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ORIGINAL RESEARCH

Biomarker Profiling for Discrimination of High-Risk Asymptomatic Carotid Artery Stenosis Patients with Ulcerated Plaques: A Pilot Study

Atif YOLGOSTEREN[1*], Ceyda COLAKOGLU BERGEL[2*], Isil Ezgi ERYILMAZ[3], Unal EGELI[3], Basak ERDEMLI GURSEL[4], Murat BICER[1], Gulsah CECENER[3], Mustafa TOK[1]

[1] Bursa Uludag University Faculty of Medicine, Cardiovascular Surgery Department, Bursa, Turkiye.
[2] Bursa Uludag University Graduate School of Medical Sciences, Medical Biology Department, Bursa, Turkiye.
[3] Bursa Uludag University Faculty of Medicine, Medical Biology Department, Bursa, Turkiye.
[4] Bursa Uludag University Faculty of Medicine, Radiology Department, Bursa, Turkiye.

ABSTRACT

Although various methods are used to treat patients with asymptomatic carotid artery stenosis (ACAS), approaches are controversial, and combining imaging of carotid plaque features with biomarkers to identify plaques prone to rupture may be crucial in identifying high-risk ACAS patients. This study aimed to investigate a blood-based biomarker for discriminating ulceration in ACAS patients by analyzing plaque surface morphology through RNA sequencing of blood samples. Peripheral blood samples were collected from ACAS patients with plaque morphology determined by Doppler ultrasonography. Then, total RNA was isolated, and RNA-Seq was performed to analyze differentially expressed genes (DEGs). The KEGG, Reactome, and Gene Ontology (GO) terms pathway enrichment analyses were performed to investigate the molecular functions and biological processes involved in plaque formation. The pilot study included 7 ACAS patients, 57.1 % exhibiting ulcerated plaques. RNA-Seq results revealed significant upregulation of genes related to immune response, cell cycle regulation, and oxidative stress in ulcerated plaques. Especially, *TP73*, *CCL3L3*, and *PXDNL* genes showed the highest fold changes, indicating their role in endothelial damage, immune activation, and oxidative stress. KEGG and Reactome analyses identified TNF and chemokine signaling pathways as key regulators in ulcerated plaque formation. Our findings indicate that *TP73*, *CCL3L3*, and *PXDNL* may be potential biomarkers for identifying high-risk ACAS patients with ulcerated plaques due to their involvement in immune system regulation and oxidative stress-related processes. Thus, these genes and the pathways may be candidate biomarkers for early diagnosis and risk stratification, improving treatment approaches for ACAS.

Keywords: Carotid stenosis. Carotid artery plaque. Biomarker. RNA-Seq. Ulcerated plaque.

Ülsere Plaklı Yüksek Riskli Asemptomatik Karotis Arter Stenozu Hastalarının Ayırımı İçin Biyobelirteç Profillemesi: Pilot Çalışma

ÖZET

Asemptomatik karotis arter stenozu (ACAS) olan hastaların tedavisinde çeşitli yöntemler kullanılsa da yaklaşımlar tartışmalıdır ve plak rüptürü eğilimli olan hastaları belirlemek için karotis plak görüntülerinin biyobelirteçlerle birleştirilmesinin, yüksek riskli ACAS hastalarının belirlenmesinde önemli olduğu düşünülmektedir. Mevcut çalışmada, plak yüzey morfolojisini analiz ederek ACAS hastalarında ülserasyonu ayırt etmek için RNA sekanslama yoluyla kan bazlı biyobelirteç araştırılması amaçlanmıştır. Bu doğrultuda, Doppler ultrasonografi ile plak morfolojisi belirlenen ACAS hastalarından periferik kan örnekleri toplandı. Daha sonra, total RNA izole edildi ve farklı şekilde ifade edilen genleri (DEG'ler) analiz etmek için RNA-Seq gerçekleştirildi. Plak oluşumunda yer alan moleküler işlevleri ve biyolojik süreçleri araştırmak için KEGG, Reactome ve Gen Ontolojisi (GO) ile yolak zenginleştirme analizleri gerçekleştirildi. Pilot çalışmaya 7 ACAS hastası dahil edildi ve %57,1'inde ülsere plaklar olduğu görüldü. RNA-Seq sonuçları ülsere plaklarda bağışıklık tepkisi, hücre döngüsü düzenlemesi ve oksidatif stresle ilgili genlerde önemli bir artış olduğunu ortaya koydu. Özellikle *TP73, CCL3L3* ve *PXDNL* genlerinin bu süreçlerde rol aldığı ve bu genlerin endotel hasarı, bağışıklık aktivasyonu ve oksidatif streste rolleri olduğu gösterildi. KEGG ve Reactome analizleri, ülserli plak oluşumunda anahtar düzenleyiciler olarak TNF ve kemokin sinyal yollarını tanımladı. Bulgularımız, *TP73, CCL3L3* ve *PXDNL*'nin bağışıklık sistemi düzenlemesi ve oksidatif stresle ilişkili süreçlerdeki katılımları nedeniyle ülserli plakları olan yüksek riskli ACAS hastalarını tanımlamak için potansiyel biyobelirteçler olabileceğini göstermektedir. Bu nedenle, ilişkili genlerin ve yolakların erken tanı ve risk sınıflandırması için aday biyobelirteçler olabileceği ve ACAS için tedavi yaklaşımlarını iyileştirebileceğini düşündürmektedir.

Anahtar Kelimeler: Karotis stenozu. Karotis arter plağı. Biyobelirteç. RNA-Seq. Ülsere plak.

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* Atif YOLGOSTEREN and Ceyda COLAKOGLU BERGEL have shared the first authorship.

Dr. Unal EGELI
Bursa Uludag University, Faculty of Medicine,
Medical Biology Department, Bursa, Turkiye,
Phone:
E-mail: egeli@uludag.edu.tr

Authors' ORCID Information:
Atif YOLGÖSTEREN: 0000-0002-4467-3915
Ceyda ÇOLAKOĞLU BERGEL: 0000-0002-7471-5071
Işıl Ezgi ERYILMAZ: 0000-0002-3316-316X
Ünal EGELİ: 0000-0001-7904-883X
Başak ERDEMLİ GÜRSEL: 0000-0002-0047-1780
Murat BİÇER: 0000-0002-9280-086X
Gülşah ÇEÇENER: 0000-0002-3820-424X
Mustafa TOK: 0000-0001-9656-537X

Atherosclerosis is a chronic and progressive vascular disorder that constitutes the primary pathophysiological basis of a significant proportion of cardiovascular diseases, which remain the leading cause of mortality and morbidity globally[1]. The disease is characterized by the accumulation of lipids within the arterial wall, chronic inflammation, fibrotic remodeling, and the progressive narrowing of the vascular lumen[2]. When this narrowing, or stenosis, occurs in the carotid arteries, it is referred to as carotid artery stenosis (CAS), a condition that significantly elevates the risk of ischemic stroke[3].

CAS patients are clinically categorized into two groups: (i) symptomatic (SCAS) and (ii) asymptomatic (ACAS). The distinction between these groups is pivotal for tailoring appropriate treatment strategies and minimizing the risk of cerebrovascular events[4]. ACAS is defined as 50% or greater stenosis in the proximal internal carotid artery at the bifurcation in individuals without a history of ischemic stroke in the ipsilateral carotid region within the last six months. Various treatment modalities are available for ACAS, including carotid endarterectomy (CEA), carotid artery stenting (CAS), and medical treatment. However, the optimal approach remains controversial. Moreover, ACAS patients often remain clinically stable for extended periods. Thus, the unpredictable progression from an asymptomatic to a symptomatic state represents a substantial clinical challenge[5,6]. Consequently, accurate clinical differentiation of high-risk ACAS patients is essential to optimize treatment approaches, balance the risks and benefits of invasive procedures, and avoid unnecessary interventions in low-risk individuals.

The surface morphology of atherosclerotic plaques plays a crucial role in the clinical outcomes of CAS. Plaque rupture is closely associated with various cerebrovascular events and is categorized into two groups based on their biological characteristics: (i) stable and (ii) vulnerable plaques[7]. Unlike stable plaques, vulnerable plaques, considered high-risk, are characterized by a thin, inflammatory fibrous cap that is particularly prone to rupture[8]. These plaques are further classified according to their surface morphology into (i) smooth, (ii) irregular, and (iii) ulcerated types. Ulceration, commonly observed in vulnerable plaques, is considered a predictive feature of plaque instability[9]. Surface characteristics, including ulceration, rupture, and erosion, are critical determinants of plaque stability and significantly influence the risk of thromboembolic events. Even when the degree of luminal stenosis is similar, variations in plaque morphology can substantially affect the patient's risk profile[10]. Therefore, the accurate assessment of plaque surface morphology is essential for effective risk stratification, treatment decisions, and optimizing clinical outcomes in patients with CAS.

The current study identified RNA-based biomarkers by performing transcriptome sequencing on blood samples obtained from ACAS patients, whose plaque surface morphologies -ulcerated or smooth- were determined through Doppler imaging. While previous research has focused on evaluating plaque biology using various biomarkers[11-14], this is the first study to utilize blood-based biomarkers to differentiate between ulcerated and smooth plaques in ACAS patients.

Material and Method

Study cohort

In the current study, peripheral blood samples were collected before CEA from ACAS patients, whose plaque surface morphologies -ulcerated or smooth- were determined using Doppler ultrasonography, and total RNA was isolated from blood materials. For the pilot group, 7 ACAS patients, of whom 57.1% had ulcerated plaques, were selected for RNA-Seq analysis.

All participants provided written informed consent for using their RNA samples in the RNA-Seq analysis conducted at the Department of Medical Biology, Bursa Uludag University. The study received ethical approval from the Bursa Uludag University Medical Ethics Committee (2021-6/38) and adhered to the Helsinki Declaration's ethical standards.

The inclusion criteria for this study were as follows: patients aged over 60 years with stenosis greater than 70%, as determined by the North American Carotid Endarterectomy Trial (NASCET) criteria, who were diagnosed with ACAS based on Doppler ultrasonography findings. Carotid plaque ulceration was assessed using Color Doppler ultrasonography (US), one of the most commonly used methods, and was defined by an expert (B.E.G.) of the carotid

arteries. The plaque surface was defined as smooth, slightly irregular, or ulcerated if there was more than a 2 mm height difference on the plaque surface. The reverse filling in the plaque was also determined. Plaques were classified as either ulcerated or smooth according to imaging results, and patients had no history of ischemic stroke in the ipsilateral carotid region within the last six months. Exclusion criteria included patients under 60, those with less than 70% stenosis according to NASCET criteria, and individuals with additional peripheral vascular diseases. Patients with a history of ischemic stroke within the last six months, systemic inflammatory or autoimmune diseases, malignancies, or other chronic illnesses that could influence gene expression profiles were also excluded.

RNA-Seq analysis

Total RNA was extracted from blood samples of ACAS patients, classified by plaque morphology (ulcerated or smooth), using the RNeasy Lipid Tissue Mini Kit (Hilden, Germany). RNA quality and concentration were evaluated using the Agilent Technologies 2100 (Santa Clara, CA, USA), and samples with DV200 scores greater than 70% were selected for RNA-Seq analysis. mRNA libraries were generated with the Ion AmpliSeq™ Chef-Ready Kit (Thermo Fisher Scientific, MA, USA) and the Ion AmpliSeq Transcriptome Human Gene Expression Panel. cDNA conversion (10 ng RNA per sample) and library preparation were performed using the Ion Chef Instrument, and sequencing was conducted on the Ion GeneStudio™ S5 System using Ion 540™ Chips at Bursa Uludag University, Medical Biology Department NGS Lab.

Determination of differentially expressed genes (DEGs)

The Kyoto Encyclopedia of Genes and Genomes (KEGG), Reactome, and Gene Ontology (GO) terms pathway enrichment analyses were used for the functional analysis of mRNA sets after correction of the enrichment results by the Benjamini-Hochberg FDR method, biological processes, and molecular pathways were considered significant with adjusted *p*-values of less than 0.05 for the genes. RPKM-normalized count data from RNA-Seq were used to identify DEGs. Significant genes were analyzed using the Web Gestalt toolkit to explore biological processes and molecular pathways. GO terms were used for biological processes, while KEGG and Reactome databases were utilized for pathway analysis.

Statistical Analysis

A *p*-value correction was performed using the false discovery rate (FDR) correction method to eliminate false positive genes, and adjusted *p*-values were determined for each gene. The cut-off value for the adjusted *p*-value for statistically significant genes was set at 0.05, and genes below this value were considered statistically significant. Down-regulated and up-regulated genes were determined by the fold changes calculated.

Results

The clinical characteristics of ACAS patients

For the pilot group, 7 patients with ACAS were included in this study. Of these, 57.2% (n=4) were male and 42.8% (n=3) were female. According to the anamnesis, 57.1% (n=4) of the patients had ulcerated plaque. The mean age was 72.4 ± 6.9 years. Additionally, 85.7% (n=6) of the patients had hypertension, 57.1% (n=4) had diabetes, and 14.3% (n=1) had hyperlipidemia. Moreover, 28.5% (n=2) of the patients had peripheral artery, and 14.3% (n=1) had coronary artery disease ($p>0.05$). The clinical characteristics of the patients are shown in Table I.

Table I. Clinical characteristics of ACAS patients.

Pilot Group (n=7)	Ulcerated (n=4)	Smooth (n=3)	*p* value
Gender (n, %)			
Female (n=3)	33.3% (n=1)	66.7% (n=2)	*0.486*
Male (n=4)	75% (n=3)	25% (n=1)	
Age (Mean ± SD)	71.25 ± 4.7	74.0 ± 10.0	*0.721*
Diabetes Mellitus	75% (n=3)	33.3% (n=1)	*0.486*
Hyperlipidemia	-	33.3% (n=1)	*0.429*
Hypertension	100% (n=4)	66.6% (n=2)	*0.429*
Coronary Artery Disease	25% (n=1)	-	*1*
Peripheral artery Disease	50% (n=2)	-	*0.429*

Evaluation of DEGs and pathway analysis for ulcerated and smooth plaques in ACAS patients

RNA-Seq analysis was performed to identify genes with significantly different expression levels among the groups and to identify blood-based biomarkers that could be used to discriminate high-risk ACAS patients. Then, the pilot group's pathway analysis was performed using KEGG, GO terms, and Reactome pathway enrichment analyses in ACAS patients with ulcerated and smooth plaques.

The DEG results indicated that genes involved in the cell cycle, such as *p73*, as well as genes related to chemokine signaling, immune response, and ROS signaling, such as *CCL3L3* and *PXDNL2*, were upregulated in ulcerated plaques ($p<0.001$) (Figure 1). The upregulation of genes associated with the immune system, ROS signaling, and cell proliferation in ulcerated plaques is consistent with endothelial damage and the enhanced immune response observed during ulceration.

Gene	Fold Change	*p* -Value
TP73	23,811	3,70E-03
CCL3L3	21,961	1,40E-03
PXDNL	16,746	3,97E-02
HOXB2	14,024	4,90E-02
BICDL2	9,805	4,30E-02
SHROOM1	9,614	3,29E-03
ABCA13	8,559	4,34E-03
ZNF215	8,540	1,25E-02
JCHAIN	7,814	2,04E-06
ADGRB2	7,470	2,31E-02
FOLR3	6,897	1,99E-07
CITED4	6,646	3,68E-02
CRABP2	5,808	2,87E-02
CDC45	5,805	4,92E-02
SLC22A1	5,092	9,00E-05
G0S2	4,968	9,63E-04
CFAP53	4,092	3,05E-02
FOSB	4,032	8,00E-03
MOB3B	3,891	1,82E-02
PTGS2	3,863	4,75E-03
DEFA3	3,718	7,75E-04
KLHL14	3,396	8,09E-03
EFCAB12	3,302	3,04E-02
H3C6	3,096	3,75E-02
PGAM4	3,055	2,58E-02
OPLAH	3,037	2,75E-02
CSRNP1	3,028	3,99E-07
LGALS9C	3,012	2,71E-02
TSHZ1	2,966	3,02E-02
TAFA2	2,943	2,34E-02
PANX2	2,907	3,99E-02
CLEC12B	2,830	4,37E-02
HMGB1	2,785	2,50E-02
CHRNE	2,775	5,10E-05
CYP4F12	2,743	1,00E-02
SPATA6	2,666	3,29E-02
PIP5K1A	2,631	2,36E-02

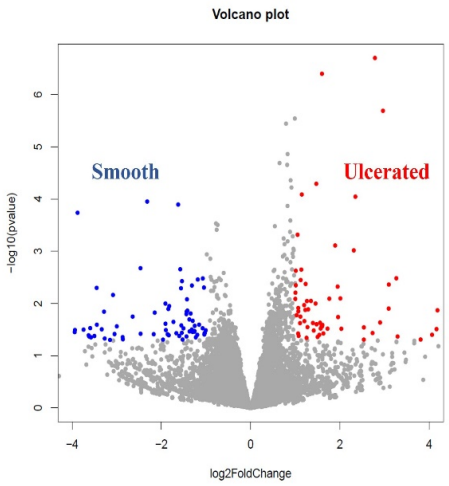

Figure 1.

DEGs and Volcano Plot in ACAS patients with ulcerated and smooth plaques. The left panel shows the table of DEGs between ulcerated and smooth plaques in ACAS patients. Fold change (log2) and corresponding p-values are presented, highlighting significant genes such as TP73, CCL3L3, and PXDNL. The right panel displays the volcano plot, with red points indicating upregulated genes in ulcerated plaques. Gray points represent non-significant genes. The plot emphasizes genes with statistical and biological significance, providing insights into the molecular differences between ulcerated and smooth plaques.

Moreover, GO terms results revealed that genes implicated in forming ulcerated plaques are primarily associated with biological regulation, metabolic processes, and responses to external stimuli. Significantly regulated genes were predominantly localized to the cell membrane, nucleus, and membrane-enclosed lumen. Moreover, these genes exhibit key molecular functions, including protein binding, ion binding, and nucleic acid binding (Figure 2A). In addition to these findings, it was observed explicitly that key immune system-related pathways, such as the cellular response to lipopolysaccharides and the activation pathways of lymphocytes, leukocytes, and T cells, were also significantly regulated.

The analysis of KEGG results also revealed that genes implicated in forming ulcerated plaques are primarily associated with biological regulation, metabolic processes, and responses to external stimuli. Significantly regulated genes were predominantly localized to the cell membrane, nucleus, and membrane-enclosed lumen. Moreover, these genes exhibit key molecular functions, including protein binding, ion binding, and nucleic acid binding. In addition to GO findings, it was observed explicitly that TNF and chemokine signaling were significantly regulated (Figure 2B).

Finally, Reactome analysis revealed that the genes' biological processes, cellular components, and molecular functions were consistent with the results of other studies. Immune system regulation emerged as a common finding across these analyses, with the Toll-like receptor cascade identified as playing a pivotal role in these pathways (Figure 2C).

Discussion and Conclusion

In the current study, RNA-Seq analysis was performed to identify DEGs and potential blood-based biomarkers to distinguish high-risk asymptomatic carotid artery stenosis (ACAS with ulcerated plaques) patients. By comparing ulcerated and smooth plaques, we aimed to uncover the molecular mechanisms underlying plaque destabilization and identify biomarkers that could guide clinical risk assessment. Our findings showed a distinct transcriptional regulation in ulcerated plaques, characterized by upregulation of genes involved in cell cycle, immune response, chemokine signaling, and reactive oxygen species (ROS) pathways. Notable the DEGs, such as *TP73*, *CCL3L3*, and *PXDNL*, were significantly upregulated in ulcerated plaques, highlighting the roles of these genes in endothelial damage, immune

Figure 2.

Functional enrichment analyses of the DEGs in ulcerated and smooth plaques. ***(A)*** *GO Pathway Analysis: Enrichment analysis of DEGs using Gene Ontology (GO) terms includes three categories: (i) Biological Processes, (ii) Cellular Components, and (iii) Molecular Functions. Bar charts on the left display the top enriched terms in each category, while the table on the bottom right lists significant GO terms, such as cellular response to lipopolysaccharide and lymphocyte activation, along with corresponding p-values and false discovery rates (FDR).* ***(B)*** *KEGG Pathway Analysis: Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis highlights enriched pathways among the DEGs. Bar charts display top pathways, such as the TNF and Chemokine signaling pathways, with detailed statistics in the accompanying table, including pathway sizes, p-values, and FDR.* ***(C)*** *Reactome Pathway Analysis: Reactome pathway enrichment analysis reveals significantly enriched pathways such as Immune System, Neutrophil Degranulation, and Cytokine Signaling in the immune system. Bar charts summarize enriched categories, while the table on the right provides details, including pathway sizes, p-values, and FDR. These analyses offer insights into the biological processes, pathways, and molecular mechanisms differentiating ulcerated and smooth plaques.*

activation, and oxidative stress. These results suggest that endothelial damage and an enhanced immune response contribute to plaque vulnerability and ulceration.

Our results revealed significant upregulation of genes involved in cell cycle regulation, immune response, chemokine signaling, and ROS pathways in ulcerated plaques compared to smooth plaques. Notably, *TP73*, a critical regulator of apoptosis and genomic stability, exhibited the highest fold change (23.8-fold, $p<0.001$). *TP73* is known to play a role in cellular proliferation and response to DNA damage, and studies conducted in recent years have highlighted that it is significantly increased in plaque tissue in atherosclerotic human carotid arteries[15,16]. In a different study, p73 was shown to initiate apoptosis in vascular smooth muscle cells (VSMCs) and was found to be highly expressed in human atherosclerotic plaques[17]. Similarly, chemokine-related genes such as *CCL3L3* were highly expressed, emphasizing the role of chemokine signaling in recruiting immune cells to sites of vascular injury. Elevated *CCL3L3* expression has been implicated in monocyte and macrophage activation, which are critical mediators of inflammation in atherosclerosis. It has also been shown that under conditions of acute inflammation, leukocyte-derived CCL3 can induce neutrophil chemotaxis toward atherosclerotic plaque and contribute to progression[18]. These findings suggest that these genes may contribute to pathological processes consistent with their upregulation in the unstable endothelial environment observed in ulcerated plaques by regulating VSMC apoptosis or chemotaxis of immune system cells.

Genes related to ROS signaling, such as *PXDNL*, were also significantly upregulated in ulcerated plaques. ROS production is a hallmark of endothelial dysfunction and contributes to oxidative stress, exacerbating plaque instability[19]. The observed upregulation of ROS-associated genes is consistent with the increased oxidative damage and inflammation reported in studies of vulnerable plaques[20]. PXDN is a peroxidase that modifies the extracellular matrix. *PXDN* expression also influences physiological processes involving redox control[21]. Furthermore, this gene has also been shown to have a pro-angiogenic role that may affect the outcome of atherosclerotic lesions[22]. These findings suggest that PXDN-mediated oxidative stress may play a pivotal role in the pathogenesis of ulcerative plaques, potentially through ROS-induced angiogenic processes.

Functional enrichment analyses provided further insights into the biological processes and pathways driving these transcriptional changes. GO analysis revealed that genes implicated in ulcerated plaques were predominantly associated with biological regulation, metabolic processes, and responses to external stimuli, significantly enriching immune response and cellular signaling pathways. Notably, cellular response to lipopolysaccharides and lymphocyte activation were highlighted, suggesting a robust innate and adaptive immune response in ulcerated plaques. From a pathway perspective, KEGG analysis emphasized the roles of TNF signaling and chemokine signaling pathways, known mediators of inflammation and vascular remodeling. The TNF signaling pathway has been implicated in promoting endothelial cell apoptosis and matrix degradation, contributing to plaque rupture[23,24]. Furthermore, given the critical role of TNF pathways in sustaining the inflammatory microenvironment, it is hypothesized that the recruitment and activation of immune cells, such as monocytes and lymphocytes, may contribute to the inflammatory milieu within ulcerative plaques. This immune cell participation is likely to exacerbate the inflammatory response, further promoting the instability and progression of the plaques.

The Reactome analysis provided complementary findings, reinforcing the role of immune system regulation in plaque ulceration. TLR cascade emerged as a pivotal pathway in ulcerated plaques. TLRs, a type of pattern recognition receptor, are associated with inflammation and the innate immune response. The dysregulation of TLRs has been implicated in developing CAS and various other cardiovascular and metabolic diseases[25]. The activation of these receptors has been identified as triggering an intracellular signaling cascade mediated through MyD88 or TRIF, leading to the production of pro- and anti-inflammatory cytokines[26]. The upregulation of TLR signaling genes in ulcerated plaques suggests their contribution to the heightened inflammatory state observed in these lesions.

Notably, the localization of DEGs to the cell membrane, nucleus, and membrane-enclosed lumen, as identified by GO analysis, highlights their functional relevance in signal transduction and transcriptional regulation. These findings suggest that plaque ulceration is associated with extracellular signaling changes and intracellular transcriptional reprogramming. Molecular functions such as protein binding, ion binding, and nucleic acid binding were also significantly enriched, underscoring the diverse roles of DEGs in modulating cellular responses to stress and injury.

Consequently, although further validation in larger cohorts is required, our preliminary findings suggest that *TP73*, *CCL3L3*, and *PXDNL* may serve as promising biomarkers for ACAS patients with ulcerative plaques. Furthermore, the results suggest that pathways such as TLR and TNF may be key pathways in these processes. Thus, noninvasive, blood-based assays targeting these biomarkers may

provide valuable tools for early detection and risk stratification in ACAS patients with ulcerated plaque. They may offer a more practical approach to personalized ACAS patient care and management.

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