



Correlation between Complement C1q A Chain (*C1QA*) and Macrophages in the Progression of Carotid Atherosclerosis

Guanglong Dong, Xiangwen Yu, Mingyu Zhao, Shusen Lin, *Qingyu Meng

Vascular Surgery Department, the Third Affiliated Hospital of Qiqihar Medical University, Heilongjiang 161000, China

*Corresponding Author: Email: mengyu2001ok@qmu.edu.cn

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Abstract

Background: There is increasing evidence that macrophages are involved in the development of carotid atherosclerosis (CAS), but the specific mechanism is still unclear. We aimed to explore the key genes that play a regulatory role on macrophages in the progression of CAS.

Methods: From 2021 August to 2023 August, GEO datasets GSE100927 and GSE43292 were downloaded and the key gene modules related to CAS were identified by weighted Gene co-expression network analysis (WGCNA). Kyoto Encyclopedia of Genes and Genes (KEGG) pathway analysis was performed on the genes of the key modules to identify common gene enrichment pathways. Differential expression analysis of pathway-related genes was performed by the "limma" package of R software. Case groups were categorized into high and low expression groups based on the expression levels of key genes, and ssGSEA immune infiltration analysis was performed.

Results: The turquoise module of GSE100924 (threshold=12) and the brown module of GSE43292 (threshold=7) were obtained through WGCNA analysis. The analysis of KEGG showed that the differentially expressed genes in the turquoise and brown modules were co-enriched in the staphylococcus aureus infection signaling pathway. Differential expression analysis identified 18 common differentially expressed genes, all of which were highly expressed in the case group. *C1QA* is the gene of interest. According to ssGSEA analysis, the high expression group of *C1QA* showed a significant increase in the number of macrophages (GSE43292, $P=0.0011$; GSE100927, $P=0.025$).

Conclusion: This study identified the key gene *C1QA* involved in regulating macrophage functional activity during the CAS process, providing new ideas for effective control of CAS.

Keywords: Carotid Atherosclerosis; Bioinformatics; Macrophages; Key modules; Differentially expressed genes

Introduction

Carotid Atherosclerosis (CAS) is a chronic inflammatory disease that commonly affects middle-aged and elderly individuals. It is a leading cause of acute cardiovascular events. The prevalence of CAS increases with age, and so does the

incidence rate of complications, which poses a serious threat to patient safety (1). Currently, clinical practice commonly employs drug therapies such as antihypertensive, hypoglycemic, lipid-lowering, and antiplatelet therapy as prevention



and treatment measures (2-4). However, due to the wide variety of antihypertensive drugs, the direct effect of various antihypertensive drugs on carotid atherosclerosis has not yet been determined (5). Moreover, studies have found a certain correlation between high insulin levels and plaque bleeding in patients, raising doubts about the therapeutic effect of hypoglycemic therapy (6). Lipid-lowering therapy has clinical side effects that can affect the prognosis of patients (7). While antiplatelet therapy can effectively inhibit thrombosis, it also increases the risk of bleeding in patients (8). Current treatment methods primarily aim to stabilize plaques and control the progression of inflammation, but they neglect the effective control of the occurrence and development mechanism of CAS (9,10).

Therefore, it is crucial to clarify the specific regulatory mechanisms responsible for the occurrence and development of CAS to achieve effective control. Macrophages, which are cells directly involved in inflammation in the body, participate in the entire process of plaque inflammation and are one of the important factors affecting changes in plaque stability (11,12). Several studies have shown that macrophages play distinct roles in different stages of carotid atherosclerosis. During the early stages of CAS, the regression of plaque is facilitated by the clearance of excessive macrophages through phagocytosis (13). In the late stage of CAS, due to local environmental damage and other reasons, excessive apoptosis of macrophages and obstacles in clearing cell debris can lead to the formation of necrotic cores in the plaque and a decline in plaque structural stability (14). Additionally, studies have shown that macrophages activation is involved in the occurrence and development of CAS. M1 macrophages can secrete high levels of inflammatory factors, leading to tissue damage. M2 macrophages secrete anti-inflammatory factors, including IL-1 receptor agonists, IL-10, and collagen (15,16). In human atherosclerotic lesions, M1 macrophages are predominantly located in unstable and easily damaged areas, while M2 macrophages are mostly found in stable plaque areas (17). Therefore, researchers believe that M1 macrophages en-

hance the inflammatory response and accelerate plaque formation, while M2 macrophages alleviate inflammation and stabilize plaques. However, the specific molecular functional changes of macrophages during the occurrence and development of CAS are not yet clear.

C1QA is a crucial component of the complement system and is closely associated with the systemic inflammatory response in patients. Previous studies have shown that *C1QA* is a prognostic factor for osteosarcoma tumor microenvironment, and is linked to hepatitis virus infection as well as the onset and progression of Alzheimer's disease (18-20). These findings suggest that *C1QA* plays a regulatory role in the patient's immune system and inflammatory response. However, no relevant research reports exist on whether there is a mutual regulatory effect between *C1QA* and macrophages.

We aimed to explore the molecular mechanisms that regulate macrophage changes during the progression of CAS through data mining combined with experimental research. Thus providing sufficient theoretical basis for effectively controlling the course of CAS in clinical practice.

Materials and Methods

Data acquisition

The gene expression matrix was downloaded for datasets of carotid artery related studies from the GEO database:

(<https://www.ncbi.nlm.nih.gov/geo/>).

GSE100927 and GSE43292 are used for screening key genes and signaling pathways, GSE21545 and GSE159677 are used to validate key genes and signaling pathways.

WGCNA Analysis

The "goodGeneSample" function from the WGCNA package was used to remove sample data with more missing values in the GSE100927 and GSE43292 data sets. Then, the sample hierarchical clustering pruning graph method was used to remove outliers. The key modules related

to atherosclerosis were screened through the "WGCNA" package of R software (21).

KEGG pathway analysis

To identify common enriched signaling pathways, perform a KEGG pathway analysis on the genes in the key modules of GSE100927 and GSE43292. The case group in the GSE21545 dataset was divided into high expression and low expression groups based on C1QA expression level. Gene signaling pathway validation was performed through KEGG pathway analysis.

Identification of key genes

By the "limma" package of R software, we obtained the upregulated genes expressed in the same enriched signal pathway from GSE100927 and GSE43292 datasets, respectively. The venn diagrams were drawn from the intersection of these upregulated genes and obtained co-expressed upregulated genes. The expression heatmap of the differentially expressed genes in the two datasets was drawn using the "heatmap" package. To visualize the relative expression levels of genes, a scatterplot was created using the "ggplot" package.

Immune infiltration analysis

The level of immune infiltration in each sample was determined using the ssGSEA algorithm based on the GSE100927 and GSE43292 datasets (22). The case group of the dataset was divided into high and low expression groups based on the expression level of C1QA, and an immune cell expression box plot was generated.

Single-cell sequencing analysis

Validation of the relationship between the target gene and macrophages was performed using the single-cell RNA sequencing dataset GSE159677. Firstly, the expression region of macrophages was localized using the macrophage marker CD68. Then, the location of the C1QA expression region was compared to the macrophage expression region, and the degree of overlap between the two regions was assessed.

Cell culture

The Raw 264.7 mouse macrophage strain used by the Institute was purchased from the cell bank of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. Cells were cultured in an incubator with saturated humidity at 37°C and 5% CO₂, using RPMI1640 medium with 10% FBS and 1% dual antibody. Cell morphology and growth density were observed daily under an inverted microscope, and cells were passaged every 2-3 days (23).

Construction of macrophages stably expressing/silencing C1QA

According to the grouping of control, C1QA-minics, C1QA-minics-con, C1QA-inhibitor, and C1QA-inhibitor-con, 293T cells were transfected with corresponding plasmids. After transfection for 10 hours, the complete culture medium was updated. After 48 hours, the supernatant was collected to obtain lentivirus. Macrophages were inoculated in Petri dish until 30% of Cell fusion. Macrophages were infected with recombinant lentivirus and control lentivirus. After 12 hours, the virus liquid is discarded, fresh culture medium is added to continue the culture, and after 48 hours, puromycin is added for drug screening. The cells surviving after 48 hours of drug screening are those infected by lentivirus.

qPCR

Total RNA was extracted from cell samples using Trizol reagent (Invitrogen). cDNA was obtained using a reverse transcription kit (Thermo Fisher Scientific). Using cDNA as a template, qRT-PCR detection was performed using the ABI Prism 7500 system (Applied Biosystems, Foster City, CA, USA) and SYBR Green Supermix (Invitrogen). Calculate gene expression levels using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

The experimental data were analyzed using SPSS software, significant difference analysis was performed using the ANOVA method, *P* less than 0.05 represents a statistically significant difference (**P*<0.05, ***P*<0.01, ****P*<0.001,

**** $P < 0.0001$). Statistical plots were generated using GraphPad Prism 8 software.

Results

WGCNA Analysis Obtains Key Modules of Carotid Atherosclerosis

Gene expression matrix data for carotid artery studies were downloaded from the GEO data-

base. GSE43292 includes 32 cases in the case group and 32 cases in the control group, while GSE100927 includes 29 cases in the case group and 12 cases in the control group. Using the "WGCNA" package of R software, WGCNA analysis was performed on the two datasets to obtain the turquoise module of GSE43292 (threshold=12) and the brown module of GSE100927 (threshold=7) (Fig. 1).

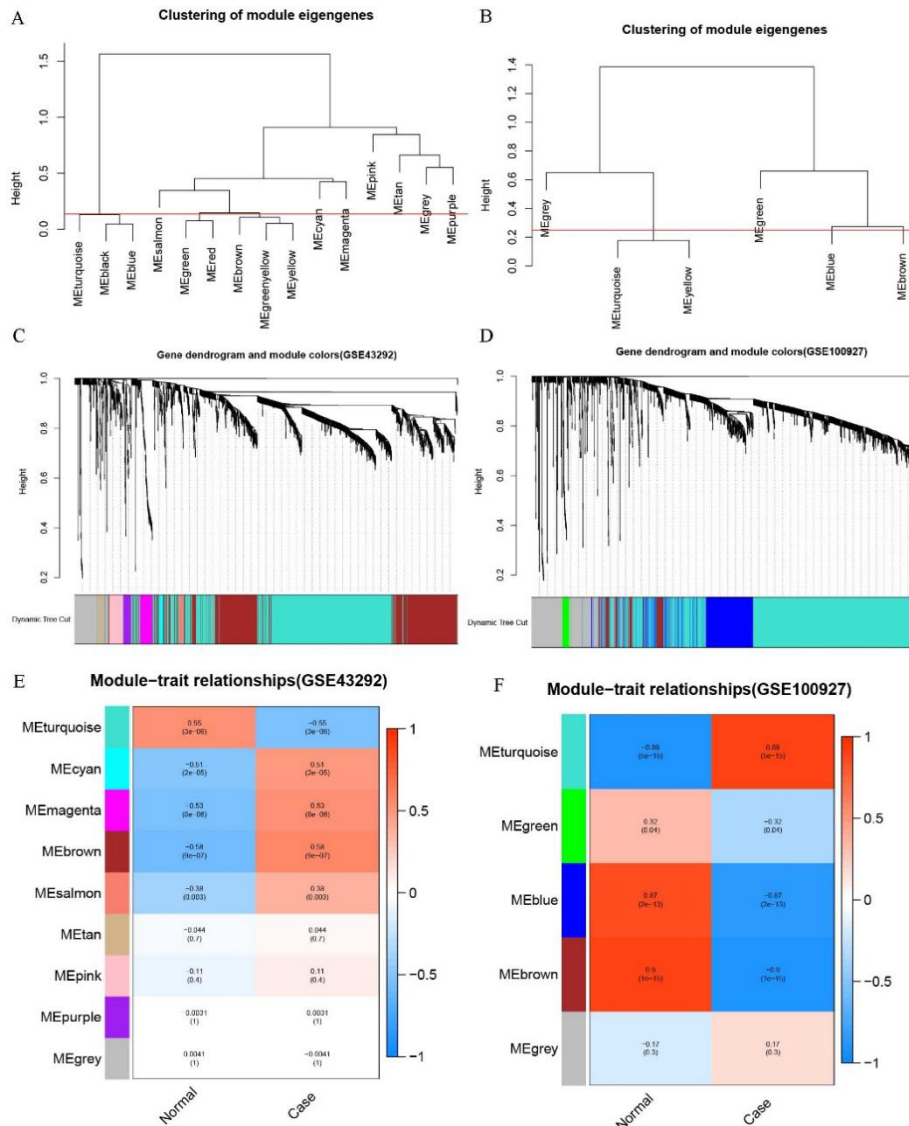


Fig. 1: WGCNA analysis

A-B: Cluster diagram of module feature genes in datasets GSE43292 and GSE100927; C-D: mRNAs clustering diagram for datasets GSE43292 and GSE100927. Different colors represent different co-expression modules; E-F: Module-trait relationship diagram for datasets GSE43292 and GSE100927. Each row represents a module intrinsic gene, and each column represents a trait. Each cell includes the corresponding correlation and P -value

Enrichment pathway screening and key gene extraction

The turquoise module of GSE43292 and the brown module of GSE100927 were subjected to KEGG pathway analysis. The results showed that the *Staphylococcus aureus* infection pathway is a common enrichment pathway of the two key modules (Fig. 2 A-B). This pathway is associated with macrophages. Differentially expressed genes of key modules were obtained in the *S. aureus* infection-signaling pathway separately. GSE43292 has 24 differentially expressed genes, while GSE100927 has 27. By creating Venn plots to

identify intersections, 18 genes related to macrophages were obtained (Fig. 2C). The heatmap of these 18 genes shows significant overexpression in the case group (Fig. 2D). Additionally, we visualized the differential expression scatter plots of these 18 genes (Fig. 2E). Based on the results presented above, we identified three genes of interest, *C1QA*, *C1QB*, and *C1QC*, which encode three subunits of the complement C1q protein, respectively. *C1QA* is considered the key gene for this study's subsequent analysis, as it has not been previously identified in research on carotid atherosclerosis.

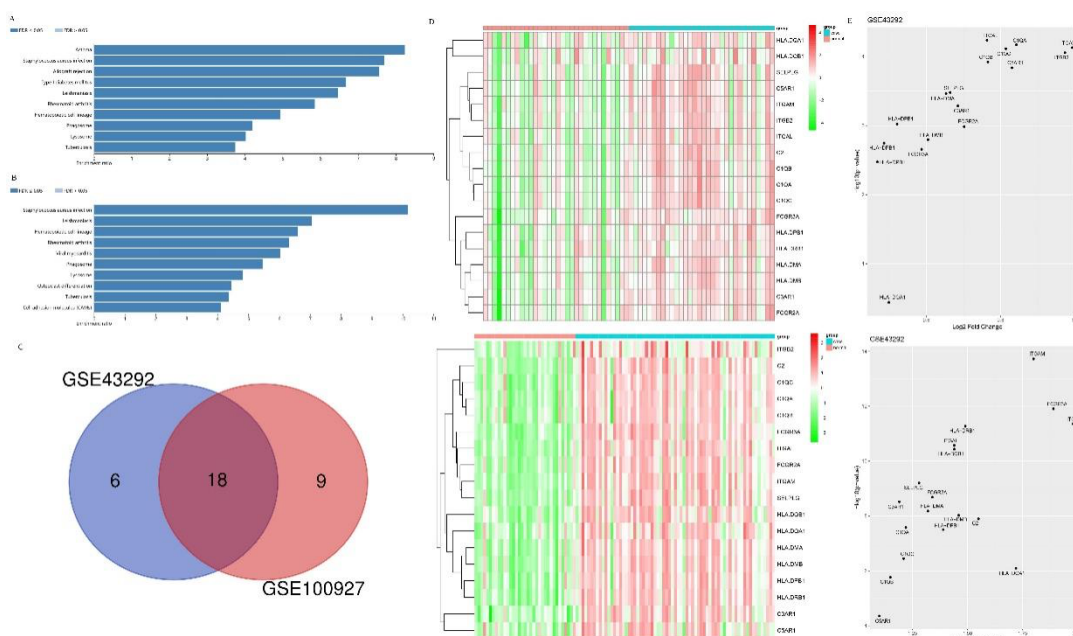


Fig. 2: Key module gene analysis

A-B: KEGG analysis of turquoise and brown modules; C: Venn diagram of differentially expressed genes in the common pathway of turquoise and brown modules; D: Differential gene expression heat map; E: Scatter plot of differential gene expression

Immunoinfiltration analysis of the relationship between *C1QA* and macrophages

We analyzed the degree of immune infiltration of each sample in the GSE43292 and GSE100927 datasets using ssGSEA analysis. The case group was divided into high and low expression groups based on the expression level of *C1QA*, and a

box plot of the total immune cell ratio was drawn. The results showed that in the carotid atherosclerosis case group, the number of macrophages was higher in the *C1QA* overexpression group than in the low expression group (Fig. 3). This suggests that the expression level of *C1QA* may be related to the number of macrophages.

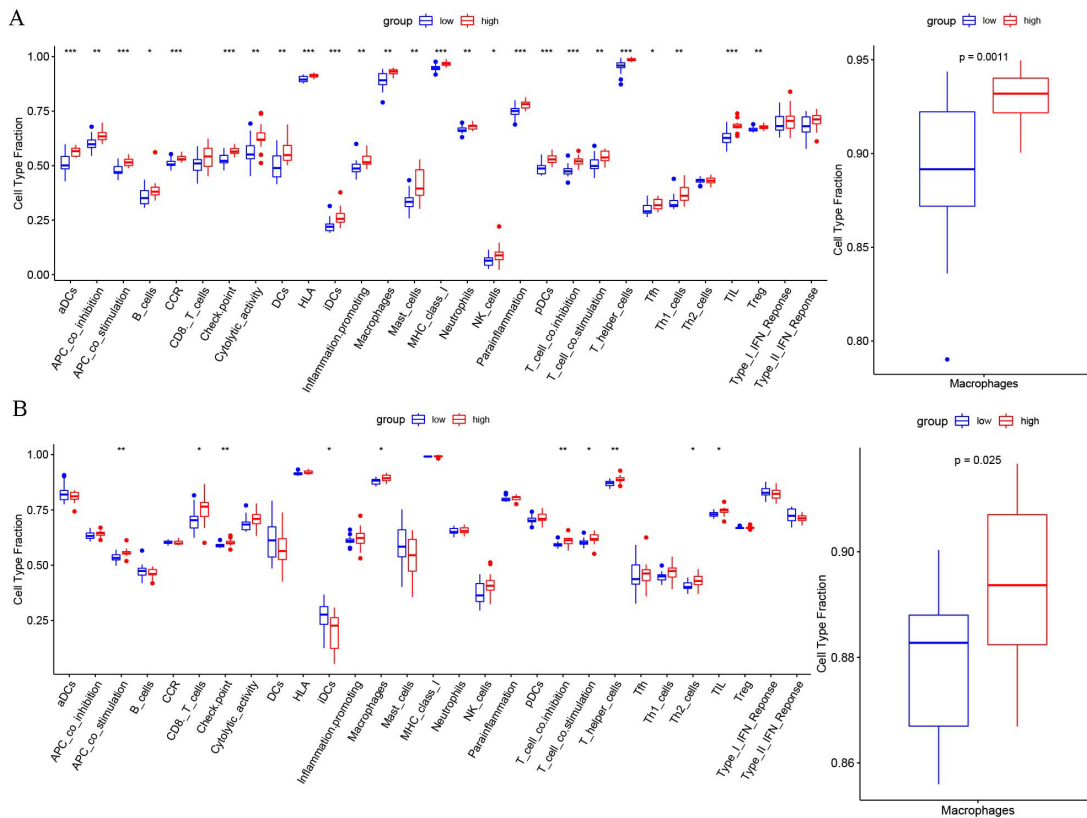


Fig. 3: Immune infiltration analysis

A: ssGSEA immune infiltration analysis of the GSE43292 dataset; B: ssGSEA Immune infiltration analysis of GSE100927 dataset

Verification of C1QA expression regions and key pathways

The *C1QA* genes related to macrophages were screened and the relationship between *C1QA* and macrophages was further verified using the single-cell sequencing dataset GSE159677. The expression and localization areas of the macrophage marker *CD68* were mainly concentrated in the core area of atherosclerosis (Fig. 4A-B). The expression and localization area of *C1QA* is highly consistent with *CD86* and is concentrated in the core area of atherosclerosis (Fig. 4C-D). *C1QA* is

a differentially upregulated gene in the *S. aureus* infection pathway. The GSE21545 dataset was downloaded, and only case group samples were used. The dataset was divided into high and low expression groups based on *C1QA* expression levels for KEGG pathway analysis. The results showed that *C1QA* was mainly enriched in the *S. aureus* infection pathway (Fig. 4E). The validation of these external data sets suggests that *C1QA* may play a crucial role in the regulation of macrophage functional activity during the development of carotid atherosclerosis.

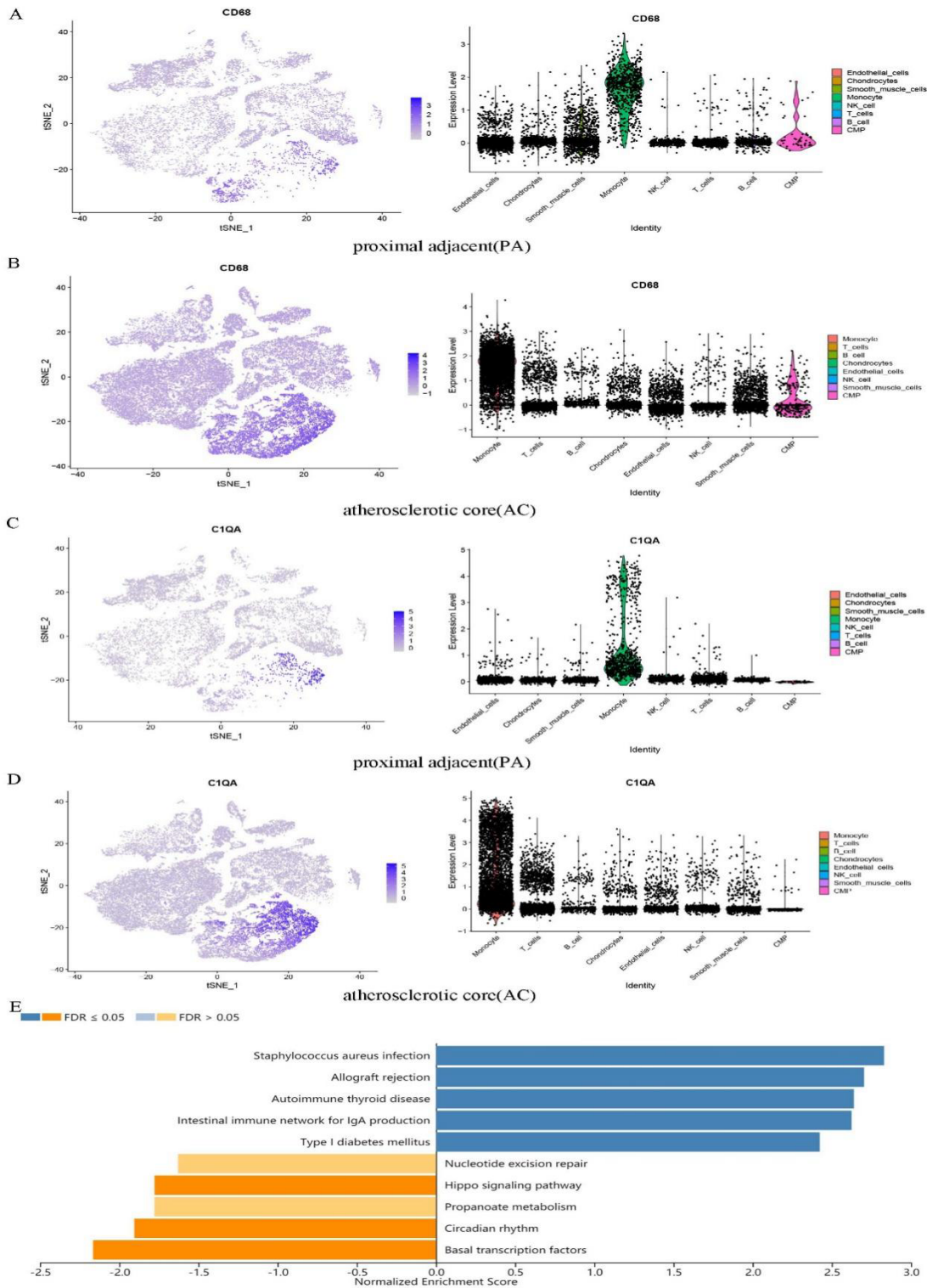


Fig. 4: External Dataset Validation *C1QA* Function

A-B: Single-cell sequencing dataset GSE159677 verifies the *CD68* expression region; C-D: Single-cell sequencing dataset GSE159677 verifies the *C1QA* expression region; E: KEGG pathway analysis of GSE21545 dataset

C1QA affects macrophage activity

The above bioinformatics analysis results show that *C1QA* is highly expressed in the carotid artery case group and in the macrophages of the case group. However, it is unclear which type of macrophage is highly expressed. M1 macrophages secrete proinflammatory factors that may promote the development of atherosclerosis, whereas M2 macrophages secrete anti-inflammatory factors that may inhibit atherosclerosis. Based on this evidence, we hypothesized that *C1QA* may contribute to the development of carotid atherosclerosis by promoting the polarization of M1 macrophage. We confirmed this hypothesis through experimental means by con-

structing macrophages that stably expressed (*C1QA*-minics) and silenced (*C1QA*-inhibitor). qPCR analysis was used to detect the expression of M1 polarization-related genes *IL-1 β* and *TNF- α* in macrophages. The results showed that the expression level of *IL-1 β* and *TNF- α* was significantly increased in *C1QA*-minics group compared to the control group. However, the expression level of *IL-1 β* and *TNF- α* in *C1QA*-inhibitor group was significantly decreased (Fig. 5). This indicates that the high expression of *C1QA* will increase the expression of M1 macrophage polarization-related genes, promote M1 macrophage polarization, and thus affect the progression of carotid atherosclerosis.

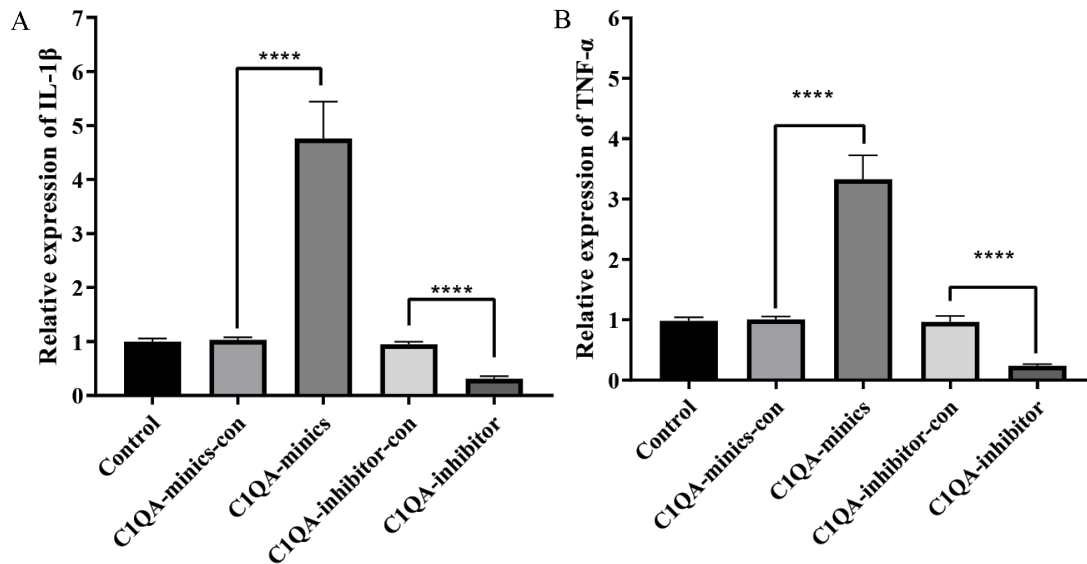


Fig. 5: Expression Analysis of *C1QA* and M1 Polarization Related Genes

Discussion

Carotid atherosclerosis is an important pathological process of cardiovascular disease, which is characterized by lipid accumulation and inflammation (24). Some studies have shown that atherosclerosis plaques are composed of smooth muscle cells, endothelial cells, fibroblasts, T cells and macrophages (25-27). Among them, changes in the function and activity of macrophages play a certain role in the development of atherosclerosis,

but the key genes involved are still unclear. After being stimulated by different signals, static macrophages M0 will polarize into two different states: M1 and M2. M1 macrophages can be activated by Toll-like receptors or NF-Kappa B pathway activation, secreting *TNF- α* , *IL-1 β* , *IL-6* and other pro-inflammatory cytokines (28). M2 macrophages secrete the anti-inflammatory cytokines *IL-10* and *TNF- β* (4). To date, targeting *CCL2*, *TNF- α* and *IL-6*, which are pro- or anti-inflammatory cytokines, has been shown to slow the progression of atherosclerosis in animal mod-

els and may improve the cardiovascular outcomes in humans in large phase III trials (29). For example, targeting *IL-1 β* with the monoclonal antibody canakinumab has been shown to reduce the risk of adverse cardiovascular events (30). These studies indicate that the research on cytokines secreted by macrophages will be another direction for the treatment of carotid atherosclerosis.

WGCNA is an important tool for identifying the correlation between key modules and phenotypes, and is widely used in the cardiovascular field (31). Some studies have used WGCNA to identify key genes in the progression of atherosclerosis. For example, Wang et al. analyzed the GSE40231 dataset through WGCNA and determined the lncRNA that plays an important role in atherosclerosis (32). Siliang et al. screened the key genes of carotid atherosclerosis in peripheral blood monocyte samples by WGCNA and DEG (33). In this study, two key modules of the CAS dataset GSE100927 and GSE43292 were analyzed by WGCNA. KEGG analysis of the key module genes revealed that they are enriched in the macrophage associated *S. aureus* infection pathway. Among these genes, *C1QA* is the key gene in this study.

C1q is the complement system recognition protein. *C1QA*, *C1QB* and *C1QC* encode the A, B and C-chain polypeptides of C1q, respectively. Some studies have shown that the removal of apoptotic cells by C1q can prevent the development of the inflammatory disease atherosclerosis (34). Based on early and late human atherosclerosis samples, Bin et al. found through comprehensive bioinformatics analysis that *C1QB* is related to CD8 of T cells in late atherosclerosis plaque samples, and *C1QC* is related to immune cells in early atherosclerosis samples (35). Our immune infiltration analysis shows that the proportion of macrophages is higher in the CAS case group, and the number of macrophages in the *C1QA* high-expression group is higher than that in the low-expression group. These results indicate that the number of macrophages is a key factor affecting the CAS process, and *C1QA* is a key gene regulating the number of macrophages. In addition, we confirmed this result through external

validation datasets GSE159677 and GSE21545. In addition, we tested the expression levels of *TNF- α* and *IL-1 β* in macrophages after changing the expression level of *C1QA*, and the results showed that when *C1QA* expression was inhibited, the expression levels of *TNF- α* and *IL-1 β* were decreased, and vice versa. Because *TNF- α* and *IL-1 β* were the inflammatory factor secreted by M1 macrophages, which also indicates that *C1QA* is related to the polarization of M1 macrophages in atherosclerosis samples.

Overall, this study identified the key gene *C1QA* of CAS through bioinformatics analysis. *C1QA* is a highly expressed gene in the CAS case group, and the samples with high expression of this gene have a higher number of macrophages. Cell experiments showed that *C1QA* could affect the expression of M1 macrophage polarization-related genes *TNF- α* and *IL-1 β* . These results suggest that *C1QA* may promote the progression of carotid atherosclerosis by promoting M1 macrophage polarization.

Conclusion

C1QA is a highly expressed gene in patients with carotid atherosclerosis, and the high expression of this gene is associated with the number of macrophages in the progression of atherosclerosis. At the same time, we confirmed that interference with *C1QA* expression could inhibit M1 macrophage polarization during the progression of carotid atherosclerosis. These results add to the body of research on the role of macrophages in the progression of atherosclerosis, but there are still limitations. For example, the number of data sets and validation sets used for analysis may not be comprehensive enough. The effect of *C1QA* on macrophage activity has only been validated using in vitro cell experiments, and there is a lack of data from clinical samples.

Journalism Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or fal-

sification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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