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Ferroptosis-Related genes *NOX4*, *PDK4*, *PRKAA2*, and *FABP4* Emerge as Novel Prognostic Biomarkers and Therapeutic Targets in Stomach Adenocarcinoma

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Abstract

Background: Stomach adenocarcinoma (STAD) is the fifth most common malignant tumor worldwide in terms of both incidence and mortality. Treatment and prognosis face great challenges. We aimed to identify potential therapeutic targets or prognostic genes by analyzing STAD RNA-seq and clinical data, as well as ferroptosis-related genes.
Methods and Results: RNA-seq and clinical data of STAD were downloaded from the Cancer Genome Atlas (TCGA) database, and ferroptosis-related gene data were downloaded from the FerrDb V2 website. Bioinformatics and statistical analyses were performed using R software, and statistical significance was set at $P<0.05$.
We screened 1384 differentially expressed genes (DEGs) between STAD and normal tissues, including 24 ferroptosis-related DEGs. Among these 24 genes, we further screened four hub genes related to survival prognosis: NOX2, PRKAA2, FABP4, and PDK4. Through single-gene survival analysis and Cox regression analysis, and by constructing a prognostic model, we found that STAD patients with low expression of *NOX2*, *PRKAA2*, *FABP4*, and *PDK4* had significantly longer survival times; the difference was statistically significant ($P<0.05$).
Conclusion: We identified four ferroptosis-related DEGs in STAD that are associated with prognosis. These four genes have the potential to become targets for STAD treatment.(**International Journal of Biomedicine. 2025;15(3):483-489.**)

Keywords: stomach adenocarcinoma • differentially expressed gene • ferroptosis • prognosis

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Abbreviations

DEGs, differentially expressed genes; **PPI**, protein-protein interaction; **STAD**, stomach adenocarcinoma.

Introduction

Stomach adenocarcinoma (STAD) is a malignant tumor originating from gastric mucosal epithelial cells. The pathological types of STAD vary. In 1965, P. LAUREN classified STAD into intestinal and diffuse types according to whether the tumor had intestinal metaplasia.[1] According to the 5th Edition of the World Health Organization (WHO) classification system in 2019, the most common pathological types of STAD include tubular, papillary, poorly cohesive, mucinous, and mixed adenocarcinomas.[2] According to data released in 2024 by the International Agency for Research on Cancer (IARC), there are 968,350 new cases of STAD worldwide, accounting for 4.9% of all new cases of 36 cancers,

and 659,853 deaths, accounting for 6.8% of the deaths of 36 cancers. Morbidity and mortality rates are ranked fifth in the world.[3] Although the incidence rate of STAD is relatively low compared to lung cancer, which has an incidence rate of 11.7%, it is still the fifth largest cause of cancer-related deaths in the world.[4,5] The incidence of STAD is associated with various factors, including Helicobacter pylori infection, genetic factors, environmental factors, dietary factors, and others. The choice of treatment strategies for patients with STAD is closely related to their clinical stage. For non-metastatic STAD, combination therapy, such as perioperative chemotherapy or postoperative chemotherapy plus chemoradiation, is usually the preferred option, and palliative treatment is typically employed for metastatic and unresectable STAD.[6] Although the incidence of STAD has declined over the past fifty years, its global 5-year survival rate remains at only about 25-30%, [7] making it a significant global health burden due to its poor prognosis. In addition, studies have shown that the incidence of STAD is increasing in younger age groups.[4,5] Therefore, the diagnosis, treatment and prognosis of STAD in the world are still facing severe challenges.

Ferroptosis is an iron-dependent, non-apoptotic form of cell death proposed by Dr. Brent R. Stockwell of Columbia University in 2012.[8] Ferroptosis is an iron-dependent, regulated, unique mode of cell death.[9] It is mediated by lipid peroxidation, resulting in a large accumulation of intracellular reactive oxygen species (ROS), which eventually leads to cell death.[9] Numerous studies have indicated that tumor suppression or growth is associated with ferroptosis. For example, Ma et al. found that CD8+ T cells with up-regulated CD36 expression promoted ferroptosis by ingestion of excessive polyunsaturated fatty acids (PUFAs), thus causing them to lose their ability to regulate tumors and promoting tumor growth.[10] Wang et al.[11] suggested that in the immunotherapy of tumors, activated CD8+T cells secrete IFNγto downregulate the expression of two subunits of the glutamate cysteine efflux system xc−, SLC3A2, and SLC7A11, thereby promoting lipid peroxidation in tumor cells, ultimately leading to ferroptosis and inhibiting tumor growth. Ferroptosis may also serve as an effective therapeutic target for the treatment of pancreatic cancer, lung cancer, and liver cancer.[12,13] These studies have shown that the pathological process of tumors is closely related to ferroptosis.

STAD cells can evade ferroptosis by up-regulating *GPX4*, thereby promoting tumor progression; however, knocking out *GPX4* or inhibiting ferroptosis resistance with drugs can significantly suppress tumor growth and delay the progression of metastasis.[14] The microprotein HCP5-132aa encoded by *HCP5* enhances the interaction between *YBX1* and *ELAVL1*, maintains SLC7A11 levels, and thereby inhibits ferroptosis to promote the proliferation and metastasis of STAD cells.[15] These studies indicate that the investigation of ferroptosis-related genes in STAD can not only uncover potential new therapeutic targets but also serve as prognostic markers. Therefore, the discovery of additional ferroptosis-related genes in STAD could provide significant benefits for both clinical practice and patients with this cancer. In this study, we aimed to perform bioinformatics analysis on the RNA-seq profiles and clinical data of STAD samples downloaded from the Cancer Genome Atlas (TCGA) database to identify ferroptosis-related genes associated with the survival prognosis of STAD patients. This study may provide a reference for the prognostic evaluation of patients with STAD or the search for therapeutic targets in clinical practice.

Materials and Methods

Data Acquisition and Processing

RNA-seq profiles and clinical data of 379 STAD samples and 34 normal tissue samples were downloaded from the TCGA website (https://portal.gdc.cancer.gov) on August 31, 2024. The GTF annotation file was then used to convert the Ensemble gene ID to a gene symbol. We downloaded 717 ferroptosis-related genes from the FerrDb V2 website *(*http://www.zhounan.org/ferrdb/current*/)*, including 369 driver and 348 suppressor genes. R software (R4.4.0 version) and online websites were used for data analysis.

Identification of Significantly Differentially Expressed Genes

R software was used to pre-process the sample data, and the "Limma" R package was used to screen for DEGs. The cut-off values were determined according to the parameters $P<0.01$ and false discovery rate < 0.01. The absolute value of log2FC > 2 and adjusted P-value < 0.01 was used to screen for significant DEGs. Visualization of DEGs using volcano plots. We then took the intersection of DEGs and ferroptosis-related genes to screen for ferroptosis-related DEGs. The intersection genes were visualized using a Venn diagram.

Functional Enrichment Analysis of Ferroptosis-Related DEGs

The ferroptosis-related DEGs were visualized using heatmaps, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed using R software. Gene Ontology (GO) functional enrichment analysis of ferroptosis-related DEGs, including biological process (BP), cellular component (CC), and molecular function (MF), was performed using the Wei Sheng Xin online website (https://www.bioinformatics.com.cn). Statistical significance was set at $P<0.05$.

Protein-Protein Interaction (PPI) Network Analysis and Screening of Hub Genes

The PPI network was predicted using the Search Tool for the Retrieval of Interacting Genes (STRING: http://string-db.org, Version12.0)，and the Cytoscape software (Version 3.10.2) was used to screen hub genes. Here, we selected the top ten genes in the node scores as hub genes.

Single-Gene Survival Analysis, Cox Regression Analysis, and Constructing Prognostic Models

We performed single-gene survival analysis on hub genes, and statistical significance was set at $P<0.05$. We then selected genes with statistical significance for Cox regression analysis using the Wei Sheng Xin online website (https://www.bioinformatics.com.cn) to create a forest map. The key genes selected were utilized to perform risk scoring and

construct a prognostic model using R software. Receiver operating characteristic (ROC) curves were plotted to assess the model's predictive accuracy for 1-year, 3-year, and 5-year survival rates. Additionally, Kaplan-Meier (K-M) curves were generated to compare survival differences between high-risk and low-risk groups.

Statistical analysis

All statistical analyses were performed using R software or specialized online tools: R software was employed for DEGs screening and single-gene survival analysis, whereas functional enrichment analysis and Cox regression analysis were conducted using the online platform Wei Sheng Xin. P< 0.05 was considered statistically significant.

Results

Differentially Expressed Genes

After standardized processing of microarray data results, we used the Limma package to screen 1384 DEGs, including 503 up-regulated genes and 881 down-regulated genes (Figure 1). Subsequently, we intersected the 1,384 differentially expressed genes with 717 ferroptosis-associated genes that we had downloaded, resulting in the identification of 25 ferroptosis-related DEGs. These genes encompassed five up-regulated driver genes, seven up-regulated suppressor genes, two down-regulated driver genes, and 11 down-regulated suppressor genes. Notably, among the down-regulated genes, *PRKAA2* emerged as an overlapping gene between driver and suppressor categories. Based on the FerrDb score and further KEGG pathway analysis, *PRKAA2* was classified as a down-regulated driver gene (Table 1).

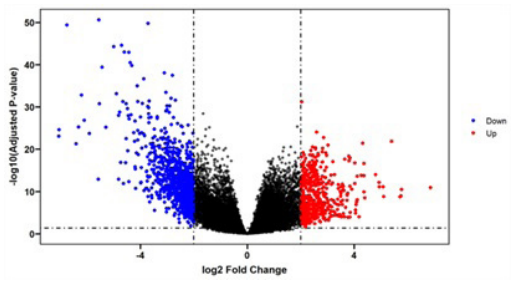

Figure 1. *Differentially expressed genes.*

Ultimately, we curated a list of 24 ferroptosis-related DEGs and visualized their expression profiles using a heatmap (Figure 2).

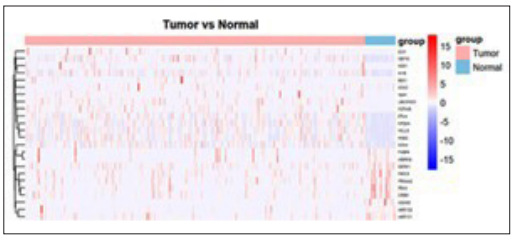

Figure 2. *Ferroptosis-related DEGs.*

Functional Enrichment Analysis of Ferroptosis-Related DEGs

Functional enrichment analysis was performed on the 24 ferroptosis-related DEGs. Through KEGG pathway analysis, we found that ferroptosis-related DEGs were mainly enriched in the chemical carcinogenesis reactive oxygen species pathway (Figure 3), including up-regulated and down-regulated genes. GO analysis results showed that the changes in BP of ferroptosis-related DEGs were significantly enriched in the fatty acid metabolic process, monocarboxylic acid biosynthetic process, cellular ketone metabolic process, fatty acid biosynthetic process, response to fatty acids, carboxylic acid biosynthetic process, organic acid biosynthetic process, response to oxygen levels, and prostanoid metabolic process.

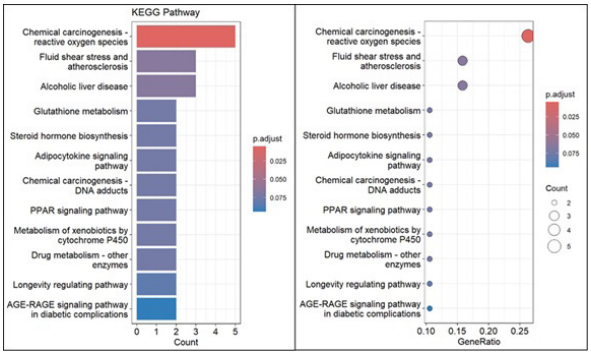

Figure 3. *KEGG pathway analysis of ferroptosis-related DEGs.*

Changes in CC were significantly enriched in the NADPH oxidase complex, oxidoreductase complex, intercellular bridge, messenger ribonucleoprotein complex, telomere cap complex, nuclear telomere cap complex, chromosome, telomeric repeat region, invadopodium, pericentric heterochromatin, and telomerase holoenzyme complex. Changes in MF were significantly enriched in oxidoreductase activity(acting on NAD(P)H), bile acid binding, monocarboxylic acid binding,

Table 1.

Ferroptosis-related DEGs.

	Up driver DEG	Up suppressor DEG	Down driver DEG	Down suppressor DEG
Gene names	*NOX1, NOX4, MYB, IDO1, HOTAIR*	*HELLS, RRM2, GDF15, KIF20A, ETV4, TERT, LINC01833*	*PRKAA2, CPEB1*	*AKR1C1, AKR1C2, FNDC5, BEX1, ASAH2, FABP4, SOX2, PDK4, ADIPOQ, GSTM1*

alditol: NADP+ 1-oxidoreductase activity, superoxide-generating NAD(P)H oxidase activity, carboxylic acid binding, organic acid binding, oxidoreductase activity(acting on NAD(P)H, oxygen as an acceptor), alcohol dehydrogenase (NADP+) activity and oxidoreductase activity(acting on the CH-CH group of donors, NAD or NADP as an acceptor) (Figure 4).

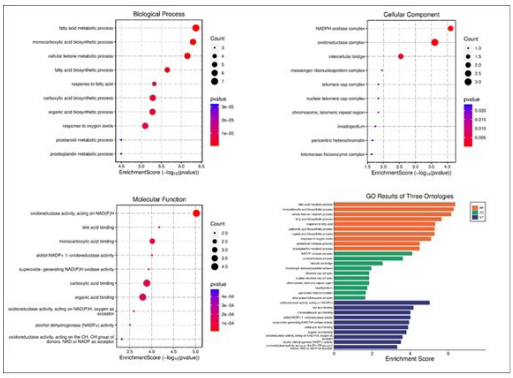

Figure 4. *GO results of three ontologies of ferroptosis-related DEGs.*

PPI Network Analysis and Hub Genes

We have constructed a PPI network comprising 24 ferroptosis-related DEGs using the STRING database. Subsequently, we employed Cytoscape software to identify the top 10 key ferroptosis-related DEGs based on their node scores. These key genes include *ADIPOQ*, *PDK4*, *FABP4*, *NOX4*, *HELLS*, *KIF20A*, *RRM2*, *FNDC5*, *PRKAA2*, and *NOX1* (Figure 5).

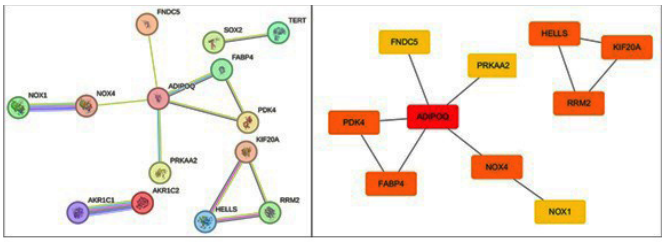

Figure 5. *PPI network and hub genes of ferroptosis-related DEGs.*

Single-Gene Survival Analysis, Cox Regression Analysis, and Prognostic Models

By conducting single-gene survival analysis on the 10 key genes, we have ultimately pinpointed four genes that are statistically significant: *FABP4*, *NOX4*, *PDK4*, and *PRKAA2* (P<0.05) (Figure 6). We further performed Cox regression analysis on these four hub genes and found that their Hazard Ratios were all < 1 (Figure 7). We constructed a prognostic model using these four key genes and plotted ROC curves. The area under the curve (AUC) values for 1-, 3-, and 5-year survival predictions were 0.587, 0.652, and 0.729, respectively. The sensitivity and specificity for 1-year prediction reached 0.53 and 0.63, 0.59 and 0.71 for 3-year, and 0.55 and 0.92 for 5-year predictions. K-M curve analysis demonstrated significantly longer survival time in the low-risk group compared to the high-risk group (P=0.0053) (Figure 8) .

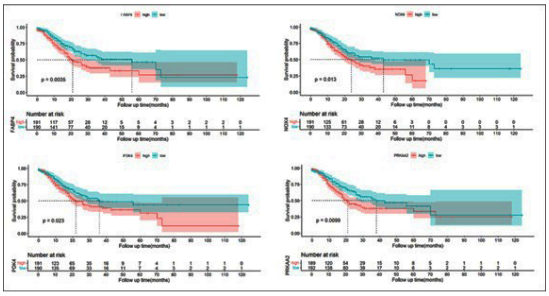

Figure 6. *Single-gene survival analysis of FABP4, NOX4, PDK4 and PRKAA2.*

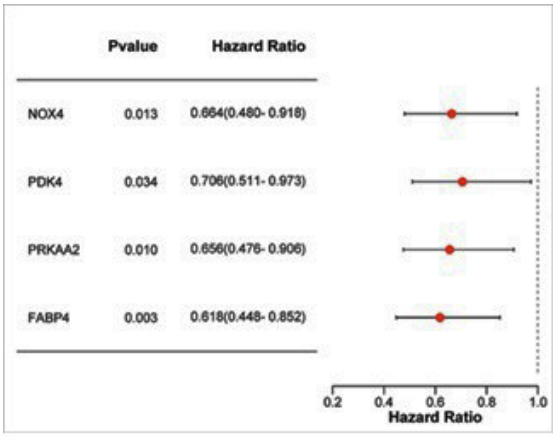

Figure 7. *Cox regression analysis of NOX4, PDK4, PRKAA2, and FABP4 genes.*

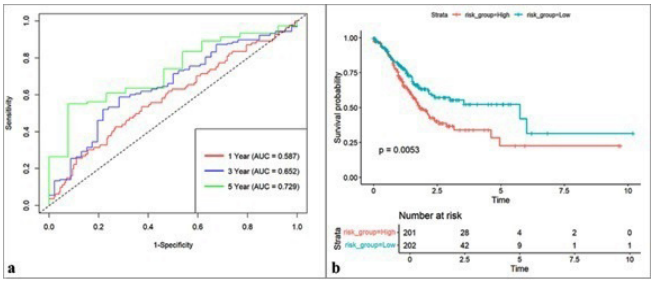

Figure 8. *(a). ROC curves and AUC values for predicting 1-year, 3-year, and 5-year survival rates; (b). Survival analysis of high-risk and low-risk groups.*

Discussion

STAD, a common and aggressive malignancy of the digestive system, holds the fifth position globally in both incidence and mortality rates. The diagnosis, treatment, and prognosis of this disease are fraught with significant challenges. As such, one of the most effective strategies in managing STAD lies in the identification of appropriate and efficacious therapeutic targets. In the past decade, ferroptosis

has been a widely studied mode of cell death. We investigated the ferroptosis-related DEGs in STAD tissues. *NOX4*, as a ferroptosis driver gene,[16,17] is highly expressed in STAD tissues, which is consistent with previous studies.[18] In our study, despite the high expression of the *NOX4* gene in STAD tissues, survival analysis revealed that patients in the high-expression group had significantly shorter survival times than those in the low-expression group, with the difference being statistically significant (P=0.013). NOX4, as a member of the Nicotinamide Adenine Dinucleotide Phosphate (NADPH) Oxidases family, primarily functions to produce ROS. These ROS participate in a multitude of physiological and pathological processes within cells, encompassing signal transduction, angiogenesis, immune responses, and cell death, thereby influencing cellular metabolism, proliferation, and apoptosis.[19] In STAD, elevated NOX4 expression may enhance the sensitivity of tumor cells to ferroptosis and facilitate its onset.[20] However, in our study, high *NOX4* expression did not lead to prolonged patient survival. On the contrary, patients in the high *NOX4* expression group had significantly shorter survival times compared to those in the low expression group. This outcome is attributed to the fact that heightened *NOX4* expression ultimately drives the progression and aggravation of STAD by modulating tumor cell metabolism, proliferation, and migration,[21,22] which is consistent with the findings of Gao et al.[23]

FABP4 and *PDK4*, as ferroptosis suppressor genes, exhibit lower expression levels in STAD tissues. Furthermore, patients in the low-expression groups of *FABP4* and *PDK4* have longer survival times compared to those in the high-expression groups, with statistically significant differences (P-values of 0.0035 and 0.023, respectively). FABP4 is a fatty acid-binding protein primarily expressed in adipocytes and macrophages. It regulates fatty acid metabolism and participates in systemic metabolic regulation through various lipid signaling pathways, being implicated in multiple diseases.[24,25] FABP4, produced by tumor endothelial cells and adipocytes in the tumor microenvironment, can induce an increase in lipid droplets within tumor cells, leading to resistance to oxidative stress and thereby avoiding the onset of ferroptosis, further contributing to tumor progression.[26] Therefore, patients with STAD who have low expression of FABP4 have a longer survival period. The study by Guo et al. demonstrated that increased expression of *FABP4* predicts poor prognosis in STAD,[27] which is consistent with our research findings. *PDK4*, as a ferroptosis suppressor gene, inhibits ferroptosis by blocking pyruvate dehydrogenase-mediated oxidation of pyruvate into the tricarboxylic acid cycle and subsequent fatty acid synthesis.[28] In both in vitro experiments and high-fat diet-induced diabetic mouse models, inhibiting *PDK4* enhances the antitumor activity of system xc-inhibitors, thereby suppressing tumor growth.[28] Therefore, patients with low *PDK4* expression have a longer survival time compared to those with high expression.

However, the other hub gene, *PRKAA2*, that we identified caused confusion during our analysis. In the data downloaded from the FerrDb V2 website, *PRKAA2* serves as both a driver and suppressor gene for ferroptosis. Its FerrDb score for the driver gene was 2, while its FerrDb score for the suppressor gene was 1. We conducted KEGG pathway analysis on *PRKAA2* through the DAVID website and found that its tumor-related pathways mainly included the PI3K-Akt signaling pathway, mTOR signaling pathway, Apelin signaling pathway, FoxO signaling pathway, and AMPK signaling pathway. The first three pathways mainly promote tumor growth through various complex mechanisms.[29-32] The FoxO signaling pathway initiates apoptosis by participating in oxidative stress-related pathways, thereby inhibiting tumor cell growth.[33-35] However, the direct role of *PRKAA2* in these four pathways is not clear, but it acts indirectly through the AMPK signaling pathway. The mechanism of action of the AMPK signaling pathway is complex. It can inhibit the growth of tumor cells by suppressing the mTOR signaling pathway.[36] DAVID pathway analysis showed that it can also synthesize eNOS through phosphorylation, catalyze the production of nitric oxide from L-arginine, and ultimately produce ROS, leading to cell death. On the other hand, AMPK can also act as an oncogene to promote the proliferation of tumor cells. [37-39] The protein encoded by *PRKAA2* is the catalytic subunit α2 of AMPK,[40] which participates in various regulatory mechanisms in the AMPK signaling pathway, including the promotion of ferroptosis.[41] Therefore, in this study, we classified *PRKAA2* as a ferroptosis driver gene. According to our single-gene Cox regression analysis, the risk ratio of the *PRKAA2* gene with low expression in STAD tissue was less than 1. We speculate that the tumor-promoting effect of the AMPK signaling pathway in STAD is greater than its inhibitory effect, and the *PRKAA2* single-gene survival analysis results showed that patients with low expression had a longer survival period, and the difference was statistically significant (P<0.01). Therefore, we believe that high expression of *PRKAA2* promotes tumor growth, whereas patients with low expression of *PRKAA2* in gastric adenocarcinoma tissues have a longer survival time compared to those with high expression.

However, the prognostic model we constructed using these four key genes yielded AUC values of only 0.587 and 0.652 for 1-year and 3-year predictions, respectively, with low sensitivity and specificity. This indicates that our prognostic model has limited ability to identify and exclude events early on. Nevertheless, the 5-year AUC value was 0.729, with sensitivity and specificity of 0.55 and 0.92, respectively. This suggests that our model has a certain predictive power for 5-year survival, although it may have insufficient ability to identify true positives, leading to missed diagnoses, it excels at excluding low-risk patients.

We have identified four ferroptosis-related DEGs associated with the prognosis of STAD, making them potential prognostic markers or therapeutic targets. However, this study lacks support from in vitro and in vivo experimental results, which is a limitation of our research. We will further validate our findings in subsequent experimental studies. Another limitation of this study is the low sensitivity of the prognostic model for 5-year survival, which results in a higher rate of missed diagnoses among high-risk patients. Therefore, a comprehensive judgment needs to be made in conjunction with other biomarkers.

Conclusion

After analyzing RNA-seq and clinical data obtained from the TCGA database, we have pinpointed four ferroptosis-related DEGs—*NOX2*, *PRKAA2*, *FABP4*, and *PDK4*—that are differentially expressed and correlated with the survival prognosis of STAD patients. Notably, patients with low expression levels of these four genes demonstrated significantly prolonged survival times, with statistical significance ($P<0.05$). Consequently, these genes hold promise as biomarkers or novel therapeutic targets for the prognosis and treatment of STAD, paving the way for innovative therapeutic strategies for patients afflicted with this malignancy.

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Competing Interests

The authors declare that they have no competing interests.

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