

A2M promotes the progression of STAD by upregulating vimentin expression and epithelial-mesenchymal transition: bioinformatics analysis and experimental verification

Wenyan Jiang^{1#}, Guowen Ma^{1,2#}, Huakai Zeng¹, Xinyue Xu¹, Junyi Liu¹, Xiaoke Wang¹, Yuyao Li¹, Xindi Huang¹, Wenjie Xu¹, Chunyan Fang¹, Wenqi Sun³, Xuejian Wang¹

¹School of Pharmacy, Shandong Second Medical University, Weifang, China; ²Department of Pharmacy, Weifang People's Hospital, Weifang, China;

³Weifang Institute of Dermatology, Weifang, China

Contributions: (I) Conception and design: Xuejian Wang, W Sun; (II) Administrative support: Xuejian Wang, C Fang; (III) Provision of study materials or patients: Xiaoke Wang, J Liu; (IV) Collection and assembly of data: H Zeng, X Xu; (V) Data analysis and interpretation: W Jiang, G Ma; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

[#]These authors contributed equally to this work.

Correspondence to: Xuejian Wang, MD. School of Pharmacy, Shandong Second Medical University, No. 7166 Baotong Road, Weifang 261053, China. Email: wangxuejian@sdsmu.edu.cn; Wenqi Sun, BS. Weifang Institute of Dermatology, No. 6708 Beihai Road, Weifang 261045, China. Email: swqfcy@126.com.

Background: Stomach adenocarcinoma (STAD) is a common malignant tumor within the digestive system, characterized by significant morbidity and mortality rates. The identification of innovative biomarkers or therapeutic targets for STAD is of utmost importance. A deeper understanding of the molecular mechanisms underlying STAD progression may facilitate the identification of novel prognostic indicators and therapeutic strategies. This investigation aims to assess the expression patterns of alpha-2-macroglobulin (A2M) across various tumors and their corresponding pathological stages, utilizing data from The Cancer Genome Atlas (TCGA) and University of Alabama at Birmingham Cancer Analysis Portal (UALCAN) databases.

Methods: To evaluate the influence of A2M on survival prognosis, we employed the Kaplan-Meier method alongside Cox and receiver operating characteristic (ROC) analysis. Additionally, Tumor Immune Estimation Resource, Version 2 (TIMER2.0) was utilized to examine its impact on the infiltration of immune cells within tumors. By employing R programming, STAD samples were divided into high-expression and low-expression groups based on A2M gene expression levels. Differentially expressed genes (DEGs) were subsequently identified, followed by enrichment analyses, including Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and gene set enrichment analysis (GSEA). We further selected STAD cells exhibiting high A2M expression and utilized CRISPR/Cas9 technology to silence A2M, to investigate its effects on cell viability, migration, invasion, and colony formation capabilities.

Results: Bioinformatics analysis indicated that A2M is highly expressed in STAD tumor tissues compared to normal gastric tissues. Patients exhibiting elevated A2M levels experienced shorter survival periods compared to those with lower expression levels, with Cox and ROC analyses suggesting A2M's potential as a prognostic biomarker. This implies that A2M plays a role in promoting STAD progression and functions as an oncogene. Pathway enrichment analyses demonstrated that A2M facilitates epithelial-mesenchymal transition (EMT) in STAD cells, showing a significant correlation with EMT marker vimentin and EMT-related genes. Furthermore, A2M exhibited a positive correlation with the infiltration of various immune cells in STAD tissues, displaying strong associations with multiple immune cell markers. A2M expression also influences the responsiveness of STAD patients to immunotherapy and small-molecule drug therapies. Cell experiments indicated that the silencing of A2M expression led to reduced STAD cell viability, migration, invasion, and colony formation, alongside a decrease in the expression of the mesenchymal marker vimentin.

Conclusions: These findings suggest that A2M promotes EMT through the upregulation of vimentin expression, thereby facilitating the malignant progression of STAD. Thus, A2M emerges as a promising

therapeutic target that warrants further investigation to refine treatment strategies and improve patient outcomes in STAD.

Keywords: Alpha-2-macroglobulin (A2M); stomach adenocarcinoma (STAD); bioinformatics; epithelial-mesenchymal transition (EMT)

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Introduction

Gastric cancer (GC) represents a prevalent form of malignancy affecting the digestive system, ranking fifth in terms of global cancer incidence and fourth in terms

of mortality rates (1). GC is a complex, progressive disease influenced by a multitude of factors, such as environmental elements (including smoking or occupational tobacco exposure), genetic predispositions, infections by *Helicobacter pylori* and Epstein-Barr virus, as well as dietary factors (notably, high consumption of smoked or high-salt foods) (2-5). Stomach adenocarcinoma (STAD) arises from the gastric mucosal epithelial glands, constituting approximately 90% of GC cases. It is marked by rapid progression, pronounced invasiveness, and malignancy (6). The treatment strategy for STAD typically involves a combination of therapies, including surgical intervention, chemotherapy, radiotherapy, molecular targeted therapy, and immunotherapy, however, the prognosis remains bleak, with a 5-year survival rate falling below 20% (7). Consequently, the identification of novel biomarkers or therapeutic targets is critical for improving the diagnosis, prevention, and management of STAD.

Alpha-2-macroglobulin (A2M) is a substantial glycoprotein predominantly produced in the liver, with plasma concentrations of 1.5 to 3.5 mg/mL, accounting for 4–10% of the total protein content in the human body. A2M has an approximate relative molecular weight of 720 kD and is composed of four identical subunits. Two subunits are interconnected by disulfide bonds to form dimers, which subsequently associate through non-covalent interactions to create a tetrameric structure. Each subunit is characterized by five functional domains: the bait region, internal thiol ester, receptor-binding domain, transglutaminase reactive site, and zinc-binding site (8). A2M functions as a broad-spectrum protease inhibitor inactivating protease molecules via a distinctive “capture” mechanism. When A2M binds to proteases, it undergoes conformational alterations, resulting in a “cage-like” structure that encases the protease, thereby inhibiting its enzymatic activity (9,10). Besides its protease-inhibiting properties, A2M binds to several key cytokines [including transforming growth factor (TGF)- β , interleukin (IL)-

Highlight box

Key findings

- Alpha-2-macroglobulin (A2M) is overexpressed in stomach adenocarcinoma (STAD) and high A2M levels robustly predict poor patient prognosis. Silencing A2M significantly inhibits STAD cell proliferation, migration, invasion, and colony formation *in vitro*. A2M promotes STAD progression by upregulating the mesenchymal marker Vimentin and driving epithelial-mesenchymal transition (EMT). A2M expression shapes the tumor immune microenvironment, correlating with specific immune cell infiltration and immune checkpoint expression.

What is known and what is new?

- STAD is a lethal malignancy with limited therapeutic options, and there is an urgent need for new biomarkers and therapeutic targets. The role of A2M, a broad-spectrum protease inhibitor, in STAD progression remains unclear.
- This study is the first to integrate bioinformatics analysis and experimental validation to comprehensively demonstrate that A2M functions as an oncogene in STAD. We newly identified that A2M drives STAD progression specifically through upregulating vimentin and activating EMT, and revealed its association with the tumor immune microenvironment and therapy response.

What is the implication, and what should change now?

- A2M is not only a robust prognostic biomarker but also a promising novel therapeutic target for STAD. Targeting A2M could potentially suppress EMT and curb tumor metastasis, while also modulating the efficacy of existing therapies.
- Future research should focus on developing and testing targeted inhibitors against A2M. Clinical studies are warranted to validate the utility of A2M as a biomarker for patient stratification, particularly in the context of immunotherapy, to guide the development of more precise and effective treatment strategies for STAD patients.

1 β , IL-6, tumor necrosis factor (TNF)- α], growth factors [such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and nerve growth factor (NGF)], and hormones, consequently modifying their biological functions. Research has demonstrated that A2M is involved in the management of various diseases such as Alzheimer's disease, sepsis, acute pancreatitis, and osteoarthritis (9).

Moreover, recent investigations have examined the function of A2M in cancer. Kurz *et al.* (11) highlighted in experiments utilizing nude mice that A2M has the capacity to inhibit tumor-promoting signaling pathways (including PI3K/AKT and SMAD), thereby modulating tumor cell adhesion, migration, and proliferation while exerting anti-tumor effects through the downregulation of miR-21 and the upregulation of PTEN. Naqa *et al.* (12) employed bioinformatics to develop a predictive model for radiation pneumonia in patients with non-small cell lung cancer undergoing radiotherapy, investigating the interactions between A2M and three additional candidate biomarkers for radiation pneumonia [angiotensin-converting enzyme (ACE), IL-6, and TGF β]. The results suggested that A2M could serve as a novel biomarker for radiation pneumonia. Lee *et al.* (13) discovered that A2M protein derived from extracellular vesicles may function as a potential biomarker for diagnosing bladder cancer. They confirmed through enzyme-linked immunosorbent assay that A2M protein expression was significantly elevated in the urinary extracellular vesicles (uEVs) of bladder cancer patients. Notably, A2M has been implicated in various cancers with context-dependent roles. For instance, it exerts tumor-suppressive effects in cholangiocarcinoma (14) and osteosarcoma (15) by inhibiting cell migration and proliferation. In contrast, in bladder cancer, it serves as a potential diagnostic biomarker (13). However, its expression pattern and biological function in STAD remain insufficiently characterized. Given the high incidence and unfavorable prognosis of STAD, there is an urgent need to identify novel molecular targets. This study aims to elucidate the role of A2M in STAD progression and its underlying mechanisms, with a focus on epithelial-mesenchymal transition (EMT) regulation.

In the present study, we conducted a bioinformatics analysis to evaluate the expression level and prognostic significance of A2M in STAD, followed by functional validation through cell experiments. We utilized The Cancer Genome Atlas (TCGA) and University of Alabama at Birmingham Cancer Analysis Portal (UALCAN)

databases to analyze A2M expression in STAD and assess its correlation with various clinical pathological characteristics. The prognostic value of A2M was predicted using Kaplan-Meier analysis, while Tumor Immune Estimation Resource, Version 2 (TIMER2.0) was employed to investigate its influence on immune cell infiltration in STAD tissues. Finally, we undertook cellular experimentation to elucidate the involvement of A2M in the progression of STAD, with the intent of offering novel perspectives for its diagnosis, therapeutic strategies, and prognostic evaluations. We present this article in accordance with the MDAR reporting checklist (available at <https://tcr.amegroups.com/article/view/10.21037/tcr-2025-aw-2449/rc>).

Methods

Bioinformatics

To discover genes related to STAD, we obtained a total of 415 tumor samples alongside 34 adjacent normal tissue samples from TCGA data portal (<https://cancergenome.nih.gov>). The expression levels of A2M were evaluated using the UALCAN database (<http://ualcan.path.uab.edu>), while the analysis of A2M expression across different immune subtypes in STAD was conducted via TIMER2.0 (<http://timer.cistrome.org/>). Patients were stratified into high and low A2M expression groups based on the median expression value for A2M. Differentially expressed genes (DEGs) were identified using thresholds of $|\log_2$ fold change (FC)| >1 and P-value <0.05. Kaplan-Meier survival curves were generated with the “survival” R package to compare overall survival rates between high- and low-risk patient groups. Furthermore, receiver operating characteristic (ROC) analysis was executed, and ROC curves were constructed to evaluate the prognostic model's efficacy in predicting the survival duration of STAD patients. Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and gene set enrichment analysis (GSEA) were conducted utilizing R package to explore the potential functions and molecular mechanisms of A2M in STAD. Additionally, the correlations between A2M and other genes, A2M and immune cell infiltration, and A2M and therapy response were assessed using R packages.

TCGA was selected due to its large-scale multi-omics datasets and comprehensive clinical annotations, making it suitable for survival and expression analyses. UALCAN was used for its user-friendly interface and its support of stratified analyses based on clinicopathological features.

However, limitations include the predominance of Western population data and the lack of protein-level validation in the TCGA cohort. To address this, we supplemented our analysis with immunohistochemistry images from the Human Protein Atlas and conducted *in vitro* experiments to validate the key findings. The study was conducted in accordance with the Declaration of Helsinki and its subsequent amendments.

Cell culture and reagents

The human STAD cell lines AGS (SCSP-5262, Cell Bank of the Chinese Academy of Sciences, Shanghai, China), BGC-823 (C6123, Beyotime Biotechnology, Shanghai, China), HGC-27 (SCSP-5263, Cell Bank of the Chinese Academy of Sciences), MKN-45 (SCSP-5473, Cell Bank of the Chinese Academy of Sciences), and SGC-7901 (TCHu46, Cell Bank of the Chinese Academy of Sciences) were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS). The cells were maintained at 37 °C in a humidified environment with 5% CO₂. All cell lines were obtained from Weifang People's Hospital, Weifang, China. gRNA was synthesized by Shanghai GenePharma.

CRISPR/Cas9-mediated A2M silencing

Two gRNAs were designed, targeting the following sequences: (I) TAAGAAGCGGACCACAGTGA; and (II) CTGCCACGGTGAAGATTCAC. The LentiCRISPR v2 plasmid underwent digestion with BsmBI and subsequent ligation with the synthesized gRNAs. The resulting constructs were co-transfected with psPAX2 and pCMV-VSV-G into 293T cells to generate lentiviral particles. The STAD cells were subsequently infected and selected using puromycin to establish stable A2M-silenced cell lines.

Western blot

This assay was performed following a previously established protocol (16). A total of 30 µg of protein from each lysate was applied to either 10% or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), subsequently transferring the proteins onto polyvinylidene fluoride (PVDF) membranes (Catalog No. IPVH00010, Millipore, Burlington, USA). The membranes were subjected to a blocking step using bovine serum albumin (BSA) and then incubated with A2M primary antibody (Catalog No. 13545-1-AP, Proteintech, Wuhan, China)

diluted at 1:1,000. Following a washing step, HRP-conjugated secondary antibodies (Catalog No. SA00001-2, Proteintech) were applied at a dilution of 1:3,000. After further washing with TBST, the bound antibodies were detected using enhanced chemiluminescence (ECL, Cat. WBKLS0050, Millipore).

Cell viability assay

A total of 1×10^3 cells per well were cultured in a 96-well plate. At 24 h intervals, the cells were treated with 10 µL of CCK8 reagent (Cat. C0037, Beyotime Biotechnology) and allowed to incubate for an additional 2 h. The optical density (OD) at 450 nm was subsequently measured using a plate reader (M5, MD). The OD values reported represent the average of three replicates for each well.

Cell migration assay

A total of 4×10^4 cells suspended in serum-free RPMI-1640 medium were introduced into the upper chamber of a Transwell apparatus (Corning, 8-µm pore size; Corning, NY, USA), while the lower chamber was filled with 500 µL of RPMI-1640 supplemented with 10% FCS. Following a 24 h incubation period, the Matrigel on the upper surface of the membrane was carefully removed, along with the non-migrated cells. The cells that had migrated to the lower side were subsequently fixed using methanol and stained with a solution of crystal violet at a concentration of 1 mg/mL. Images of the invaded cells were captured using a microscope (IX81, Olympus, Tokyo, Japan).

Cell invasion assay

The invasion assay was conducted in accordance with previously established protocols (17). Initially, Matrigel (Catalog No. 356234, BD Biosciences, Franklin Lakes, USA) was diluted in a ratio of 1:19 using serum-free RPMI-1640. Subsequently, 50 µL of this solution was employed to coat the upper chamber of a Transwell insert (Corning, with an 8-µm pore size; Corning) for a duration of 2 h. Following this, 4×10^4 cells suspended in serum-free RPMI-1640 were introduced into the upper chamber, while the lower chamber was filled with 500 µL of RPMI-1640 containing 10% FCS. After a 24 h incubation period, the matrigel remaining on the upper surface of the membrane was carefully removed, along with the non-invading cells. The cells that had migrated to the lower side of the membrane

were subsequently fixed using methanol and stained with a 1 mg/mL solution of crystal violet dye. Images of the invaded cells were captured using a microscope (IX81, Olympus).

Colony formation assay

This experiment was performed following a previously established protocol (16). A total of 1,000 cells were seeded in each well of a six-well plate with 2 mL of medium. The experiment was concluded once each colony developed to a size exceeding 50 cells. The cells were subsequently washed twice with phosphate-buffered saline (PBS) and fixed in methanol for a duration of 10 min. Then the cells were stained with 1% crystal violet for 5 min. Lastly, the colonies were rinsed with PBS, and images were captured using a microscope (IX81, Olympus).

Statistical analysis

All statistical analyses pertaining to bioinformatics and R packages were performed using R software version 4.4.1. The data are expressed as mean $\pm$ mean of standard error (SEM), and the analysis was conducted utilizing GraphPad Prism 6.0 statistical software. The student's *t*-test was employed to assess differences between two groups, with significance levels defined as $P < 0.05$. All cell experiments were repeated at least three times in biological replicates.

Results

Abnormal transcription of A2M in various tumors

The complete workflow was shown in *Figure 1*. Firstly, we extracted clinical data for STAD patients from the TCGA database, comprising 285 male and 158 female patients, with a median age of 65.7 years. Comprehensive details are available in *Table S1*. Subsequent analyses of gene expression and survival primarily employed TCGA STAD data. Additionally, we assessed the expression levels of A2M in various tumors utilizing the UALCAN database. The findings revealed an upregulation of A2M in STAD, glioblastoma (GBM), kidney renal clear cell carcinoma (KIRC), and thyroid carcinoma (THCA), while a downregulation was observed in bladder urothelial carcinoma (BLCA), breast invasive carcinoma (BRCA), cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC), cholangiocarcinoma (CHOL), colon adenocarcinoma (COAD), kidney chromophobe

(KICH), kidney renal papillary cell carcinoma (KIRP), liver hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), pancreatic adenocarcinoma (PAAD), rectum adenocarcinoma (READ), and uterine corpus endometrial carcinoma (UCEC), with these differences achieving statistical significance (*Figure 2*).

Upregulation of A2M expression in STAD correlates with poor prognosis

We examined the transcriptional levels of A2M mRNA in STAD via the UALCAN database. As illustrated in *Figure 3A*, the A2M mRNA levels in the tumor group were significantly elevated when compared to the normal group. In an analysis of paired gastric adenocarcinoma and adjacent non-tumor tissues, a majority (21/32) of the tumor samples exhibited higher mRNA levels relative to their normal counterparts, which was statistically significant (*Figure 3B*). Furthermore, we downloaded immunohistochemistry images of A2M expression in both normal and GC tissues from The Human Protein Atlas. The results confirmed that A2M expression was markedly higher in GC tissue compared to normal tissue (*Figure 3C*). Meanwhile, we analyzed the single-cell sequencing data (GSE183904) of normal and GC tissues. Following data quality control (*Figure 3D*), dimensionality reduction, and clustering, we identified distinct cell populations of normal tissues (*Figure 3E*) and GC tissues (*Figure 3F*). Subsequently, we compared the expression levels of the A2M gene between epithelial cells in normal tissues and tumor cells in GC tissues. The findings revealed that both the number of positive cells and the average expression level of A2M gene in tumor cells were higher than those in normal epithelial cells (*Figure 3G, 3H*). Consistent results were also obtained from the single-cell sequencing analysis of the GSE112302 dataset (data not shown). Subsequently, we explored the influence of A2M transcription levels on survival outcomes for STAD patients. Kaplan-Meier curve analysis indicated that patients with elevated A2M transcription exhibited significantly poorer survival compared to those with lower transcription levels, with pronounced significant differences (*Figure 3I*). Univariate Cox analysis identified A2M, age, grade, and stage as significant factors influencing STAD patient survival ($P < 0.05$). Multivariate Cox analysis further indicated that Age and Stage were notably correlated with patient survival ($P < 0.05$), whereas A2M did not demonstrate a statistically significant relationship with the survival outcomes ($P = 0.06$, *Figure 3J*). Lastly, ROC curve analysis

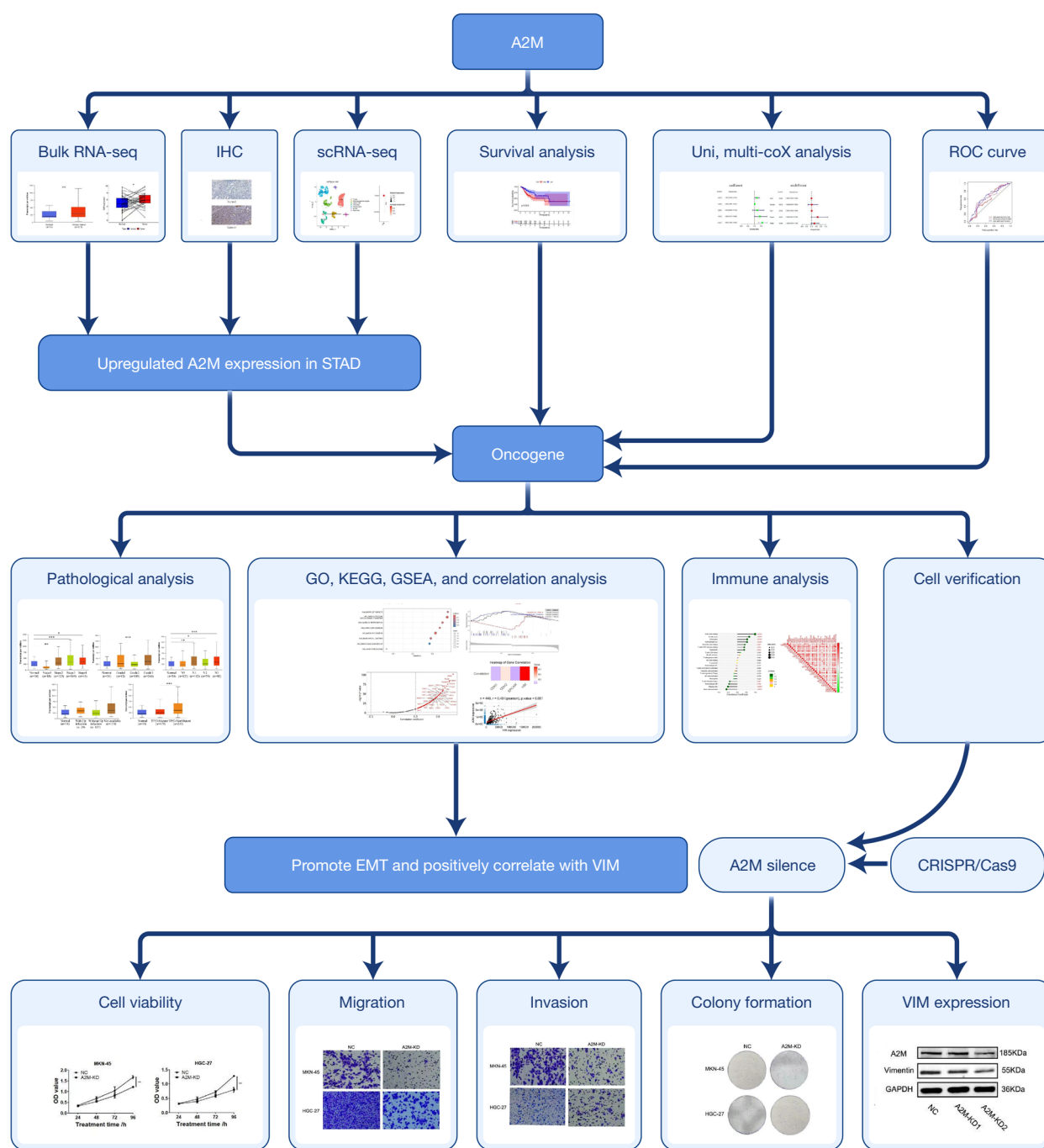


Figure 1 Schematic diagram of the present study. A2M, alpha-2-macroglobulin; EMT, epithelial-mesenchymal transition; GO, Gene Ontology; GSEA, gene set enrichment analysis; IHC, immunohistochemistry; KEGG, Kyoto Encyclopedia of Genes and Genomes; ROC, receiver operating characteristic; STAD, stomach adenocarcinoma; VIM, vimentin.

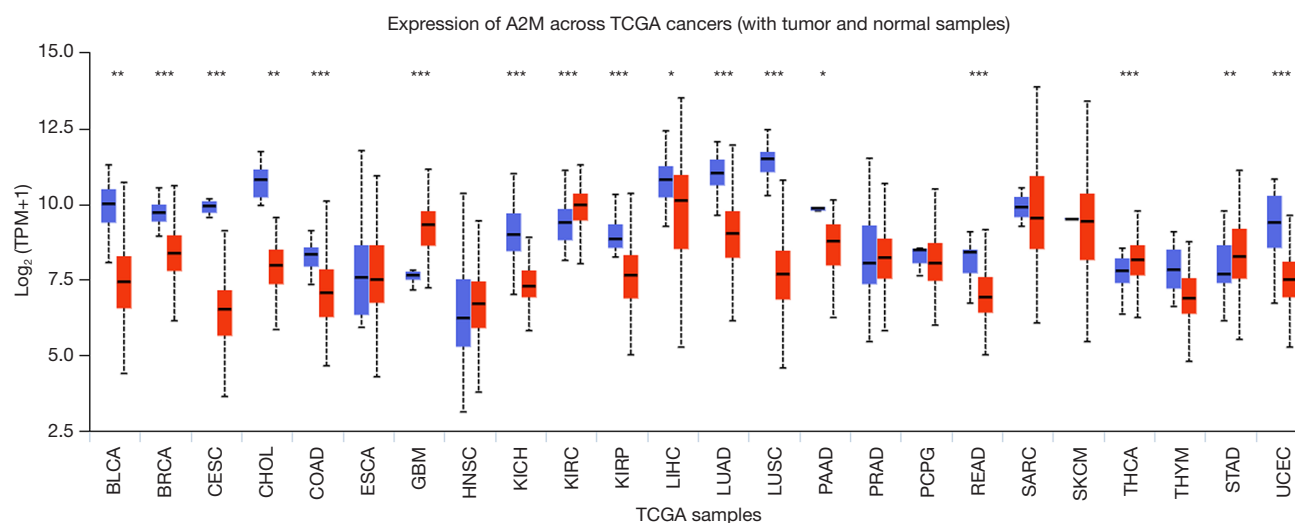


Figure 2 A2M mRNA expression in pan-cancer. Blue: normal tissue. Red: tumor tissue. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. A2M, alpha-2-macroglobulin; TCGA, The Cancer Genome Atlas; TPM, transcript per million.

was conducted to evaluate 1-, 3-, and 5-year survival, with the 1-year ROC curve yielding the highest area under the curve (AUC) value of 0.663, thus suggesting the diagnostic potential of A2M in STAD (Figure 3K).

Correlation between A2M expression and clinicopathological features in STAD

We further analyzed the differences in A2M mRNA transcription levels across different STAD patient groups using the UALCAN database. The data, presented in Figure 4A, indicated that A2M mRNA levels in stages 2, 3, and 4 were significantly higher than those in the normal group. In Grade 3 tumors, A2M mRNA levels surpassed those in the normal group, exhibiting high statistical significance (Figure 4B). Moreover, A2M mRNA levels in N1, N2, and N3 classifications were also higher than those in the normal group, with statistically significant differences (Figure 4C). These observations imply that A2M expression correlates positively with tumor malignancy. Notably, no significant differences were identified in A2M transcription levels in STAD tissues from patients infected with *H. pylori* (Figure 4D), suggesting a lack of association between A2M expression and *H. pylori* infection. Nevertheless, the transcriptional levels of A2M were markedly elevated in the TP53 non-mutated group when compared to the mutated group (Figure 4E). This observation implies that a mutation in TP53 may lead to a reduction in A2M mRNA transcription levels. Furthermore, a comprehensive analysis

using the R package ComplexHeatmap demonstrated that A2M expression was significantly associated with race, stage, grade, and T (Figure 4F). These findings collectively suggest that A2M expression plays a critical role in the malignant progression of STAD.

A2M function and pathway enrichment analysis

Using R programming, STAD samples from the TCGA were categorized into high- and low A2M expression groups. DEGs were identified through the limma package, with 1,326 genes that were upregulated and 1,501 genes that were downregulated (table available at <https://cdn.amegroups.cn/static/public/tcr-2025-aw-2449-1.xls>). These DEGs served as a basis for subsequent enrichment analysis. The GO analysis revealed significant enrichment of processes involving extracellular matrix (ECM) organization and intermediate filament organization (Figure 5A), both of which are closely linked to EMT and cytoskeletal remodeling during tumor metastasis. KEGG pathway analysis further identified enrichments of ECM-receptor interaction, focal adhesion, and cell adhesion molecules (Figure 5B), which are pathways known to regulate tumor cell adhesion, migration, and invasion in GC (18). GSEA demonstrated that the HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION gene set was significantly enriched in the high A2M expression group (Figure 5C, 5D), directly supporting the involvement of A2M in EMT activation. Collectively, these findings indicate that

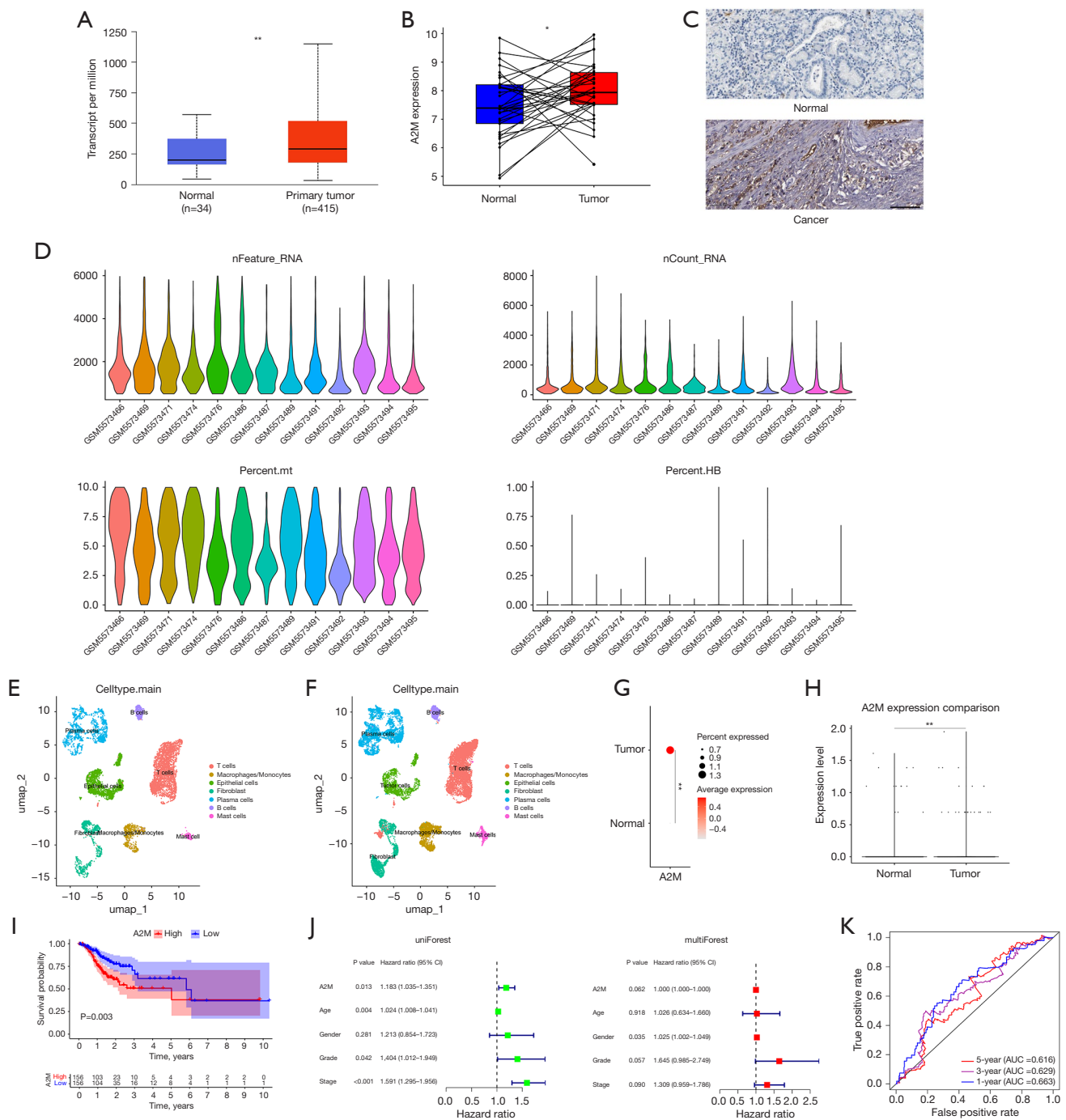


Figure 3 Integrated analysis of A2M in STAD patients. (A) A2M mRNA expression levels in normal gastric tissues versus STAD tissues from TCGA cohort. (B) The mRNA expression of A2M in gastric cancer and paracancerous tissues. (C) Immunohistochemistry (scale bar: 100 μm) image of A2M in stomach downloaded from the Human Protein Atlas website (normal: image available from <https://www.proteinatlas.org/ENSG00000175899-A2M/tissue/stomach#img>; cancer: image available from https://images.proteinatlas.org/2265/7606_B_2_3.jpg). (D-H) Single-cell sequencing data were subjected to quality control, dimensionality reduction, and clustering to analyze the expression of A2M gene. (I) Kaplan-Meier curve analysis of the overall survival rate of high-risk and low-risk groups. (J) Cox analysis forest plot. (K) ROC curve analysis of the prognostic model for predicting the survival of STAD patients at 1, 3, and 5 years. *, $P < 0.05$; **, $P < 0.01$. A2M, alpha-2-macroglobulin; AUC, area under the curve; HB, hemoglobin; ROC, receiver operating characteristic; STAD, stomach adenocarcinoma; TCGA, The Cancer Genome Atlas; umap, uniform manifold approximation and projection.

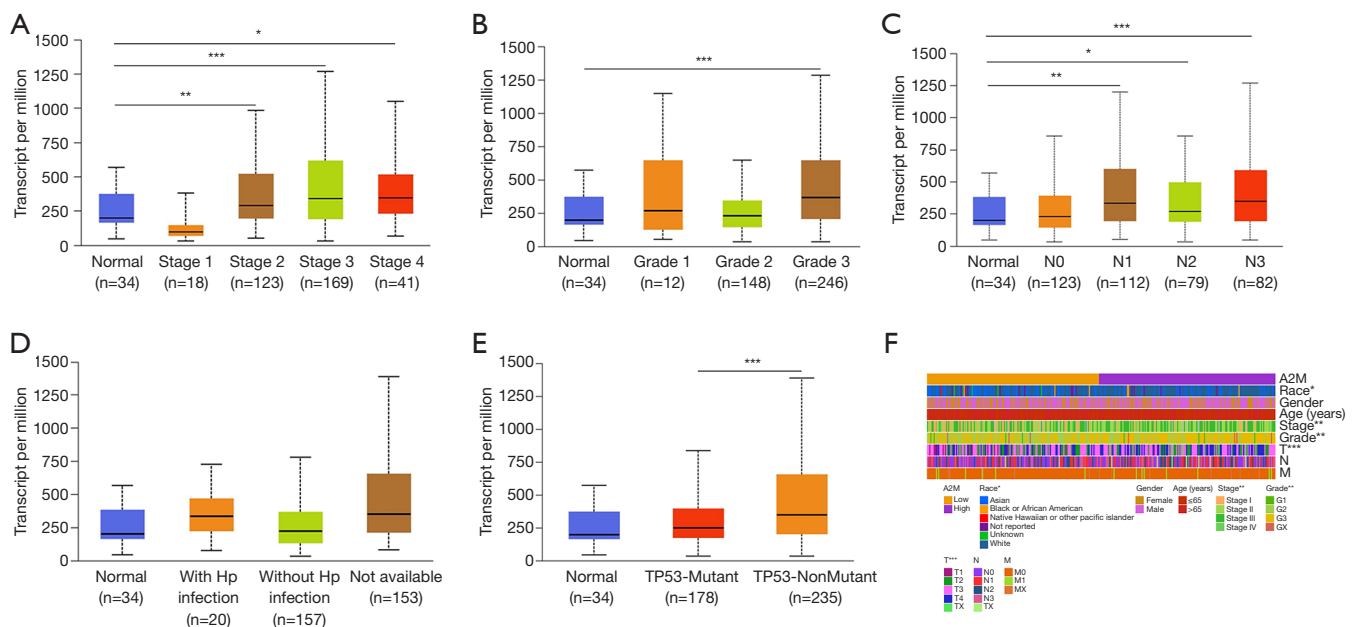


Figure 4 Expression of A2M in different STAD patient groups. The mRNA expression of A2M among the stages (A), grades (B), tumor nodal metastasis (C), with or without Hp infection (D), TP53 mutation (E) in the TCGA cohort, and a comprehensive analysis using the R package ComplexHeatmap (F). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. A2M, alpha-2-macroglobulin; Hp, *H. pylori*; M, metastasis; N, node; STAD, stomach adenocarcinoma; T, tumor; TCGA, The Cancer Genome Atlas.

A2M may influence the malignant progression of STAD by modulating EMT.

Subsequently, we retrieved EMT-related genes from the GSEA database and conducted a Pearson correlation analysis between A2M and EMT-related genes using R programming. The analysis revealed that 118 out of 200 genes exhibited correlation coefficients > 0.3 with A2M, with the strongest correlation observed for LAMA2, which had a correlation coefficient of 0.8 (Figure 5E, table available at <https://cdn.amegroups.cn/static/public/tcr-2025-aw-2449-2.xlsx>). Additionally, we examined the correlation between A2M and EMT markers, finding a positive correlation with mesenchymal markers (Vimentin and N-cadherin/CDH2) and a negative correlation with epithelial markers (Epcam and E-cadherin/CDH1), with a correlation coefficient of 0.49 with Vimentin (Figure 5F, 5G, Table S2). These results further support the notion that A2M facilitates STAD cell metastasis through EMT modulation.

A2M and immune cell infiltration

We investigated the association between A2M expression and immune cell infiltration in STAD tissues utilizing R

and the TIMER2.0 database. As depicted in Figure 6A, the expression level of A2M significantly affected the tumor microenvironment, with significant differences in Stromal Score, Immune Score and ESTIMATE Score. A2M expression exhibited a positive correlation with the infiltration of resting mast cells, naive B cells, monocytes, M2 macrophages, resting dendritic cells, resting CD4 memory T cells and eosinophils, with statistically significant differences. Conversely, A2M expression demonstrated a negative correlation with the infiltration of follicular helper T cells, M0 macrophages, plasma cells and activated mast cells, which was also statistically significant (Figure 6B, 6C). Analysis of immune cell markers indicated significant correlations with A2M expression, such as CCR7 (0.245), ETS1 (0.557), ITK (0.263), RGS1 (0.285), SAMSN1 (0.281), SELL (0.3), TRAT1 (0.267) for CD4⁺ T cells; CD3E (0.23), CD3G (0.282), CD8A (0.226), CD37 (0.263), CD69 (0.319), GZMK (0.292), IL2RB (0.252), MPZL1 (0.248), PIK3IP1 (0.55), ZAP70 (0.208) for CD8⁺ T cells; CYBB (0.348), FCRL5 (0.201), HDAC9 (0.216), HVCN1 (0.346), NCF1 (0.205), P2RY10 (0.308), SP100 (0.275), TAGAP (0.363), TXNIP (0.498), ZCCHC2 (0.236) for B cells; AIF1 (0.361), BASP1 (0.263), CCL14 (0.34), CD4

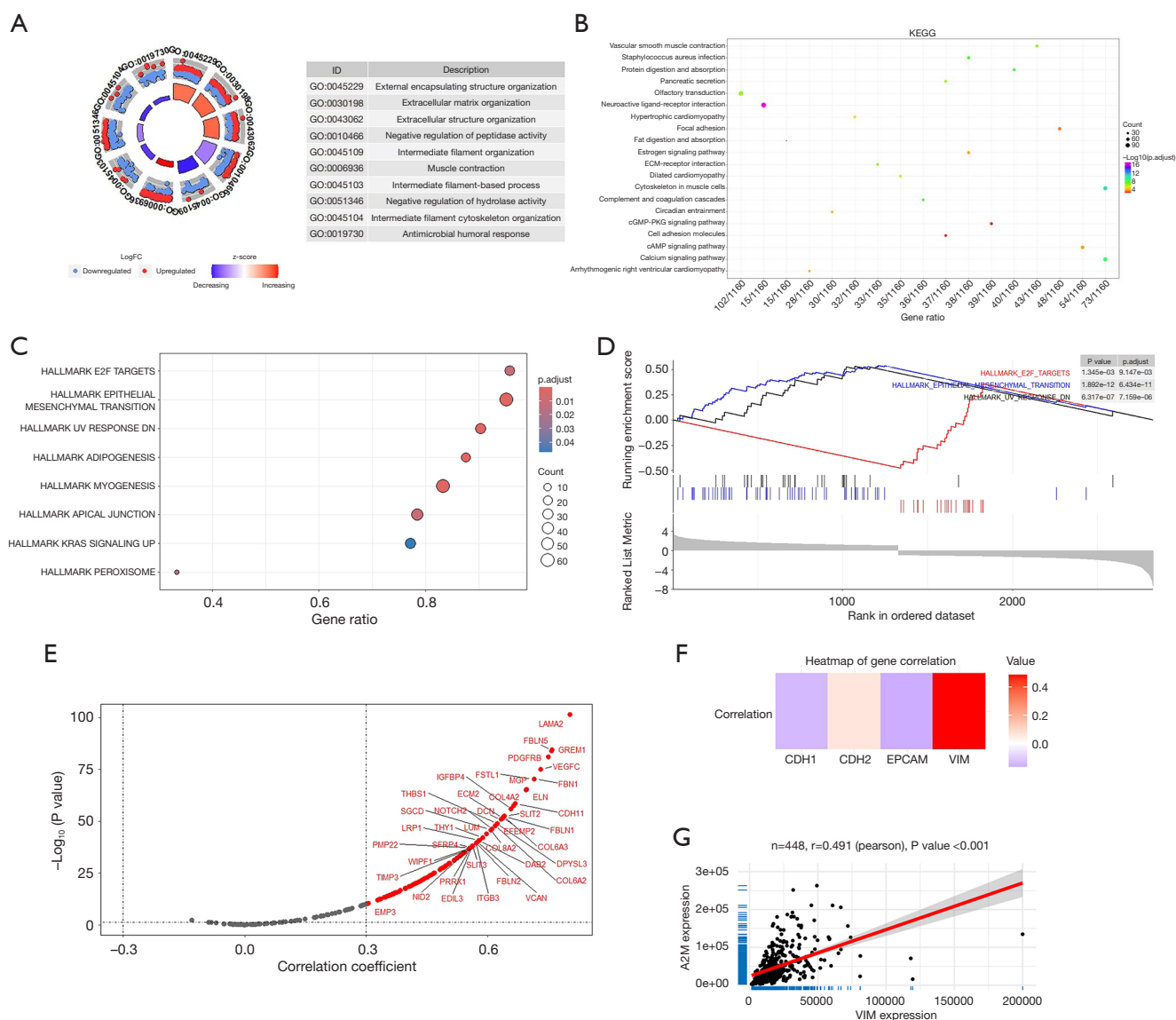


Figure 5 Functional enrichment and correlation analysis. (A,B) GO and KEGG pathway enrichment analysis for the DEGs in STAD. (C,D) GSEA for A2M in STAD. (E-G) Correlation analysis of A2M in STAD. A2M, alpha-2-macroglobulin; DEG, differentially expressed gene; GO, Gene Ontology; GSEA, gene set enrichment analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes; STAD, stomach adenocarcinoma; FC, fold change; VIM, vimentin.

(0.405), CD300LB (0.524), CNR1 (0.355), CNR2 (0.228), CRYBB1 (0.379), FES (0.369), FPR1 (0.336), FPR2 (0.215), FRMD4A (0.556), HNMT (0.3), HRH1 (0.344), IGSF6 (0.307), MS4A2 (0.563), NPL (0.216), SLC15A3 (0.236) for macrophages; CHST15 (0.303), CREB5 (0.301), CXCR1 (0.205), STEAP4 (0.431), TNFRSF10C (0.209) for neutrophils; and CCR2, 0.418; CD2, 0.251; CD14, 0.36; CD86, 0.336; CXCR4, 0.34; FCGR2A, 0.408; FCGR2B

(0.419), FCGR3A (0.228), GPSM3 (0.267), IL4R (0.437), IL18BP (0.236), ITGAL (0.3), ITGAM (0.328), PARVG (0.347), PSAP (0.334) for myeloid-derived suppressor cells (MDSCs) (Figures S1-S6). At the same time, we analyzed the correlation between A2M expression and immune checkpoints. As shown in Figure 6D, A2M expression was positively correlated with a large number of immune checkpoints. These findings suggest that A2M may play a

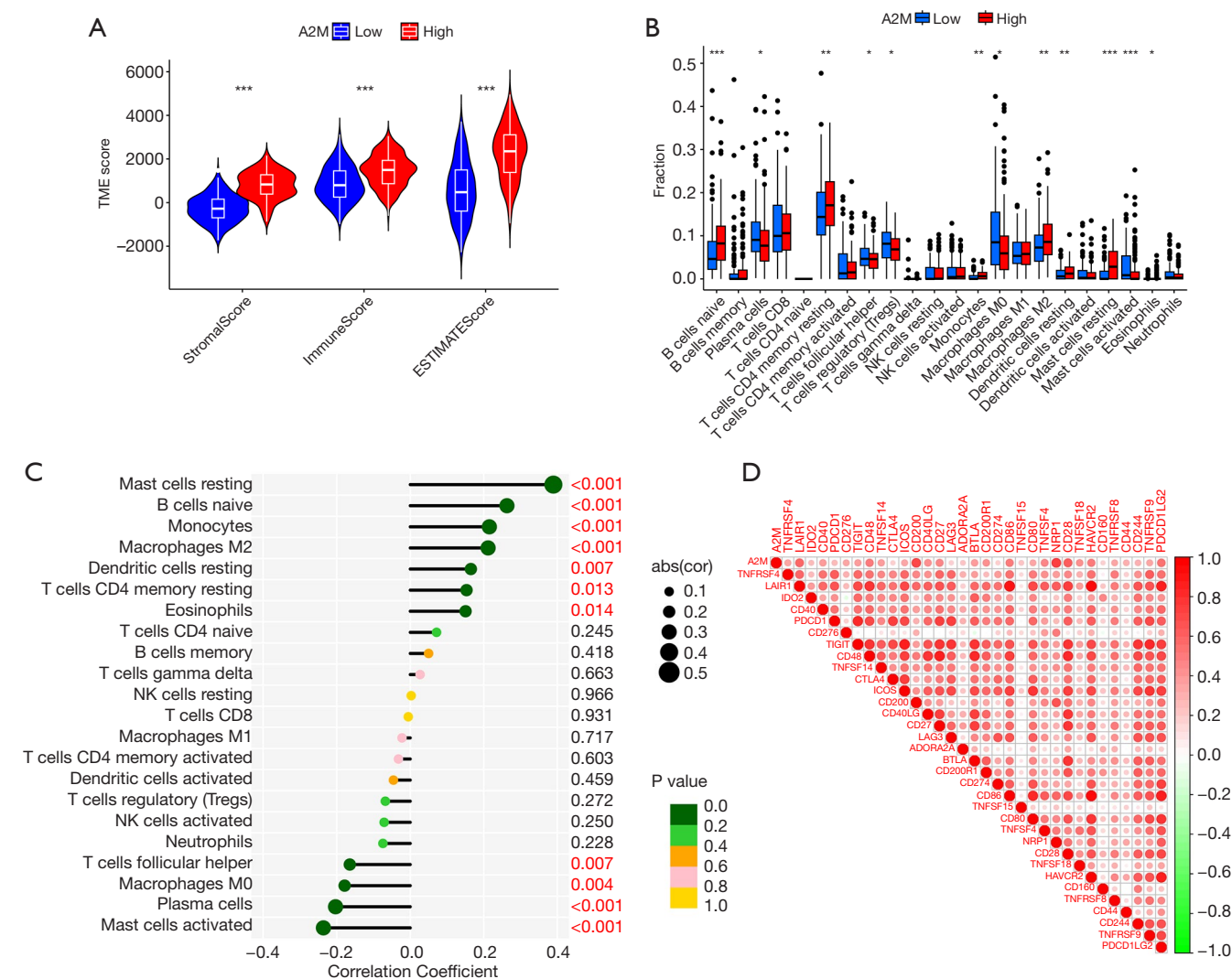


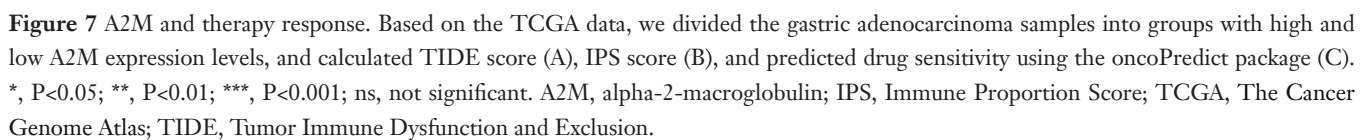
Figure 6 Correlation of A2M mRNA expression and immune cell infiltration. (A) A2M expression and immunoscore; (B,C) bar charts and correlation plots of A2M expression and immune cell infiltration; (D) correlation between A2M expression and immune checkpoints. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. A2M, alpha-2-macroglobulin; TME, tumor microenvironment.

crucial role in facilitating tumor immune escape.

A2M and therapy response

Based on TCGA data, we divided gastric adenocarcinoma samples into high and low A2M expression groups. We then analyzed their immune microenvironment and drug sensitivity. Regarding immune evasion, the A2M high-expression group showed significantly higher Tumor Immune Dysfunction and Exclusion (TIDE) scores (Figure 7A), indicating that A2M is associated with the inhibition of anti-tumor immune response in the tumor

microenvironment, thereby facilitating immune evasion by tumor cells. In terms of immunotherapy, the A2M high-expression group had significantly higher Immune Proportion Score (IPS) for cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) antibody alone (Figure 7B), suggesting that high A2M expression may serve as a positive predictive marker for response to CTLA-4 inhibitors, and gastric adenocarcinoma patients with high A2M expression might achieve better therapeutic outcomes. In the drug sensitivity analysis, the A2M high-expression group showed significantly higher predicted sensitivity to multiple targeted and chemotherapeutic drugs (Figure 7C),



Silencing A2M reduces STAD cell growth, migration, and invasion

Following our bioinformatics analysis, we proceeded to evaluate the effects of A2M silencing on the viability, migration, and invasion of STAD cells. Initially, we measured the expression levels of A2M protein across five STAD cell lines. Notably, A2M protein levels were highest in MKN-45 and HGC-27 cells (*Figure 8A*), which were

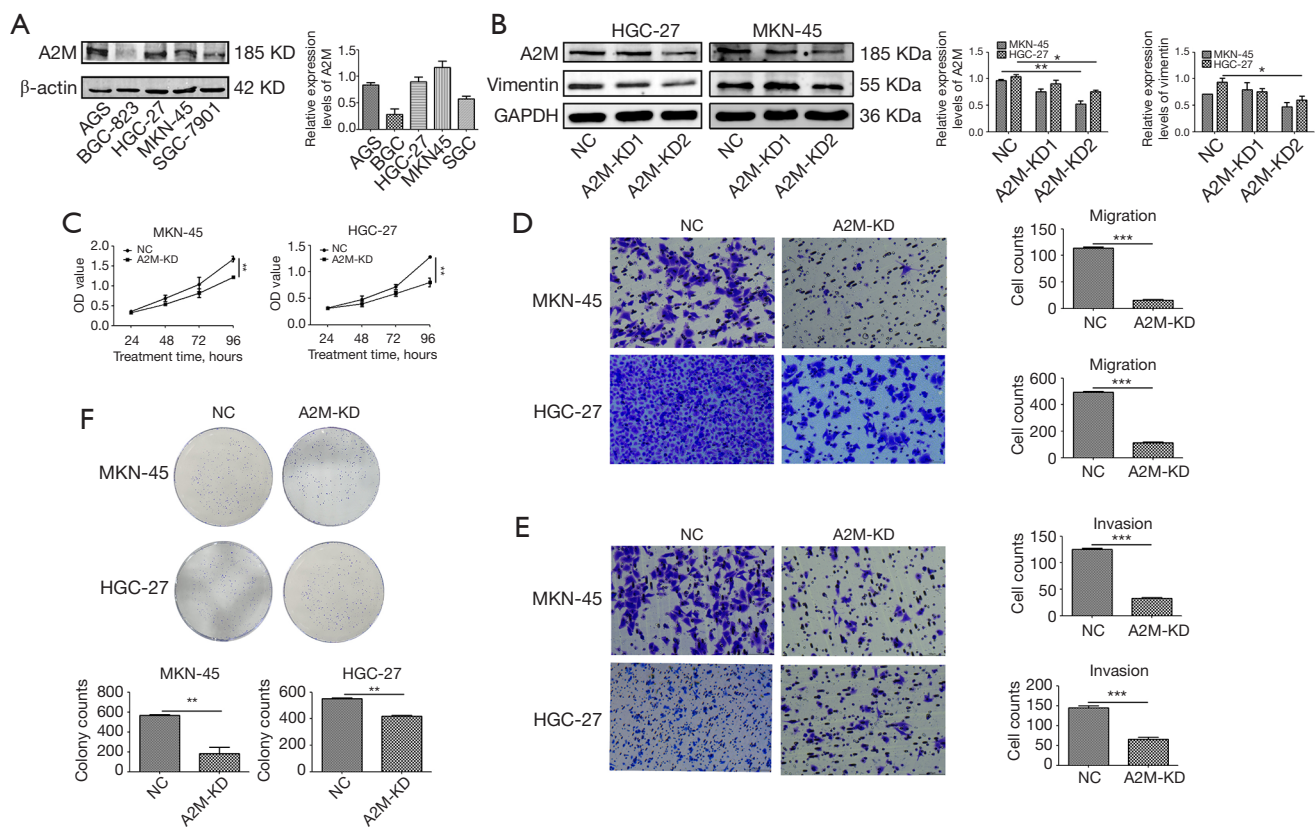


Figure 8 Silencing A2M expression suppresses the viability, migration, invasion, and colony-forming ability of STAD cells. (A) Five types of STAD cells were subjected to WB to detect A2M expression. (B) After silencing A2M expression in STAD cells using CRISPR/Cas9 technology, WB was performed to detect the expression of A2M and Vimentin. Cell viability (C), migration (D; crystal violet, $\times 400$), invasion (E; crystal violet, $\times 400$), and colony formation (F) were assessed in both control cells and A2M-silenced cell lines. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. A2M, alpha-2-macroglobulin; KD, knockdown; NC, negative control; STAD, stomach adenocarcinoma; WB, western blot.

subsequently chosen for CRISPR/Cas9-mediated silencing experiments. Among the gRNAs tested, the second gRNA exhibited superior silencing efficiency (Figure 8B) and was selected for further experimentation. Our results demonstrated that the silencing of A2M expression led to a substantial decrease in STAD cell viability, migration, invasion, and colony formation (Figure 8C-8F), with differences that were statistically significant. Moreover, A2M silencing was found to reduce Vimentin expression, aligning with the bioinformatics prediction. These findings imply that A2M may modulate the EMT in STAD cells through the regulation of Vimentin expression. Thus, A2M appears to function as an oncogene in STAD, with its silencing mitigating malignant progression.

Discussion

In this study, we utilized bioinformatics methodologies alongside cellular experiments to explore the expression characteristics of the A2M gene in STAD and its association with immune cell infiltration and patient prognosis. Our analysis revealed that A2M is overexpressed in STAD and is correlates with unfavorable prognosis outcomes for patients. Furthermore, we observed that A2M may facilitate the migration of STAD cells by promoting EMT, and its expression positively correlates with the infiltration of various immune cells. These observations suggest that A2M functions as an oncogene in STAD, and its suppression may hinder malignant progression.

A2M is a protein widely present in plasma, primarily known for its ability to bind and inhibit various proteases, thus playing a crucial role in both physiological and pathological processes, including inflammation (19), cardiovascular diseases (20), and osteonecrosis (21). While previous studies have indicated that A2M may exhibit tumor-suppressive properties in different cancer types (14,22), such as inhibiting migration in canine osteosarcoma cells (15) and impairing cell adhesion, migration, and proliferation in naked mole-rat cells through the PI3K/AKT signaling pathways (11), our current study indicates a contrasting oncogenic role for A2M in STAD. This functional divergence could stem from the heterogeneity of the tumor microenvironment, the interaction of A2M with various signaling pathways, and the tissue-specific expression patterns.

Our enrichment analyses revealed that A2M is significantly associated with several key pathways involved in tumor metastasis and EMT. Specifically, the pronounced enrichment of ECM-related processes and focal adhesion pathways aligns with the established role of the tumor microenvironment in promoting cancer cell dissemination (18). ECM-receptor interactions and focal adhesions are critical for integrating extracellular signals which drive cytoskeletal reorganization and cell motility—key features of EMT (23). Furthermore, the positive correlation between A2M and vimentin, and its negative correlation with E-cadherin, further supports a mesenchymal shift in A2M-high STAD cells. Vimentin is a well-established driver of EMT and metastasis in gastric and other cancers (24,25). Moreover, the enrichment of KRAS signaling and E2F targets suggests that A2M may also contribute to proliferation and cell cycle progression, which are hallmarks of aggressive tumors. These findings collectively suggest that A2M is a potential master regulator of EMT in STAD, consistent with recent reports linking A2M to cytoskeletal dynamics and metastatic behavior in other malignancies (11,15).

Our study shows that A2M is highly expressed in STAD tissues and correlates with poor patient prognosis. Additionally, the silencing of A2M expression was shown to significantly diminish the viability, migration, invasion, and colony formation of STAD cells. These findings suggest that A2M may be a promoter of STAD progression by influencing critical intracellular signaling pathways. Specifically, we observed a close relationship between A2M and EMT, with A2M demonstrating a significant positive correlation with multiple EMT-related genes,

and underscoring the importance of EMT in tumor cell metastasis (26,27). Importantly, the positive correlation between A2M and Vimentin observed in bioinformatics analysis ($R=0.49$, $P<0.001$) was consistent with Western blot results showing reduced Vimentin protein levels upon A2M silencing in STAD cells. In triple-negative breast cancer and non-small cell lung cancer, Vimentin is recognized as a key promoter of EMT and tumor metastasis (25). These findings reinforce the hypothesis that A2M promotes EMT via Vimentin upregulation and validate the reliability of our bioinformatics predictions. Consequently, these results suggest that A2M may promote EMT through the upregulation of Vimentin expression, thereby enhancing the metastatic potential of STAD cells, and thus contributing to tumor malignancy.

Beyond its direct influence on the malignant advancement of tumor cells, A2M also plays a vital role in shaping the tumor immune microenvironment. We analyzed the correlation between A2M expression and immune cell infiltration within STAD tissues, utilizing the TIMER2.0 database. Our findings revealed that A2M expression is positively correlated with the infiltration of various immune cell types, including CD4⁺ T cells, CD8⁺ T cells, B cells, macrophages, myeloid dendritic cells, and neutrophils. This observation suggests that A2M may play a crucial role in regulating the tumor immune microenvironment. Notably, the strong association between A2M and immune cell markers indicates that A2M could potentially influence tumor immune escape by modulating the infiltration of immune cells. Additionally, the negative correlation observed between A2M and MDSCs bolsters this hypothesis, as MDSCs are recognized for their ability to hinder anti-tumor immune responses (28,29). These findings underscore the significant role of A2M in the tumor immune microenvironment, potentially regulating tumor immune escape and progression through its effects on immune cell infiltration and function. Further investigations are needed to elucidate the mechanisms by which A2M alters the functions of specific immune cell populations, thereby clarifying its precise role in tumor immune escape.

Patients with high expression of A2M show significant sensitivity to anti-CTLA-4 therapy, indicating a strong immune response within the tumor. Moreover, patients with high A2M expression have a higher TIDE score. This suggests that the tumor may activate a stronger compensatory immunosuppressive mechanism to counter the immune attack in order to survive. This suggests that

tumors with high A2M expression may be “immune-hot tumors”, but they are also rich in immunosuppressive factors. Such patients may particularly need immunotherapy to remove this immunosuppressive inhibition and thereby release the full potential of existing anti-tumor immunity.

Conclusions

In summary, this study demonstrates that A2M is significantly upregulated in STAD and is associated with unfavorable patient prognosis. Integrated bioinformatics and experimental evidence reveal that A2M promotes STAD cell proliferation, migration, invasion, and EMT through upregulation of Vimentin. Furthermore, A2M expression is closely associated with immune cell infiltration and immune checkpoint proteins, suggesting its role in tumor immune modulation. These findings highlight A2M as a potential prognostic biomarker and therapeutic target in STAD. Future studies should focus on elucidating the upstream regulatory mechanisms of A2M and assessing its translational potential in preclinical models.

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None.

Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at <https://tcr.amegroups.com/article/view/10.21037/tcr-2025-aw-2449/rc>

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki and its subsequent amendments.

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