International Journal of General Medicine

Dovepress

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ORIGINAL RESEARCH

Identification of Biomarkers of Autophagy-Related Genes Between Early and Advanced Carotid Atherosclerosis

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Background: Accumulating evidence demonstrates that autophagy is important in inhibiting inflammation and cholesterol efflux. It suggested the autophagy may be a treatment of atherosclerosis. Thus, we screened autophagy-related mRNA to explore their mechanism of scientific basis for early diagnosis and therapy of atherosclerosis.

Methods: The GSE28829 datasets were assessed to analyze differentially expressed genes by GEO2R. And autophagy-related hub genes were identified by HADb. The biological function of autophagy-related DEmRNAs was examined by Metascape. The construction of a protein–protein network was explored by String. Cytohubba was utilized to screen hub genes. Analysis of DEmiRNA-mRNA pairs was executed by DIANA microT-CDS database. Finally, correlation analysis was carried out to identify the relationship between DEARGs and clinical and prognostic factors.

Results: A number of 1087 DEGs and 19 autophagy-related DEmRNAs were identified in advanced carotid atherosclerotic plaque compared with the early. The biological function containing development and growth was enriched. Moreover, we screened the top hub nodes with the highest degrees. MicroRNAs (miRNAs) are confirmed to participate in genesis and progression of atherosclerosis, so we further analyzed the miRNA–mRNA regulatory network genes with four hub genes to explore their potential mechanism in atherosclerosis. Then, we revealed co-expression of four key genes *CTSB*, *ITGB1*, *CXCR4*, *TNFSF10* and autophagy-related genes. As for the clinical factors, hypertension factor showed higher expression of *ITGB1*. The probability of coronary heart disease factor was significantly increased with high expression of *CTSB* and *CXCR4*, as well as low expression of *ITGB1* and *TNFSF10*. Diabetes factor tended to express distinguished levels of *CTSB* and *ITGB1*. *TNFSF10* was highly expressed in both hyperlipidemia and ischemic stroke factor.

Conclusion: *CTSB*, *ITGB1*, *CXCR4* and *TNFSF10* may be critical in atherosclerosis development and were thought to be potential diagnostic biomarkers for atherosclerosis.

Keywords: atherosclerosis, autophagy, bioinformatics analysis, genes, miRNA

Introduction

Atherosclerosis is the main pathophysiological process responsible for atherosclerotic cardiovascular disease (ASCVD), such as coronary, cerebrovascular, and peripheral arterial diseases.[1] Atherosclerosis is a systemic disease, and the carotid atherosclerosis may reflect atherosclerosis elsewhere in the body, including coronary artery, cerebral artery, renal artery and so on.[2] Vulnerable atherosclerotic plaques are principally responsible for thromboembolic events in various arterial territories such as carotid, coronary, and lower limb vessels.[3] Nowadays, it is widely accepted that the rupture of the atherosclerotic plaque in coronary and carotid arteries plays a fundamental role in the development of acute myocardial infarctions or cerebrovascular events.[4] Carotid intima-media thickening and fatty streak represent an earlier stage of the disease, while carotid atherosclerotic plaque represents an advanced stage of the disease. Moreover, the advanced carotid atherosclerotic plaque is more likely to rupture, leading to adverse events such as Ischemic stroke.[5–8] Carotid atherosclerosis is a complex and time-dependent process. While atherosclerosis develops early in life, the clinical manifestations of atherosclerosis usually do not occur until

Received: 14 December 2021
Accepted: 4 April 2022
Published: 31 May 2022

middle age.[9] New diagnostic methods, which can targetedly identify advanced carotid plaques in vivo, are therefore needed for a more precise targeting of prophylactic treatment of ASCVD.[10]

Recently, more attention has been paid on the role of autophagy in atherosclerosis. Some researchers believed that autophagy is a protective mechanism to promote cell survival rather than cell death, suggesting that autophagy induced in smooth muscle cells (SMCs) of advanced plaque is potentially an important mechanism to maintaining plaque stability. Autophagy protects vascular cells in plaques against oxidative stress and apoptosis by degrading damaged organelles.[11] Fucoidan, a marine sulfated polysaccharide derived from brown seaweeds, can inhibit inflammasome activation by enhancing autophagy to alleviate carotid atherosclerosis.[12] Inhibition of macrophage apoptosis and necrotic core formation by autophagy-mediated reduction of oxidative stress is one mechanism of the suppression of carotid plaque progression and destabilization by K-80003 (a non-canonical RXRα modulator).[13,14] While hsa_circ_0030042 upon overexpression, it inhibits abnormal autophagy of coronary arteries and human umbilical vein endothelial cells (HUVECs) and maintain plaque stability in vivo.[15] Beclin 1 localized in major intracranial artery plaques is an essential autophagic protein, and the Beclin-1-interacting complex promotes the formation of autophagosomes and exerts protective roles in atherosclerosis.[16] DR-NPs (DEX and RAPA-coloaded mPEG2k-DSPE calcium phosphate nanoparticles) efficiently aggregated at atherosclerosis plaques in the abdominal artery in mice and exhibited excellent plaque regression ability by inducing autophagy, with smaller necrotic cores and lipid core areas observed after in vivo treatment.[17] Mechanistically, miR-100 indirectly stimulated endothelial autophagy in vitro and in vivo, and attenuated aortic atherogenesis, resulting in a decrease of plaque area by 45%.[18] The occurrence process of carotid atherosclerosis includes four pathological stages: intimal thickening, intimal xanthoma, thin fiber cap atheroma and thick fiber cap atheroma. The first two stages represent the early stage and the last two stages represent the late stage.[19] Many clinical complications and risk factors are also closely related to carotid plaque. Studies have shown that whether symptomatic or asymptomatic, subjects with advanced carotid atherosclerosis have a higher risk of coronary heart disease.[20,21] In addition, the recurrence of ischemic stroke is related to advanced carotid atherosclerosis.[22] Thromboembolism of carotid atherosclerotic plaque is the most common mechanism leading to ischemic stroke.[23] In addition, old age, male, hypertension, diabetes, frequent smoking, long-term smoking and lack of exercise are all risk factors of carotid atherosclerosis. Hypertension (69.1%), dyslipidemia (26.0%) and diabetes (16.1%) are highly prevalent among carotid atherosclerosis participants.[24] However, there are few studies about autophagy-related biomarkers on the differences between advanced and early carotid atherosclerosis. Thus, we screened key autophagy-related DEmRNAs and analyze target functions and related signal pathways to explore their mechanism of action and provide important theoretical references and scientific basis for early diagnosis and targeted therapy of atherosclerosis.

In this study, a protein–protein interaction (PPI) network of differentially expressed autophagy-related genes (DEARGs) was constructed, and hub genes were revealed. To determine the functions of DEARGs in carotid atherosclerosis, we constructed a atherosclerosis-related mRNA/miRNA network interactions. Finally, correlation analysis was carried out to identify the relationship between differentially expressed autophagy-related genes (DEARGs) and clinical and prognostic factors to identify the underlying regulatory mechanisms in advanced carotid atherosclerosis.

Materials and Methods

Identification of DEGs

In the present study, we sifted datasets from the publicly available Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/). We are committed to studying the difference between early and late atherosclerosis, so we searched the GEO database, entering search term "early and advanced atherosclerotic plaque". By selecting "series", a total of four series were screened out. The dataset GSE28829 contained 16 advanced carotid atherosclerosis *Homo sapiens* specimens and 13 early carotid atherosclerosis samples. The Platform was GPL570 [HG-U133_Plus_2]-Affymetrix Human Genome U133 Plus 2.0 Array. Then, in order to analyze the miRNA-mRNA regulatory network, we added the dataset GSE34647 (GPL15053-Applied Biosystems TaqMan Array Rodent MicroRNA Cards v2.0) which contained three advanced carotid atherosclerosis Musculus specimens and four early carotid atherosclerosis samples. MiRNAs are widely present in a variety of eukaryotes, from lower organisms

to humans, with traces of their existence. Its biological characteristics are mainly as follows: high conservation, temporal expression specificity and tissue expression specificity. Its high degree of conservation indicates that various miRNAs can find homologues in their germlines. The dataset GSE34647 was to explore stage-specific microRNA signatures in the progress of atherosclerosis in hyperlipidemia mouse model, which may help to identify the critical miRNAs contributing atherosclerotic development and stabilization. As for data processing, the online analysis tool GEO2R (https://www.ncbi.nlm.nih.gov/geo/geo2r/) from NCBI's GEO online analysis tool was used to obtain differential mRNAs and screen the differential genes. GEO2R (https://www.ncbi.nlm.nih.gov/geo/info/geo2r.html) is an interactive web tool that allows users to compare two or more groups of Samples in a GEO Series in order to identify genes that are differentially expressed across experimental conditions. Results are presented as a table of genes ordered by significance, and as a collection of graphic plots to help visualize differentially expressed genes and assess data set quality. And GEO2R was used for screening DEGs out between advanced and early carotid atherosclerosis samples in GSE28829 with $|logFC|>0.5$ and $adj.p<0.05$ and GSE34647 with $|logFC|>4$ and $p<0.05$ for top 65 miRNAs.

Identification of Differentially Expressed Autophagy-Related Genes (DEARGs)

We extracted 222 human autophagy-related genes from the HADb. Then, we obtained the DEARGs by intersecting the 222 ARGs with DEmRNAs identified in the GSE28829 dataset and we got 19 autophagy-related DEmRNAs for the further study.

Functional Annotation and Pathway Enrichment of DEGs

To obtain the underlying biological function and signaling pathways of DEGs, the Metascape database was used for Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis.[25] Only those terms or pathways with p-value <0.05 were selected as substantially enriched. KEGG pathway enrichment analysis for these DEARGs was carried out to reveal the autophagy gene-associated pathways.

Protein–Protein Interaction Network Construction

Search Tool for the Retrieval of Interacting Genes (STRING) database[26] was used to retrieve the predicted interactions between proteins encoded by all screened DEGs and other proteins. STRING (https://string-db.org) aims to collect, score, and integrate all publicly available sources of protein–protein interaction (PPI) data. In addition to experimental data, results of text from PubMed abstracts and synthesis of data from other databases, it also contains the potential functions predicted results using bioinformatics methods. We conducted a PPI network analysis of DEARGs to explore the interactions among them and their neighbor genes with STRING. The PPI network basic Settings was constructed with active interaction sources from text mining, experiments, databases, co-expression, neighborhood, gene Fusion, co-occurrence and the minimum required interaction score no less than 0.7. Then, the PPI network was visualized by the Cytoscape Software (version 3.7.1). Subsequently, the plugin cytohubba[27] of the Cytoscape software was used to select important hub genes among these genes. The hub genes were selected with the criteria of top 2 scores according to eccentricity, which was one of cytohubba ranking algorithms.

Hub Genes Regulated by miRNA

Herein, the miRNA-target prediction tool DIANA microT-CDS (http://www.microrna.gr/microT-CDS, v5.0) was implemented to forecast the target genes of carotid atherosclerosis-related miRNAs with threshold set to 0.4. DIANA-microT-CDS is the latest version of DIANA-microT, which was one of the first miRNA target prediction systems to predict targets.[28] MiRNAs are widely present in a variety of eukaryotes, from lower organisms to humans, with traces of their existence. Its biological characteristics are mainly as follows: high conservation, temporal expression specificity and tissue expression specificity. Its high degree of conservation indicates that various miRNAs can find homologues in their germlines. The dataset GSE34647 was to explore stage-specific microRNA signatures in the progress of atherosclerosis in hyperlipidemia mouse model, which may help to identify the critical miRNAs contributing atherosclerotic development and stabilization. Only the target genes overlapped with hub genes were chosen for further validation.

Simultaneously, forecasting target genes predicted by different miRNAs were regarded as key genes. Then, these carotid atherosclerosis-related miRNAs and four key genes interaction network was constructed and visualized by Cytoscape 3.7.1. These four genes *CTSB*, *ITGB1*, *CXCR4*, *TNFSF10* all have mouse orthologs, Gene ID: 13030 Ctsb cathepsin B [Mus musculus (house mouse)]; Gene ID: 16412 Itgb1 integrin beta 1 (fibronectin receptor beta) [Mus musculus (house mouse)]; Gene ID: 12767 Cxcr4 chemokine (C-X-C motif) receptor 4 [Mus musculus (house mouse)]; Gene ID: 22035 Tnfsf10 tumor necrosis factor (ligand) superfamily, member 10 [Mus musculus (house mouse)].

Correlation Analysis of the ARGs and Clinical Factors in Advanced Carotid Atherosclerosis

Ultimately, we revealed co-expression of four key genes *CTSB*, *ITGB1*, *CXCR4*, *TNFSF10* and autophagy-related genes. The expression correlation of two genes was analysed with Spearman. The abscissa represents the expression distribution of the first gene, and the ordinate represents the expression distribution of the second gene. The density curve on the right represents the trend in distribution of the second gene, the upper density curve represents the trend in distribution of first gene expression. The value on the top represents the correlation *p* value, correlation coefficient and correlation calculation method. Moreover, the association between four key genes expression and clinical factors including coronary heart disease (GSE40231), ischemic stroke (GSE22255 and GSE16561) and hypertension-diabetes-hyperlipidemia (GSE90074) was evaluated with univariate logistic regression to screen independent predictive indicators. All statistical analysis was conducted and processed using the SPSS statistical software version 17.0. All covariates were set as categorical data. Statistical significance was identified at $P<0.05$, as well as $\alpha=0.05$ was taken as the significant level.

Dataset GSE40231 contained 40 coronary heart disease samples and 238 normal samples; dataset GSE22255 contained 20 ischemic stroke samples and 20 control samples; dataset GSE16561 contained 39 ischemic stroke samples and 24 control samples. At last, dataset GSE90074 contained 143 samples, which included 126 hypertension samples, 55 diabetes samples and 102 hyperlipidemia samples. Both of dataset GSE40231 and GSE22255 were acquired on the platform Affymetrix Human Genome U133 Plus 2.0 Array. In datasets GSE16561 and GSE90074, samples were acquired on the platform Illumina HumanRef-8 v3.0 expression beadchip and Agilent-014850 Whole Human Genome Microarray 4x44K G4112F (Probe Name version), respectively.

The study of GSE16561 received approval for human subject research from the institutional review boards of the National Institute of Neurological Disorders and Stroke and National Institute on Aging at NIH and Suburban Hospital, Bethesda, MD. Written informed consent was obtained from all subjects or their authorized representatives prior to performing any study procedures. The study of GSE22255 was approved by the ethics committees of the participating institutions. All participants were informed of the study and provided informed consent. The study of GSE90074 was approved by The University of North Carolina Institutional Review Board (number 05–1798) and conducted according to institutional guidelines; all participants provided written informed consent. The studies of GSE40231 were approved by the Ethics Committee of Karolinska University Hospital. All patients gave written informed consent.

Statistical Analysis

All statistical analyses conducted and processed were analyzed using SPSS 17.0 software (IBM Corp., USA). We analyzed the four hub genes mRNA expression between groups using Wilcoxon tests. All covariates were set as categorical data. Statistical significance was identified at $P<0.05$, as well as $\alpha=0.05$ was taken as the significant level.

Results

The Flowchart of the Analysis Process

The bioinformatics analysis flowchart was carried out as shown in Figure 1. Taking advantage of the microarray dataset GSE28829, we calculated DEmRNAs with |logFC| >0.5 and adj. *P*-value <0.05. Consequently, a number of 1807 DEGs were identified using GEO2R. Secondly, we identified 19 autophagy-related hub genes from PPI network and constructed the autophagy-related mRNA/miRNA regulatory network. Next, we conducted co-expression analysis of four key genes

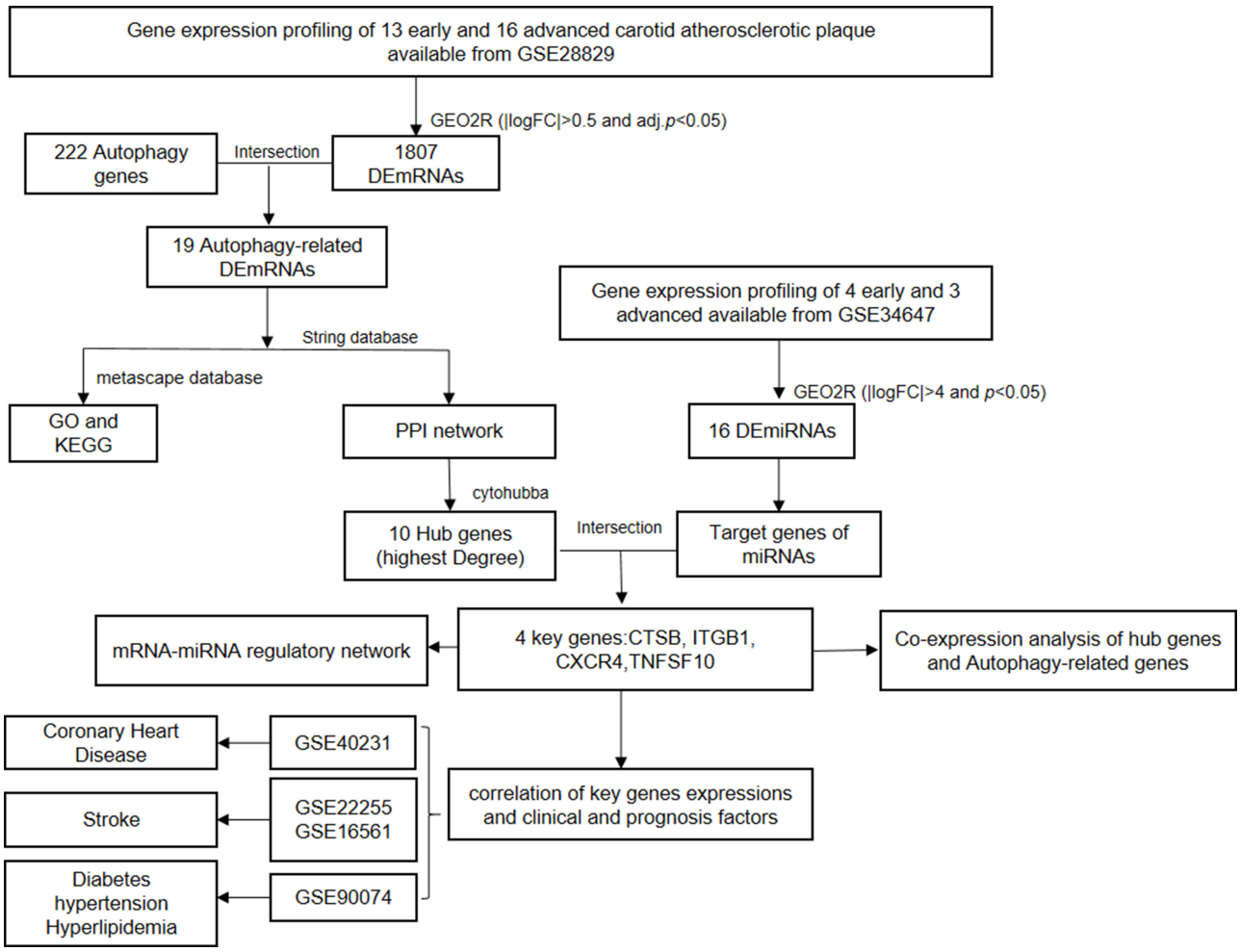

Figure 1 The flowchart of the analysis process.

and autophagy-related genes. Finally, correlation of key genes expressions and clinical and prognostic factors was conducted to explore the clinical significance.

To conclude, we identified that four DEARGs (*CTSB*, *ITGB1*, *CXCR4*, *TNFSF10*) may be critical in atherosclerosis development and provide potential predictive markers and therapeutic targets for determining a treatment strategy for advanced carotid atherosclerosis.

Identification of DEARGs in Advanced Carotid Atherosclerosis

We identified 1807 mRNAs in 13 early and 16 advanced carotid atherosclerotic plaque available from GSE28829 for significantly differential expression (Figure 2A and B). Simultaneously, 222 autophagic genes were obtained from HADb database. Then, the 222 autophagic genes were intersected with the 1807 DEmRNAs identified in the GSE28829 dataset. The results showed that 19 DEARGs were selected for further analysis (|logFC| >0.5 and adj. *P*-value <0.05; Figure 2D). Besides, gene expression of 1807 DEARGs was shown through Volcano in Figure 2C.

Functional Annotation and Pathway Enrichment of DEARGs

To explore the possibility of biological function of these 19 DEARGs, we performed GO and pathway enrichment analysis. A *P* value of <0.05 was recognized as significant. The top 10 enriched GO terms ontology were demonstrated as shown in Figure 3A and B. GO analysis results showed that DEARGs were significantly enriched in developmental

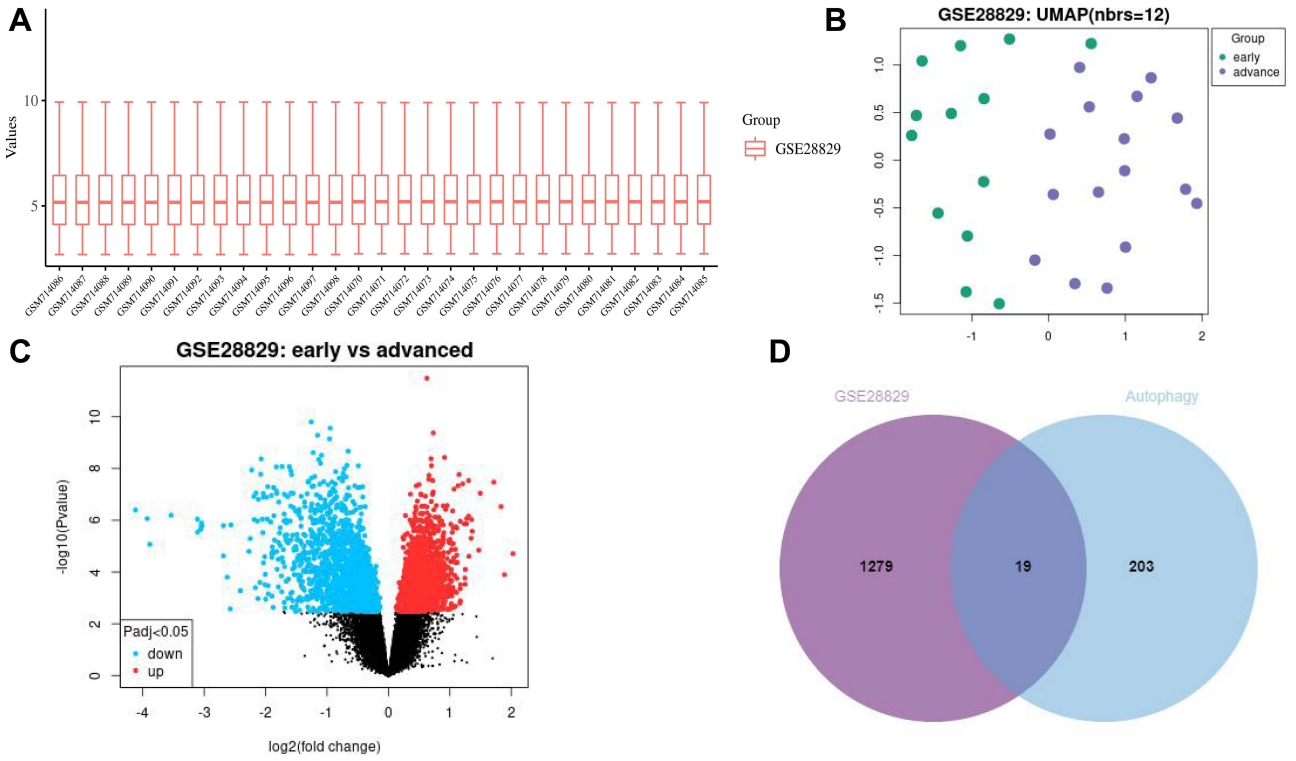

Figure 2 Identification of DEARGs in advanced carotid atherosclerosis. (**A**) The boxplot was used to view the distribution of values of the selected samples of GSE28829. (**B**) Uniform Manifold Approximation and Projection (UMAP) is a dimension reduction technique useful for visualizing how Samples are related to each other. UMAP was performed on all carotid atherosclerotic plaque samples. (**C**) The volcano of DEGs in GSE28829. (**D**) Venn diagram was used to explore and download the overlap in the significant genes between multiple contrasts. The results showed that 19 DEARGs were selected.

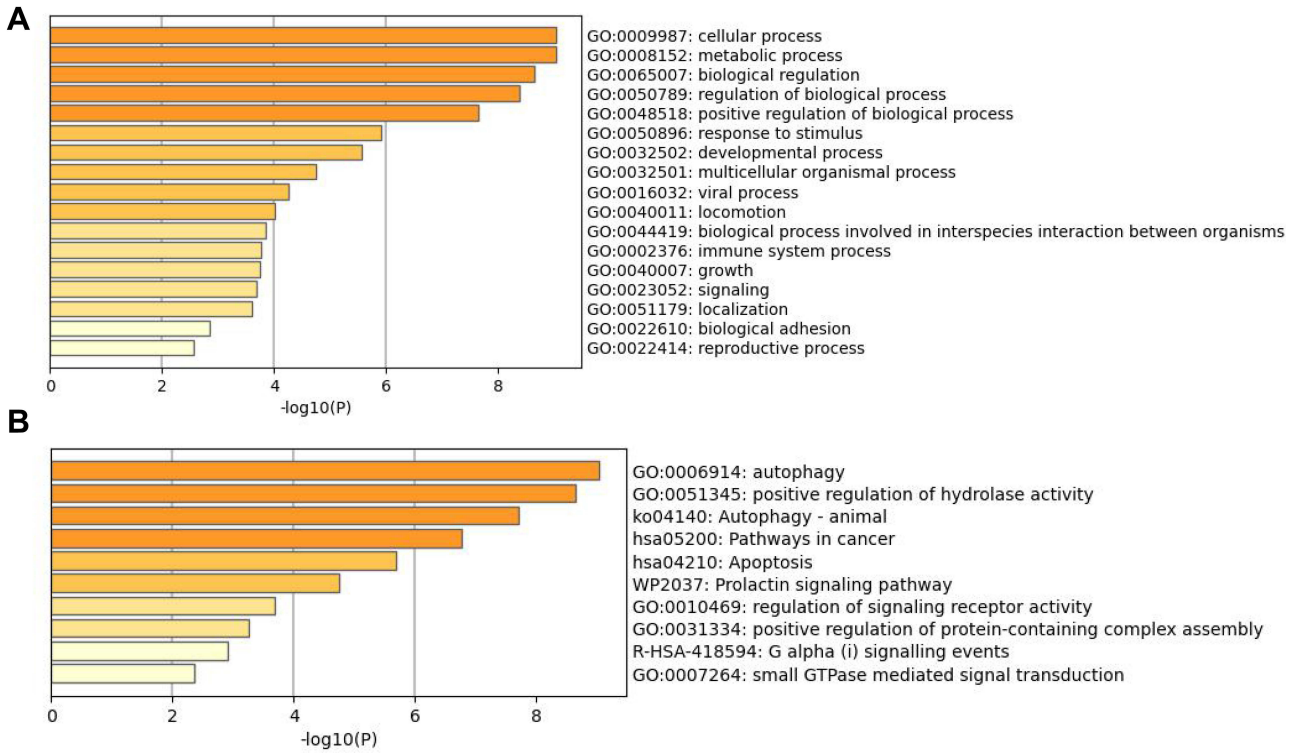

Figure 3 Functional annotation and pathway enrichment of DEARGs. (**A** and **B**) Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment.

process, growth and reproductive process (Figure 3A). It can be observed that the majority of enriched KEGG functional categories, including pathway in cancer, prolactin signaling pathway and apoptosis (Figure 3B).

PPI Network Construction and Hub Genes Selection

To acquire the communication among these 19 DEARGs which helps identify the key modules and hub genes in advanced carotid atherosclerosis encoded proteins in GSE28829 and additional proteins, PPI network was investigated and visualized by Cytoscape (Figure 4A). Moreover, we screened the top ten hub nodes with highest degrees from the PPI network. The 10 hub nodes, including CTSB, ITGB1, CXCR4, TNFSF10, BID, CTSD, SERPINA1, CASP1, VEGFA and ERBB2, were considered as hub genes concerning atherosclerosis genesis and progression (Figure 4B).

Search for miRNAs and Construction of a mRNA/miRNA Regulatory Network in Carotid Atherosclerosis

To screen the carotid atherosclerosis progression of related miRNAs, the miRNA-disease associations was derived from GSE34647. As a consequence, for the carotid atherosclerosis associated miRNAs, expression of 16 DEmiRNA target genes was observed (Figure 5A). To further understand the regulatory relationship between hub genes and disease-related DEmiRNAs, known targets of the above 16 DEmiRNAs were utilized using DIANA microT-CDS database. The target gene number and hub genes regulated by these 16 DEmiRNAs were indicated. Subsequently, only four target hub genes predicted by DEmiRNAs were regarded as key genes, including *CTSB*, *ITGB1*, *CXCR4* and *TNFSF10*. Additionally, the regulatory network of key miRNA-target interaction was presented in Figure 5B.

Analysis of the Expression Level of Four Key Genes and Correlation Analysis of DEARGs and Clinical Factors

Furthermore, we analyzed the expression of the above four potentially significant DEARGs in the data set GSE28829 and we found that the expression levels of *CTSB*, *CXCR4* and *TNFSF10* were up-regulated in the advanced carotid atherosclerosis patients. ($P < 0.001$) (Figure 6A–D), while *ITGB1* was down-regulated. The four genes may be potentially suitable therapeutic targets for advanced carotid atherosclerosis patients. To determine the relationship

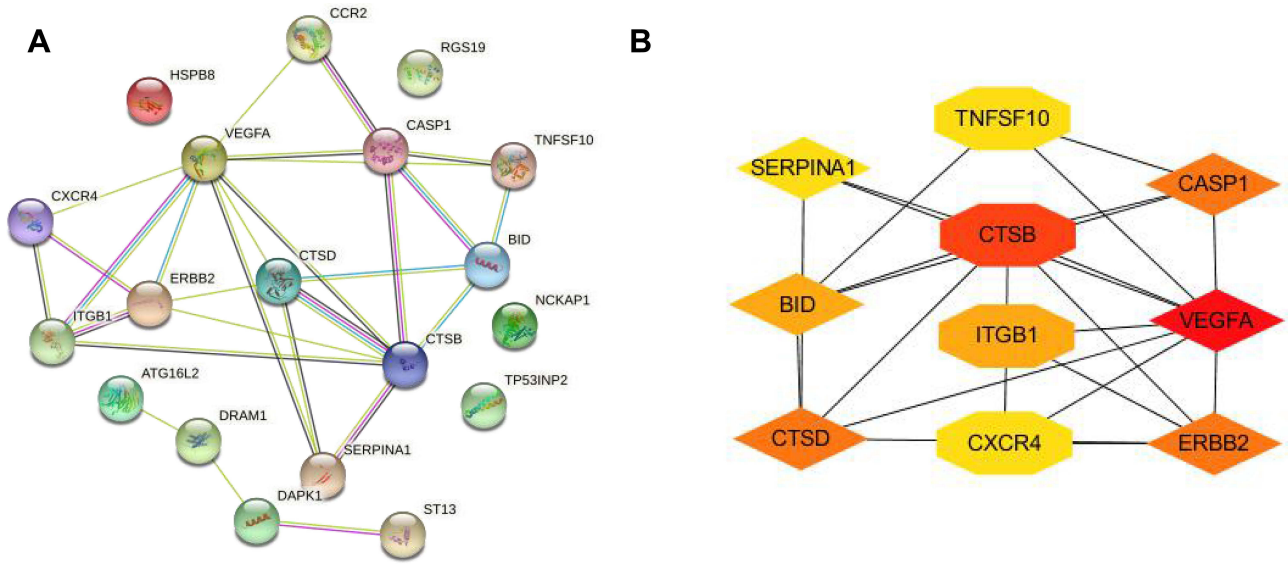

Figure 4 PPI network construction and hub genes selection. (**A**) The PPI network constructed using STRING database for DEARGs. (**B**) The most significant genes obtained from PPI network with cytohubba.

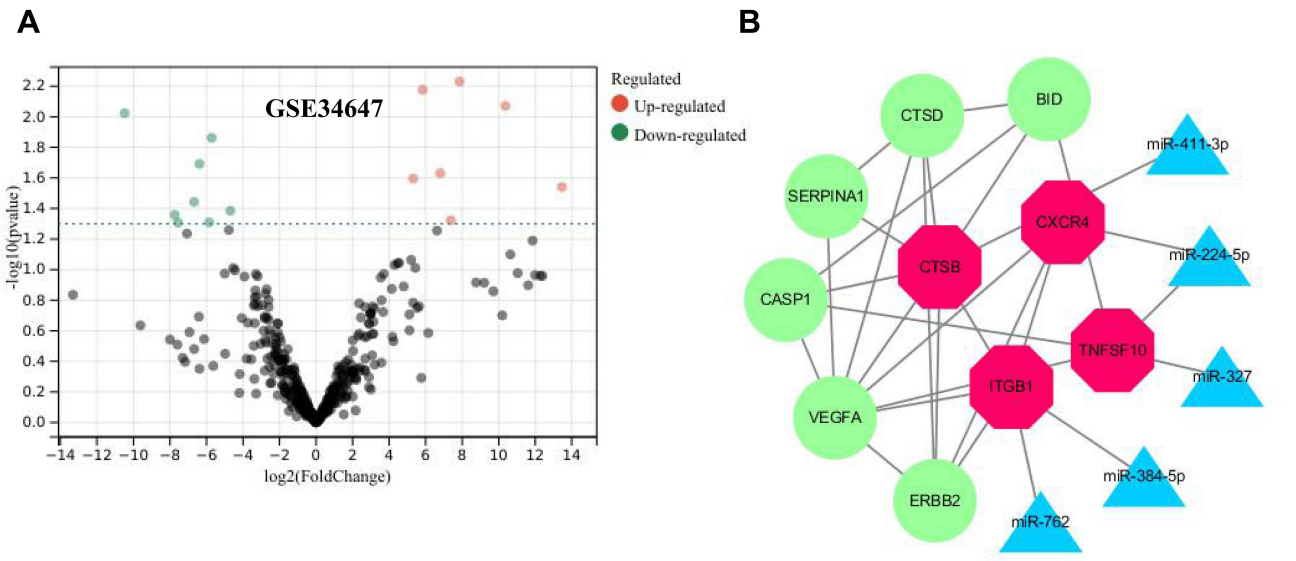

Figure 5 Search for miRNAs and Construction of a mRNA/miRNA regulatory network in carotid atherosclerosis. (**A**) The volcano of DEGs in GSE34647. (**B**) The mRNA–miRNA network of top 10 hub genes. (The size of each node was proportional to |log (Fold Change)| value. The red color indicated top hub genes and the green color indicated hub genes. The blue color indicated DE-miRNAs.

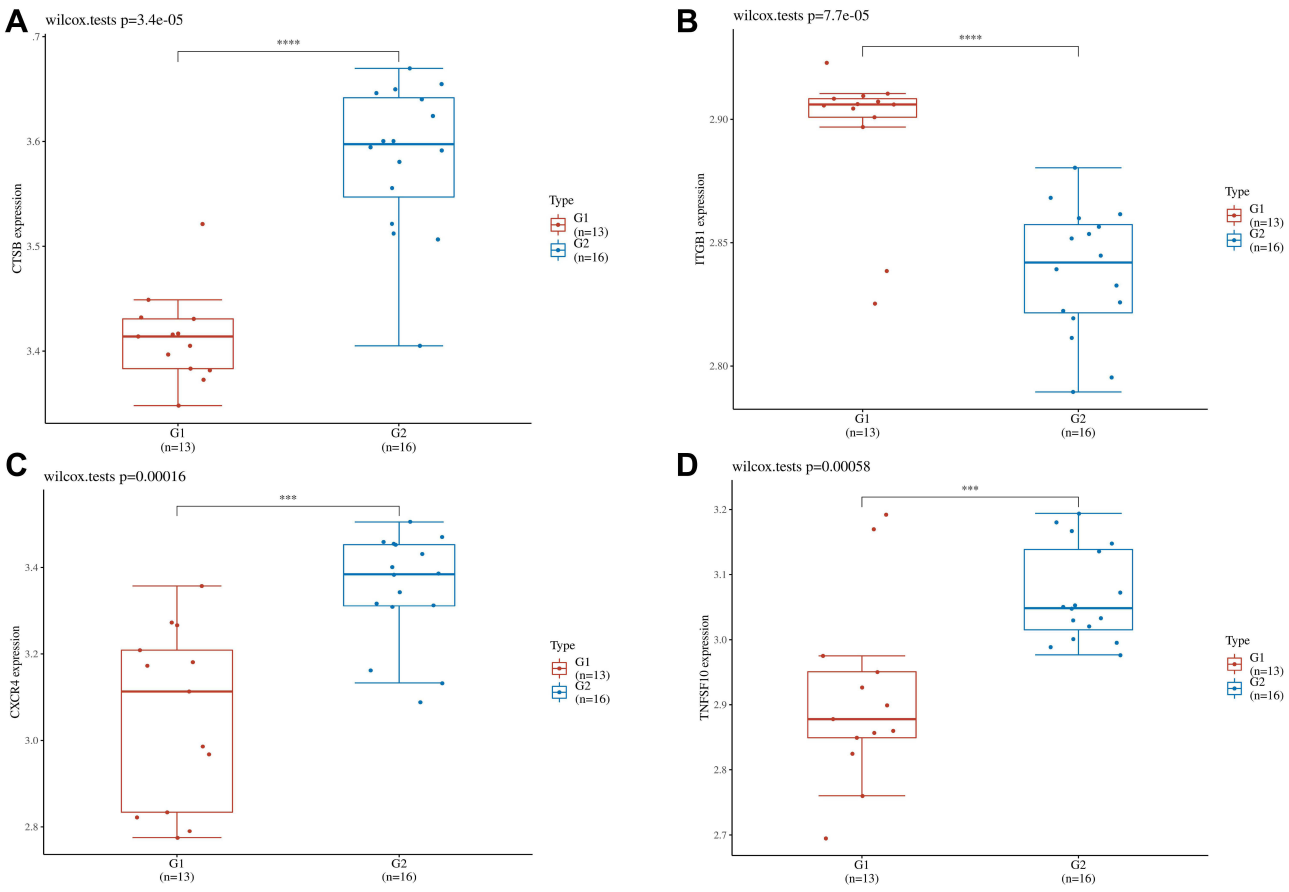

Figure 6 Analysis of the expression level of four key genes and correlation analysis of DEARGs and clinical factors. (**A**) The mRNA expression of CTSB. (**B**) The mRNA expression of ITGB1. (**C**) The mRNA expression of CXCR4. (**D**) The mRNA expression of TNFSF10. G1 group was for the early carotid atherosclerotic plaque while G2 was for advanced carotid atherosclerotic plaque. Asterisks (*) stand for significance levels, ****for $p < 0.0001$, ***for $p < 0.001$.

between key gene expressions and clinical complications and risk factors related to atherosclerosis, four datasets including GSE40231, GSE22255, GSE16561 and GSE90074 were performed by logistic regression analysis.

As shown in Table 1, hypertension factor showed higher expression of ITGB1 than Normal blood pressure persons. The probability of coronary heart disease was significantly increased in factor with high expression of CTSB and CXCR4, while the expression of ITGB1 and TNFSF10 were significantly reduced. Diabetes factor tended to express distinguished levels of CTSB and ITGB1 compared with non-diabetic factor. In addition, ischemic stroke was positively associated with ITGB1 and TNFSF10 status in GSE16561, and negatively associated with ITGB1 status in GSE22255. Finally, TNFSF10 was highly expressed in hyperlipidemia factor. It worth noting, ITGB1 was found to have significant correlation with four clinical complications and risk factors related to atherosclerosis, including coronary heart disease, ischemic stroke, hypertension and diabetes. Combined with this information, logistic regression identified ITGB1 as potentially suitable therapeutic targets for atherosclerosis patients.

Co-Expression Analysis of DEARGs and Autophagy-Related Genes

The underlying communication mechanisms of DEARGs and autophagy were explored by the Pearson correlation analysis. We found 23 autophagy-related proteins, which are involved in the formation of autophagosome membrane in mammalian, including ULK1, ULK2, ATG2A, ATG2B, ATG3, ATG4A, ATG4B and so on. As shown in Figure 7, the resulting heatmap showed that the percentages of different autophagy-related genes represented weak to moderate correlation. The results revealed that the expression level of CTSB, had significant associations with ATG12 and ATG9A. The correlation relationship between CXCR4 and ATG3 represented a good correlation. These findings suggested that DEARGs, including *CTSB*, *ITGB1*, *CXCR4* and *TNFSF10*, may play a specific regulatory role in formation of autophagosome membrane.

Discussion

Cardiovascular disease is the leading cause of morbidity and mortality in developed countries.[29] In clinical medicine, coronary artery disease, carotid artery disease and peripheral vascular disease are common manifestations of cardiovascular diseases.[30] Extent of atherosclerosis in the carotid arteries has been shown to be a risk factor or marker for symptomatic coronary artery disease.[31] The rupture of advanced carotid atherosclerotic plaques even can directly lead to ischemic strokes.[32] Autophagy has emerged in recent years as a critical cellular survival mechanism for cell homeostasis and may play a protective role in atherosclerosis.[33] Autophagy-related biomarkers have been found in many atherosclerotic diseases, including hsa_circ_0030042 in coronary arteries, Beclin 1 in major intracranial artery, DR-NPs in the abdominal artery and miR-100 in aorta. However, there are few autophagy-related studies, comparing early carotid atherosclerosis with advanced carotid atherosclerosis samples. Therefore, it is necessary to observe new biomarkers by microarrays, which will enhance the development of strategies for carotid atherosclerosis progression.

Table 1 The Correlation Analysis of DEARGs (*CTSB*, *ITGB1*, *CXCR4*, *TNFSF10*) and Clinical Factors

Dataset	Factors	Genes	B	SE	Wald	Sig.	OR	95% CI for EXP (B)
GSE40231	Coronary heart disease	*CTSB*	−0.007	0.001	33.498	0.000	0.993	0.990–0.995
		ITGB1	0.023	0.007	9.435	0.002	1.023	1.008–1.038
		CXCR4	−0.009	0.001	45.868	0.000	0.991	0.989–0.994
		TNFSF10	0.020	0.006	12.952	0.000	1.020	1.009–1.032
GSE22255	Ischemic stroke	*ITGB1*	6.093	2.889	4.446	0.035	442.591	1.536–127502.698
		CXCR4	−1.503	0.678	4.917	0.027	0.222	0.059–0.84
GSE16561	Ischemic stroke	*ITGB1*	−1.93	0.751	6.607	0.01	0.145	0.033–0.632
		TNFSF10	−4.179	1.033	16.348	0	0.015	0.002–0.116
GSE90074	Hypertension	*ITGB1*	−0.921	0.457	4.072	0.044	0.398	0.163–0.974
	Diabetes	*CTSB*	−1.04	0.511	4.137	0.042	0.354	0.13–0.963
		ITGB1	−1.052	0.361	8.484	0.004	0.349	0.172–0.709
	Hyperlipidemia	*TNFSF10*	−1.039	0.514	4.087	0.043	0.354	0.129–0.969

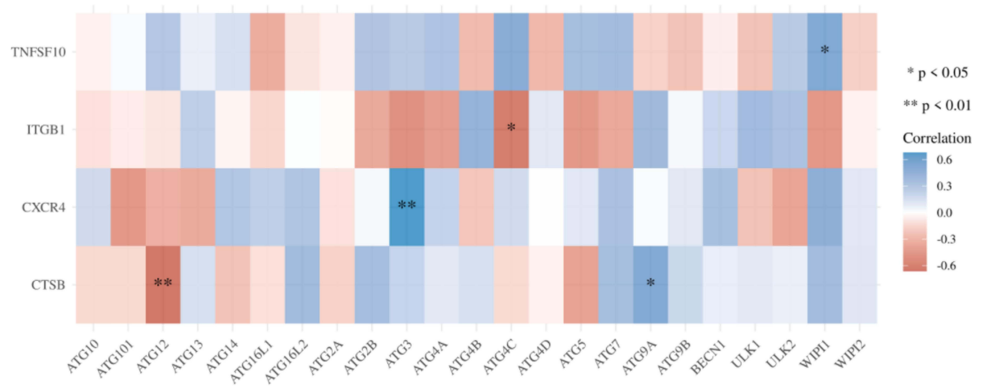

Figure 7 Co-expression analysis of DEARGs and autophagy-related genes. The negative correlation of DEARGs were marked as red color whereas the positive correlation of DEARGs were marked as blue color. A heatmap of the correlation between multiple genes and multiple genes (or one gene). The abscissa and ordinate represent genes, different colors represent different correlation coefficients (blue represents positive correlation whereas red represents negative correlation), the darker the color, the stronger the relation. Asterisks (*) stand for significance levels, **for $p < 0.01$, *for $p < 0.05$.

In the present study, we obtained GSE28829 and GSE34647 datasets from GEO by repurposing microarray data and compared gene and miRNA expression profiles between early and advanced carotid atherosclerosis samples. A protein–protein interaction (PPI) network of differentially expressed autophagy-related genes (DEARGs) was constructed, and four hub genes (*CTSB*, *ITGB1*, *CXCR4* and *TNFSF10*) were revealed. Finally, correlation analysis was carried out to identify the relationship between differentially expressed autophagy-related genes (DEARGs) and clinical and prognostic factors to identify the underlying regulatory mechanisms in advanced carotid atherosclerosis.

CTSB encodes a member of the C1 family of peptidases. Overexpression of the encoded protein has been associated with esophageal adenocarcinoma and other tumors. CTSB was up-regulated in atherosclerotic plaques of rabbit carotid arteries through an atherogenic diet and balloon injury.[34] It has been argued that the expression levels of CTSB which was associated with autophagy was upregulated in coronary heart disease tissues.[35] In the CTSB knockout mice, the susceptibility to attenuated enterovirus infection, replication, and disease of food restriction (FR) required functional autophagy and lysosomal biogenesis was blunted.[36] It was reported that elevated blood sugar and obesity are recognized risk factors for atherosclerotic diseases. CTSB plays a key role in angiogenesis and cholesterol absorption from the intestine, such as obesity, diabetes, non-alcoholic fatty liver disease and cancer. Recently, it was described that CTSB is essential in the protective cellular mechanism called autophagy.[37] Furthermore, the degradative capacity restoration of autophagy decreased the expression of inflammatory factors and VEGF (vascular endothelial growth factor), and protected against apoptosis in retinal pigment epithelial cells in the early stages of diabetic retinopathy.[38] In line with the above study, the present study revealed a causal relationship between increased CTSB levels and advanced carotid atherosclerosis, which conformably occurred with coronary heart disease and diabetes. Similarly, we observed that the GO categories related to autophagy, pathways in cancer, apoptosis and viral process were all most significantly enriched in carotid atherosclerosis progression. Therefore, we can infer that CTSB may slow down the development of advanced carotid atherosclerosis and coronary heart disease through autophagy protective cellular mechanism.

ITGB1 is involved in cell adhesion and recognition in a variety of processes including tissue repair, immune response and metastatic diffusion of tumor cells. It was necessary to demonstrate that *ITGB1* was one of the down regulated differentially expressed genes between primary and advanced atherosclerotic plaque tissues.[39] Miao et al identified the expression of *ITGB1* was lower in coronary artery disease samples.[40] Furthermore, ITGB1 was predicted as a surface protein in cultured brain microvascular endothelial cells mimicking early cerebral ischemia in vitro.[41] The induction of autophagy, resulted from decreased ITGA3-ITGB1 function, functions as a key mediator of anoikis resistance in mammary epithelial cells during extracellular matrix detachment.[42] Moreover, there was less expression of ITGB1 and higher autophagy in herpesvirus-infected cells, and the integrin-dependent signalosome mediated or coactivated numerous inflammatory responses and signaling transductions, showing a better ability to maintain immune homeostasis in both steady and inflammatory states.[43] In addition, the decreased ITGB1 level was extremely related to advanced carotid atherosclerosis, coronary heart disease and stroke, as well as

GO categories related to apoptosis, small GTPase mediated signal transduction, viral process and immune system process were all most significantly enriched in advanced carotid atherosclerosis, which was supported by the present study. Therefore, we can conclude that the decrease of ITGB1 may play an anti-apoptosis role in advanced carotid atherosclerosis by strengthening autophagy, activating inflammatory reaction and signal transduction. In the present study, we also found that ITGB1 was highly expressed in diabetes and hypertension patients, but those findings remain controversial. Indeed, it has been argued that ITGB1 was down regulated in brain microvascular pericytes isolated from spontaneously hypertensive rats compared with wild-type Wistar Kyoto rats.[44] Adipose-selective deletion of ITGB1, similar to loss of adipose insulin receptors, results in a lipodystrophy-like phenotype and systemic insulin resistance.[45] This might be attributed to the extent to which autophagy in atherosclerosis may exert "detrimental" or "beneficial" effects, since in the literature, both labels have been associated with atherosclerosis processes. Autophagy may initially be an adaptive mechanism occurring in atherosclerotic plaque to recycle cellular damaged components for cell survival, and for that reason, autophagy in atherosclerosis has been traditionally tagged as "beneficial". However, when autophagic activity becomes continuously activated in an excessive manner, the plaque may become unstable due to detrimental cell death occurring in some cells, such smooth muscle cells and macrophages.

CXCR4 encodes a CXC chemokine receptor specific for stromal cell-derived factor-1. It acts with the CD4 protein to support HIV entry into cells and is also highly expressed in breast cancer cells. It acts with the CD4 protein to support HIV entry into cells and is also highly expressed in breast cancer cells. It was found that CXCR4-expression on inflammatory cells was more evident in symptomatic carotid stenosis plaques compared to asymptomatic carotid stenosis plaques and was associated with vulnerability-criteria.[46] Additional studies suggested that in case of high expression of CXCR4, macrophagic PI3K/Akt signaling was suppressed, and autophagy was decreased, rendering cells susceptible to apoptosis or death.[47] Previous studies showed that the biological functions of CXCR4 were related to inflammation, immunity, chemokine and cell adhesion molecule, such as PIK-Akt signaling pathway, Rap1 signaling pathway, MAPK signaling pathway, NOD-like receptor signaling pathway and B cell receptor signaling pathway.[48] Our study found that CXCR4 was highly expressed in advanced carotid atherosclerosis, ischemic stroke and coronary heart disease, and enriched in immune process, signal transduction and bioadhesion. Therefore, we can conclude that CXCR4 may aggravate the development of carotid atherosclerosis by reducing autophagy, promoting apoptosis, participating in immune process and increasing bioadhesion.

TNFSF10 was also called TRAIL. The protein encoded by TNFSF10 is a cytokine that belongs to the tumor necrosis factor (TNF) ligand family. This protein preferentially induces apoptosis in transformed and tumor cells, but does not appear to kill normal cells although it is expressed at a significant level in most normal tissues. It was found that an increase of TRAIL on the surface of CD4 T cells in carotid atheroma tissues correlated strongly with plaque instability.[49] Additional studies suggested that TRAIL exhibits pleiotropic activities on endothelial, vascular smooth muscle and inflammatory cells.[50] And activation of TRAIL induces apoptosis in carotid artery cells in vitro, which is an important feature in atherosclerosis, contributing to necrotic core formation, and plaque vulnerability.[51] Furthermore, TRAIL was originally isolated as an inducer of apoptosis and was associated with carotid intima-media thickness in carotid and femoral arteries.[52] Moreover, apoptosis and autophagy are two forms of programmed cell deaths. They can interact with each other through TRAIL. Excessive apoptosis can promote myocardial ischemia, ischemia/reperfusion injury, post-ischemia cardiac remodeling and coronary atherosclerosis.[53] Previous studies also showed that multiple cell death pathways, including extrinsic apoptosis and autophagy, are implicated in the pathogenesis of ischemic stroke. The activation of immune cells during inflammation initiated by ischemic stroke may release several factors that trigger neuronal cell death through the extrinsic apoptotic pathway, including pro-inflammatory cytokines such as TRAIL receptor.[54] Besides, activation of autophagy contributes to adipose tissue and metabolic dysfunction in obesity. In human adipocytes, TRAIL enhanced basal and impaired insulin-inhibitable lipolysis and altered adipokine secretion; and TRAIL expression is associated with higher diabetes status.[55] This outcome was supported by the current study, who demonstrated that TNFSF10 was simultaneously up-regulated in advanced carotid atherosclerosis, stroke and obesity patients and down-regulated in coronary heart disease patients. Therefore, we can conclude that TNFSF10 may worsen carotid plaque through autophagy, apoptosis and immune system processes.

In the current study, we discussed four potential crucial DEARGs involved in the occurrence and development of carotid atherosclerosis, suggesting that these genes may serve as potential biomarkers and therapeutic targets for carotid

atherosclerosis. However, there are still some limitations in this study. Firstly, due to the small sample size of this study and the heterogeneity of carotid atherosclerosis, the interpretation of the study results needs to be cautious. In addition, the specific pathophysiological mechanisms of ARGs regulating the initiation and progression of carotid atherosclerosis need to be further study. Finally, the working mechanism of these genes is not yet fully understood, so more evidence is needed to discover its biological basis and further exploration is needed to lucubrate the exact mechanism and function of these four biomarkers in carotid atherosclerosis progression.

Conclusions

Overall, our study shows that these four ARGs have great potential as biomarkers and therapeutic targets in carotid atherosclerosis. In addition, the crucial genes *CTSB*, *ITGB1*, *CXCR4* and *TNFSF10* may provide new possibilities for further identifying the susceptibility of advanced carotid atherosclerosis and finding useful therapeutic targets. These results expand our understanding of advanced carotid atherosclerosis and ARGs, which could help to distinguish advanced carotid atherosclerosis from carotid atherosclerosis progression from the perspective of genetics and improve accurate diagnosis and therapies of advanced carotid atherosclerosis.

Data Sharing Statement

The data used to support the findings of this study are publicly available in the Figshare repository (https://figshare.com/s/a8826786938d3cb4e879, DOI:10.6084/m9.figshare.16830670).

Acknowledgments

The study was approved by the Ethics Committee of Shengjing Hospital (2021PS862K), with the exemption of further consent from participants. This study was not funded. He Zhang conceived and supervised the study and designed experiments; Yuanyuan Zhang, Jiake Wu and Na Sun performed experiments and analyzed data; Yuanyuan Zhang wrote the manuscript and made manuscript revisions.

Disclosure

The authors declare that they have no conflicts of interest.

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