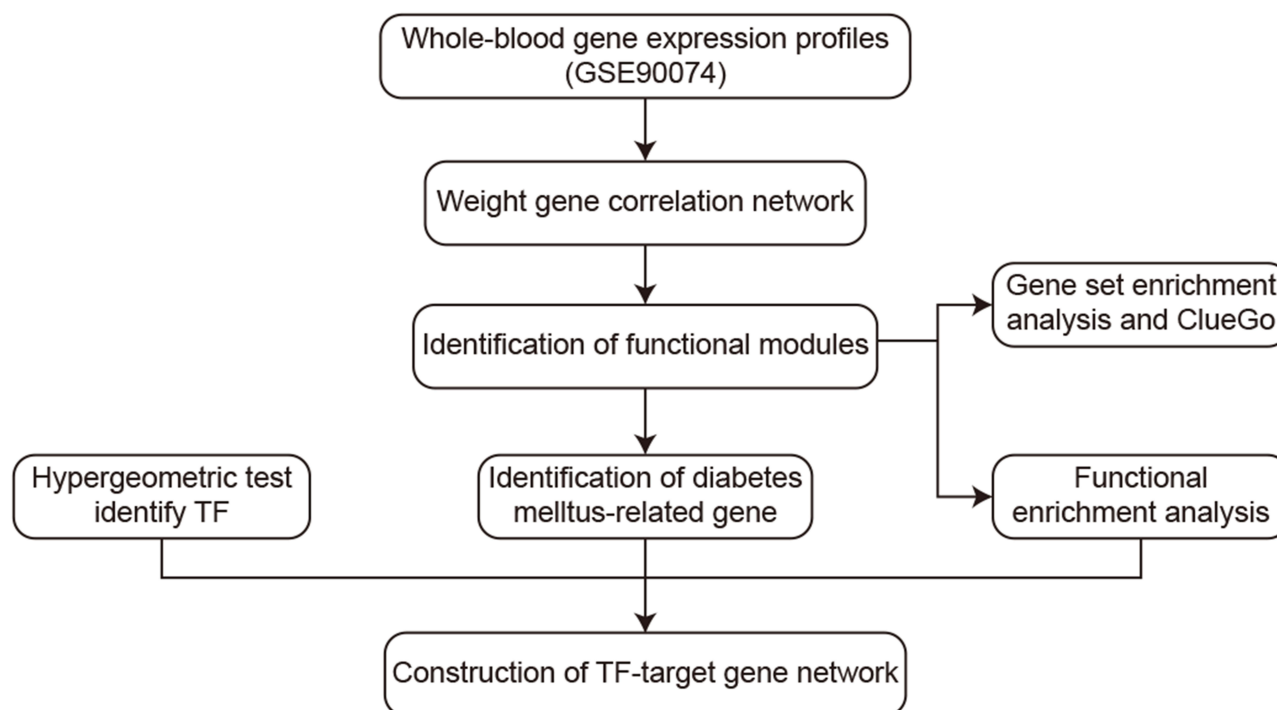


Figure 1 Study workflow.

Abbreviation: TF, transcription factor.

connectivity degrees of different modules and their independence. If the degree of independence was >0.85 , the power value was selected. Hierarchical clustering analysis was performed using the *hclust* function in the WGCNA package. Furthermore, we explored the correlation between modules and phenotype, and focused on modules that correlated with progression from a healthy state through diabetes mellitus to DMCAD.

Functional Enrichment Analysis of Gene Modules

To explore the signaling pathways and biological characteristics of genes in modules of interest, we analyzed the genes for enrichment in Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways using the “clusterProfiler” package²⁸ in R and the Clue GO plug-in²⁹ in Cytoscape.³⁰ We also performed gene set enrichment analysis (GSEA)³¹ on the entire gene expression profiles using the software GSEA (www.gsea-msigdb.org/gsea/download.jsp). The reference gene sets were *c5.bp.v7.0.symbols.gmt* and *c2.cp.kegg.v7.0.symbols.gmt*, downloaded from the Molecular Signature Database.³² Results associated with $P < 0.05$ were considered significant.

Prediction of TFs in Modules and Construction of a Global Regulatory Network

Based on the interaction of human TFs with their target genes in the TRRUST v2 database,³³ the hypergeometric test within the “igraph” package in R (<https://igraph.org/r/>) was used to predict TFs that may regulate functional modules. Only TFs within modules were retained. Then the genes targeted by those TFs were extracted, and a map of TFs and target genes was constructed. Results associated with $P < 0.05$ were considered significant.

Results

Gene Modules Related to DMCAD

WGCNA was performed on 996 genes, and soft-thresholding was determined to be 6 based on the scale-free fit index and average connectivity (Figure 2A). Average linkage hierarchical clustering and module merging divided the genes into four modules (Figure 2B). Three modules were considered of interest (Figure 2C): turquoise, which showed a negative correlation with phenotype ($r = -0.39$, $p = 2e-02$); and blue and brown modules, which showed a positive correlation with phenotype ($r = 0.37$, $p = 4e-04$). The grey module was reserved for unassigned genes and was not analyzed further.

DMCAD is Associated with Dysfunction in Multiple Modules

Gene expression across the three groups was analyzed by clustering analysis (Figure 3A). Genes in the three modules were involved in GO biological processes including responses to virus, to type I interferon, and to interferon-gamma (Figure 3B). Genes in the three modules were also significantly involved in pathways related to viral myocarditis, phagosomes, tight junctions, type 1 diabetes mellitus and chemokine signaling (Figure 3C). GSEA identified several pathways enriched in DMCAD samples: leukocyte transendothelial migration, RNA destabilization, the proteasome and type 1 diabetes mellitus (Figure 3D). In contrast, GSEA identified pathways related to cellular defense responses as well as antigen processing and presentation in individuals with diabetes mellitus (Figure 3E). ClueGo analysis indicated that genes in the three modules were involved in granulocyte migration and responses to type 1 interferon and interferon-gamma (Figure 3F).

TFs Potentially Involved in DMCAD

Hypergeometric tests identified the TFs IRF9, PML and RUNX1 in the turquoise, blue, and brown modules as potentially involved in DMCAD. These TFs strongly correlated with their respective target genes *CCL3*, *FLT3*, *PML* and *SP100* (Figure 4A). In GSE90074, the three TFs were expressed at higher levels in DMCAD patients than in individuals with diabetes mellitus or in healthy controls (Figure 4B).

Global Regulatory Landscape in DMCAD

A network of TFs and their target genes in DMCAD was constructed (Figure 5A), providing a first glimpse into the global transcriptional regulatory landscape. *CCL3* was significantly related to pathways involving chemokine signaling,

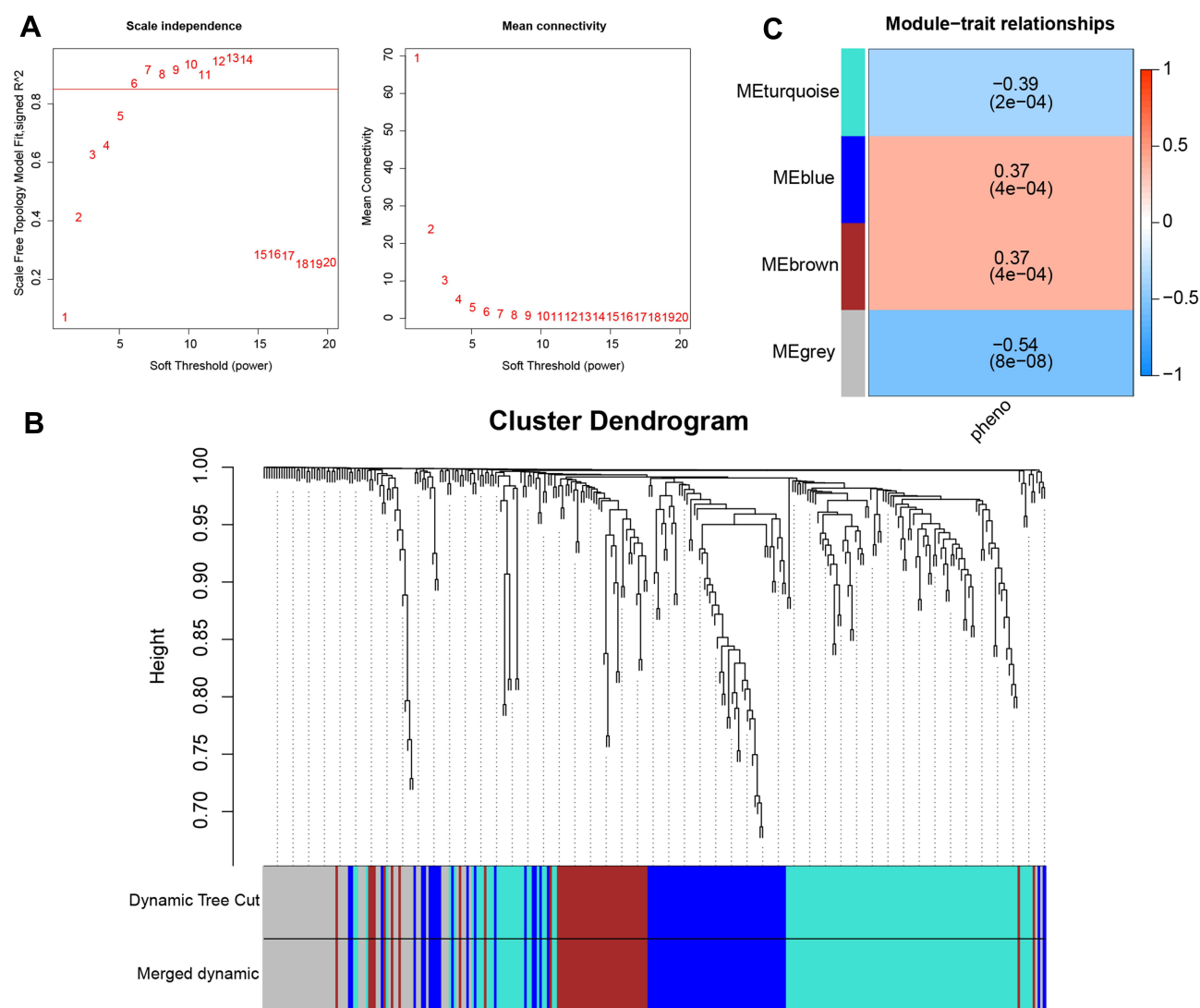


Figure 2 Weighted gene correlation network analysis (WGCNA). **(A)** Selection of soft-thresholding power based on scale-free fit index (left) and mean connectivity (right). **(B)** Division of co-expression modules. **(C)** Correlations between module and phenotype.

Abbreviation: ME, module.

cytokine-receptor interactions and Toll-like receptor signaling (Figure 5B), which therefore may be involved in DMCAD (Figure 5C).

Discussion

Diabetes mellitus is a chronic disease that endangers human health and poses a severe socioeconomic burden. DMCAD is associated with greater risk of adverse events than diabetes on its own,^{34,35} but how DMCAD occurs is poorly understood. In the present study, we performed WGCNA on whole-blood gene expression profiles in the GSE90074 database in order to identify gene modules involved in DMCAD pathogenesis. Leukocyte transendothelial migration has already been linked to DMCAD,³⁶ suggesting that our analysis is reliable and that LDB2, a previously identified inhibitor of leukocyte transendothelial migration, may be a useful therapeutic target.³⁷ We also found evidence that RUNX1 is up-regulated during progression from a healthy state to diabetes mellitus and then to DMCAD. This suggests that RUNX1 is a key TF in DMCAD. It has been proposed that RUNX1 mutations cause cardiac developmental defects and coronary vascular dysplasia, and a direct role of RUNX1 in upregulating mesenchymal markers in epicardial cells and promoting EMT cannot be excluded,³⁸ which endocardial cells play an important role in coronary artery development.³⁹

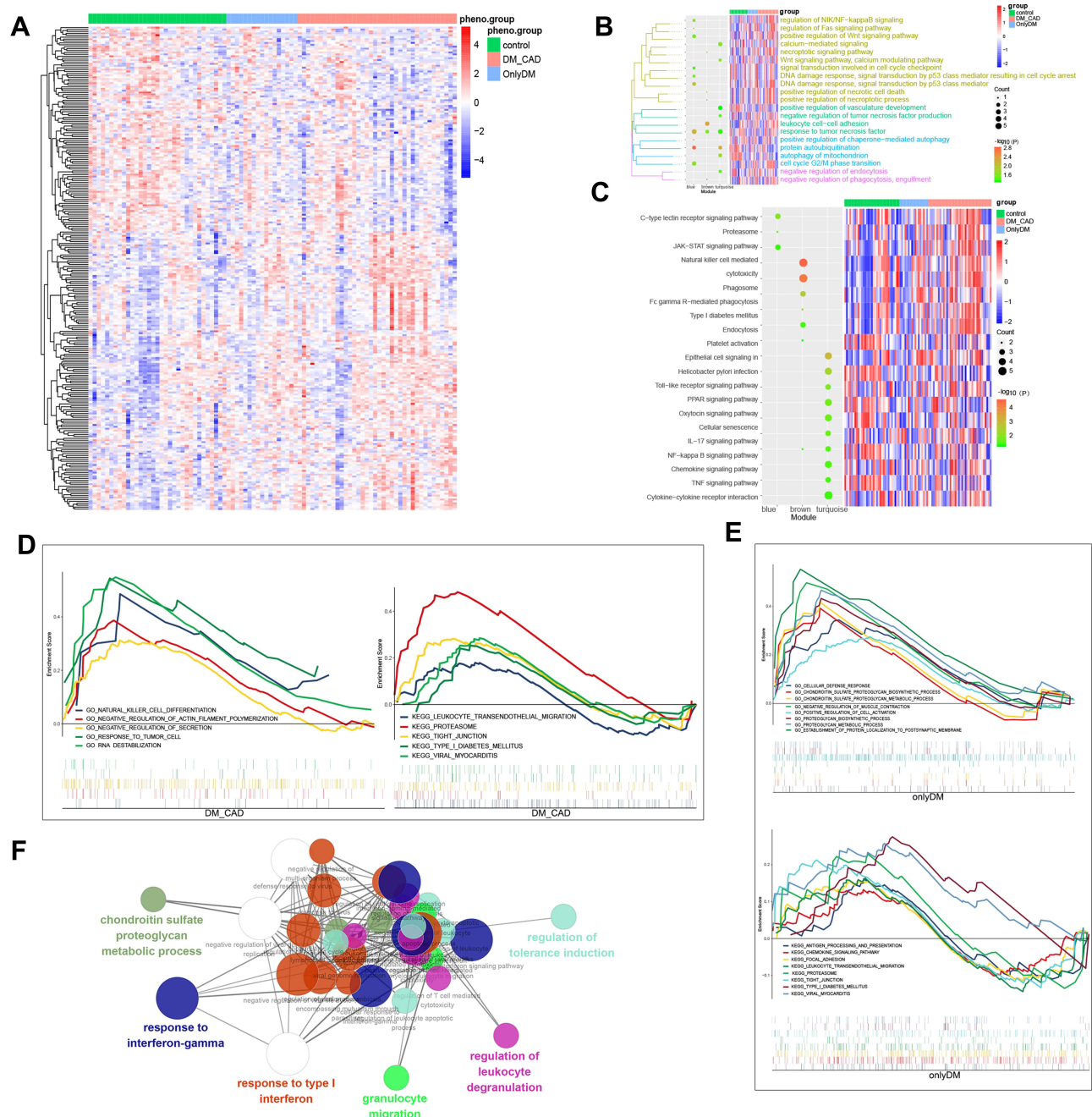


Figure 3 Functional enrichment analysis of phenotype-related modules. **(A)** Hierarchical clustering analysis to distinguish control, diabetes mellitus and DMCAD samples. **(B)** Enrichment of BPs and heatmap of gene expression in the modules of interest. **(C)** Enrichment of KEGG pathways and heatmap of gene expression in the modules of interest. **(D)** GSEA showing enrichment of BPs and pathways in DMCAD samples. **(E)** GSEA showing enrichment of BPs and pathways in diabetes mellitus samples. **(F)** ClueGo analysis of BP enrichment.

Abbreviations: BP, biological process; DMCAD, diabetes mellitus-related coronary artery disease; GSEA, Gene Set Enrichment Analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes.

Our global transcriptional regulatory network in DMCAD suggests that CCL3 may be a marker of the condition. In fact, chemokines and chemokine receptors are important mediators in coronary artery disease.⁴⁰ CCL3, for example, can indirectly affect cytokine production, cellular growth and differentiation, apoptosis and migration along a chemokine gradient.⁴¹ Indeed, monocytes from individuals with diabetes mellitus show a defect in chemotaxis.^{42,43} CCL3 acts as an inflammatory cytokine through Toll-like receptor signaling pathways,⁴⁴ and diabetes mellitus involves systemic low-grade inflammation, which may increase risk of coronary artery disease. Our observation of a link between CCL3 and

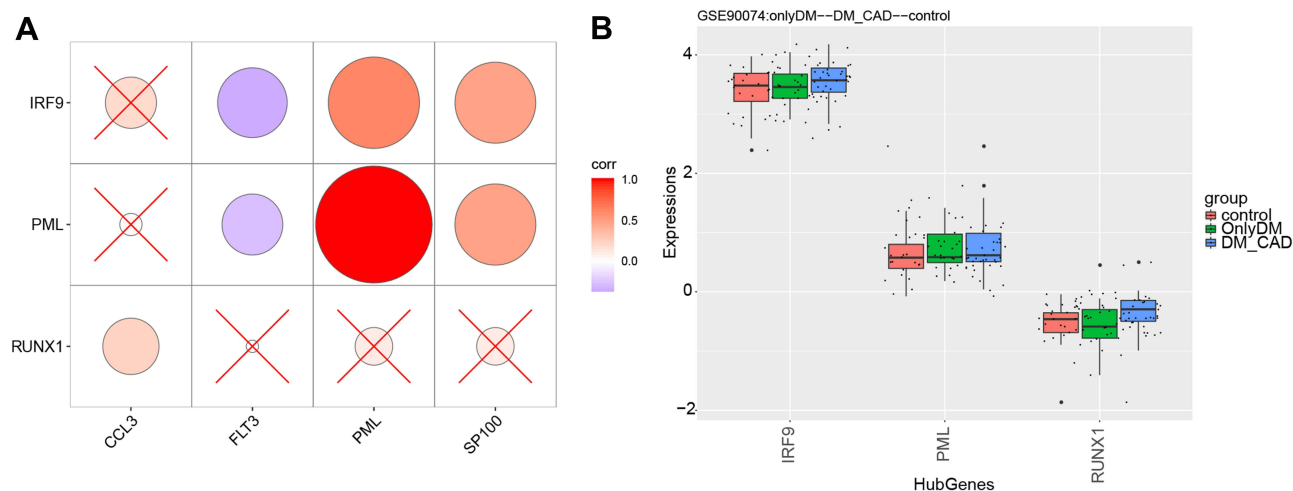


Figure 4 Potential key TFs in DMCAD. (A) TFs correlated with target genes. (B) TFs were up-regulated in DMCAD relative to individuals with diabetes mellitus and healthy controls. **Abbreviations:** DMCAD, diabetes mellitus-related coronary artery disease; TF, transcription factors.

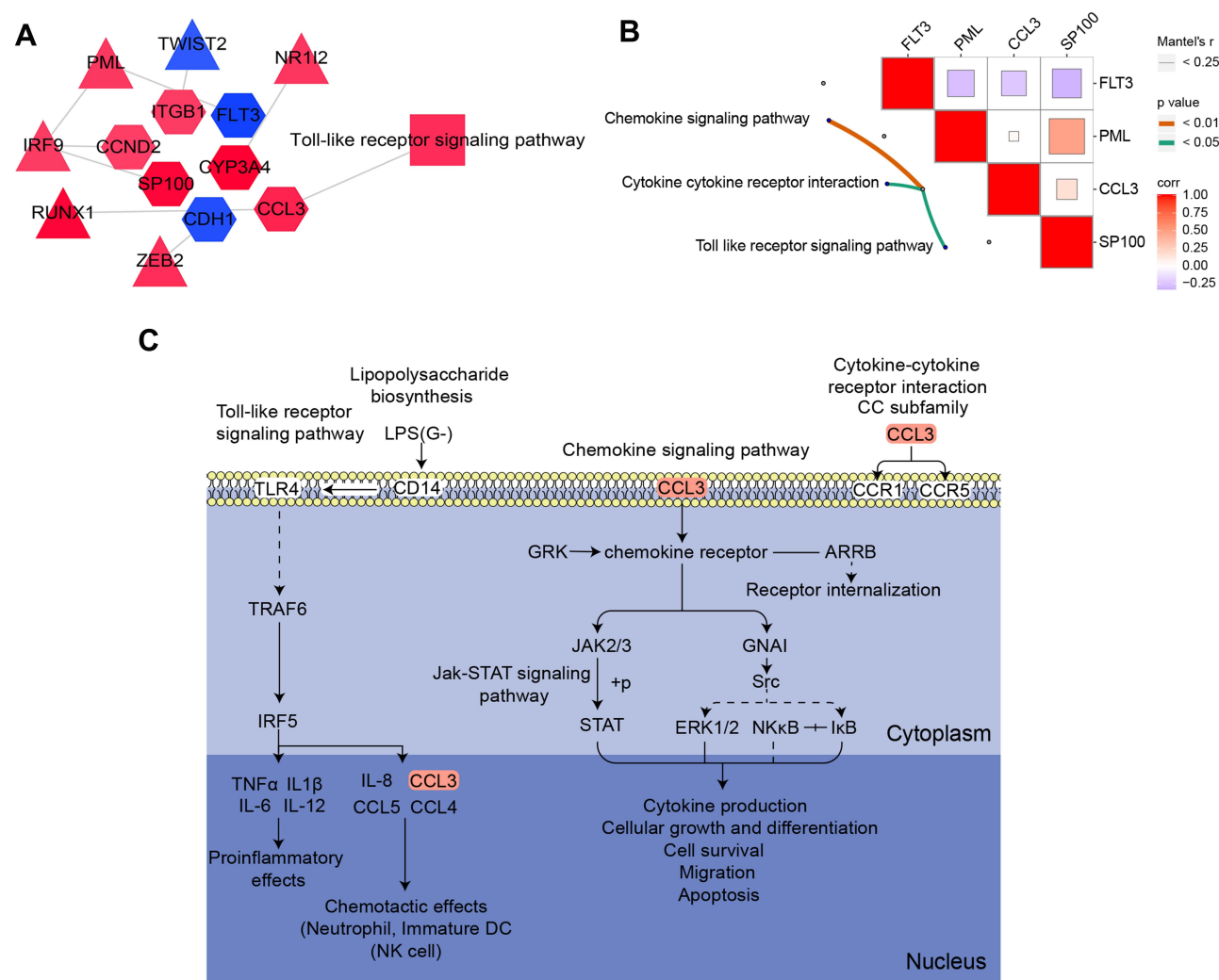


Figure 5 Global regulatory landscape of DMCAD. (A) Network diagram of TF-target gene pathways related to DMCAD. Hexagons represent target genes; rectangles, pathways; triangles, TFs. (B) Target genes correlated with pathways. (C) CCL3-related KEGG pathways potentially involved in DMCAD.

Abbreviations: DC, dendritic cell; DMCAD, diabetes mellitus-related coronary artery disease; KEGG, Kyoto Encyclopedia of Genes and Genomes; NK, natural killer; TF, transcription factor; LPS, lipopolysaccharide.

DMCAD provides testable hypotheses about which signaling pathways are involved in the condition, since CCL3 is known to interact with the receptors CCR1 and CCR5.⁴⁵ CCR1 mediates pro-fibrotic effects in hematopoietic cells,⁴⁶ while CCR5 regulates obesity, insulin resistance and glucose homeostasis.^{47,48} Notably, it has been proposed that CCL3 causes migration and invasion of cancer cells through CCR5,²² associated with activation of PI3K-Akt/PKB and ERK MAPK pathways.¹⁹ New signaling pathways were obtained in the present study, so this deserves further deeper investigation as potential therapeutic targets to prevent or mitigate DMCAD.

Our findings should be interpreted with caution in light of several limitations. First, our analyses were based entirely on bioinformatics, so our results should be verified and extended in cellular and biochemical experiments. Second, our sample was relatively small, so larger studies are needed to determine whether RUNX1 and CCL3 or their downstream targets can be considered DMCAD biomarkers.

Conclusion

Our work links DMCAD to the TF RUNX1 and the regulatory factor CCL3. Further work should explore these molecules and their downstream pathways in DMCAD, which may elucidate pathogenesis and suggest therapeutic strategies.

Data Sharing Statement

The raw data GSE90074 was download from GEO (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE90074>), analyses and codes in this study can be obtained from the corresponding author Dr Wei and Dr Huang upon reasonable request.

Ethics Approval and Informed Consent

Not applicable because GEO belongs to public databases, the patients involved in the database have obtained ethical approval, users can download relevant data for free for research and publish relevant articles, and our study is based on open-source data. Ethics committee from the Fifth Affiliated Hospital of Guangxi Medical University specifically do not require research using publicly available data to be submitted for review to their ethics committee, so this study does not require approval.

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Disclosure

The authors have no potential conflicts of interest to declare.

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